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Constituents of Pine Heartwood

XXIV.* Investigations on Strobopinin, Cryptostrobin, and Two New Substances, Strobobanksin and Strobochrysin, from the Heartwood of *Pinus Strobus* L.

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In Part V of this series, the isolation of a new compound, strobopinin, from the heartwood of *Pinus strobus* was described by Erdtman [1]. This compound was almost colourless, had m. p. 225—227°**, and was optically active, showing $[\alpha]_D^{20}$—61° (methanol). Its composition, $C_{16}H_{14}O_4$, corresponds to a C-methyl dihydroxyflavanone (methoxyl absent), and the presence of a C—C_6H_5 grouping was proved by the fact that benzoic acid was formed on oxidation with permanganate. Strobopinin has also been isolated by one of us [2] from the heartwood of *P. monticola.*

Later, a compound called cryptostrobin was also isolated from *P. strobus* [3]. It is an isomer of strobopinin, melting at 202—203° and having $[\alpha]_D^{20}$—33° (methanol). Both substances were found to contain one C—CH_3 group, but further investigations on their structures were not carried out.

Strobopinin and cryptostrobin are rather difficult to separate by crystallisation, but a fairly good separation is achieved on the paper chromatogram [4]. The R_F values in standard solvent are approximately 0.48 for cryptostrobin and 0.65 for strobopinin. Diazotised benzidine gives somewhat different colours with the two isomers, the cryptostrobin spot having a deeper colour (orange) than the strobopinin spot (yellow to orange yellow). Both substances give yellow colours when reduced with magnesium and hydrochloric acid.

Both strobopinin and cryptostrobin yield methylphloroglucinol on degradation with strong alkali. Sufficient quantities were not available to allow

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** All melting points uncorrected.

isolation of this phenol, but a micro method was worked out, involving boiling with 50 % potassium hydroxide and identification of the phenol by paper chromatography. A good separation of phloroglucinol and methylphloroglucinol is obtained, using ethyl ether as the solvent (R_F values about 0.5 and 0.7 respectively). By this method, very good chromatograms were obtained starting from 0.5 mg of each flavanone. The method can of course be extended to any hydroxylated flavone or flavanone, if the right solvent is chosen for the identification of the resulting phenol.

If either strobopinin or cryptostrobin is heated with dilute alkali, a mixture of both substances is obtained. This rearrangement was studied by the aid of paper chromatography, using a few milligrams of the flavanones. It is thus evident that strobopinin and cryptostrobin must be very intimately related, and on the basis of the experiments hitherto discussed, the most probable structures for the two substances would be 5,7-dihydroxy-6-methylflavanone (I) and 5,7-dihydroxy-8-methylflavanone (II). In alkaline solution, these two flavanones would be in equilibrium with the same chalkone (III) and could thus be converted into each other.

These structures have been confirmed by synthesis. By condensing methylphloroglucinol with cinnamoyl chloride as described by Fujise and Tatsuta for phloroglucinol [5], a mixture of cryptostrobin and strobopinin was obtained in low yield. From this mixture, pure *d,l*-strobopinin, m. p. 231—233°, could be isolated after filtering through a magnesium oxide column. (Pure *d,l*-cryptostrobin could not be isolated.)

With diazomethane, strobopinin and cryptostrobin yielded monomethyl ethers, m. p. 96—97° and 134—136° respectively in the crude state. Further methylation with dimethyl sulphate and potassium carbonate in acetone led in the case of strobopinin to an orange red product which was identified as 2′-hydroxy-3′-methyl-4′,6′-dimethoxychalkone (V), and in the case of cryptostrobin to a chalkone-flavanone mixture which could not be separated. (Only very small amounts were available.)

The structure of the chalkone follows from its synthesis. Curd and Robertson [6] have proved the structure (IV) for the ketone obtained by nuclear methylation of phloroacetophenone, and from this ketone Brockmann and Maier[7] have prepared the chalkone (V) by condensation with benzaldehyde. The same authors stated that they were unable to rearrange the chalkone to the hitherto unknown 5,7-dimethoxy-8-methylflavanone (VI); the present authors, however, did succeed in obtaining a 10 % yield of the flavanone, m. p. 141—142°, on boiling with sulphuric acid in ethanol for five days. The chalkone can also be demethylated and rearranged in one operation by heating with pyridine hydrochloride [8], yielding a mixture of strobopinin and cryptostrobin.

The formation of the chalkone (V) by methylation of strobopinin suggests that the latter most probably has structure (II), but it is no rigid proof since the pyranone ring may have opened before methylation occurred. Further investigation in this field was made impossible by the small quantities of material at our disposal.

From crude fractions of strobopinin and cryptostrobin from *P. strobus*, a yellow substance, almost insoluble in ether and melting at 285—288°, was isolated in very small quantities (12 mg). It has the composition $C_{16}H_{12}O_4$ and all the characteristic properties of a flavone. Methylphloroglucinol can be identified among its alkali fission products. This new heartwood phenol must evidently be a 6- or 8-methyl chrysin (VII, VIII), and the name strobochrysin is proposed for it.

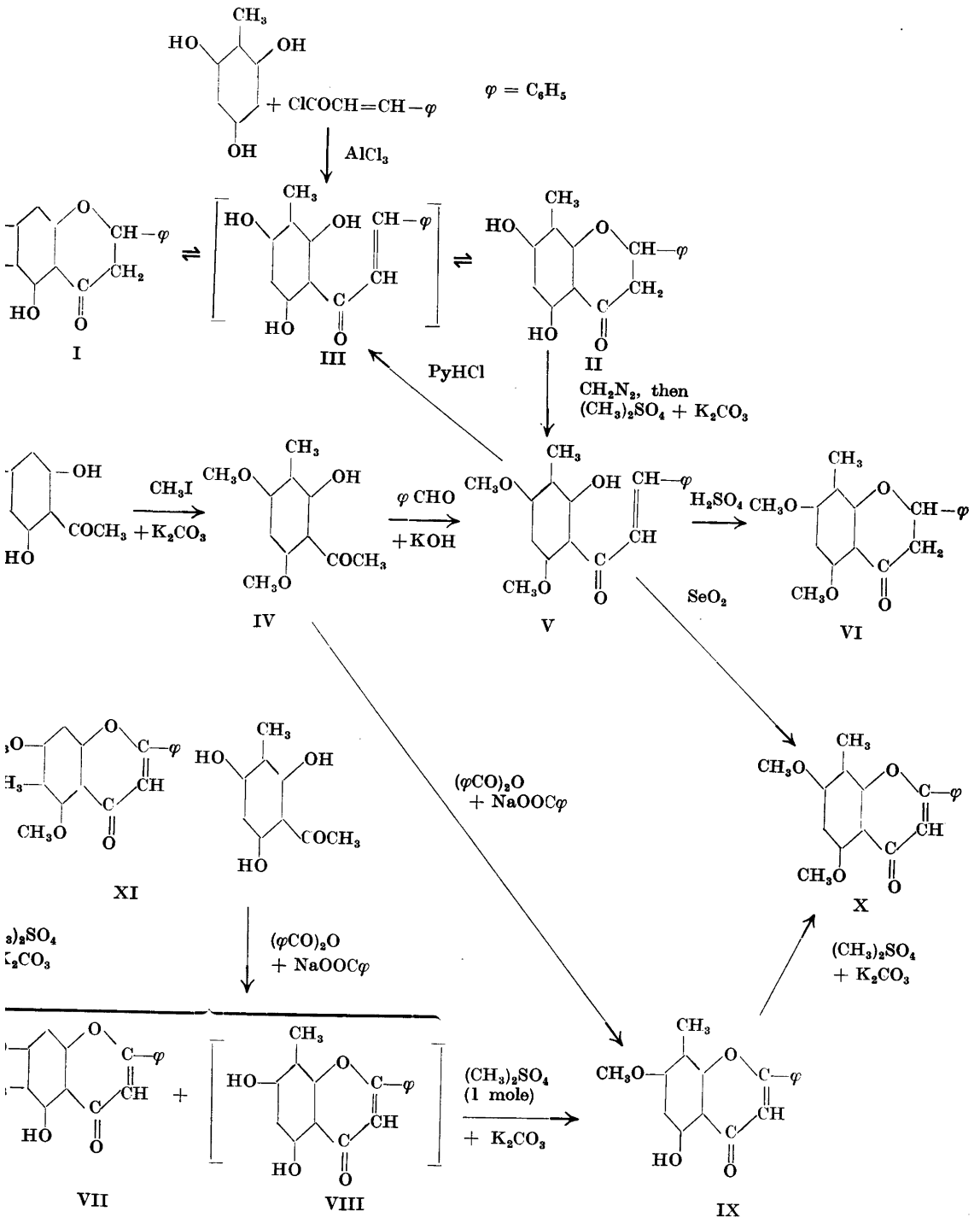
To synthesise strobochrysin, methylphloroacetophenone was heated with benzoic anhydride and sodium benzoate according to the method of Allan-Robinson. A mixture of yellow, high-melting products was obtained, from which small amounts of a pure substance could be isolated, which was identical with natural strobochrysin, as established by the mixed m. p. of their acetates (m. p. 190—191°) *.

The remainder of the reaction mixture was methylated with one mole of dimethyl sulphate by the Seshadri method [9], and the crude monomethyl ether extracted with strong alkali. The alkali fraction contained a monomethyl ether (IX), m. p. 230—231° (in the crude state), which on further methylation yielded a dimethyl ether, m. p. 230—231°. This compound was also obtained in 60 % yield, when the chalkone (V) was oxidised with selenium dioxide for 40 hours by the method of Mahal, Rai, and Venkataraman [10]. It must therefore be identical with 5,7-dimethoxy-8-methylflavone (X).

Synthetic strobochrysin, on methylation, yielded a dimethyl ether, m. p. 170—171°. The available quantities of this compound were too small to permit complete purification, but the large difference in melting point between the dimethyl ether and 5,7-dimethoxy-8-methylflavone (X) indicates that the former compound must be the 6-methyl isomer (XI). Their colour reactions on reduction with magnesium and hydrochloric acid are also different. Thus, the most probable structure for strobochrysin is 6-methyl chrysin (VII). It may be identical with a high-melting product, a few milligrams of which were isolated by Erdtman from *P. strobus* in 1944 [1].

We first tried to synthesise the dimethyl ether (X) from the ketone (IV)

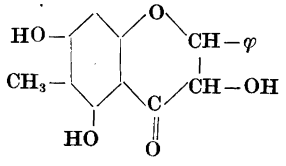
* After the completion of this work we became aware that the same synthesis had already been carried out by S. Furukawa [*Bull. Inst. Phys. Chem. Research (Tokyo)* **13** (1934) 1098; *Chem. Zentr.* (1935 I) 1708]. The m.p.s of his reaction products are different from those recorded by us. It is our intention to return to this question later.


φ = C6H5
+ ClCOCH=CH−φ
AlCl3
I
III
II
PyHCl
CH2N2, then
(CH3)2SO4 + K2CO3
CH3I
+K2CO3
IV
φCHO
+KOH
V
H2SO4
VI
SeO2
X
(φCO)2O
+ NaOOCφ
XI
(φCO)2O
+ NaOOCφ
(CH3)2SO4
+ K2CO3
(CH3)2SO4
(1 mole)
+ K2CO3
VII
VIII
IX

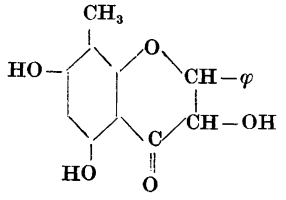
by the Allan-Robinson synthesis. This condensation, however, was accompanied by partial demethylation in the 5-position, and 5-hydroxy-7-methoxy-8-methylflavone (IX) was obtained as the main product. Similar demethylations are previously recorded in the literature [11]. Demethylation of (X) with pyridine hydrochloride is accompanied by partial rearrangement to the 6-methyl series, and a small yield of strobochrysin is obtained.

Another new compound was detected on the paper chromatogram as an orange yellow spot ($R_F \sim 0.35$ in standard solvent). It seemed to accumulate in the 0.2 % alkali fraction from *P. strobus*. The new compound was comparatively easy to isolate from this fraction, owing to the fact that it formed an insoluble sodium salt in sodium carbonate solution, just like pinobanksin [12]. The pure product is colourless, melts at 177—178°, and is optically active, having $[\alpha]_{20}^{D}$ + 17° (methanol). It yields methylphloroglucinol on alkali fission, and has the composition $C_{16}H_{14}O_5$, corresponding to a C-methyl pinobanksin. Oxidation with alkaline permanganate leads to benzoic acid, thus indicating the presence of a C—C_6H_5-group. It gives yellow reduction products with both magnesium and zinc in hydrochloric acid, which is reported to be characteristic for 3-hydroxyflavanones [13]. The name strobobanksin is proposed for this new compound.

Pinobanksin can easily be dehydrogenated to galangin by palladium and cinnamic acid [12]. (Flavanones having no hydroxyl in the 3-position do not react under these conditions.) The same method was also applied to strobobanksin and a yellow product, melting between 220° and 225°, was obtained. It gave a yellow colour reaction with magnesium and hydrochloric acid. An attempt was made to prepare its trimethyl ether, but no pure product was obtained. The monomethyl ether of strobobanksin, m. p. 129—131°, was prepared by the action of diazomethane. Further methylation under mild conditions yielded a product which must be the 5,7-dimethyl ether. This was not purified, but on dehydrogenation by the above-mentioned method, a yellow crystalline substance was obtained, melting at 211—213°. Even here, the quantity was too small to allow a pure methyl ether to be prepared. There is no doubt, however, that strobochrysin yielded a flavonol on dehydrogenation, and thus it must be a 6- or 8-methylpinobanksin (XII, XIII). Further investigations could not be carried out owing to lack of material.



XII



XIII

Flavones or flavanones containing a methylphloroglucinol nucleus have not previously been found in nature. Recently, Schmid and Bolleter have found three chromones in *Eugenia caryophyllata* (L.) Thunb., which all contain a methylphloroglucinol nucleus [14]. They are called eugenitin, (2,6-dimethyl-5-hydroxy-7-methoxychromone), isoeugenitin (2,8-dimethyl-5-hydroxy-7-methoxychromone) and isoeugenitol (2,8-dimethyl-5,7-dihydroxychromone). These substances are thus closely related to those found in *P. strobus*, and here too the 6- and 8-methyl derivatives occur together in the same plant.

Matteucinol and desmethoxymatteucinol, occurring in a fern, are flavanones containing a dimethylphloroglucinol nucleus [15].

EXPERIMENTAL

The isolation of strobopinin and cryptostrobin from the heartwood of *P. strobus* has already been described [1,3].

Alkali fission of the flavanones on a micro scale

The substance to be tested (0.5—2 mg) was boiled in a small test tube with 50 % potassium hydroxide (0.5 ml) for ten minutes. The solution was then cooled, acidified, and extracted with ether (0.5 ml). One drop of the ether solution was applied to a filter paper strip (Munktell OB); one drop of a solution containing phloroglucinol (1 %) and methylphloroglucinol (1 %) was also applied to the same strip as a reference mixture. The chromatogram was run in the usual manner, using water-saturated ether as the solvent. The paper strip was then sprayed with or dipped into diazotised benzidine reagent [4]. The phenols gave immediate colorations, brownish-red for phloroglucinol ($R_F \sim 0.5$) and brick red for methylphloroglucinol ($R_F \sim 0.7$). The spots were circular and very sharp.

Strobopinin, cryptostrobin, strobochrysin and strobobanksin gave strong spots for methylphloroglucinol and sometimes also a very faint phloroglucinol spot, especially when the boiling time was prolonged. This was due to the fact that methylphloroglucinol itself gives a small yield of phloroglucinol on alkali treatment. Chrysin, pinocembrin, and pinobanksin gave strong spots for phloroglucinol. All substances, in addition, gave a strong brick-red spot with $R_F \sim 0.9-0.95$, which must have been due to some other degradation product.

Interconversion of the flavanones

Strobopinin or cryptostrobin (1—2 mg) was heated with 0.5 ml of 1 % sodium hydroxide on the water bath for one and a half hours. The solution was then acidified and extracted with ether (0.5 ml). One drop of the ether solution was investigated on the paper chromatogram by the standard procedure [4]. In both cases, the chromatogram showed the presence of the two isomers.

The same result was obtained by heating the flavanones with pyridine hydrochloride at 180° for one and a half hours.

Synthesis of *d,l*-strobopinin

Solution A: Dry methylphloroglucinol (5 g) was suspended in nitrobenzene (60 ml) and the mixture cooled with ice. A solution of cinnamoyl chloride (6 g) in nitrobenzene (50 ml) was then added.

Solution B: Dry aluminium chloride (7.5 g) was dissolved in nitrobenzene (115 ml), and four drops of thionyl chloride were added to the solution.

Solution A was cooled with ice, and about 90 ml of B were added dropwise in the course of two hours under anhydrous conditions. Next day the remainder of B was added, and the solution left at room temperature for four days. It was then heated to 50—60° for one hour, while a current of carbon dioxide was passed through it. Ice (100 g) and hydrochloric acid (10 ml) were then added, and all nitrobenzene removed by steam distillation. The remaining reddish-brown oil was taken up in ether, the solution thoroughly washed with sodium bicarbonate and partly decolourised by filtration through aluminium oxide. The filtrate, on concentration, deposited crystals. The first fraction, melting at 200—220°, gave no spots on the chromatogram. The second fraction, m. p. 185—205°, gave the spots of strobopinin and cryptostrobin. Additional amounts of this mixture were extracted from the remainder of the filtrate by boiling water.

The entire strobopinin-cryptostrobin mixture was dissolved in ether, and the solution filtered through a magnesium oxide column. Two zones were visible on the column, one yellowish-brown in the upper part, and below that zone a broad pale yellow one, which slowly moved downwards when the column was washed with ether. After it had reached the bottom of the column, the filtrate only contained strobopinin as long as the washing was continued with ether. Each fraction was tested on a paper chromatogram. When the column was washed with methanol, the upper zone moved rapidly downwards, and a mixture of both strobopinin and cryptostrobin could be found in the filtrate.

From the ether fractions, pure *d,l*-strobopinin (0.23 g) was obtained after two recrystallisations from dilute acetic acid as colourless leaflets, m. p. 231—233°. A mixture with (—)strobopinin from *P. strobus* (m. p. 224—226°) melted at 226—229°. The two substances gave identical spots on the paper chromatogram.

$C_{16}H_{14}O_4$ (270.3)	Calc.	C	71.1	H	5.22
	Found	»	71.1	»	5.13

No pure product could be obtained from the methanol fraction.

Methylation of strobopinin and cryptostrobin

Strobopinin from *P. strobus* (0.2 g) was treated with diazomethane in ether solution. Next day, the ether was evaporated and the residue recrystallised once from methanol. A colourless crystalline product, m. p. 96—97°, was obtained. It gave a brownish-violet colour with ferric chloride in ethanol.

The crude monomethyl ether (0.15 g) was boiled with dimethyl sulphate (0.06 ml) and dry potassium carbonate (1 g) in acetone (10 ml) for four hours. The acetone solution was filtered, and the solvent evaporated to dryness. The residue was taken up in ether, and the ether solution washed with 2 *N* sodium hydroxide. It was then dried over anhydrous sodium sulphate and concentrated, depositing orange red crystals, which were filtered off and recrystallised from methanol. They melted at 140—141° alone or on

admixture with a sample of 2′-hydroxy-3′-methyl-4′,6′-dimethoxychalkone, synthesised according to Brockmann and Maier [7]. Yield of pure product, 24 mg.

$C_{16}H_{12}O_2$ $(OCH_3)_2$ (298.3)	Calc.	OCH_3	20.8
	Found	»	21.0

Cryptostrobin (0.1 g) was methylated in the same way as described above. With diazomethane, a monomethyl ether melting at 134—136° was obtained, which gave a brownish-violet ferric chloride reaction. The crude monomethyl ether, on further methylation, yielded a mixture of yellow and colourless crystals, which could not be separated. The mixture gave a yellowish-brown colour with ferric chloride.

Synthesis of 5,7-dimethoxy-8-methylflavanone (VI)

2′-Hydroxy-3′-methyl-4′,6′-dimethoxychalkone (1.0 g) was dissolved in ethanol (250 ml); sulphuric acid (12 ml) was added and the solution boiled under reflux for five days. The solution, which was still orange in colour, was concentrated by vacuum distillation, and water was then added to the residue. The precipitate so obtained was recrystallised several times from methanol. Each time both yellow and colourless crystals were formed, but finally it was possible to collect 0.1 g of the colourless product. It melted at 141—142° and gave the following colour reactions: Ferric chloride, none; magnesium-hydrochloric acid, very pale pink.

$C_{16}H_{12}O_2(OCH_3)_2$ (298.3)	Calc.	C	72.5	H	6.08	OCH_3 20.8
	Found	»	72.4	»	6.15	» 21.0

Isolation of strobochrysin

Crude fractions of strobopinin and cryptostrobin from *P. strobus*, when dissolved in ether, left a small yellow insoluble residue. This residue was recrystallised several times from ethanol. Finally, pale yellow needles, m. p. 285—288°, were obtained (12 mg). Colour reactions: Hot nitric acid, pale yellow; ferric chloride, greenish-brown; magnesium-hydrochloric acid, pale yellow; diazotised benzidine, very pale orange. The R_F value of the compound in standard solvent is about 0.1. (The chromatogram is rather diffuse and not suitable for identification.)

$C_{16}H_{12}O_4$ (268.3)	Calc.	C	71.6	H	4.51
	Found	»	71.3	»	4.52

5 mg of the flavone were acetylated with acetic anhydride-perchloric acid. The acetate, recrystallised from ethanol, was colourless and melted at 190—191°.

Synthesis of strobochrysin and its isomer

Methylphloroacetophenone [6] (1 g), benzoic anhydride (8 g) and sodium benzoate (2.5 g) were heated to 180—190° in an evacuated sealed Pyrex tube for five hours. The contents of the tube were then refluxed with potassium hydroxide (5 g) in ethanol (50 ml) for half

an hour, the solution diluted with water and neutralised with carbon dioxide. The yellowish-brown precipitate was separated, dried, and extracted with ether in a Soxhlet apparatus. The ether extract, on concentration, deposited a yellowish precipitate, melting at 270—280°. After recrystallisation from ethanol, it yielded yellow crystals, m. p. 284—286°. The acetate melted at 190—191° alone or on admixture with strobochrysin acetate.

The remainder of the reaction product could not be separated. Part of it (90 mg) was methylated with 1.1 moles of dimethyl sulphate and potassium carbonate in acetone for two hours. After purifying the reaction product, the mixture of monomethyl ethers was dissolved in chloroform and extracted three times with 4 *N* sodium hydroxide. A yellow precipitate was formed in the alkali phase. The latter phase was acidified and extracted with ether. The ether solution, after drying and concentration, yielded a crystalline residue, which was recrystallised from methanol giving yellow crystals, m. p. 230—231°.

Colour reactions: Ferric chloride, greenish; magnesium-hydrochloric acid, pale pink.

$C_{16}H_{11}O_3(OCH_3)$ (282.3)	Calc.	OCH_3	11.0
	Found	»	10.1

This product was not purified further but was methylated once again for 16 hours in the way described above. A pale yellow crystalline product, m. p. 230—231°, was obtained. It gave no colour with ferric chloride and did not depress the m. p. of synthetic 5,7-dimethoxy-8-methylflavone (see below).

Synthesis of 5,7-dimethoxy-8-methylflavone (X)

The chalkone (V, 1.0 g) was refluxed with selenium dioxide (1.3 g) in *iso*-amyl alcohol (15 ml) for 40 hours. The selenium precipitate was filtered off, and the alcohol removed by steam distillation. The residue was taken up in chloroform, washed with 2 *N* sodium hydroxide, dried over anhydrous sodium sulphate, and the solvent evaporated. The crystalline residue was recrystallised from ethanol, yielding pale yellow needles (0.66 g) which had m. p. 230—231°; they gave no colour with ferric chloride, but gave a pink colour with magnesium-hydrochloric acid.

$C_{16}H_{10}O_2(OCH_3)_2$ (296.3)	Calc.	C	73.0	H 5.44	OCH_3	20.9
	Found	»	72.8	» 5.35	»	20.6

Methylation of strobochrysin

Synthetic strobochrysin (60 mg) was refluxed for 24 hours with dimethyl sulphate (0.10 ml) and potassium carbonate (0.5 g) in acetone (25 ml). The reaction product was worked up as described earlier. A small amount of colourless crystals, m. p. 170—171°, was obtained; these gave no colour with ferric chloride, but gave a yellow colour with magnesium-hydrochloric acid.

Strobochrysin (30 mg) was also methylated with one mole of dimethyl sulphate and potassium carbonate in acetone for fifteen hours. Pale yellow needles, m. p. 170—173°, were obtained, with which ferric chloride gave a greenish-brown colour. This product was not further investigated.

Synthesis of 5-hydroxy-7-methoxy-8-methylflavone (IX)

2-Hydroxy-3-methyl-4,6-dimethoxyacetophenone (IV, 0.4 g) was heated with benzoic anhydride (3.2 g) and sodium benzoate (0.95 g) to 225—230° for six hours. The reaction mixture was worked up as described for strobochrysin, giving a low yield of 5-hydroxy-7-methoxy-8-methylflavone, m. p. 230—231° alone or on admixture with the product obtained before (see under "Synthesis of strobochrysin . . .").

Demethylation of 5,7-dimethoxy-8-methylflavone (X)

The flavone (X, 0.3 g) was heated to 200° with pyridine hydrochloride (1.5 g) for six hours. The reaction product was then treated with water, and the precipitate filtered off, dried and sublimed in a vacuum. The sublimate, on recrystallisation from acetic acid and from ethanol, yielded a small amount of a yellow crystalline product, m. p. 280—282°. The acetate melted at 190—191°, alone or on admixture with strobochrysin acetate.

Demethylation for two hours at 180° yielded 5-hydroxy-7-methoxy-8-methylflavone (IX) as the main product.

Isolation of strobobanksin

The 0.2 % sodium hydroxide fraction of the heartwood extract from *P. strobus* contains five or six different phenols, according to the paper chromatogram. One of these has $R_F \sim 0.35$ in standard solvent and gives an orange yellow colour with benzidine reagent. The other spots are due to substances already known.

The entire fraction was recrystallised from dilute acetic acid, the crystals separated, and the mother liquor precipitated with water. The sticky precipitate was extracted several times with boiling water. Each water extract was cooled and shaken with ether. The ether extracts, on concentration, yielded yellow oils, which partly crystallised. From the first extract, colourless crystals were obtained, m. p. 168—170°. This product, when investigated on the paper chromatogram, seemed to consist mainly of the unknown substance. After recrystallisation from benzene and from dilute acetic acid and drying at 100° the compound had m. p. 177—178°, $[\alpha]_D^{20} + 17° \pm 1°$ (methanol, $c = 3.0$). Colour reactions: Ferric chloride, brownish-violet; magnesium or zinc and hydrochloric acid, bright yellow; diazotised benzidine, orange yellow.

$C_{16}H_{14}O_5$ (286.3)	Calc.	C	67.1	H	4.93
	Found	»	67.3	»	4.90

Additional quantities of the new substance were obtained from the other aqueous extracts by shaking with saturated sodium carbonate. Strobobanksin was then precipitated as a colourless sodium salt, which was filtered off, decomposed by acid, and the phenol recrystallised. Total yield, 0.25 g of pure strobobanksin from 7.0 kg of air-dry heartwood.

Strobobanksin (20 mg) was oxidised with potassium permanganate in alkaline solution as described by Erdtman [1]. Benzoic acid was obtained and identified by a mixed m. p. determination.

Methylation and dehydrogenation of strobobanksin

Strobobanksin (0.15 g) was treated with diazomethane in ether solution. The reaction product, on recrystallisation from methanol, melted at 129—131°. It gave a brownish-violet colour with ferric chloride, and, hence, was clearly a monomethyl ether.

The crude monomethyl ether (0.15 g) was further methylated for four hours with one mole of dimethyl sulphate and potassium carbonate. The reaction product was a yellow syrup, which slowly deposited crystals. The entire product was heated to 180° for two hours with cinnamic acid (0.25 g), palladium-carbon catalyst [16] (0.05 g) and water (5 ml) in a sealed tube. The reaction product was dissolved in ether and washed with sodium bicarbonate. Evaporation of the ether solution gave a yellow residue, which was recrystallised from chloroform-light petroleum. Brownish-yellow crystals, m. p. 211—213°, were obtained. Yield, 14 mg. Ferric chloride gave a brownish-violet colour.

The entire yield of this product was further methylated with dimethyl sulphate and potassium carbonate in acetone. The quantity of crude reaction product obtained was too small to allow purification, but the absence of a colour reaction with ferric chloride indicated that it was a fully methylated substance. Magnesium and hydrochloric acid gave a strong yellow colour.

Strobobanksin (33 mg) was also dehydrogenated in the way described above. A yellow crystalline product (13 mg) was obtained which melted at 220—225°. Colour reactions: Ferric chloride, greenish-brown; magnesium-hydrochloric acid, yellow.

Methylation of this compound did not lead to any pure product.

SUMMARY

Four substances from the heartwood of *Pinus strobus* L., the previously isolated strobopinin and cryptostrobin and the new compounds strobochrysin and strobobanksin, have been investigated. They are all flavones or flavanones containing a methylphloroglucinol nucleus. Strobopinin and cryptostrobin are 5,7-dihydroxy-8-and-6-methyl-flavanones. The position of the C-methyl group in each isomer has not definitely been determined, but the most probable structure for strobopinin seems to be 5,7-dihydroxy-8-methylflavanone. Strobochrysin it probably 5,7-dihydroxy-6-methylflavone and strobobanksin 3,5,7-trihydroxy-6-or-8-methylflavanone.

In connection with this work some new flavones and flavanones containing a methylphloroglucinol nucleus have been synthesised.

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Potentiometric Studies on the Equilibria of Some Copper(II)-hydroxysalts in Aqueous Salt Solutions, and Involved Complex Formation

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The solubility equilibria of copper(II)-hydroxysalts have recently been dealt with in a series of papers [1] from this laboratory. It has been shown that copper trihydroxysalts are precipitated from copper chloride, nitrate and sulphate solutions. These precipitates have a definite character and the law of mass action may be applied to their equilibria in aqueous solutions. The fact that copper hydroxyperchlorate is much more soluble than the other copper hydroxysalts, makes it possible to investigate the effect of alkali perchlorate on the solubility equilibria of the copper hydroxysalts. These studies have been continued, paying especial attention to complex formation; and in the following, results on copper hydroxybromide and some additional data concerning copper hydroxychloride and sulphate are presented.

EXPERIMENTAL

The chemicals used were of the best qualities obtainable (Kahlbaum for analysis) except the copper bromide and sodium perchlorate, which were recrystallized many times. The experiments were performed at 20° C or 25° C as indicated. For further experimental details the reader is referred to the previous papers [1].

COPPER(II)-TRIHYDROXYBROMIDE

When alkali hydroxide is added to copper bromide solution, trihydroxybromide is precipitated [2]. In Table 1 the results of experiments for the determination of the solubility product in potassium bromide solutions are recorded. The experiments were made in Pyrex flasks into which the copper bromide and potassium bromide as well as the required amount of water and

sodium hydroxide solution were measured. After four — nine weeks the pH of the solutions was measured. In the calculations the following equations, derived earlier [1], were used:

$$[Cu^{++}]\ (OH^-)^{1.5}\ [Br^-]^{0.5} = S \quad (1)$$
$$[Cu^{++}] = c_{Cu} - x \quad (2)$$
$$[Br^-] = c + 2c_{Cu} - 0.5x \quad (3)$$
$$x = 0.667\ (c_B + [H^+] - [OH^-]) \quad (4)$$

Table 1. Solubility product of copper(II)-trihydroxybromide in potassium bromide solutions at 20° C.

$\sqrt{I}$	$c_{Cu} \cdot 10^2$	$c_B \cdot 10^2$	c_{KBr}	pH	pS
0.086	0.390	0.448	—	5.63	16.96
0.093	0.393	0.335	—	5.44	16.95
0.099	0.396	0.219	—	5.31	16.96
0.130	0.767	0.653	—	5.18	16.91
0.139	0.779	0.431	—	5.08	16.86
0.175	1.31	0.889	—	4.90	16.88
0.248	1.31	0.889	0.0308	4.82	16.80
0.260	1.35	0.446	0.0318	4.71	16.79
0.339	1.27	1.30	0.0896	4.94	16.72
0.350	1.31	0.889	0.0924	4.77	16.71
0.363	1.35	0.446	0.0954	4.67	16.69
0.569	1.27	1.30	0.299	4.87	16.59
0.582	1.31	0.889	0.308	4.72	16.56
0.595	1.35	0.446	0.318	4.59	16.58
0.788	1.27	1.30	0.597	4.84	16.50
0.803	1.31	0.889	0.616	4.67	16.50
0.820	1.35	0.446	0.636	4.55	16.49
0.959	1.27	1.30	0.896	4.79	16.49
0.977	1.31	0.889	0.924	4.60	16.51
0.995	1.35	0.446	0.954	4.49	16.51
1.23	1.27	1.30	1.493	4.72	16.48
1.25	1.31	0.889	1.540	4.52	16.52
1.46	1.40	1.82	2.105	4.85	16.54
1.53	1.53	0.619	2.306	4.29	16.58
1.78	1.46	1.25	3.144	4.36	16.66
1.83	1.53	0.619	3.294	4.17	16.68

where the symbols in brackets denote concentrations, $(\overline{OH})$ is the activity of hydroxyl ion, c_{Cu} the total copper molarity, c_B that of sodium hydroxide and c that of the added alkali bromide. In Table 2 the results of a similar series

in sodium perchlorate solutions are recorded. The results in both cases may be represented by the Debye-Hückel equation

$$pS = pS_0 - \frac{2.26\sqrt{I}}{1 + a\sqrt{I}} + BI \qquad (5)$$

and the following values for the parameters at 20° were obtained:

	KBr	$NaClO_4$
pS_0	17.145	17.145
a	1.939	2.50
B	0.128	0.380

Table 2. Solubility product of copper(II)-trihydroxybromide in sodium perchlorate solutions at 20° C.

$\sqrt{I}$	$c_{Cu} \cdot 10^2$	$c_B \cdot 10^2$	c_{NaClO_4}	pH	pS
0.217	1.40	1.82	0.0227	5.33	16.82
0.236	1.46	1.25	0.0237	4.97	16.79
0.254	1.53	0.619	0.0248	4.80	16.77
0.264	1.40	1.82	0.0453	5.35	16.78
0.282	1.46	1.25	0.0474	4.97	16.79
0.300	1.53	0.619	0.0496	4.80	16.77
0.304	1.40	1.82	0.0680	5.35	16.78
0.321	1.46	1.25	0.0710	4.98	16.78
0.339	1.53	0.619	0.0744	4.81	16.76
0.500	1.40	1.82	0.227	5.39	16.72
0.518	1.46	1.25	0.237	5.02	16.72
0.711	1.46	1.25	0.474	4.99	16.77
0.732	1.53	0.619	0.496	4.80	16.77
0.839	1.40	1.82	0.680	5.32	16.84
0.862	1.46	1.25	0.710	4.94	16.84
0.886	1.53	0.624	0.744	4.76	16.84
0.990	1.46	1.25	0.947	4.90	16.91
1.08	1.40	1.82	1.133	5.26	16.93
1.10	1.46	1.25	1.184	4.86	16.96
1.13	1.53	0.619	1.240	4.68	16.96
1.27	1.40	1.82	1.585	5.18	17.04
1.30	1.46	1.25	1.657	4.79	17.06
1.36	1.40	1.82	1.812	5.08	17.20
1.42	1.53	0.619	1.984	4.51	17.21
1.51	1.40	1.82	2.264	5.02	17.29
1.55	1.46	1.25	2.367	4.60	17.35
1.59	1.53	0.619	2.480	4.37	17.42

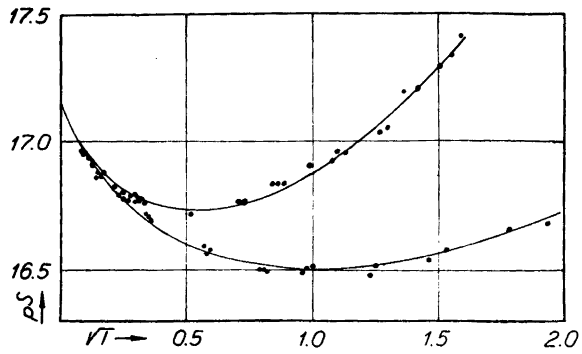

Fig. 1. Solubility product of copper(II)-trihydroxybromide in solutions of potassium bromide (lower curve) and sodium perchlorate (upper curve) at 20° C as a function of ionic strength.

In Fig. 1 the solubility product of copper hydroxybromide is represented as a function of ionic strength. The solubility product is seen to be smaller in sodium perchlorate solutions than in potassium bromide solutions. Such a difference may be interpreted in terms of complex formation between cupric and bromide ions or of the difference of the ion activity coefficients. It is, however, impossible by means of potentiometric measurements to distinguish between these two effects. But since the activity coefficients are, so far as is known, larger in perchlorate solutions than in the solutions of other electrolytes, it is possible to calculate the upper limits for the complexity constants

$$k_\nu = \frac{[\mathrm{CuBr}_\nu^{(\nu-2)-}]}{[\mathrm{Cu}^{++}][\mathrm{Br}^-]^\nu} \tag{6}$$

For this purpose we have carried out a series of measurements in mixed solutions of sodium perchlorate and bromide at unit ionic strength. The results are presented in Table 3; each pS value is a mean value of three measurements.

Table 3. Solubility product of copper(II)-trihydroxybromide in the mixed solutions of sodium bromide and perchlorate at 25° C. $I = 1$.

c'_{Br}	pS	c'_{Br}	pS
0.146	16.677	0.510	16.617
0.267	16.646	0.630	16.591
0.389	16.632	0.751	16.557
		0.992	16.501

pS is seen to decrease nearly linearly with bromide concentration. From equation (6) and the stoichiometric equations

$$c'_{Cu} = [Cu^{++}] + [CuBr^+] + [CuBr_2] + \ldots\ldots \quad (7)$$

and

$$c'_{Br} = [Br^-] + [CuBr^+] + 2[CuBr_2] + \ldots\ldots \quad (8)$$

where

$$c'_{Cu} = c_{Cu} - x \text{ and } c'_{Br} = c + 2c_{Cu} - 0.5x$$

we obtain

$$\sum k_\nu [Br^-]^{\nu-1} + (1 - S/S')/[Br^-] = 0 \quad (9)$$

and

$$[Br^-] = c'_{Br} - (c'_{Cu} \sum \nu k_\nu [Br^-]^\nu)/(1 + \sum k_\nu [Br^-]^\nu) \quad (10)$$

where S' refers to the solution of sodium perchlorate. The solution of equation (9) by the method of least squares gives in this case

$$k_1 \leqq 0.4,\ k_2 \leqq 0.1 \text{ and } pS' = 16.703$$

COPPER(II)-TRIHYDROXYCHLORIDE

The solubility product of copper(II)-trihydroxychloride in potassium chloride solutions has been determined by Näsänen and Tamminen [1]. A series of measurements was carried out by the present authors in mixed solutions of sodium perchlorate and chloride at unit ionic strength. The results are recorded in Table 4; each pS value is a mean value of three measurements. By means of equation (9) the following values for the constants were obtained:

$$k_1 \leqq 0.4,\ k_2 \leqq 1.4 \text{ and } pS' = 17.163$$

Table 4. Solubility product of copper (II)-trihydroxychloride in mixed solutions of sodium chloride and perchlorate at 25° C. $I = 1$.

c'_{Br}	pS	c'_{Br}	pS
0.154	17.126	0.656	16.883
0.280	17.081	0.782	16.824
0.405	17.023	0.993	16.725
0.531	16.967		

COPPER(II)-TRIHYDROXYSULPHATE

The solubility equilibria of copper(II)-trihydroxysulphate has been investigated by Näsänen and Tamminen [1] in potassium sulphate solutions and by Näsänen [1] in sodium perchlorate solutions. Assuming again that the great difference between the solubility products in these solutions is due entirely to complex formation, one can calculate the upper limits for the complexity constants. In Table 5 a series of measurements in mixed solutions of sodium sulphate and perchlorate at unit ionic strength is recorded. Each value in the table is a mean value of three measurements. Equation (9) may be used in the calculation, however, for this case

$$[Cu^{++}](OH^{-})^{1.5}[SO_4^{=}]^{0.25} = S \tag{11}$$

and

$$c'_{SO_4} = c + c_{Cu} - 0.25x \tag{12}$$

Table 5. Solubility product of copper(II)-trihydroxysulphate in mixed solutions of sodium sulphate and perchlorate at 20° C. I = 1.

c'_{SO_4}	pS	c'_{SO_4}	pS
0.0365	16.716	0.1982	16.207
0.0684	16.600	0.3270	15.854
0.1336	16.416		

The following values were obtained:

$$k_1 \leqq 11,\ (k_2 \sim 3),\ k_3 \leqq 150 \text{ and } pS' = 16.863$$

FORMATION OF COPPER(II)-TETRAHYDROXYSALTS

According to Weiser, Milligan and Cook [3] tetrahydroxysulphate is precipitated, in addition to the hydroxide or trihydroxysalt, when the ratio of the equivalents of copper sulphate and sodium hydroxide in a mixture is between 1 and 1.33. In corresponding conditions tetrahydroxychloride and bromide should exist according to Tobler [4]. We have investigated the precipitation curve of copper hydroxychloride and bromide and have found that, when sodium hydroxide solution is added to copper salt solution in such an amount that the ratio of moles of sodium hydroxide to copper salt exceeds 1.5, the formation of copper oxide does not begin until this ratio is about 1.6. The hydroxychloride corresponding to the value 1.6 gave, however, the same X-ray diffraction pattern as was obtained by Feitknecht [5] for trihydroxychloride

(II γ modification). The precipitate investigated was equilibriated for about one year. It is therefore obvious that trihydroxychloride and very probably also the trihydroxybromide is metastable between the values 1.5 and 1.6. Trihydroxysulphate has not been studied in this respect. This does not imply, however, that tetrahydroxychloride or bromide could not be formed in some other ways, for instance by digesting copper oxide in dilute copper chloride or bromide solutions.

DISCUSSION

The accuracy obtainable in the determination of the solubility products of hydroxysalts is not very high. On the other hand when chloride concentration is sufficiently small hydroxyperchlorate is precipitated and therefore the measurements in such conditions are impossible. As a result, the values obtained above for the complexity constants cannot be very accurate. Nevertheless the k_1 values are in satisfactory agreement with the spectrophotometric results of Näsänen [6].

For the complexity constant of the copper(II)-monochlorocomplex McConnell and Davidson [7] have recently found spectrophotometrically the value $k_1 = 1.30$. This value must, however, be considered as an upper limit, as will be shown below. The authors have measured the extinction of a solution with a small chloride concentration and much larger copper ion concentration and have used the equation

$$f(c_{Cu}) = c_{Cu}c_{HCl}/e = c_{Cu}/\varepsilon_1 + 1/\varepsilon_1 k_1 \qquad (13)$$

where c_{Cu} denotes the total copper concentration, c_{HCl} that of hydrochloric acid, and e is the extinction of a 1 cm layer of the solution investigated compared to a similar solution without hydrochloric acid. The ionic strength has been kept constant by addition of perchloric acid. The authors conclude from the linear character of the curve, $f(c_{Cu})$ versus c_{Cu}, that k_1 is constant in mixed solutions of copper perchlorate and perchloric acid at unit ionic strength. This is not, however, necessarily true. The linear character implies only that $1/k_1$ (or log k_1, which within the limits of the experimental error means the same in the narrow concentration range investigated), is a linear function of c_{Cu}. If we write

$$1/k_1 = 1/k_1' + bc_{Cu} \qquad (14)$$

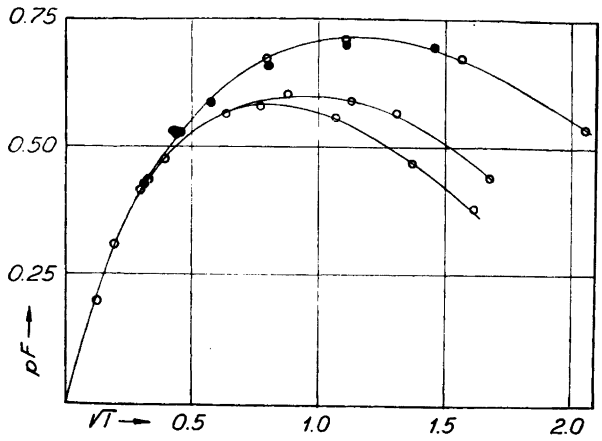

Fig. 2. Activity coefficient of the copper(II)-monochlorocomplex in perchloric acid (lower curve), sodium (middle curve), barium and calcium (upper curve) perchlorate solutions at 25° C. ●, barium perchlorate.

where k_1' is the value of k_1 in perchloric acid solution and b a parameter, we obtain from equation (13)

$$f(c_{Cu}) = c_{Cu}/\varepsilon_1 + (1/k_1' + bc_{Cu})/\varepsilon_1 \tag{15}$$

When $c_{Cu} = 0$, $f(0) = 1/\varepsilon_1 k_1'$, and the first derivative in regard to c_{Cu} is

$$f'(c_{Cu}) = (1 + b)/\varepsilon_1$$

Thus

$$k_1' = \frac{f'\ (c_{Cu})}{f(0)\ (1 + b)} \tag{16}$$

According to McConnell and Davidson

$$f'(c_{Cu})/f(0) = 1.30$$

which would be the value of k_1 if $b = 0$. When b is positive, which means that k_1 is smaller in copper perchlorate solution than in perchloric acid solution, k_1' is smaller than 1.30. Because it seems likely that k_1 is in fact smaller in copper perchlorate than in perchloric acid solution, this value must be considered as an upper limit for k_1. In fact the activity coefficient is generally different in solutions of different electrolytes. This is seen from Fig. 2, in

which the activity coefficient of the copper(II)-monochlorocomplex is represented as a function of ionic strength in perchloric acid, sodium, barium and calcium perchlorate solutions. The activity coefficient

$$F = f_{Cu^{++}} f_{Cl^-} / f_{CuCl^+} \tag{17}$$

was determined by the spectrophotometric method of Näsänen [6], using the equation

$$\log \frac{e}{[Cu^{++}][Cl^-]} = \log \frac{1}{\varepsilon_1 k_{1,0}} - pF \tag{18}$$

where $k_{1,0}$ is the thermodynamic constant. For $[Cu^{++}]$ and $[Cl^-]$ the values of c_{Cu} and c_{Cl} may be used. The differences in pF at unit ionic strength are as follows:

	$HClO_4$	$NaClO_4$	$Ba(ClO_4)_2$ and $Ca(ClO_4)_2$
ΔpF	0.00	0.03	0.13

Thus the complexity constant can vary considerably with the composition of the solution. We have calculated some values for the constant k_1 in perchloric acid and copper perchlorate solution at unit ionic strength from McConnell and Davidson's data using equations (14) and (16) and adopting arbitrary values for b. We obtained

	b	k_1	ΔpF	b	k_1	ΔpF	b	k_1	ΔpF
$Cu(ClO_4)_2$		0.534			0.336			0.245	
	1		0.089	2		0.110	3		0.123
$HClO_4$		0.650			0.433			0.325	

These values, as compared to the above data obtained spectrophotometrically for ΔpF, seem to explain the discrepancy between McConnell and Davidson's results and ours. Further if barium or calcium perchlorate is used instead of perchloric acid in such experiments as those carried out by McConnell and Davidson, a value of nearly the same magnitude as our value will obviously be obtained for k_1 by means of their method of calculation.

In the copper sulphate system Fronaeus [8], by two potentiometric methods, has obtained the values:

$$k_1 = 11,\ k_2 = 10\text{—}17,\ k_3 = 200$$

and

$$k_1 = 9,\ (k_2 \sim 0),\qquad k_3 = 80$$

Our values are seen to be in satisfactory agreement with these values. On the other hand the spectrophotometric method gives lower values. Fronaeus [8] has obtained at unit ionic strength $k_1 = 5$ and Näsänen [9] $k_1 = 4$. Probably in this case also, the potentiometric values must be considered as upper limits of the constants.

SUMMARY

The solubility equilibria of copper(II)-trihydroxy chloride, bromide and sulphate have been investigated. The solubility products of these compounds were determined in solutions of some alkali chlorides, bromides, sulphates and perchlorates. The complex formation of copper(II)-ion with chloride, bromide and sulphate ions has been studied potentiometrically and upper limits for the complexity constants have been calculated. The discrepancies in the values obtained by different methods are discussed in terms of activity coefficients. The activity coefficient of the copper(II)-monochloro complex has been determined spectrophotometrically in perchloric acid, sodium, barium and calcium perchlorate solutions as a function of ionic strength.

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Hydrolysis of Benzoylphosphates *

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The mixed anhydrides of phosphoric acid have in recent years attracted the interest of several biochemists, and their work [1-3] has brought forward several facts about their reactivities which are of considerable interest also from the point of view of pure chemistry.

Chantrenne [3] has shown that at pH = 7.4 dibenzoylphosphate and phenylbenzoylphosphate will readily benzoylise glycine, ammonia and aniline, while monobenzoylphosphate will not. The latter, however, reacts much faster with water than the disubstituted compounds. Lipman [1] has investigated monoacetylphosphate and Lynen [2] has hydrolysed both mono-, di- and triacetylphosphate. The triacetyl compound is destroyed almost momentarily by water and the behaviour of the two others seems closely analogous to that of the benzoyl compounds.

Bentley [4] has hydrolysed acetylphosphate in nearly neutral and in strongly alkaline solutions in ^{18}O enriched water and he traces the water's oxygen to the phosphate ions in the former case and to the acetate in the latter.

The purpose of the present investigation is to find the variation with pH of the rate of hydrolysis of mono- and dibenzoylphosphate in order to get a better basis for speculations about the mechanism of these interesting reactions.

EXPERIMENTAL

Preparation of dibenzoylphosphate was done as described by Chantrenne [3], but not in a "cold room". Possibly for this reason no monobenzoylphosphate could be isolated.

Reaction took place in test tubes in thermostat at 37.0° C and samples were withdrawn at intervals and analysed photometrically through the formation of a red ferric ion complex with hydroxamic acid, formed by reaction of the residual amount of mixed an-

* The results of this work were communicated at the *Syvende nordiske kemikermöde* in Helsinki, August 1950.

hydride with hydroxylamine. The method was a slight modification of Lipmans procedure: Three stock solutions were prepared. A: 28 % (4 *M*) hydroxylamine hydrochloride; B: 14 % (3.5 *M*) sodium hydroxide and C: 1.66 % ferric chloride in 1 *N* HCl. Just before use equal volumes of A and B were mixed and 1 ml of the mixture was added to a 1 ml sample in a 10 ml measuring flask. After standing for an hour in thermostat at 37° C 2 ml C-solution was added and the volume adjusted to 10 ml with 1 *N* HCl. The optical density was measured in 10 mm cells in a Spekker photometer with green filters (604). The modification in the methods of Lipman and Tuttle [5] and Chantrenne [3] were necessitated partly by the wider range of pH throughout which a uniform procedure was desired, and partly by the fact that the undiluted solution was not stable; the colour faded and bubbles were formed on the windows of the cell. Diluted according to the above procedure, the solution kept so well that it faded only about 2 % in an hour.

On the Spekker photometer the optical density is read directly, and so long as the same cell is used, the reading is proportional to the concentration of unhydrolysed anhydride bonds, *i. e.*

$$\varepsilon = \text{const} \cdot (2\ [B_2P] + [B_1P]) \tag{1}$$

where $[B_2P]$ and $[B_1P]$ are the concentrations of dibenzoyl- and monobenzoylphosphate, respectively.

This analytical method is not accurate and convenient enough to make it feasible to investigate the variation of rate constants with temperature and ionic strength, but it is quite accurate enough for an orientation with regard to the dependence on pH.

At high pH-values it is convenient to follow the reaction by automatic titration of the liberated acid, and a few runs were made with a "pH-stat", built in this institute by Mr. Arne E. Nielsen, chemical engineer. In acid solution this method is not so suitable, as the buffering capacity of the weak acids formed makes the readings less accurate, and eventually under the p*K* of benzoic acid the consumption of base becomes negligible.

Calculation of the Rate Constants presents the familiar difficulties in cases where the two steps of the reaction proceed with comparable velocities. At constant pH both steps should be expected to proceed as first-order reactions, and no deviations from this could be detected.

The differential equations,

$$d[B_2P]/dt = -k_2[B_2P] \qquad \text{and} \tag{2}$$

$$d[B_1P]/dt = -k_1[B_1P] + k_2[B_2P] \tag{3}$$

can be solved and give:

$$[B_2P] = [B_2P]^\circ \exp(-k_2t) \qquad \text{and} \tag{4}$$

$$[B_1P] = \{ \exp(-k_2t) - \exp(-k_1t) \}\ [B_2P]^\circ\ k_2/(k_1 - k_2) \tag{5}$$

(4) and (5) can be inserted in (1) to give an equation for ε as a function of time.

Fig. 1 shows as an example a plot of $\ln\varepsilon$ against time for an experiment in a buffer of pH = 9.8. The later part of the curve forms a straight line as it should do, when all B_2P has disappeared and only B_1P is being hydrolysed. From the equations above we deduce for the asymptote of the curve for $t \to \infty$, when $k_2 > k_1$:

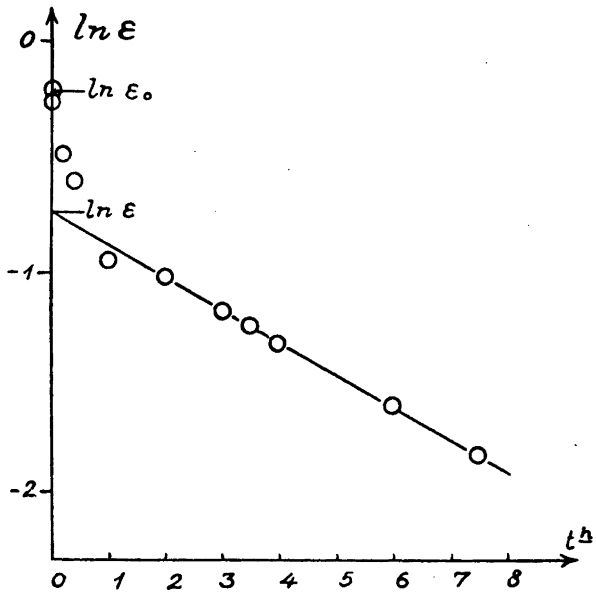


Fig. 1. Dibenzoylphosphate (B_2P) hydrolysis at pH = 9.8.

$$y = \ln \varepsilon' - k_1 t \tag{6}$$

$$\text{where } \varepsilon' = \varepsilon_0 \cdot k_2/2(k_2 - k_1) \tag{7}$$

Taking the best straight line through the last points of the curve to represent the asymptote, we find a value for ε' from its point of intersection with the ordinate-axis and a value for k_1 from its slope. k_2 can then be found either from (7) or from the slope of the tangent of the curve at $t = 0$. With a small amount of trial and error a set of values is obtained with an accuracy corresponding to that of the analytical method.

When $k_2 \ll k_1$, the ln ε curve becomes a straight line after a short while, during which the "stationary" concentration of B_1P is building up. From the slope of this line k_2 is easily inferred.

k_1 was determined by letting B_2P react for an hour with 0.1 mol glycine at pH = 9.5, which is approximately pK_2 for glycine, and subsequently following the hydrolysis of the formed monobenzoylphosphate after adjustment of the desired pH.

All pH-values are given as read on a valve potentiometer with a glass electrode standardized on Sørensen-phosphatebuffer.

Discussion of experimental results. Within the not very great experimental accuracy the results show the usual appearance of reactions which proceed both acid- and base-catalysed as well as uncatalysed, except for a few values of k_2 at pH = 7.0 and 7.4. The most probable explanation why these values are 3 or 4 times too high, is "general base catalysis" by HPO_4^{--} ions. If the "pH-stat" had been at hand at an earlier date, it would have been easy to investigate this discrepancy by using that instead of buffersolutions

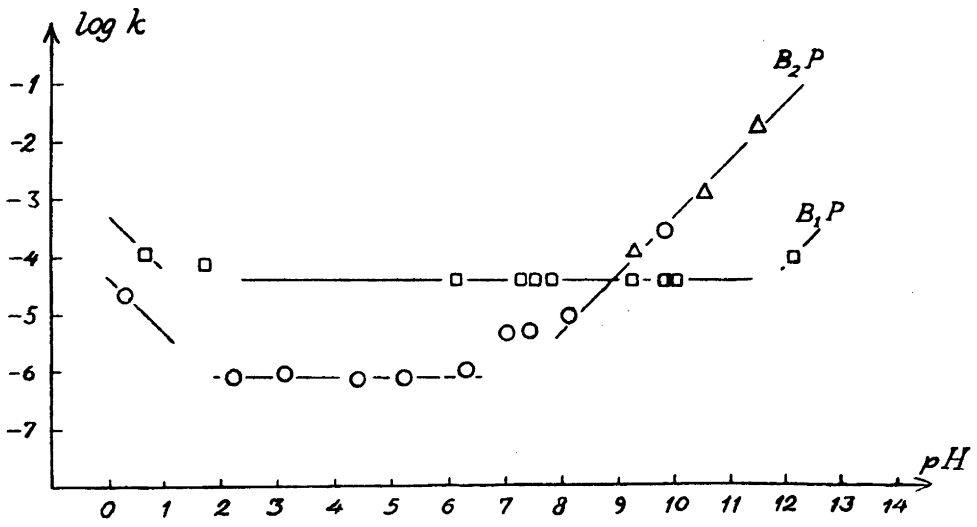


Fig. 2. Hydrolysis of benzoylphosphates.

Table 1. Determinations of k_1 (the unimolecular velocity constant of hydrolysis of monobenzoylphosphate at 37° C). All experiments are carried out with 0.01—0.02 M sodium-dibenzoylphosphate in solutions of indicated composition: ½ McI and ½ Sør. mean buffer solutions according to Mc Ilvaine (citrate-phosphate) and Sørensen (phosphate) respectively, diluted to half concentrations. Glyc. = aminoacetic acid. The pH-values are given as read on a valve potentiometer with glass-electrode, standardized on Sørensens phosphate buffer solution. Ionic strength is calculated approximately and with neglection of amfoions.

pH	Solution	Ionic strength	k_1 (sec^{-1})	log k_1
0.65	NaCl/HCl 0.1 *M* Glyc.	0.4	$1.2 \cdot 10^{-4}$	— 3.92
1.7	NaCl/HCl 0.1 *M* Glyc.	~ 0.5	$7.8 \cdot 10^{-5}$	— 4.11
6.1	½ McI 0.1 *M* Glyc.	0.2	$3.9 \cdot 10^{-5}$	— 4.41
7.3	½ Sør. 0.2 Glyc.	0.1	$4.1 \cdot 10^{-5}$	— 4.39
7.5	½ Sør. 0.03 *M* Glyc.	0.1	$4.1 \cdot 10^{-5}$	— 4.39
7.8	½ Sør. 0.1 *M* Glyc.	0.1	$4.0 \cdot 10^{-5}$	— 4.39
9.0	0.1 *M* Na_2CO_3 0.1 *M* Glyc.	0.25	$4.0 \cdot 10^{-5}$	— 4.39
9.25	NaOH from pH-stat	<0.03	$3.5 \cdot 10^{-5}$	— 4.46
9.8	0.1 *M* Na_2CO_3	0.25	$4.0 \cdot 10^{-5}$	— 4.39
10.0	0.1 *M* Glyc. + NaOH	0.05	$3.5 \cdot 10^{-5}$	— 4.46
12.1	NaOH	0.1	$9.1 \cdot 10^{-5}$	— 4.04

Table 2. Determinations of k_2 (the unimolecular velocity constant of the hydrolysis of dibenzoylphosphate at 37° C) see text of Table 1.

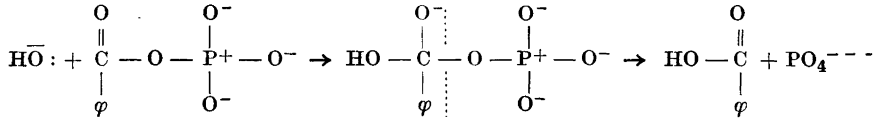
pH	Solution	Ionic strength	k_2 (sec^{-1})	log k_2
0.32	HCl	0.1	$1.9 \cdot 10^{-5}$	− 4.72
2.2	½ McI	0.02	$7.65 \cdot 10^{-7}$	− 6.12
3.1	½ McI	0.04	$9.05 \cdot 10^{-7}$	− 6.04
4.4	½ McI	0.1	$7.43 \cdot 10^{-7}$	− 6.13
5.2	½ McI	0.12	$7.49 \cdot 10^{-7}$	− 6.13
6.3	½ McI	0.2	$1.04 \cdot 10^{-6}$	− 5.98
7.0	½ McI	0.2	$4.22 \cdot 10^{-6}$	− 5.37
7.4	½ Sør	0.08	$9.5 \cdot 10^{-6}$	− 5.02
8.1	½ McI	0.3	$9.35 \cdot 10^{-6}$	− 5.03
9.25	NaOH from pH-stat	< 0.01	$1.2 \cdot 10^{-4}$	− 3.92
9.8	0.1 *M* Na_2CO_3	0.25	$2.6 \cdot 10^{-4}$	− 3.59
10.5	NaOH from pH-stat	< 0.03	$1.22 \cdot 10^{-3}$	− 2.91
11.5	NaOH from pH-stat	< 0.03	$1.75 \cdot 10^{-2}$	− 1.76

also in ranges where the burette readings were of little or no use analytically. Working with buffer solutions we met considerable difficulties in finding suitable substances. All substances with amino-groups were ruled out *a priori* and also borate, for instance, turned out to falsify the results, reacting either with B_2P or with hydroxylamine.

Some effort was invested in attempts to refine the analytical technique, but with little success. We would not deem it worth while to attempt more accurate kinetic measurements with these substances unless it is possible to find some better analytical procedure.

DISCUSSION

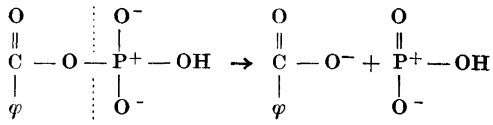
In conformity with the present views on the hydrolysis of carbonic esters, acetic anhydride etc., it is a natural assumption that reaction of the benzoylphosphates with bases takes place as displacement reaction at the carbon atom, the phosphate ion being displaced by a hydroxyl ion or another base. The mentioned tracer experiments by Bentley with acetylphosphate support this hypothesis:



(8)

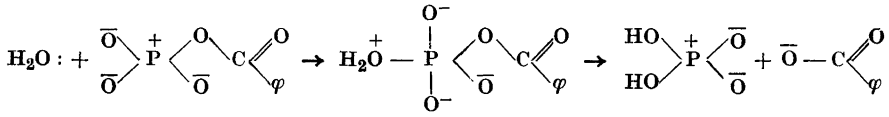
According to the tracer experiments the remarkably rapid reaction of B_1P in acid or neutral solution must proceed by some other path, however, and Bentleys results leave as the two most probable possibilities for the initial step:

a) fission of bond between O and P:



(9)

b) displacement at the P atom utilizing its fifth covalent orbital for the formation of an intermediate bond:

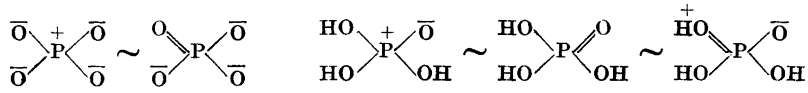


(10)

Now from this investigation we know that the reaction is virtually uncatalysed within a long range of pH-values, and it seems improbable that water-molecules should react so easily when much stronger bases as NH_3 are not known to be phosphorylised in this way.

a) then seems the more feasible, and probably it is a general feature that not too large *central atoms, surrounded by four tetrahedrically arranged oxygen atoms, are very well shielded against intruding bases.* We know for instance that sulfate and orthophosphate ions do not exchange ^{18}O in alkaline solution and also that pyrophosphate and hypophosphate ($P_2O_6^{-4}$) ions as well as ortho-carbonic esters are completely stable in alkaline solution, while the hydrolysis of such compounds is strongly catalysed by acids.

Another interesting phenomenon is the much greater reactivity of B_2P than of B_1P in reactions with bases, such as (8). Chantrenne's work [3b] has made it probable that the power to acylise glycine, ammonia, aniline *etc.* in aqueous solution is a characteristic of the disubstituted phosphoric acids, no matter what the other substituent might be. This seems to indicate that we will have to look at the engagement of the fifth covalent bond orbital of the phosphorus atom for an explanation.



I II III IV V

In the orthophosphate ion structures with pentacovalent phosphorus (II) are supposed to participate to some unknown extent, being responsible for the stability of the ortho-acid relative to the meta-acid in contradistinction to

nitric acid. In the ion the four oxygen atoms will obviously take an equal share of the fifth bond, and even in the partly or totally undissociated acid the OH groups will be able to take a considerable part of it due to the small electronegativity of hydrogen (V). When phenyl, alkyl or acyl groups are substituted for the proton this will not happen to any comparable degree, and consequently the remaining O^- and OH groups will get an increased share of the fifth bond, resulting in a relative stabilisation of the dissociated ion and hence in a stronger acid character. This may account for the fact that substituted phosphoric acids are stronger than orthophosphoric acid itself. Ethyl- and diethylphosphoric acids are reported to have first dissociation constants which are 3 and 9 times that of orthophosphoric acid.

Considering next the intermediate steps in the displacement reaction (8), we notice that it involves an analogous rupture of a bond from the acyl radical to oxygen and here again: the better chance the oxygen atom has for getting a double bond to phosphorus, the more readily will it let loose the acyl group.

It is in agreement with this hypothesis that acetyl metaphosphate is an even stronger acylating reagent than diacetylphosphate, as shown by Heyman and Rosenberg [6].

The acyl groups and the proton are "acids" in Lewis' sense or "antibases" in the language of J. Bjerrum [7] and the parallelism between the acylating potency and the normal acid strength is interesting from the point of view of generalized acid-base terminology. The extent to which such a parallelism will prove to exist, will determine the feasibility and applicability of such terminologies.

That the ability to react through fission as in (9) is reversed for B_2P and B_1P in comparison with the reactivity towards bases (8), is a natural consequence of the fact, that the P—O bonds are made stronger through the same superposed structures which loosen the O—R bond (cp. V).

Trisubstituted phosphoric esters and mixed anhydrides are known to be highly unstable in aqueous solution. According to the present hypothesis they should react by splitting one of the O—R bonds. Experiments with ^{18}O would prove or disprove this mechanism.

SUMMARY

The rates of hydrolysis of benzoylphosphate (B_1P) and of dibenzoylphosphate (B_2P) have been measured over the normal range of pH-values. The rapid hydrolysis of B_1P is shown to be virtually uncatalysed by acids and bases, while B_2P reacts slowly with water and rapidly with bases (Fig. 2).

Structural speculations lead to the possible explanation that substitution of a proton in orthophosphoric acid derivatives through the fifth covalent bond

of the P-atom influences the other bonds in such a way as to strengthen the P—O bonds and to loosen other O—R bonds.

This work was inspired mainly through discussions with Dr. Th. Rosenberg, formerly of this institute and now of *Nordisk Insulinlaboratorium.*

The Spekker photometer was generously presented to the institute by the maker, Adam Hilger Ltd. through the Royal Society of London.

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Addition of Maleic Anhydride to α-Methyl-*p*-methylstyrene and some Related Compounds. I

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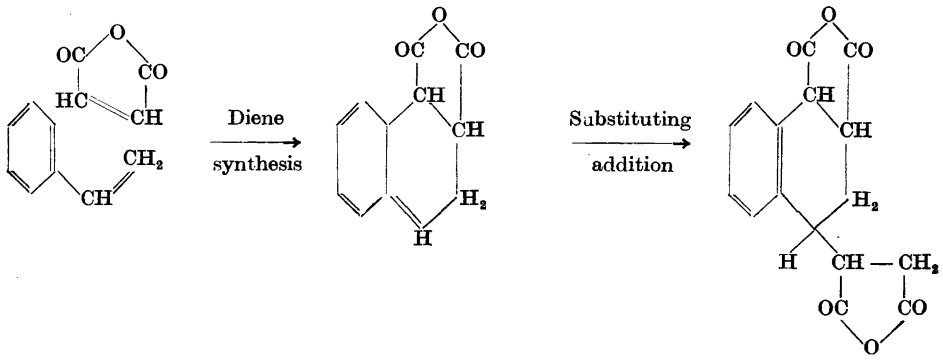
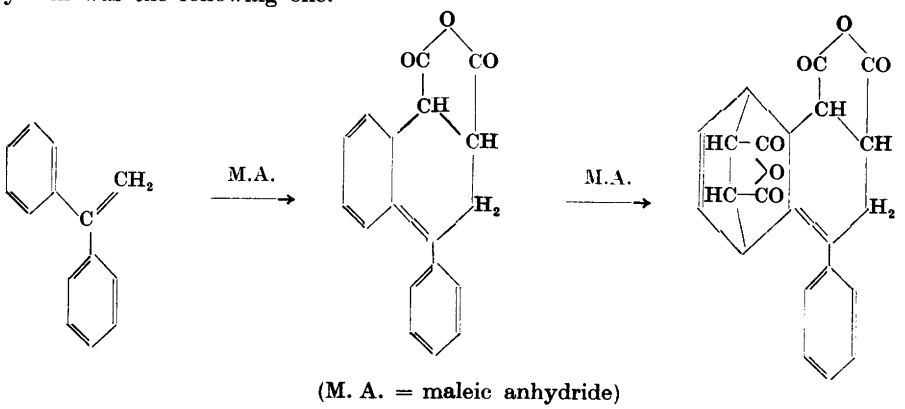
The conjugation of an aromatic double bond with an exocyclic one gives rise to a system capable of undergoing the diene synthesis. Particularly the styrene derivatives as the simplest types have been objects of investigation in this respect. However, their great tendency in general to polymerize and to form heteropolymers with maleic anhydride (or other philodienes) restricts their applicability as diene components. In the diene synthesis of this type either one or two molecules of maleic anhydride participate in the adduct formation. In the first case the primary addition product stabilizes through aromatization, in the second case one additional maleic anhydride molecule is added resulting in the formation of a stable bis-adduct. This latter case is known as the Wagner-Jauregg reaction.

On dealing with the addition of maleic anhydride to styrene and its nuclear — substituted derivatives, it can be stated that no reports about regular bis-adducts are to be seen in the literature except a preliminary one given by Alder and Schmitz-Josten [1]. According to these authors, by means of some unrevealed inhibitors the tendency of styrene to polymerization and heteropolymerization could be so far suppressed that a simple bis-adduct resulted. The following reaction mechanism was suggested:



1,1-Diphenylethylene seems to be the favoured α-substituted styrene derivative to undergo the bis-adduct formation with maleic anhydride. Wagner-Jauregg [2], the discoverer of this reaction type, obtained this bis-adduct in good yield by simple heating the components together or in benzene solution. The reaction mechanism at first proposed by him was the following one:



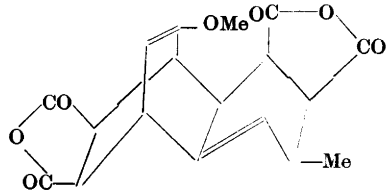
(M. A. = maleic anhydride)

In spite of numerous observations in favour of the above structure of the bis-adduct he was not able to prove it decisively. Later he himself distrusted it and was inclined to accept a concept of substituting addition.

Bergmann *et al.*[3] prepared several new bis-adducts by condensing nuclear-substituted 1,1-diphenylethylenes with maleic anhydride. These authors accepted the above reaction mechanism without giving any conclusive arguments in its favour.

Other reports of α-substituted styrenes undergoing the bis-adduct formation with maleic anhydride are not to be seen in the literature.

Among the β-substituted styrene derivatives *p*-methoxypropenylbenzene (anethole) and *o*-methoxypropenylbenzene are the only ones that have been reported to form bis-adducts with maleic anhydride. By using dimethylaniline as polymerization inhibitor in the condensation of anethole with maleic anhydride Bruckner and Kovács [4,5] could repress the formation of heteropolymers and obtained a crystalline bis-adduct (m. p. 241°) with a 61 per cent yield. The following formula was given by them to this bis-adduct:



If the spatial arrangement is omitted, this formula is completely analogous to that proposed by Wagner-Jauregg to the corresponding 1,1-diphenylethylene adduct. In order to prove the existence and position of the double bond belonging to the bicyclo-octene ring in the above formula, the enol ether character of the methoxyl group was

made use of. The other double bond was demonstrated by hydrogen peroxide addition to a hydroxy lactone tricarboxylic acid obtained from the bis-adduct by saponification and lactonization resulting in the disappearance of the first double bond. The experimental observations made by Bruckner and Kovács may agree with the above structure, but that the bis-adduct in question must have just this structure, is, on the basis of the arguments put forward by these authors, rather difficult to become convinced of.

By using hydroquinone as inhibiting agent towards polymerization, Lora Tamayo [6] obtained a crystalline anethole — maleic anhydride bis-adduct (m. p. 232°), which he thaught to be identical with the Bruckner product. At first he accepted the structure proposed by Bruckner and Kovács. Because the ultra-violet absorption spectrum of this adduct proved to be of the same type as that of tetralin, Lora Tamayo later [7] took into account another possibility, a substituting addition of the second molecule of maleic anhydride to the intermediate. Accordingly the bis-adduct would have a tetrahydronaphthalene structure analogous to that suggested by Alder and Schmitz-Josten [1] for the corresponding styrene adduct.

A maleic anhydride bis-adduct of *o*-methoxypropenylbenzene is also reported by Lora Tamayo [8]. He suggested a tetrahydronaphthalene structure for this adduct too.

As a summary it can be stated, that two divergent opinions exist upon the mechanism of the Wagner-Jauregg reaction. All authors agree in the addition of the first molecule of maleic anhydride. The stabilization of the intermediate is thaught to proceed either through a second 1,4-addition of maleic anhydride resulting in the formation of a bicyclooctene ring (Bruckner and Kovács [4,5]) or through a substituting addition (Alder and Schmitz-Josten [1]). Both possibilities are put forward by Wagner-Jauregg [2] and Lora Tamayo [6,7]). The main argument in favour of the latter reaction mechanism has been the "saturated character" of the bis-adducts, a fact pointed out by almost all authors. The great tendency to resist catalytic hydrogenation and the negative double bond test with different reagents have been common features of these compounds. If both reaction mechanisms are possible by starting with the same styrene derivative, cannot be said at this time. So far only one bis-adduct of each styrene has been reported.

The purpose of the present investigation is:

1) to study, if α-substituted styrenes other than 1,1-diphenylethylene would react with maleic anhydride according to the Wagner-Jauregg reaction.

2) to elucidate the mechanism of the Wagner-Jauregg reaction by establishing the structure of one bis-adduct, if succeeded to obtain.

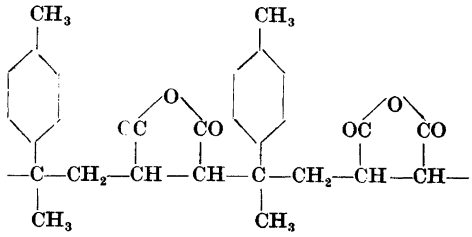
Most of the experiments for preparing bis-adducts were made with α-methyl-*p*-methylstyrene, a few ones with α-methylstyrene. For comparison some experiments were carried out with styrene.

I. Addition of maleic anhydride to α-methyl-*p*-methylstyrene

When α-methyl-*p*-methylstyrene was allowed to act on maleic anhydride, the reaction could take three different courses depending on the conditions:

1. *Heteropolymerization*

By warming the reactants together in a molecular ratio of 1 : 1 without solvents and inhibitors a heteropolymerization took place resulting in the formation of a clear, almost colorless, brittle resin with a fusion range of 105—120°. If more than one equivalent of maleic anhydride was used, the excess remained unconverted. The structure of the heteropolymer may be written, in analogy with that suggested by Hersberger *et al* [9]. for the corresponding heteropolymer of α-methylstyrene, as follows:



No more research was made within this field.

2. *Bis-adduct formation of α-methyl-p-methylstyrene with maleic anhydride*

In order to prevent the dimerization of α-methyl-*p*-methylstyrene and its heteropolymerization with maleic anhydride some inhibitor must be used. Dimethylaniline, the inhibitor used successfully by Bruckner and Kovács [4] in the case of anethole, was first experimented with. It proved to be effective in this case too. On the contrary hydroquinone, which according to Lora Tamayo [7] was usefull in respect of anethole, failed to prevent the dimerization of α-methyl-*p*-methylstyrene. Benzene and acetic anhydride were employed as solvents, the latter with better result. Besides, by starting with dimethyl-*p*-tolylcarbinol acetic anhydride acted also as a dehydration agent. The optimum temperature was 80°. The yield of the adduct was 40 per cent irrespective if α-methyl-*p*-methylstyrene or dimethyl-*p*-tolylcarbinol was used as starting material. The yield reported comprised only that part of the adduct that crystallized directly from the reaction mixture on cooling (cf. Bruckner and Kovács [4]).

Structure of the adduct: The adduct was obtained in the form of coarse, colorless prisms, m.p. 255—256° (dec.). When dissolved in glacial acetic acid the adduct did not absorb bromine, whereas its alkaline solution decolorized readily potassium permanganate. On the basis of analysis the compound

must be a maleic anhydride bis-adduct of monomeric α-methyl-p-methylstyrene. A structural formula (III) analogous to that suggested by Wagner-Jauregg [2] for the corresponding bis-adduct of 1,1-diphenylethylene, was taken as working hypothesis.

For establishing the structure of the bis-adduct it was first aromatized by heating with sulfur according to Bergmann et al. [3]. The molecular formula of the yellow reaction product obtained in a yield of 66 per cent was proved by analysis to correspond that of a dimethylnaphthalene dicarboxylic acid anhydride. It was converted into the free dicarboxylic acid, the barium salt of which was decarboxylated. An oil resulted, which was identified as 1,6-dimethylnaphthalene (XIII). Because the expected aromatization product of the adduct, 1,6-dimethylnaphthalene-3,4-dicarboxylic acid anhydride (XI) is not reported in the literature, the position of the anhydride grouping had to be determined. It is done later.

For locating the double bonds (or one of them) ozonization as one of the most reliable methods in this respect was made use of. The amount of ozone absorbed by the adduct in ethyl acetate solution corresponded to one double bond in the molecule. Plenty of time was consumed with efforts to isolate the ozonide decomposition products (or product) obtained by various methods. A resinous matter always resulted, from which no single compounds could be separated, not even in the form of derivatives by means of carbonyl reagents.

The bis-adduct was now converted into the corresponding tetracarboxylic acid and the same ozonization experiments were repeated with this compound but unfortunately with the same result.

The next effort was made with the tetramethylester obtained from the above acid by diazomethane. The consumption of ozone corresponded exactly to one double bond. On decomposition of the ozonide by means of catalytic dehydrogenation, the hydrogen consumption was 80 per cent of that calculated for a mono-ozonide. A crystalline decomposition product resulted in high yield.

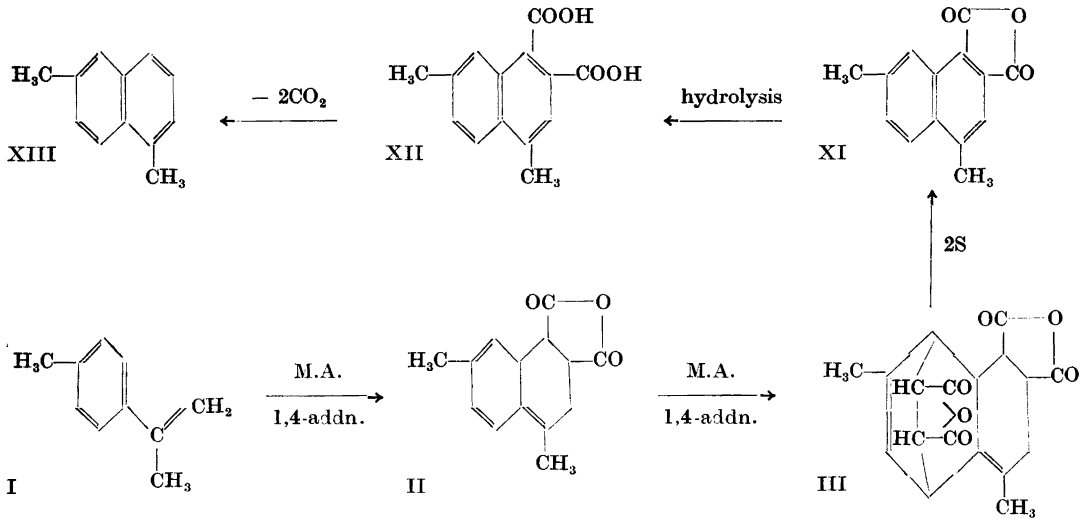
The molecular formula of the decomposition product corresponded to that of the tetramethylester added by two oxygen atoms. It formed dicarbonyl-derivatives with hydroxylamine and semicarbazide. The Schiff test for aldehydes was negative. Because, in addition, the same compound was obtained in another ozonization experiment, in which the decomposition of the ozonide was carried out by boiling with a dilute hydrogen peroxide solution, it must be a diketone. Assuming the tentative structure (III) of the bis-adduct to be correct, only one of the two double bonds would have been attacked by ozone resulting in the formation of the diketone (VI).

When the diketone obtained by ozonization was heated in a vacuum distillation apparatus at 220—230° (bath temperature), it decomposed smoothly and all except a little residue distilled slowly over. The distillate proved to be a mixture, from which two compounds were isolated. One of them, an oil, was identified as dimethylmaleate (yield 73 per cent). The other was a crystalline solid, for which the molecular formula $C_{15}H_{16}O_5$ was obtained by analysis (yield 36 per cent). If dimethylmaleate ($C_6H_8O_4$) is thaught to be cleaved off from the diketone ($C_{22}H_{28}O_{10}$), a residue of $C_{16}H_{20}O_6$ remains. This means that a compound with the formula CH_4O, *e.g.* methanol, would have been escaped during the decomposition. When in another experiment the vacuum distillation apparatus was provided with a trap cooled in liquid air, methanol really was caught in a yield of 74 per cent.

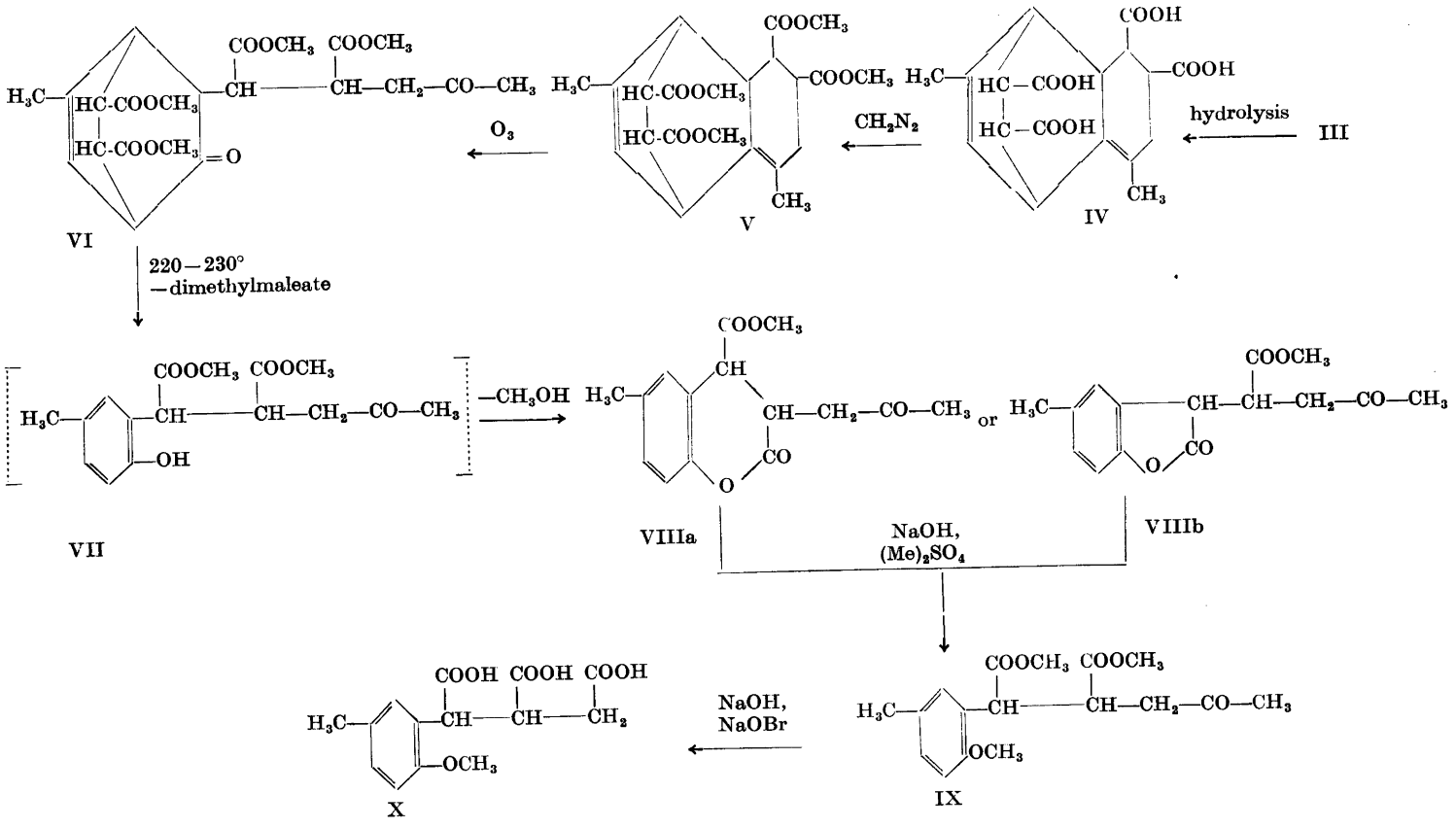
Considering the tentative structure (VI) of the diketone, the decomposition may be thaught to proceed as presented in the reaction chart. The cleavage of methanol from the intermediate (VII) resulting in the formation of a dihydrocoumarin derivative (VIIIa) has an analogy in the behaviour of ethyl 2-hydroxyphenylpropionate reported by Pschorr and Einbeck [10].

That the decomposition product ($C_{15}H_{16}O_5$) must be one of the two products (VIIIa and VIIIb) is proved by the following consideration:

1) It dissolved quickly in dilute alkali by warming on a water bath. By methylating in this solvent with dimethyl sulphate an oil ($C_{17}H_{22}O_6$) was



(M. A. = maleic anhydride)


COOH
COOH
COOH
hydrolysis
III
IV
CH3
CH2N2
COOCH3
COOCH3
COOCH3
V
CH3
O3
VI
220–230°
–dimethylmaleate
COOCH3 COOCH3
–CH3OH
VII
COOCH3
VIIIa
or
COOCH3
VIIIb
NaOH,
(Me)2SO4
COOCH3 COOCH3
IX
NaOH,
NaOBr
COOH COOH COOH
X

obtained, which was proved to be a mixture of two isomers * of dimethyl α-(2-methoxy-5-methylphenyl)-β-acetonylsuccinate (IX) by mixed melting point of its semicarbazones (two isomers) with the corresponding ones of a synthetic sample. In addition, when the oil was saponified and the resulting acid treated with sodium hypobromite, carbon tetrabromide and an acid ($C_{14}H_{16}O_7$) were obtained. The latter was identified by synthesis as α-(2-methoxy-5-methylphenyl)-tricarballylic acid (X).

2) When the decomposition product was hydrolyzed with a solution of potassium hydroxide in methanol, the solution acidified with concd. hydrochloric acid and the resulting free acid methylated by means of diazomethane, the starting material was recovered.

3) No free phenolic hydroxyl group could be present in the decomposition product, because its methanol solution did not decolorize an ethereal solution of diazomethane.

Which of the above two possibilities (VIIIa and VIIIb) is the correct one, is still uncertain, but the primary purpose of this work is not suffered by it.

Because the decomposition product contained only one carbonyl group (it formed a mono-semicarbazone), one of the two carbonyl groups of the diketone had disappeared during the heat treatment. It is clear that the carbonyl-oxygen of this latter had converted into a phenolic one with subsequent lactonization of the phenolic hydroxyl group. The aromatization of the diketone by mere cleavage of dimethyl maleate without any dehydrogenation is fully understood in this way.

By the formation of the above heat decomposition product from the diketone, the position of the dicarboxylic acid anhydride grouping in the sulfur dehydrogenation product of the bis-adduct is fixed too. Accordingly the sulfur dehydrogenation product must be 1,6-dimethylnaphthalene-3,4-dicarboxylic acid anhydride (XI) as expected. In addition, it must be the same maleic anhydride (or ester) molecule that has cleaved off in these two cases.

If the possible structure of the diketone is discussed in the light of the results obtained above, some conclusions may be drawn:

1) At least one ring must be present in the diketone molecule. If none, there would be owing to the molecular formula ($C_{22}H_{28}O_{10}$) three double bonds in an aliphatic compound. This is, however, impossible in reference to the saturated character of the diketone towards the common double-bond reagents.

2) The ring (or rings) must be carbocyclic, because the functions of all the oxygen atoms are strictly determined.

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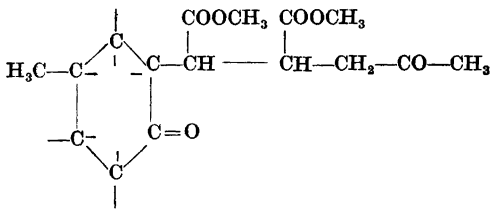
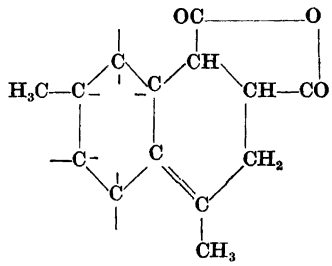
* The existence of two isomers with a structural formula IX has to be ascribed to the presence of two dissimilar asymmetric carbon atoms in the molecule.

3) The ring (or one of them) must be a six-carbon ring on the basis of the following consideration: If in the compound (IX) the methoxyl group later introduced is thaught to be hydrolyzed, a phenol results with the formula (VII). In this formula one molecule of maleic anhydride (as esterified) and the fragments of α-methyl-p-methylstyrene are still clearly to be seen. Because it is impossible to assume that the six-carbon ring of α-methyl-p-methylstyrene would have been ruptured during the maleic anhydride addition and reformed on the degradation process, this six-carbon ring skeleton must be present in the diketone.

4) By omitting the grouping that cleaves off as dimethyl maleate during the heat decomposition, the diketone must contain the following carbon skeleton (XIV):



XIV



XV

How the omitted grouping is being thaught to attach to the above skeleton (XIV), is still uncertain. If the diketone is monocyclic, the ring must contain two double bonds and a lateral chain consisting of the grouping $H_3COOC\text{-}CH\text{-}CH_2\text{-}COOCH_3$. Another possibility, which is to be preferred, is a bicyclo-octene structure represented by the formula VI.

Considering that the diketone has been obtained from the bis-adduct through reactions, the course of which is strictly determined, the above conclusions can be directly made use of in deducing the structure of the bis-adduct. Accordingly the bis-adduct must contain the skeletal structure XV. If this ring system is compared with the working hypothesis (formula III), it can be seen that the latter really is quite possible. The only thing impossible to explain at this stage is, why the other double bond in the formula III has not been attacked by ozone.

The research is being continued for establishing the definite structure of the bis-adduct.

3. *Dimerization of α-methyl-p-methylstyrene followed by bis-adduct formation of the resulting dimer with maleic anhydride*

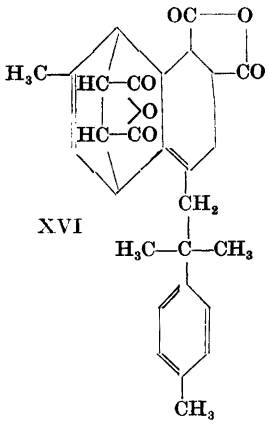
If the mixture of α-methyl-*p*-methylstyrene and maleic anhydride in benzene solution was heated at 80°, large crystals slowly separated at the bottom of the reaction flask with simultaneous thickening of the liquid. It appeared that two processes, *viz.* adduct formation and heteropolymerization were going side by side. The molecular formula ($C_{28}H_{28}O_6$) found by analysis for the crystalline product corresponds to that calculated for a maleic anhydride bis-adduct of a dimeric α-methyl-*p*-methylstyrene. That this was really the case, was confirmed by reacting the unsaturated dimer of α-methyl-*p*-methylstyrene reported in a previous communication[11] with maleic anhydride. The same product was obtained, and this time in good yield (67 per cent). No corresponding report is to be seen in the literature. The same adduct could be prepared also from dimethyl-*p*-tolylcarbinol and maleic anhydride in the presence of formic acid (yield 70 per cent).

On the basis of the above observations it is quite clear that α-methyl-*p*-methylstyrene is at first dimerized owing to the action of maleic anhydride (or maleic acid) probably in the same way as by formic acid [11]. The dimer subsequently reacts with maleic anhydride according to the Wagner-Jauregg reaction.

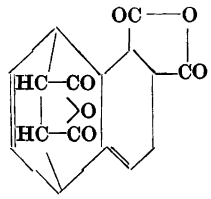
On considering the possible structure of the reaction product it must be taken into account, that the unsaturated dimer of α-methyl-*p*-methylstyrene used above is a mixture of two isomers, *viz.* 2,4-di-(*p*-tolyl)-4-methyl-pentene -1 and 2,4-di-(*p*-tolyl)-4-methyl-pentene-2 in a ratio of 5:1. A question naturally arises, which of these two isomers participates in the adduct formation. It is hardly probable that any marked change would occur in the ratio of both isomers during the reaction with maleic anhydride, since the mixture of the isomers was obtained by means of formic acid under more drastic conditions. Thus considering the high yield of the adduct, the chief isomer component, 2,4-di-(*p*-tolyl)-4-methyl-pentene-1 must be the diene constituent of the bis-adduct. If the other isomer is also able to form a bis-adduct with maleic anhydride, is not known. Only one crystalline product could be isolated from the reaction mixture. As it may be seen, the above case is one additional example of α-substituted styrenes undergoing the Wagner-Jauregg reaction.

In order to cast some light upon the structure of the bis-adduct, it was ozonized in ethyl acetate solution. The amount of ozone absorbed and similarly that of hydrogen required for the catalytic hydrogenation of the ozonide both corresponded to one double bond. It was, however, impossible to isolate any crystalline decomposition product of the ozonide.

In addition, the bis-adduct was converted into the corresponding tetracarboxylic acid ($C_{28}H_{32}O_8$) and the latter into its tetramethyl ester ($C_{32}H_{40}O_8$) A great similarity in behaviour was to be seen between these derivatives and the corresponding ones of the maleic anhydride bis-adduct of the monomeric α-methyl-p-methylstyrene. So it exists some reason to suggest for the bis-adduct of the dimer the following structural formula (XVI) in analogy with that proposed for the corresponding adduct of the monomer (III):



XVI



XVII

II. Addition of maleic anhydride to α-methylstyrene

By reacting α-methylstyrene with maleic anhydride in the presence of dimethylaniline under the same reaction conditions as in the case of α-methyl-p-methylstyrene, no bis-adduct could be isolated from the reaction mixture. This remarkable difference in these two homologous styrenes obviously has to be ascribed to the effect of p-substitution. This hypothesis is supported by the observation made by Hudson and Robinson [12] that in certain styrene derivatives an alkoxyl group in the p-position to the unsaturated side chain enhances the tendency to undergo a diene synthesis with maleic anhydride.

III. Addition of maleic anhydride to styrene

Some experiments were carried out in order to see, if it would be possible to prepare a regular maleic anhydride bis-adduct of styrene by the method used for obtaining that of α-methyl-p-methylstyrene. It turned out, however, that the Wagner-Jauregg reaction of styrene does not proceed as readily as that of α-methyl-p-methylstyrene. A black tar always resulted accompanied

by unchanged styrene and maleic anhydride. Only minute amounts (about 1 per cent) of a highly crystalline compound could be separated from the tar. Its molecular formula was stated by analysis to be $C_{16}H_{12}O_6$, so it must be the product pursued. It seems out, that a higher temperature is required for this reaction, but any increase in temperature is followed also by a greater tendency to polymerization.

In order to elucidate the structure of the above bis-adduct, so far only an aromatization with sulfur was carried out. It gave a light yellow compound, which was identified as naphthalene-1,2-dicarboxylic acid anhydride.

As it may be seen, the behaviour of this bis-adduct on heating with sulfur agrees with that of the corresponding adduct of α-methyl-*p*-methylstyrene. In addition, because these two compounds are formed under the same reaction conditions, it is quite possible that they have analogous structures. Accordingly the structural formula XVII is suggested for the maleic anhydride bis-adduct of styrene. If the bis-adduct obtained is identic with that reported by Alder and Schmitz-Josten [1], is still in doubt, because no constants of the latter are to be seen. The structure proposed by these authors for their product is represented in the beginning of the present paper.

EXPERIMENTAL

Heteropolymerization of α-methyl-p-methylstyrene with maleic anhydride

A mixture of 14.5 g (0.11 mole) of α-methyl-*p*-methylstyrene (b. p. 189—191°) and 10.8 g. (0.11 mole) of maleic anhydride was warmed on a water bath. At about 70° a vigorous reaction occurred the temperature rising to the boiling point of the liquid. After cooling, the reaction product was a clear, brittle, almost colorless resin with a fusion range of 105—120°.

Maleic anhydride bis-adduct of the monomeric α-methyl-p-methylstyrene

1) From α-methyl-*p*-methylstyrene and maleic anhydride:

A mixture of 5.0 g (0.038 mole) of α-methyl-p-methylstyrene, 7.4 g (0.076 mole) of maleic anhydride and 0.5 g of dimethylaniline dissolved in 10 ml of acetic anhydride was kept at 80° for 24 hours. Great, colorless crystals separated from the dark liquid already in the hot. After cooling, the crystalline product was separated and washed with cold acetic anhydride. The yield was 5.0 g (40 per cent), m. p. 252—254°. After recrystallization from 16 ml of acetic anhydride, 4.3 g of colorless prisms, m. p. 255—256° (dec.), were obtained. If the temperature was raised very slowly, a melting point of 250—251° (dec.) was recorded. The product was moderately soluble in boiling ethyl acetate, well in boiling acetic anhydride but only slightly in other usual solvents. From xylene it crystallized as small, white needles.

$C_{18}H_{16}O_6$	Calc.	C	65.9	H	4.9	Mol.wt.	328
	Found	»	65.7	»	4.9	» »	338

2) From dimethyl-*p*-tolylcarbinol and maleic anhydride:

A mixture of dimethyl-*p*-tolylcarbinol and *p*-methylacetophenone obtained by catalytic oxidation of cymene with air in liquid phase [11] was used as starting material. It contained 62 per cent of dimethyl-*p*-tolylcarbinol.

8.1 g of the above mixture (0.033 mole of dimethyl-*p*-tolylcarbinol), 9.7 g (0.099 mole) of maleic anhydride, 0.5 g of dimethylaniline and 15 ml of acetic anhydride were mixed and heated at 80° for 24 hours. After cooling, 4.4 g (40 per cent) of a crystalline compound, m. p. 252—3° were separated from the reaction mixture. It was proved to be identic with the previous product by mixed melting point.

Aromatization of the bis-adduct $C_{18}H_{16}O_6$

An intimate mixture of 5.0 g of the bis-adduct and 0.98 g (2 equiv.) of sulfur was heated at 220—30° for 1 ½ hours, after which the temperature was raised to 270—80° for 5 minutes. The black reaction product was extracted with boiling ethyl acetate and the solution concentrated. On cooling, black crystals separated, yield 2.76 g, m. p. 228—231°. By subliming in vacuo at 210° (bath temperature) 2.28 g (66 per cent) of yellow needles, m. p. 235.5—6.5° were obtained.

$C_{14}H_{10}O_3$	Calc.	C	74.2	H	4.4
	Found	»	74.4	»	4.5

Hydrolysis of the aromatization product $C_{14}H_{10}O_3$

2.0 g of the yellow product obtained above were converted into the free acid by heating on a water bath with a solution of 1.4 g of sodium hydroxide in 25 ml of water until all had dissolved. After cooling, the solution was acidified with concd. hydrochloric acid. A microcrystalline, white compound precipitated, yield 2.1 g (97 per cent), m. p. 236—7°. On crystallization from methanol, coarse, light yellow, transparent crystals were obtained, the melting point being unaltered.

$C_{14}H_{12}O_4$	Calc.	C	68.7	H	4.9	Neut.equiv.	122
	Found	»	68.8	»	5.0	» »	125

Decarboxylation of the acid $C_{14}H_{12}O_4$

1.8 g of the acid were dissolved in 28 ml of 5 % sodium bicarbonate solution. By adding 2 g of barium chloride in 8 ml of water, the acid was precipitated as the barium salt, which was separated, washed with water and dried at 100°. It was mixed with half its weight of copper bronze and ground thoroughly. By heating the mixture on a sand bath in vacuo, 440 mg of a red oil distilled slowly over. It gave on fractionation in vacuo 240 mg (21 per cent) of an almost colorless oil, b. p. 135—8°/15 mm, the odor of which resembled that of naphthalene. The oil was proved to be 1,6-dimethylnaphthalene by mixed melting point of its picrate and trinitrobenzolate with authentic specimens *.

* I am indebted to Dr. J. Gripenberg for these samples.

Hydrolysis of the bis-adduct $C_{18}H_{16}O_6$.

30.0 g of the bis-adduct were dissolved in a solution of 34 g of potassium hydroxide in 300 ml of water by heating on a water bath. After cooling, the solution was acidified by slowly adding 84 ml of cold, concd. hydrochloric acid with stirring and cooling. The solution remained clear during this operation but on standing colorless crystals soon begin to separate. After standing overnight, the crystals were filtrated off, washed with water and dried. The yield was 33 g (theoretical), m. p. about 200° (dec.). The acid crystallized from glacial acetic acid in colorless microcrystals, m. p. 210—15° (dec.) after sintering already at 200°.

$C_{18}H_{20}O_8$	Calc.	C	59.3	H	5.5	Neut.equiv.		91
	Found	»	59.7	»	5.6	»	»	91

The acid could not be titrated directly with 0.1 N sodium hydroxide. The neut.equiv. value reported above was obtained by a lactone titration.

If the alkaline solution was acidified with concd. hydrochloric acid in the hot and the solution concentrated on a water bath, another acid was obtained. Once crystallized from glacial acetic acid it formed colorless microcrystals, m. p. 307—8° (dec.).

$C_{18}H_{18}O_7$	Calc.	C	62.4	H	5.2
	Found	»	62.4	»	5.3

Accordingly it must be an anhydride-acid formed from the tetracarboxylic acid by the elimination of one molecule of water.

Methylation of the tetracarboxylic acid $C_{18}H_{20}O_8$ by diazomethane

30.0 g of the tetracarboxylic acid prepared above were dissolved in 120 ml of methanol and methylated with an ethereal solution of diazomethane by cooling. After concentration on a water bath, coarse, colorless crystals separated. The yield was 34.0 g (98 per cent), m. p. 120—2°. Once recrystallized from methanol, the compound formed large prisms, m. p. 121—3°.

$C_{22}H_{28}O_8$	Calc.	C	62.7	H	6.6
	Found	»	62.3	»	6.4

Ozonization of the bis-adduct $C_{18}H_{16}O_6$

5.0 g of the bis-adduct were dissolved in 250 ml of ethyl acetate. Ozonized oxygen containing 4.9 vol. % of O_3 was passed through this solution with cooling. The amount of ozone absorbed corresponded to one double bond. The solvent was evaporated at room temperature in vacuo. The residue was a porous, light yellow resin. On decomposition of the ozonide no single products could be isolated.

Ozonization of the tetracarboxylic acid $C_{18}H_{20}O_8$

5.0 g of the tetracarboxylic acid were dissolved in 100 ml of glacial acetic acid and ozonized as above. The behaviour of the free acid on ozonization was similar to that of the anhydride (bis-adduct).

Ozonization of the tetramethyl ester $C_{22}H_{28}O_8$

Ozonized oxygen containing 4.9 vol. % of ozone was passed through a solution of 10.0 g of the tetramethyl ester in 100 ml of chloroform by cooling in an ice-salt mixture. A wash bottle charged with potassium iodide solution was connected to the reaction bottle. The completion of the ozonization was sharply indicated by a sudden separation of iodine. At this moment an amount of ozone equivalent to one double bond had been absorbed. The solvent was evaporated at room temperature in vacuo. The residue was a porous, white resin.

The ozonide was dissolved in 100 ml of methanol and decomposed by catalytic hydrogenation with Adams' platinum oxide catalyst. In the beginning, the hydrogenation vessel was cooled with cold water. After 18 hours, the amount of hydrogen consumed was 80 per cent of that calculated for a mono-ozonide. Plenty of colorless crystals had separated during the hydrogenation. The crystals were dissolved by warming, the platinum catalyst was filtrated off and the filtrate concentrated on a water bath. On cooling, coarse crystals separated. The yield was 7.53 g (70 per cent), m. p. 151—4°. After recrystallization from 20 ml of methanol, 6.5 g of large, clear prisms were recovered, m. p. 153—4°. The compound gave a negative Schiff test for aldehydes.

$C_{22}H_{28}O_{10}$	Calc.	C	58.3	H	6.2
	Found	»	58.2	»	6.3

In another experiment, where the decomposition of the ozonide was carried out by boiling with dilute hydrogen peroxide solution, the same compound was obtained in a yield of 24 per cent.

Semicarbazone: Colorless crystals from 50 % ethanol, m. p. 230—1° (dec.).

$C_{24}H_{34}N_6O_{10}$	Calc.	N	14.8
	Found	»	14.5

Oxime: Small plates from ethanol, m. p. 211—3° (dec.).

$C_{22}H_{30}N_2O_{10}$	Calc.	N	5.8
	Found	»	5.5

Decomposition of the ozonization product $C_{22}H_{28}O_{10}$ by heating

3.45 g of the ozonization product were placed in a Claisen flask and heated in a metal bath in vacuo. A trap cooled in liquid air for catching the readily volatile products was inserted between the receiver and the water pump. At 220—30° (bath temperature) a pale yellow, towards the end thickening oil distilled slowly over. A residue of only 0.14 g remained in the Claisen flask. The trap contained 0.18 g of a colorless, mobile liquid (solid at the temperature of liquid air), b. p. 65—70°. It was identified as methanol (yield 74 per cent) by mixed melting point of its 3,5-dinitrobenzoate with an authentic sample.

The viscous distillate (3.05 g) was fractionated in vacuo:

Fraction I, b.p. 60—3°/1mm, 0.805 g, a mobile, colorless oil.
» II, » 180—6°/1 » 1.715 » » viscous, yellowish oil.

When Fraction I was refluxed for 15 minutes with a small crystal of iodine it was almost quantitatively converted into a solid compound, which formed large plates from ether, m. p. 101—2°.

$C_6H_8O_4$	Calc.	C	50.0	H	5.6
	Found	»	50.2	»	5.7

This compound was identified as dimethyl fumarate by mixed melting point. In accordance, the oily compound (Fraction I) must be dimethyl maleate (yield 73 per cent).

Fraction II deposited on standing 0.76 g of colorless crystals, m. p. 94—108°. This substance turned out to be a mixture of two components with the same empirical formula. The higher-melting compound only could be isolated pure by fractional crystallization from methanol. It was obtained as small plates, m. p. 114—5°, which did not decolorize an ethereal solution of diazomethane.

$C_{15}H_{16}O_5$	Calc.	C	65.0	H	5.8
	Found	»	65.1	»	5.8

Semicarbazone: Clusters of small needles from ethanol, m. p. 197—8° (dec.).

$C_{16}H_{19}N_3O_5$	Calc.	N	12.6
	Found	»	12.8

Saponification of the decomposition product $C_{15}H_{16}O_5$

1.7 g of the crude decomposition product (m. p. 94—108°) were dissolved in 3 ml of methanol. A solution of 2.5 g of potassium hydroxide in 12 ml of water was added and the mixture refluxed for 6 hours. Methanol was evaporated and the residual solution acidified with 4 ml of concd. hydrochloric acid. The solution remained quite clear. It was extracted with ether. After evaporation of the solvent, a porous resin (1.7 g) was obtained, which could not be crystallized. It was very soluble in water.

Methylation of the saponification product with diazomethane

The resin (1.7 g) obtained above was dissolved in 15 ml of abs. ether and methylated with an ethereal solution of diazomethane. The solvent was evaporated and the residue triturated with methanol. Colorless crystals soon separated, yield 0.56 g, m. p. 92—114°. On fractional crystallization from methanol, 0.38 g of a compound, m. p. 114—5° were obtained. By mixed melting point it was proved to be identic with the higher-melting component of the starting material $C_{15}H_{16}O_5$.

In this connexion it may be mentioned that the uncrystallizable part (mother liquor) of the decomposition product, when saponified and methylated as above, yielded an appreciable amount of the isomer mixture $C_{15}H_{16}O_5$ in addition to the part that crystallized directly.

Methylation of the decomposition product $C_{15}H_{16}O_5$ by dimethyl sulfate in alkaline solution

0.8 g of the crude decomposition product (m. p. 94—108°) were heated with a solution of 0.5 g of sodium hydroxide in 2 ml of water on a water bath until all had dissolved (about 10 minutes). The methylation was carried out in the usual way with dimethyl

sulfate. A colorless, viscous oil, b. p. 182—192°/3 mm, was obtained, yield 0.7 g (75 per cent).

$C_{17}H_{22}O_6$	Calc.	C	63.2	H	6.8
	Found	»	63.0	»	6.8

Semicarbazones: A semicarbazone of the above oil was prepared in the customary manner. It proved to be a mixture that by fractional crystallization from ethanol could be divided into two isomers I and II, the latter being more readily soluble.

Isomer I, small plates, m. p. 183—5°.

$C_{18}H_{25}N_3O_6$	Calc.	N	11.1
	Found	»	11.2

Isomer II, microcrystalline, m. p. 157—160°.

$C_{18}H_{25}N_3O_6$	Calc.	N	11.1
	Found	»	11.0

These two semicarbazones gave no depression when mixed with the corresponding semicarbazones of dimethyl α-(2-methoxy-5-methylphenyl)-β-acetonyl-succinate prepared by synthesis (see later).

Hypobromite oxidation of the methylation product $C_{17}H_{22}O_6$

0.65 g of the oil (b. p. 182—92°/3 mm) obtained above were at first saponified by heating for 5 hours on a water bath with a solution of 0.44 g of sodium hydroxide in 4 ml of water. The resulted clear solution was cooled to room temperature. A cold solution of sodium hypobromite prepared at 0° by dissolving 1.5 g of sodium hydroxide and 2.0 g of bromine in 8.5 ml of water, was added during 10 minutes with stirring. The temperature rose hereby to about 35°. After the addition, the stirring was continued for 15 minutes. A clear, almost colorless solution resulted with some heavy particles at the bottom of the reaction vessel. The solid compound was filtrated off, yield 0.19 g, m. p. 86—9°. It was identified as carbon tetrabromide.

A few drops of acetone were added to the filtrate for destroying the excess of hypobromite. After removing all neutral matter by ether extraction, the alkaline solution was acidified with concd. hydrochloric acid and extracted with ether. After evaporation of the solvent, a brownish syrup remained. When it was triturated with cold ether, small crystals slowly separated. They were filtrated off and washed with ether. The yield was 74 mg, m. p. 157—60°. After recrystallization from a mixture of benzene and acetone, microscopical needles were obtained, m. p. 165—7°.

$C_{14}H_{16}O_7$	Calc.	C	56.8	H	5.4
	Found	»	57.0	»	5.6

This compound was identified as α-(2-methoxy-5-methylphenyl)-tricarballylic acid by mixed melting point with a sample prepared by synthesis (see later).

Trimethyl ester: It was obtained in the customary manner with diazomethane. It crystallized from dilute methanol as long needles, m. p. 73—5°. It gave no depression when mixed with the methyl ester of the synthetic acid (see later).

Synthesis of dimethyl α-(2-methoxy-5-methylphenyl)-β-acetonylsuccinate

2-Methoxy-5-methylbenzaldehyde cyanohydrin: By starting from *p*-cresol, this was converted by known methods into 2-methoxy-5-methylbenzaldehyde, from which the corresponding cyanohydrin was obtained according to a method used by Levine et al.[13] for the preparation of *o*-methoxybenzaldehyde cyanohydrin. Coarse crystals from benzene, m. p. 57—9°, yield 95 per cent.

$C_{10}H_{11}NO_2$	Calc.	C	67.8	H	6.2	N 7.9
	Found	»	68.1	»	6.2	» 7.6

2-Methoxy-5-methylmandelic acid: 100 g of 2-methoxy-5-methylbenzaldehyde cyanohydrin were placed in a porcelain dish and 100 ml of concd. hydrochloric acid (d. 1.19) were added. The hydrolysis was allowed to proceed in the cold for 14 hours. The mixture was concentrated by heating for 6 hours on a water bath. When cold, the residue was triturated with ether and the unsoluble part consisting of ammonium chloride was filtrated off. The yield of ammonium chloride on hydrolysis was 93 per cent.

The ethereal filtrate was shaken with a saturated sodium bicarbonate solution for removing any unhydrolyzed cyanohydrin (7 g). The bicarbonate solution was washed several times with ether and acidified with concd. hydrochloric acid. A colorless oil separated. It was taken in ether. The ethereal extract was washed three times with water and the solvent was evaporated. The residue was a viscous oil, which did not crystallize on standing.

In order to purify the crude acid, it was converted into the methyl ester by means of diazomethane. The reaction product was fractionated in vacuo. Only 38 g of a yellowish oil distilled over, b. p. 170°/12 mm. The bulk of the methylation product remained in the distilling flask as a red resin. The distillate in the receiver solidified almost immediately, m. p. 85—9°. It crystallized from methanol in colorless plates, m. p. 89—91°.

$C_{11}H_{14}O_4$	Calc.	C	62.8	H	6.7
	Found	»	62.7	»	6.7

36.9 g of the methyl ester were saponified by refluxing for 5 hours with a solution of 8 g of sodium hydroxide in 80 ml of water. After cooling, the resulted solution was extracted with ether for removing any unhydrolyzed ester. The alkaline solution was acidified with dilute hydrochloric acid. An oil separated which was taken in ether. After evaporation of the solvent, the remainder (38 g) solidified on long standing. It crystallized from benzene in tiny needles, m. p. 105—6°. The yield was 34 g (31 per cent calculated from the cyanohydrin, 98 per cent calculated from the methyl ester). The melting point of 2-methoxy-5-methylmandelic acid prepared by Kindler et al.[14] by catalytic hydrogenation of the corresponding phenylglyoxylic acid, was 106°.

Ethyl (2-methoxy-5-methylphenyl)-bromoacetate: 23 g of powdered 2-methoxy-5-methylmandelic acid were placed in a 150-ml round-bottomed flask provided with a reflux condenser. 107 g of powdered phosphorus pentabromide were added gradually through the condenser. A vigorous reaction occurred. Finally the reaction flask was heated on a water bath until the evolution of hydrogen bromide had ceased. After cooling, 150 ml of abs. ethanol were added in small portions through the condenser. A violent reaction followed. At last the reaction mixture was heated on a water bath for a short

while, after which the ethyl bromide formed and a part of ethanol were distilled off. After cooling, the residue was poured into water. A brown, heavy oil separated. It was taken in ether. The ethereal solution was washed once with dilute sodium carbonate solution and several times with water. Ether was evaporated and the residue distilled in vacuo. 24 g of a pale yellow oil, b. p. 135—6°/2mm were obtained. In spite of the narrow boiling range, the oil was a mixture containing along with the ester expected plenty of ethyl 2-methoxy 5-methylmandelate. A lot of 10 g of this oil was used for the preparation of the following compound. The remainder (14 g) on long standing deposited crystals, which were filtrated off. The yield was 5.9 g (30 per cent), m. p. 53—6°. On recrystallization from ethanol great, colorless prisms were obtained, m. p. 57—8°.

$C_{12}H_{15}BrO_3$	Calc.	C	50.2	H	5.2	Br	27.8
	Found	»	49.9	»	5.4	»	28.1

Dimethyl α-(2-methoxy-5-methylphenyl)-β-acetonylsuccinate: 0.8 g of sodium were dissolved in 10 ml of abs. ethanol. 7.5 g of diethyl acetonylmalonate prepared according to Gault and Salomon [15] and 10.0 g of crude ethyl (2-methoxy-5-methylphenyl)-bromoacetate (see before) were added. The mixture was heated for 3 hours on a water bath, after which the reaction product was poured into water and extracted with ether. The ethereal solution was washed once with dilute hydrochlorid acid and several times with water. After evaporation of the solvent, a red, viscous oil (13.4 g) remained, which did not crystallize on standing.

The above condensation product was saponified by refluxing for 3 hours with a solution of 10.6 g of potassium hydroxide in 21 ml of water. The free acid was separated in the usual way. It was a red, viscous oil (10.9 g), which could not be crystallized.

The decarboxylation of the malonic acid derivative obtained above was carried out by refluxing for 3 hours with dilute sulfuric acid (1 ml of concd. sulfuric acid in 17 ml of water). By treating in the customary manner, the reaction mixture yielded 9.3 g of a red, resinous acid, which did not crystallize on standing.

The acid (9.3 g) was converted into the methyl ester by refluxing for 4 hours with a mixture of 4 ml of concd. sulfuric acid and 100 ml of methanol. The ester formed was separated as usually and fractionated in vacuo:

Fraction I, b.p. 160—200°/14 mm, 2.6 g
» II, » 210—220°/14 » 3.4 »

Fraction II was assumed to contain the product expected. For purifying, it was converted into a semicarbazone, yield 2.4 g, m. p. 156—62°. By repeated fractional crystallization the semicarbazone was divided into two isomeric compounds:

Isomer I, small plates, 0.92 g, m. p. 184—5°.

$C_{18}H_{25}N_3O_6$	Calc.	N	11.1
	Found	»	10.9

Isomer II, microcrystalline, 1.21 g, m. p. 158—60°.

$C_{18}H_{25}N_3O_6$	Calc.	N	11.1
	Found	»	10.8

In order to obtain the corresponding carbonyl compounds, the semicarbazones were hydrolyzed separately by heating with a concd. oxalic acid solution on a water bath.

Isomer I, a colorless, viscous oil, b.p. 179—81°/3 mm.

$C_{17}H_{22}O_6$	Calc.	C	63.2	H	6.8
	Found	»	62.9	»	7.0

Isomer II, a colorless, viscous oil, b.p. 188—90°/3 mm.

$C_{17}H_{22}O_6$	Calc.	C	63.2	H	6.8
	Found	»	63.0	»	6.8

The corresponding free acids were also prepared by saponification of the above esters. Both acids were non-crystallizable resins.

Synthesis of α-(2-methoxy-5-methylphenyl)-tricarballylic acid

Ethyl 2-methoxy-5-methylphenylacetate: It was prepared according to known methods by starting from *p*-tolyl methyl ether. This was converted into 2-methoxy-5-methylbenzyl chloride, from which 2-methoxy-5-methylphenylacetic acid was obtained through the corresponding cyanide. The acid was esterified in the customary manner with ethanol.

α-(2-Methoxy-5-methylphenyl)-tricarballylic acid: The condensation of the above ethyl ester (b. p. 136—8°/5 mm) with diethylmaleate was carried out in analogy with the directions given by Borsche and Schmidt [16] for the preparation of α-phenyltricarballylic acid. The ester obtained was a pleasant-smelling, viscous, colorless liquid, b. p. 217—8°/5 mm. The yield was 58 per cent.

$C_{20}H_{28}O_7$	Calc.	C	63.1	H	7.4
	Found	»	63.1	»	7.3

The free acid was obtained from the ethyl ester by hydrolysis (yield 54 per cent). It crystallized from a mixture of benzene and acetone in microscopical needles, m. p. 167°.

$C_{14}H_{16}O_7$	Calc.	C	56.8	H	5.4
	Found	»	56.8	»	5.6

Trimethyl ester: It was prepared from the acid by diazomethane. It crystallized from dilute methanol in long needles, m. p. 74—6°.

Maleic anhydride bis-adduct of the dimeric α-methyl-p-methylstyrene

1) From α-methyl-*p*-methylstyrene and maleic anhydride:

A mixture of 2.3 g of α-methyl-*p*-methylstyrene and 1.7 g of maleic anhydride in 2 ml of benzene was heated for 24 hours at 80°. Large, colorless crystals separated slowly at the walls of the reaction vessel with simultaneous thickening of the liquid part. After cooling, the crystalline product was separated and washed with acetone. The yield was 0.8 g, m. p. 220—30°. The compound was well soluble in hot acetic anhydride, moderately in hot ethyl acetate, slightly in other common organic solvents. It crystallized from ethyl acetate in semispherical clusters of small needles, m. p. 231—3°.

$C_{28}H_{28}O_6$	Calc.	C	73.0	H	6.1	Mol.wt.	460
	Found	»	72.9	»	5.9	» »	435

It showed no depression when mixed with the bis-adduct obtained from the unsaturated dimer of α-methyl-*p*-methylstyrene and maleic anhydride. In order to obtain a better yield of the bis-adduct by starting from the monomeric α-methyl-*p*-methylstyrene and maleic anhydride, it is advisable to proceed in analogy with the third method (see later).

2) From the unsaturated dimer of α-methyl-*p*-methylstyrene and maleic anhydride:

A mixture of 15.0 g (0.057 mole) of the dimer [11] (b. p. 181—6°/7 mm) and 16.7 g (0.171 mole) of maleic anhydride was heated for 24 hours on a boiling water bath. Plenty of coarse crystals had separated. The liquid part was removed from the hot reaction product by suction. It was mainly maleic anhydride with some unconverted dimer as the upper layer. The crystals were washed with ether. The yield was 17.6 g (67 per cent), m. p. 225—31°. After recrystallization from 35 ml of acetic anhydride, 14.8 g of large, colorless prisms, m. p. 231—3°, were obtained.

3) From dimethyl-*p*-tolylcarbinol and maleic anhydride:

8.1 g of the mixture of dimethyl-*p*-tolylcarbinol and *p*-methylacetophenone (see the corresponding preparation of the maleic anhydride bisadduct of the monomeric α-methyl-*p*-methylstyrene), 16.4 g of maleic anhydride and 5.0 ml of 95 % formic acid were mixed and allowed to stand for 72 hours at room temperature. The crystalline reaction product was separated and washed with ether. The yield was 5.4 g (70 per cent), m. p. 230—2°.

Hydrolysis of the bis-adduct $C_{28}H_{28}O_6$

10.0 g of the bis-adduct were heated on a water bath with a solution of 8 g of potassium hydroxide in 200 ml of water until all had dissolved. After cooling, the solution was acidified with concd. hydrochloric acid. A white gum separated. This was very soluble in ether but when extracted with this solvent, it began after a while to separate as small, white crystals. After standing overnight, these were filtrated off and washed with ether. On drying in a vacuum desiccator, the product decreased continuously in weight. Only after keeping for some hours at 100° the weight became constant. The yield was 10.7 g (98 per cent), m. p. 210—5° (dec.) after previous sintering.

$C_{28}H_{32}O_8$	Calc.	C	67.6	H	6.4	Neut.equiv.	124
	Found	»	67.5	»	6.3	» »	127

On direct titration of the acid in ethanol solution with 0.5 N sodium hydroxide, no sharp end point could be observed. The value reported above was obtained by a lactone titration.

Methylation of the tetracarboxylic acid $C_{28}H_{32}O_8$ by diazomethane

5.0 g of the above tetracarboxylic acid were dissolved in 20 ml of methanol and methylated by an ethereal solution of diazomethane. Ether and the bulk of methanol were distilled off. On long standing, the remainder deposited a compact layer of crystals at the bottom of the flask. After filtering and washing with methanol, 5.1 g (92 per cent) of a crystal pulver was obtained, m. p. 118—21°. It crystallized from methanol as a white, fine-grained pulver, m. p. 120—3°.

$C_{32}H_{40}O_8$	Calc.	C	69.5	H	7.2
	Found	»	69.9	»	7.3

Ozonization of the bis-adduct $C_{28}H_{28}O_6$

7.0 g of the bis-adduct were dissolved in 280 ml of ethyl acetate and ozonized with cooling in an ice-salt mixture. The amount of ozone absorbed by the bis-adduct corresponded to one double bond. About 200 ml of the solvent were evaporated at room temperature in vacuo. The ozonide in the remaining solution was decomposed by catalytic hydrogenation. The hydrogen consumption was about 90 per cent of that calculated for a mono-ozonide. After removing the catalyst and evaporating the solvent, the decomposition product was obtained as a porous resin.

In another experiment the tetracarboxylic acid $C_{28}H_{32}O_8$ was ozonized according to the same method. No definit ozonization products could be isolated in either case.

Maleic anhydride bis-adduct of styrene

A mixture of 5.0 g (0.048 mole) of styrene (prepared by dehydration of β- phenylethyl alcohol), 14.2 g (0.145 mole) of maleic anhydride, 0.5 g of dimethylaniline and 10 ml of acetic anhydride was heated for 24 hours at 80°. On long standing in an open Erlenmeyer flask at room temperature, crystals separated from the dark liquid. They were filtrated off and washed with cold acetic anhydride. Upon treating with water, almost all dissolved (maleic acid). Only a little, crystalline residue (0.17 g) remained, m. p. 252—5° (dec.). It crystallized from acetic anhydride in clusters of small, colorless needles, m. p. 254—6° (dec.).

$C_{16}H_{12}O_6$	Calc.	C	64.0	H	4.0
	Found	»	63.9	»	4.0

Aromatization of the bis-adduct $C_{16}H_{12}O_6$

The maleic anhydride bis-adduct of styrene was aromatized in the same way as that of α-methyl-*p*-methylstyrene. By starting with 0.29 g of the bis-adduct, 0.13 g (67 per cent) of dark crystals, m. p. 155—60°, were obtained. After sublimation in vacuo and subsequent crystallization from xylene, the product was obtained in pale yellow needles, m. p. 166—7°.

$C_{12}H_6O_3$	Calc.	C	72.7	H	3.0
	Found	»	72.6	»	3.1

The compound showed no depression when mixed with authentic naphthalene-1,2-dicarboxylic acid anhydride.

SUMMARY

I. α-Methyl-*p*-methylstyrene as well as one of its dimers, *viz.* 2,4-di-(*p*-tolyl)-4-methylpentene-1, were stated to form, each one separately, a bis-adduct with maleic anhydride in a good yield (40 and 70 per cent respectively). It was also possible to prepare a maleic anhydride bis-adduct of styrene, although in a poor yield. On the contrary, α-methylstyrene failed to give a bis-adduct under the same reaction conditions.

II. Depending on the reaction conditions, the action of maleic anhydride on α-methyl-*p*-methylstyrene can lead to three different end products: 1) a heteropolymer, 2) a maleic anhydride bis-adduct of the monomeric α-methyl-*p*-methylstyrene and 3) that of the dimeric α-methyl-*p*-methylstyrene.

III. The structure of the maleic anhydride bis-adduct of the monomeric α-methyl-*p*-methylstyrene has been examined by means of ozonization. The results obtained agree with a formula deduceable from α-methyl-*p*-methylstyrene through two successive 1,4-additions of maleic anhydride (formula III). A reaction mechanism involving a diene synthesis followed by a substituting addition, which would lead to a tetralin berivative, has been excluded.

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Die Geschwindigkeit der Umwandlung von festem 1,2-Dinitrito- in 1,2-Dinitrodiäthylendiaminkobalt(III)-nitrat, extinktiometrisch untersucht

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Im Anschluss an einen früheren Aufsatz [1] über die Umwandlung von festem 1,6-Dinitritodipyridindiamminkobalt(III)-nitrat in die entsprechende Dinitroverbindung wird in dieser Arbeit die analoge Isomerisation von 1,2-Dinitritodiäthylendiaminkobalt(III)-nitrat kinetisch mit Hilfe von Extinktionsmessungen untersucht.

Zur Herstellung des zu benutzenden Salzes wurde nach Werner folgendes Syntheseschema verwendet.

$$CoSO_4 + en + H_2O + O_2 \longrightarrow [(H_2O)_2Co\ [(HO)_2Co\ en_2]_2]\ (SO_4)_2 \cdot 7\ H_2O \longrightarrow$$
$$\left[\begin{matrix}(1)\ H_2O \\ (2)\ H_2O\end{matrix}\ Co\ en_2\right] Br_3 \cdot 2\ H_2O \longrightarrow \left[\begin{matrix}(1)\ ONO \\ (2)\ ONO\end{matrix}\ Co\ en_2\right] Br \longrightarrow$$
$$\left[\begin{matrix}(1)\ ONO \\ (2)\ ONO\end{matrix}\ Co\ en_2\right] NO_3$$

Durch Luftoxydation einer äthylendiaminhaltigen wässerigen Lösung von Kobaltsulfat wurde die rote, komplizierte Verbindung $[(H_2O)_2Co[(HO)_2Co\ en_2]_2]\ (SO_4)_2 \cdot 7H_2O$ hauptsächlich nach Werner und Jantsch [2] dargestellt. Es zeigte sich aber, dass man eine höhere und von Synthese zu Synthese konstantere Ausbeute erreichen konnte, wenn das Reaktionsgefäss in einem Wasserbade von etwa 50° C stand. Letztgenanntes Salz gab, mit konzentrierter Bromwasserstoffsäure verrieben, 1,2-Diaquodiäthylendiaminkobalt(III)-bromid (Werner [3]), das seinerseits mit Eisessig und einer gesättigten Natriumnitritlösung in 1,2-Dinitritodiäthylendiaminkobalt(III)-bromid übergeführt werden konnte (Werner[4]). Das entsprechende Nitrat wurde aus der konzentrierten Lösung des Bromids mit festem Ammoniumnitrat ausgefällt. Um für kinetische Messungen hinreichend rein zu werden, muss das Nitrat aber noch zweimal aus wässeriger Lösung mit Ammoniumnitrat umgefällt werden (vgl. Werner [5]). Da die Isomerisation $\left[\begin{matrix}(1)\ ONO \\ (2)\ ONO\end{matrix}\ Co\ en_2\right]^+ \longrightarrow \left[\begin{matrix}(1)\ O_2N \\ (2)\ O_2N\end{matrix}\ Co\ en_2\right]^+$ erheblich schneller in Lösung als in fester Phase vor sich geht, bringen die erwähnten Umfällungen wie die Ausfällung des ursprünglichen Nitrats leider den grossen Nachteil

mit sich, dass die Dinitritoverbindung in zu hohem Grade isomerisiert wird, ehe man mit dem festen Endprodukt kinetische Messungen anfangen kann.

Deshalb wurde viel Arbeit daran verwendet, auf einem etwas veränderten Syntheseweg, nämlich

$$[(H_2O)_2Co\ [(HO)_2Co\ en_2]_2]\ (SO_4)_2\ ,\ 7\ H_2O \rightarrow \left[\begin{matrix}(1)\ H_2O\\(2)\ H_2O\end{matrix}\ Co\ en_2\right] Br_3\ ,\ 2\ H_2O \rightarrow$$

$$\left[\begin{matrix}(1)\ H_2O\\(2)\ H_2O\end{matrix}\ Co\ en_2\right] (NO_3)_3\ ,\ H_2O \rightarrow \left[\begin{matrix}(1)\ ONO\\(2)\ ONO\end{matrix}\ Co\ en_2\right] NO_3,$$

ein festes Endprodukt zu erreichen, das einerseits rein, andrerseits aber wenigst möglich isomerisiert war. Nach diesem Verfahren würde die Umwandlung von Bromid in Nitrat ja schon in der Diaquostufe geschehen und wenigstens eine Umfällung von instabilem Dinitritosalz aus wässeriger Lösung könnte erübrigt werden. Es zeigte sich aber als schwierig, das sehr leichtlösliche Diaquonitrat, das nach Werner[6] dargestellt wurde, genügend rein zu erhalten. Das daraus hergestellte Dinitritonitrat hatte auch keinen hinreichenden Reinheitsgrad.

Es schien deshalb ratsam, auf das ursprüngliche Syntheseschema zurückzugehen und die Isomerisation in wässeriger Lösung dadurch zu begrenzen, dass man wenigstens eine Umfällung bei Eiskälte durchführt.

Zur Orientierung über die Absorptionsverhältnisse wurde erstens die Extinktion zweier Versuchslösungen von der Zusammensetzung

$$0{,}002 \text{ bzw. } 0{,}004\ M \left[\begin{matrix}(1)\ O_2N\\(2)\ O_2N\end{matrix}\ Co\ en_2\right] NO_3 + 0{,}01\ M\ HOCOCH_3 + 0{,}01\ M\ NaOCOCH_3$$

bestimmt. Diese Messungen wurden bei der Schichtdicke $\lambda = 3{,}00$ cm und für eine Reihe von Wellenlängen in dem Gebiete 380 m$\mu \leqq \lambda \leqq$ 560 mμ und mit Hilfe des in der früheren Arbeit[1] erwähnten Beckman-Quarz-Spektrophotometers ausgeführt. Das dabei benutzte Dinitronitrat wurde aus möglichst reinem Dinitritonitrat durch 21-stündige Erwärmung bei 60° C erhalten. Zwei Proben desselben enthielten 17,70 bzw. 17,72 % Kobalt. Der theoretische Gehalt ist 17,69 %. Aus den Extinktionswerten wurde für jede Wellenlänge der molare, dekadische Extinktionskoeffizient α berechnet. In Fig. 1 wird α graphisch gegen λ wiedergegeben. Man findet, dass das Dinitronitrat bei etwa 390 mμ ein Absorptionsminimum und bei rund 435 mμ ein Maximum besitzt.

Um ein wenigstens annähernd richtiges Bild der Extinktionskurve des instabilen Dinitritonitrats zu erhalten, wurden drei berechnete, gleich grosse Mengen des neudargestellten Salzes mit dem Kobaltgehalt 17,71 bzw. 17,69 % eingewogen. Mit Intervallen von etwa 30 Minuten wurden aus ihnen drei Lösungen der Zusammensetzung

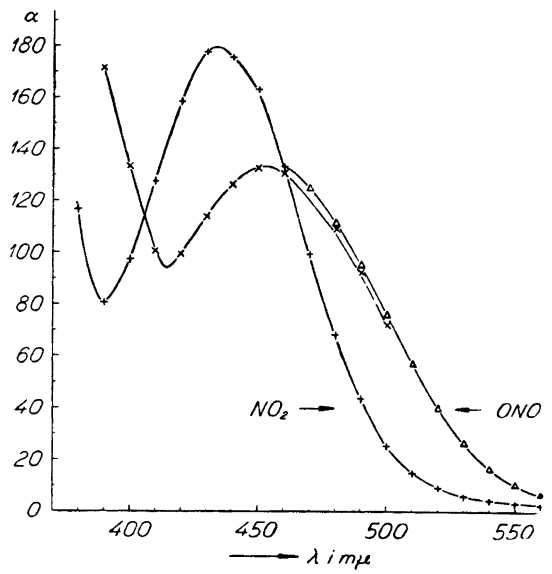


Fig. 1. Der molare dekadische Extinktionskoeffizient α als Funktion der Wellenlänge λ für Lösungen von 1,2-Dinitrodiäthylendiaminkobalt(III)-nitrat und von neu dargestelltem, aber teilweise isomerisiertem 1,2-Dinitritodiäthylendiaminkobalt(III)-nitrat.

0,004 *M* Salz + 0,01 *M* $HOCOCH_3$ + 0,01 *M* $NaOCOCH_3$

bereitet, die unmittelbar für Extinktionsmessungen bei der Schichtdicke 3,00 cm zu verwenden waren. Die mit den einzelnen Lösungen untersuchten Wellenlängengebiete waren 390—500 mμ; 460—540 mμ und 490—570 mμ. Sie überdeckten sich somit teilweise. In Fig. 1 sind auch für das Dinitritosalz die gefundenen α-Werte gegen die Wellenlänge eingezeichnet. Hauptsächlich wegen der Isomerisation des gelösten Salzes während der Messungen fallen die α-Werte in den gemeinsamen Wellenlängengebieten im allgemeinen nicht zusammen. Das Bild gibt jedoch in grossen Zügen die Extinktionskurve des neuhergestellten Dinitritonitrats wieder. Das Absorptionsminimum liegt hier bei 415 mμ und das Maximum bei 455 mμ. Beide sind also im Verhältnis zu denjenigen des Dinitrosalzes gegen grössere Wellenlängen verschoben, und das Maximum ist erheblich kleiner als das der Dinitroverbindung. (Kuroya [12] fand mit einer neu bereiteten Lösung von $[Co(NH_3)_5ONO]Cl_2$ für das langwelligere Absorptionsmaximum bei $\lambda = 471$ mμ den Wert $\log \alpha_{max} = 1{,}740$. Nach dem Verlaufe von etwa sieben Stunden, wo die Isomerisation des Salzes somit weit fortgeschritten war, lag das entsprechende Maximum derselben Lösung bei $\lambda = 459$ mμ, und $\log \alpha_{max}$ war 1,835. Die die Isomerisation begleitenden

Veränderungen der Lage und Höhe des Maximums dieser Verbindung stimmen also qualitativ mit den oben für $\left[\begin{matrix}(1)\ \mathrm{ONO} \\ (2)\ \mathrm{ONO}\end{matrix}\ \mathrm{Co\ en_2}\right] \mathrm{NO_3}$ angegebenen gut überein. Vgl. auch Adell [13].)

Bei den kinetischen Messungen war die Versuchsmethode mit der der vorigen Untersuchung [1] fast identisch. Für alle Extinktionsbestimmungen wurde das modifizierte Beckman-Quarz-Spektrophotometer benutzt. Die Wellenlänge des Lichtes war durchgehend 510 mμ und die Versuchstemperaturen 20°, 30° und 40° C. Das 1,2-Dinitritodiäthylendiaminkobalt(III)-nitrat und das entsprechende Dinitrosalz sind den 1,6-Dinitrito- und Dinitrodipyridindiamminkobalt(III)-nitraten in der Weise voraus, dass sie kristallwasserfrei sind. Sie brauchten somit nicht entwässert zu werden. Vor jeder Kinetikreihe wurde die Reinheit des zu benutzenden Dinitritosalzes durch gravimetrische Bestimmung des Kobaltgehaltes geprüft (vgl. Adell und Thölin [7]). Die eingewogenen Proben des festen, trocknen Salzes wurden über Phosphorpentoxyd in einem luftdicht verschlossenen, vernickelten Messingzylinder verwahrt, der in einem Paraffinthermostaten stand. Aus jeder Probe wurde eine Versuchslösung mit der Zusammensetzung

$$0{,}004\ M\ \text{Komplexsalz} + 0{,}01\ M\ \mathrm{HOCOCH_3} + 0{,}01\ M\ \mathrm{NaOCOCH_3}$$

bereitet. Ihre Extinktion wurde immer bei der Schichtdicke 3,00 cm bestimmt. Die Bereitung der Versuchslösungen machte hier keine Schwierigkeiten, da das Dinitrito- wie das Dinitronitrat sich fast momentan lösten.

Die Extinktion $(E_V)_T$ der Versuchslösung im Auflösungsaugenblicke T des Salzes wurde durch graphische Extrapolation aus 10 bis 12 innerhalb etwa 20 Minuten und t Minuten nach T gemessenen Extinktionswerten $(E_V)_{T+t}$ bestimmt. Diese Extrapolation konnte ziemlich leicht durchgeführt werden, denn die $(E_V)_{T+t}$-Werte gaben, gegen t eingezeichnet, Punkte, die sehr nahe an einer Gerade lagen. Im folgenden wird $(E_V)_T$ nur mit E_V bezeichnet.

Es zeigte sich, dass je älter die festen Proben des Dinitritonitrats wurden, um so mehr näherte sich die Extinktion der Versuchslösung E_V an diejenige E_G einer Lösung mit der Zusammensetzung

$$0{,}004\ M\ \text{Dinitronitrat} + 0{,}01\ M\ \mathrm{HOCOCH_3} + 0{,}01\ M\ \mathrm{NaOCOCH_3}$$

Dann blieb die Extinktion konstant. (Vgl. unten die Tabellen 8 und 9.)

Die primären Versuchsresultate sind in den ersten und zweiten, bzw. vierten und fünften Kolumnen der Tabellen 1—9 verzeichnet. t wird vom Auflösungszeitpunkte der ersten Probe jeder Reihe gerechnet.

Tabelle 1. Versuchsreihe Nr. 62 a. Temperatur 20,0° C.

Formel: $E_V - 0{,}213 = 0{,}093 \cdot 10^{-0{,}0215 \cdot t} + 0{,}442 \cdot 10^{-0{,}0027 \cdot t}$

t(Stdn)	$(E_V)_{gef.}$	$(E_V)_{ber.}$	t(Stdn)	$(E_V)_{gef.}$	$(E_V)_{ber.}$
0,00	0,751	0,748	44,68	0,565	0,557
0,517	0,744	0,745	68,68	0,501	0,505
1,17	0,730	0,740	97,17	0,454	0,455
2,10	0,725	0,733	116,73	0,436	0,427
4,63	0,714	0,716	141,43	0,396	0,396
6,65	0,691	0,704	165,20	0,369	0,371
20,73	0,638	0,634	189,43	0,350	0,349
22,79	0,630	0,626	213,44	0,330	0,330
27,03	0,617	0,610	237,39	0,312	0,314
30,71	0,607	0,597	286,33	0,284	0,287

Tabelle 2. Versuchsreihe Nr. 67 a. Temperatur 20,0° C.

Formel: $E_V - 0{,}199 = 0{,}099 \cdot 10^{-0{,}0155 \cdot t} + 0{,}459 \cdot 10^{-0{,}0023 \cdot t}$

t(Stdn)	$(E_V)_{gef.}$	$(E_V)_{ber.}$	t(Stdn)	$(E_V)_{gef.}$	$(E_V)_{ber.}$
0,00	0,757	0,757	69,03	0,522	0,526
0,967	0,748	0,752	77,33	0,508	0,504
4,25	0,730	0,733	93,08	0,474	0,480
6,70	0,723	0,721	120,67	0,438	0,441
9,05	0,715	0,708	140,36	0,417	0,417
20,53	0,658	0,659	189,17	0,376	0,368
27,52	0,630	0,633	213,00	0,346	0,348
31,26	0,620	0,620	237,42	0,326	0,330
44,17	0,586	0,583	268,67	0,311	0,310
54,33	0,560	0,558			

Tabelle 3. Versuchsreihe Nr. 71 a. Temperatur 20,0° C.

Formel: $E_V - 0{,}199 = 0{,}089 \cdot 10^{-0{,}0215 \cdot t} + 0{,}422 \cdot 10^{-0{,}0026 \cdot t}$

t(Stdn)	$(E_V)_{gef.}$	$(E_V)_{ber.}$	t(Stdn)	$(E_V)_{gef.}$	$(E_V)_{bet.}$
0,00	0,710	0,710	55,90	0,502	0,501
1,00	0,701	0,703	70,15	0,479	0,476
3,50	0,681	0,687	93,50	0,432	0,440
6,00	0,663	0,671	117,40	0,413	0,408
9,50	0,650	0,653	142,50	0,380	0,379
21,33	0,605	0,602	165,55	0,358	0,356
24,17	0,593	0,591	190,35	0,332	0,334
28,50	0,582	0,577	223,50	0,308	0,310
33,50	0,572	0,561	237,55	0,303	0,301
45,55	0,528	0,529	260,50	0,288	0,288

Tabelle 4. Versuchsreihe Nr. 60 a. Temperatur 30,0° C.

Formel: $E_V - 0{,}205 = 0{,}114 \cdot 10^{-0{,}044 \cdot t} + 0{,}436 \cdot 10^{-0{,}0097 \cdot t}$

t(Stdn)	$(E_V)_{gef.}$	$(E_V)_{ber.}$	t(Stdn)	$(E_V)_{gef.}$	$(E_V)_{ber.}$
0,00	0,757	0,755	5,78	0,639	0,653
0,367	0,751	0,747	8,50	0,610	0,614
0,850	0,734	0,738	10,49	0,588	0,589
1,60	0,725	0,723	21,82	0,481	0,485
2,61	0,705	0,704	29,60	0,444	0,436
4,10	0,683	0,678			

Tabelle 5. Versuchsreihe Nr. 61 a. Temperatur 30,0° C.

Formel: $E_V - 0{,}171 = 0{,}110 \cdot 10^{-0{,}044 \cdot t} + 0{,}418 \cdot 10^{-0{,}0097 \cdot t}$

t(Stdn)	$(E_V)_{gef.}$	$(E_V)_{ber.}$	t(Stdn)	$(E_V)_{gef.}$	$(E_V)_{ber.}$
0,00	0,696	0,699	31,69	0,386	0,382
0,875	0,687	0,681	34,51	0,364	0,368
1,83	0,663	0,664	46,55	0,313	0,320
2,84	0,638	0,646	51,63	0,306	0,304
5,58	0,594	0,603	58,74	0,280	0,285
8,48	0,562	0,564	69,56	0,260	0,260
10,58	0,546	0,539	80,49	0,236	0,240
22,14	0,443	0,438	93,53	0,220	0,223
25,58	0,419	0,416	124,33	0,198	0,197
28,38	0,399	0,399			

Tabelle 6. Versuchsreihe Nr. 70 a. Temperatur 30,0° C.

Formel: $E_V - 0{,}179 = 0{,}142 \cdot 10^{-0{,}054 \cdot t} + 0{,}416 \cdot 10^{-0{,}0085 \cdot t}$

t(Stdn)	$(E_V)_{gef.}$	$(E_V)_{ber.}$	t(Stdn)	$(E_V)_{gef.}$	$(E_V)_{ber.}$
0,00	0,737	0,737	32,50	0,404	0,401
1,00	0,714	0,711	45,00	0,348	0,352
2,75	0,675	0,674	52,67	0,326	0,327
5,50	0,619	0,624	56,50	0,309	0,317
7,00	0,610	0,601	68,50	0,281	0,288
9,50	0,572	0,568	77,50	0,274	0,270
20,50	0,467	0,469	93,25	0,252	0,246
23,50	0,450	0,449	117,78	0,221	0,220
26,67	0,430	0,431	141,60	0,210	0,205
29,50	0,405	0,416			

Tabelle 7. Versuchsreihe Nr. 74 a. Temperatur 30,0° C.

Formel: $E_V - 0{,}201 = 0{,}110 \cdot 10^{-0{,}054 \cdot t} + 0{,}448 \cdot 10^{-0{,}00975 \cdot t}$

t(Stdn)	$(E_V)_{gef.}$	$(E_V)_{ber.}$	t(Stdn)	$(E_V)_{gef.}$	$(E_V)_{ber.}$
0,00	0,759	0,759	33,20	0,424	0,415
1,00	0,736	0,736	45,25	0,370	0,363
2,58	0,705	0,704	49,30	0,340	0,349
5,30	0,655	0,655	57,25	0,312	0,325
7,60	0,618	0,622	72,25	0,297	0,290
10,25	0,592	0,588	79,25	0,285	0,277
21,35	0,485	0,486	94,05	0,253	0,255
24,35	0,466	0,465	104,65	0,240	0,244
27,58	0,447	0,448	118,32	0,233	0,232
30,20	0,436	0,432	141,35	0,225	0,220

Tabelle 8. Versuchsreihe Nr. 73 a. Temperatur 40,0° C.

Formel: $E_V - 0{,}194 = 0{,}110 \cdot 10^{-0{,}160 \cdot t} + 0{,}403 \cdot 10^{-0{,}0375 \cdot t}$

t(Stdn)	$(E_V)_{gef.}$	$(E_V)_{ber.}$	t(Stdn)	$(E_V)_{gef.}$	$(E_V)_{ber.}$
0,00	0,708	0,707	24,50	0,243	0,243
0,50	0,675	0,670	27,50	0,233	0,232
1,25	0,620	0,624	30,08	0,225	0,224
2,25	0,577	0,574	46,00	0,203	0,202
4,85	0,474	0,477	55,65	0,195	
5,85	0,446	0,450	71,05	0,194	
6,50	0,437	0,434	95,90	0,194	
8,52	0,393	0,392	190,30	0,194	
9,92	0,367	0,368	248,80	0,195	
22,15	0,248	0,253			

Tabelle 9. Versuchsreihe Nr. 75 a. Temperatur 40,0° C.

Formel: $E_V - 0{,}199 = 0{,}062 \cdot 10^{-0{,}165 \cdot t} + 0{,}409 \cdot 10^{-0{,}040 \cdot t}$

t(Stdn)	$(E_V)_{gef.}$	$(E_V)_{ber.}$	t(Stdn)	$(E_V)_{gef.}$	$(E_V)_{ber.}$
0,00	0,669	0,670	22,80	0,251	0,249
0,50	0,639	0,640	25,53	0,234	0,238
1,65	0,581	0,583	28,50	0,228	0,229
4,35	0,480	0,485	31,50	0,220	0,221
5,48	0,456	0,454	44,03	0,208	0,206
6,52	0,432	0,428	68,57	0,199	
7,60	0,410	0,405	92,60	0,199	
8,50	0,392	0,388	115,67	0,200	
20,22	0,259	0,262	188,33	0,198	

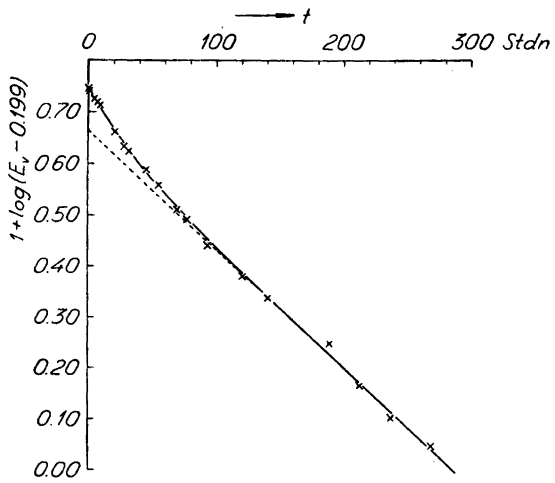


Fig. 2. $1 + \log (E_V - E_G)$ als Funktion der Zeit t für 1,2-Dinitritodiäthylendiamin-kobalt(III)-nitrat. Versuchsreihe Nr. 67 a. Isomerisationstemperatur der festen Verbindung: 20.0° C.

Wie in der früheren Arbeit[1] wurde so log (E_V—E_G) gegen t graphisch aufgetragen. Durch die resultierenden Punkte jeder Versuchsreihe liess sich eine ausgleichende Kurve von dem in Fig. 2 wiedergegebenen Typ ziehen. Es ist eine Linie, die für kleinere t-Werte gegen die Abscissenachse schwach gekrümmt ist. Für grössere t-Werte geht sie in eine Gerade über. In jeder einzelnen Kinetikreihe wurde dabei für E_G der Extinktionswert benutzt, den eine auf 60° über Nacht erhitzte Probe des fraglichen Salzpräparates unter den oben S. 57 angegebenen Bedingungen gab.

Die E_G-Werte aller Reihen schwanken zwischen den Extremwerten 0,213 und 0,171. Das hängt sicherlich mit der Gegenwart kleiner Mengen von schwer zu entfernenden Verunreinigungen in dem festen, leichtlöslichen Dinitritonitrat zusammen. Der Kobaltgehalt der benutzten Präparate dieses Salzes wechselte zwischen 17,57 und 17,72 % mit dem Durchschnittswert 17,64 %. (Theoretischer Wert 17,69 %). Man kann aber die Reinigung des Ausgangsmaterials kaum länger treiben, als es hier geschehen ist, denn bei weiteren Umfällungen aus wässeriger Lösung liegt die Gefahr sehr nahe, dass beim Anfang der kinetischen Messungen mit dem festen Salze die erste Stufe der Isomerisation schon vorüber sei. Dann würde der gekrümmte Teil der log (E_V—E_G)-Kurve gar nicht zum Vorschein kommen. Wenn zur Bereitung einer Versuchslösung ein Salzpräparat benutzt wurde, das nach der Erhitzung auf 60° C ausserdem mit festem Ammoniumnitrat umgefällt war, zeigte die Lösung unter den obigen Bedingungen (vgl. S. 57) eine Extinktion E_G, die sich nur sehr wenig von dem Extinktionswert E_G unterschied, den das nicht umgefällte, aber erhitzte Salz gab. Für die Versuchsreihe Nr. 71 a war z. B. E'_G 0,196 und E_G 0,199; für Nr. 70 a 0,178 und 0,179; für Nr. 74 a 0,200 und 0,201 und für Nr. 75 a 0,200 und 0,199. Noch eine Umfällung ist also nicht hinreichend, um die Unterschiede in E_G zu eliminieren.

Die Form der log $(E_V—E_G)$-Kurven und die Erfahrungen aus der vorigen Untersuchung [1] machten es naheliegend, zu prüfen, ob der Zusammenhang zwischen $E_V—E_G$ und der Zeit t durch eine Gleichung vom Typ

$$E_V—E_G = C \cdot 10^{-\lambda_1 \cdot t} + D \cdot 10^{-\lambda_2 \cdot t} \quad (1)$$

ausgedrückt werden könne. C, D, λ_1 und λ_2 sind dabei Konstanten, deren Werte empirisch zu bestimmen sind. Eine Relation dieser Art wäre zu erwarten, falls die Isomerisation in zwei Stufen nach dem Schema

$$\left[\begin{matrix}ONO\\ONO\end{matrix}\ Co\ en_2\right] NO_3 \longrightarrow \left[\begin{matrix}O_2N\\ONO\end{matrix}\ Co\ en_2\right] NO_3 \longrightarrow \left[\begin{matrix}O_2N\\O_2N\end{matrix}\ Co\ en_2\right] NO_3 \quad (2)$$

vor sich geht und wenn beide Stufenreaktionen erster Ordnung sind, aber verschiedene Geschwindigkeitskonstanten haben. (Vgl. Adell [1] S. 14—17.) Der geradlinige Verlauf der log $(E_V—E_G)$-Kurve für grosse t-Werte wird zwanglos durch die Annahme erklärt, dass eine der Konstanten, z. B. λ_1, erheblich grösser als die andere λ_2 sei. Dann lässt sich in diesem t-Gebiete $C \cdot 10^{-\lambda_1 \cdot t}$ neben $D \cdot 10^{-\lambda_2 \cdot t}$ vernachlässigen, und man hat

$$\log\ (E_V—E_G) = \log\ D—\lambda_2 \cdot t \quad (3)$$

log D und $—\lambda_2$ können somit als die Ordinate im Nullpunkt und der Winkelkoeffizient für diejenige Gerade bestimmt werden, in die die log $(E_V—E_G)$-Kurve übergeht.

Wenn die Gleichung (1) gültig sei, müsste weiter

$$\log\ [E_V—E_G—D \cdot 10^{-\lambda_2 \cdot t}] = \log\ C—\lambda_1 \cdot t \quad (4)$$

sein, und wenn man die nach der obigen Methode bestimmten Werte von D und λ_2 benutzt, sollte dieser Zusammenhang für kleinere t-Werte in einem t-Diagramm Punkte ergeben, die auf einer neuen Gerade liegen. Das war auch mit befriedigender Annäherung der Fall, und die Ordinate im Nullpunkt und der Winkelkoeffizient dieser Gerade gaben die gesuchten Werte von log C und $—\lambda_1$. Auf der zweiten Zeile jeder der Tabellen 1—9 wird die für die fragliche Versuchsreihe auf die obige Weise empirisch bestimmte Formel vom Typ (1) angegeben.

Für eine Isomerisation nach dem Schema (2) oben, die also als zwei konsekutive Reaktionen erster Ordnung mit verschiedenen Geschwindigkeitskonstanten verläuft, kann man folgenden theoretischen Ausdruck für $E_V—E_G$ herleiten (Adell [1]):

$$E_V - E_G = A \cdot 10^{-K_1 t} + B \cdot 10^{-K_2 t} \quad (5)$$

mit
$$A = ad \cdot \left(\alpha_1 - \alpha_2 \cdot \frac{K_1}{K_1 - K_2} + \alpha_3 \cdot \frac{K_2}{K_1 - K_2}\right) \text{ und} \quad (6)$$

$$B = \left(\frac{aK_1}{K_1 - K_2} + b\right) \cdot d \cdot (\alpha_2 - \alpha_3) \quad (7)$$

Hier sind α_1, α_2 und α_3 die molaren Extinktionskoeffizienten des Dinitrito-, des Nitronitrito- und des Dinitronitrats, a und b die Konzentrationen der beiden ersten Isomeren bei $t = 0$, d. h. beim Auflösungszeitpunkte der ersten Probe, wie K_1 und K_2 die Geschwindigkeitskonstanten auf dekadische Logarithmen bezogen für die erste bzw. zweite Stufe der Isomerisation. d ist die absorbierende Schichtdicke der Versuchslösung.

Wenn man nun die empirische Gleichung (1) mit der theoretischen (5) identifizieren will, d. h. wenn man (1) durch die Annahme einer zweistufigen Isomerisation zu erklären versucht, so entsteht die Frage, ob man K_1 gleich dem grösseren λ_1 und A gleich C bzw. K_2 gleich dem kleineren λ_2 und B gleich D zu setzen hat oder umgekehrt. Die Frage kann durch Vergleich des gefundenen Quotienten C/D mit dem theoretischen A/B beantwortet werden. Zwar kennt man die Werte von a, b, α_1 und α_2 nicht; wird b aber in erster Annäherung neben $a \cdot \frac{K_1}{K_1 - K_2}$ vernachlässigt und α_2 gleich $\frac{1}{2} \cdot (\alpha_1 + \alpha_3)$ gesetzt, so kommt man zu dem approximativen Ausdruck

$$\frac{A}{B} \simeq 1 - 2 \cdot \frac{K_2}{K_1} \quad (8$$

Tabelle 10 gibt einen Überblick über die gefundenen Werte von C/D und die nach (8) berechneten Werte von A/B. Die letzteren werden mit A′/B′ bzw A″/B″ bezeichnet, je nachdem $K_1 = \lambda_1$ und $K_2 = \lambda_2$ oder $K_1 = \lambda_2$ und $K_2 = \lambda_1$ gesetzt wurden. ($\lambda_1 > \lambda_2$)

Tabelle 10. Gefundene Werte von C/D und nach (8) berechnete Werte von A/B.

Versuchsreihe Nr.	Temp.	$(C/D)_{gef.}$	$(A'/B')_{ber.}$	$(A''/B'')_{ber.}$
62 a	20° C	0,21	0,75	— 14,9
67 a	20° C	0,22	0,70	— 13,5
71 a	20° C	0,21	0,76	— 15,5
61 a	30° C	0,26	0,56	— 8,1
70 a	30° C	0,34	0,69	— 11,7
74 a	30° C	0,25	0,64	— 10,1
73 a	40° C	0,27	0,53	— 7,5
75 a	40° C	0,15	0,52	— 7,2

Man findet, dass, wenn man von einer stufenweisen Isomerisation ausgeht, so lassen sich die empirischen Daten nur mit der Annahme $K_1 > K_2$ vereinen. Für $K_1 < K_2$ stimmt C/D an Vorzeichen mit B''/A'' nicht überein. Dass C/D zwar grössenordnungsmässig A'/B' nahe kommt, aber jedoch nicht unbedeutend kleiner als A'/B' ist, wird verständlich, wenn man bedenkt, dass man bei der Berechnung von A/B in dem Ausdruck für B b vernachlässigt (vgl. (7) oben) und somit sicherlich einen zu kleinen B-Wert verwendet hat. Wegen der unvermeidlichen Umfällungen des Dinitritosalzes ist b sicherlich > 0 für $t = 0$, lässt sich aber nicht berechnen.

Die mit der zu jeder Versuchsreihe gehörigen Formel vom Typ (1) berechneten Werte von E_V sind in den Kolumnen 3 und 6 der Tabellen 1 bis 9 den gefundenen an die Seite gestellt. Die Übereinstimmung ist bei jedem Zeitpunkte sehr gut, und die Differenzen betragen im Mittel 0,9 %. Nur selten liegen sie zwischen 1 und 2 %. Sie überschreiten somit nicht die Grenzen der Versuchsfehler. Man muss also schliessen, dass die Veränderung von E_V mit der Zeit t durch die Annahme gut erklärt wird, dass die Isomerisation des Dinitritonitrats stufenweise als zwei konsekutive Reaktionen erster Ordnung verläuft.

Tabelle 11. Übersicht über die Geschwindigkeitskonstanten K_1 und K_2 (in h^{-1}, auf dekadische Logarithmen bezogen) für die erste und zweite Isomerisationsstufe.

Versuchsreihe Nr.	Tabelle Nr.	Temp.	K_1	K_2	A	B
62 a	1	20,0° C	0,0215	0,0027	0,093	0,442
67 a	2	20,0° C	0,0155	0,0023	0,099	0,459
71 a	3	20,0° C	0,0215	0,0026	0,089	0,422

Mittelwerte: $K_1 = 0{,}019_5 \pm 0{,}002$ $K_2 = 0{,}0025 \pm 0{,}0001$.

Versuchsreihe Nr.	Tabelle Nr.	Temp.	K_1	K_2	A	B
60 a	4	30,0° C	0,044	(0,0097)	0,114	0,436
61 a	5	30,0° C	0,044	0,0097	0,110	0,418
70 a	6	30,0° C	0,054	0,0085	0,142	0,416
74 a	7	30,0° C	0,054	0,00975	0,110	0,448

Mittelwerte: $K_1 = 0{,}049 \pm 0{,}003$ $K_2 = 0{,}0093 \pm 0{,}0004$.

Versuchsreihe Nr.	Tabelle Nr.	Temp.	K_1	K_2	A	B
73 a	8	40,0° C	0,160	0,0375	0,110	0,403
75 a	9	40,0° C	0,165	0,040	0,062	0,409

Mittelwerte: $K_1 = 0{,}162 \pm 0{,}003$ $K_2 = 0{,}039 \pm 0{,}001$.

In Tabelle 11 sind die gefundenen Werte von K_1, K_2, A und B nebst den Mittelwerten von K_1 und K_2 verzeichnet. Die erste Versuchsreihe 60 a war zu kurz, um eine graphische Bestimmung von K_2 zu erlauben. Dort wurde der K_2-Wert der Reihe 61 a verwendet. Er wird deshalb bei der Reihe 60 a in Klammern gesetzt und bei der Berechnung des mittleren Wertes von K_2 ausgeschlossen. K_1 und K_2 sind ebenso gut reproduzierbar wie in der vorigen Arbeit [1] (mittlerer Fehler des Mittelwertes rund 6 % bzw. 4 %). Der Einfluss kleiner Mengen schwer zu entfernender Verunreinigungen in den benutzten Salzpräparaten (vgl. S. 61 oben) muss deshalb sehr unbedeutend sein. (Wenn eine etwaige Verunreinigung sich während der Isomerisation des Dinitritonitrats nicht verändert, übt es natürlich auf den (E_V—E_G)-Wert keinen Einfluss aus.)

Wenn man die drei Mittelwerte von K_1 graphisch gegen $\frac{1}{T}$ einzeichnet, wo T die absolute Versuchstemperatur ist, und durch die resultierenden Punkte die bestmögliche Gerade zieht, findet man für diese die Gleichung

$$\log K_1 = 12{,}660 - \frac{4220}{T} \tag{9}$$

Für K_2 erhält man in ähnlicher Weise

$$\log K_2 = 16{,}061 - \frac{5476}{T} \tag{10}$$

Der ersten Gleichung entspricht eine Aktivierungsenergie q' von 19,30 kcal und eine Aktionskonstante α' von $4{,}57 \cdot 10^{12}$ h^{-1}; der zweiten die analogen Werte $q'' = 25{,}05$ kcal und $\alpha'' = 1{,}15 \cdot 10^{16}$ h^{-1}. Dass K_1 4,2 bis 7,8 mal grösser als K_2 ist und dass der Quotient K_1/K_2 mit steigender Temperatur sinkt, wird durch die grössere Aktivierungsenergie in der zweiten Isomerisationsstufe bedingt.

Durch Tabelle 12 wird ein Vergleich zwischen den Daten für das 1,2-Dinitritodiäthylendiaminkobalt(III)-nitrat dieser Untersuchung mit denjenigen der vorigen Arbeiten [7,1] über Nitritopentamminkobalt(III)-nitrat und 1,6-Dinitritodipyridindiamminkobalt(III)-nitrat ermöglicht. Man findet erstens, dass für die obige *Trans*verbindung K_1 zwar von derselben Grössenordnung, aber etwa 35 % kleiner als für die *Cis*verbindung ist, dass K_2 andrerseits für das *Trans*nitrat rund zehnmal kleiner als für das *Cis*nitrat ist. Für die erste Isomerisationsstufe underscheiden sich die Werte der Aktivierungs-

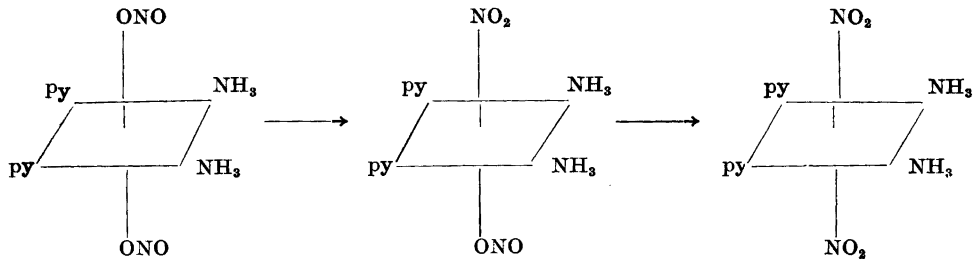
Tabelle 12. Die gefundenen kinetischen Daten der bisher untersuchten Nitrate mit einer oder zwei Nitritogruppen. (Dekadische Logarithmen; Zeit in Stunden angegeben.)

Komplexsalz	20° C		30° C		40° C	
	K_1	K_2	K_1	K_2	K_1	K_2
$[ONO \cdot Co(NH_3)_5]\ (NO_3)_2$	0,016	—	0,051	—	0,192	—
trans-$[(ONO)_2Co\ py_2(NH_3)_2]NO_3$	0,012	0,000254	0,033	0,00089	0,105	0,0039
cis-$[(ONO)_2Co\ en_2]\ NO_3$	0,0195	0,0025	0,049	0,0093	0,162	0,039

Komplexsalz	$K_1 : K_2$			q'	q''	α'	α''
	20° C	30° C	40° C	kcal	kcal	sec^{-1}	sec^{-1}
$[(ONO)Co(NH_3)_5](NO_3)_2$	—	—	—	21,89	—	$9,5 \cdot 10^{10}$	—
trans-$[(ONO)_2Co\ py_2(NH_3)_2]NO_3$	47,2	37,1	26,9	19,89	24,70	$2,2 \cdot 10^{9}$	$1,8 \cdot 10^{11}$
cis-$[(ONO)_2Co\ en_2]\ NO_3$	7,8	5,3	4,2	19,30	25,05	$1,3 \cdot 10^{9}$	$3,2 \cdot 10^{12}$

energie q' und der Aktionskonstante α' der beiden Salze nur wenig. Auch für die zweite Stufe ist die Aktivierungsenergie q'' der Dinitritosalze fast dieselbe, ist aber erheblich grösser als q'. Die zehnmal kleineren K_2-Werte der Transverbindung werden zum grössten Teil durch die bedeutend niedrigere Aktionskonstante dieses Salzes erklärt.

Das Kation von *cis*-$[(ONO)_2Co\ en_2]NO_3$ hat ja einen unsymmetrischen Bau, und das benutzte Salz war das Racemat zweier optischen Antipoden. Sowohl bei diesem Salze wie bei *trans*-$[(ONO)_2Co\ py_2(NH_3)_2]NO_3$ wäre es aus statistischen Gründen zu erwarten, dass, falls die beiden Nitritogruppen gleichwertig sind und ihre Isomerisation in ganz derselben Weise durchlaufen, K_1 gleich $2\ K_2$ sei. Ansatzweise lassen sich K_1 und K_2 für die erwähnte *Trans*verbindung mit Ausgangspunkt von der Konfiguration der Dinitrito-, Nitronitrito- und Dinitroionen:



in folgender Form schreiben:

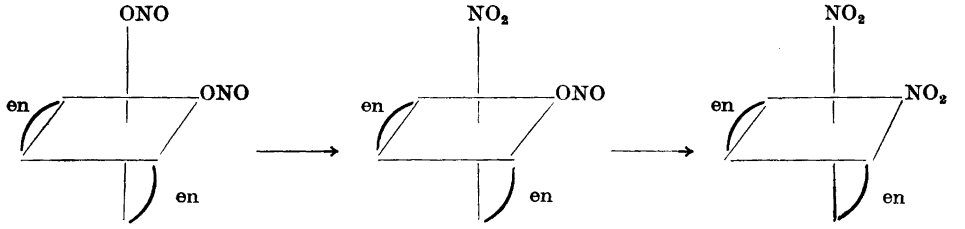
$$K_1 = 2\ k \cdot (f_{ONO})_a \cdot (f_{NH_3})_k^2 \cdot (f_{py})_k^2 \tag{11}$$

$$K_2 = k \cdot (f_{NO_2})_a \cdot (f_{NH_3})_k^2 \cdot (f_{py})_k^2 \tag{12}$$

k ist ein Proportionalitätsfaktor. $(f_{ONO})_a$, $(f_{NO_2})_a$, $(f_{NH_3})_k$ und $(f_{py})_k$ sind Koeffizienten, die den Einfluss auf die Geschwindigkeitskonstanten von bzw. einer Nitritogruppe in Axialstellung, einer Nitrogruppe in Axialstellung, einem Ammoniakmolekül in Kantenstellung und einem Pyridinmolekül in Kantenstellung im Verhältnis zu der sich umwandelnden Nitritogruppe ausdrücken sollen. (Es ist nicht mit Sicherheit festgestellt, ob die beiden Pyridinmoleküle der obigen Ionen sich in Axial- oder Kantenstellung zu einander befinden [8]. Das spielt hier aber keine Rolle.) Es scheint sehr wahrscheinlich, dass f_{NH_3} wie f_{py} dieselben Werte in (11) und (12) haben. Daraus gibt sich

$$\frac{K_1}{2\ K_2} = \frac{(f_{ONO})_a}{(f_{NO_2})_a} \tag{13}$$

Für eines der Spiegelbildisomeren vom *cis*-$[(ONO)_2Co\ en_2]^+$-Ion erhält man entsprechend



und

$$K_1 = 2\ k \cdot (f'_{ONO})_k \cdot (f'_{en})_{kk} \cdot (f'_{en})_{ka} \tag{14}$$

$$K_2 = k \cdot (f'_{NO_2})_k \cdot (f'_{en})_{kk} \cdot (f'_{en})_{ka} \tag{15}$$

$(f'_{en})_{kk}$ und $(f'_{en})_{ka}$ sind Masse der Einwirkung von Äthylengruppen, deren Koordinationsstellen sich in Kanten-Kanten- bzw. Kanten-Axialstellung zu der sich isomerisierenden ONO-Gruppe befinden. (Die Umwandlung der einen oder anderen Nitritogruppe im Dinitritoion gibt identische Nitronitritoionen.) Für das zweite Spiegelbildisomere vom *cis*-$[(ONO)_2Co\ en_2]^+$-Ion kommt man natürlich auch zu den Gleichungen (14) und (15). Wenn man dieselben Werte von $(f'_{en})_{kk}$ bzw. $(f'_{en})_{ka}$ bei der ersten und zweiten Isomerisationsstufe voraussetzt, so findet man für die Isomerisation des Racemates, der Gleichung (13) entsprechend.

$$\frac{K_1}{2K_2} = \frac{(f'_{ONO})_k}{(f'_{NO_2})_k} \tag{16}$$

In der Abweichung des Quotienten $K_1/2K_2$ vom Werte eins würde man somit einen Ausdruck für die verschiedenartige Einwirkung einer zweiten ONO- und einer NO_2-Gruppe auf die Isomerisationsgeschwindigkeit einer ONO-Gruppe haben. Für das *Trans*dinitritodipyridindiamminnitrat mit $K_1/2K_2$ gleich etwa 18,5 macht sich dieser Unterschied viel kräftiger bemerkbar als für das *Cis*dinitritodiäthylendiaminnitrat, wo $K_1/2K_2$ den ungefähren Wert 2,9 hat, und dies trotz dem grösseren, räumlichen Abstand zwischen der ONO- bzw. NO_2-Gruppe und der sich isomerisierenden Nitritogruppe bei der *Trans*verbindung. (Vgl. Tabelle 12.)

In diesem Zusammenhange ist es von Interesse, dass Grünberg [9] und Jensen [10] bei stereoisomeren Diaquodiamminplatoionen eine sogenannte Transwirkung gefunden haben. Jensen hatte für *cis*-$[Pt(H_2O)_2(NH_3)_2]^{2+}$ die Säuredissoziationskonstanten $K'_s = 2{,}76 \cdot 10^{-6}$ und $K''_s = 4{,}79 \cdot 10^{-8}$ wie für das stereoisomere *Trans*-ion die Werte $K'_s = 4{,}79 \cdot 10^{-5}$ und $K''_s = 4{,}17 \cdot 10^{-8}$ bei 20° C und in wässeriger Lösung bestimmt. Für diese symmetrischen, zweibasischen Kationsäuren macht die Abweichung der Grösse $K'_s/4\ K''_s$ vom Werte eins ein Mass aus für die Verschiedenheit der Einwirkung einer Aquo- und einer Hydroxogruppe auf die Dissoziation einer zweiten Aquogruppe. Für das *Cis*-ion wird $K'_s/4K''_s$ gleich 14,5, für das *Trans*-ion 288 berechnet. Die erwähnte Verschiedenheit macht sich also bei der Transverbindung viel stärker geltend. Das wird mit der Transwirkung in Zusammenhang gesetzt, d. h. damit, dass bei der Wechselwirkung koordinierter Gruppen die quantenmechanische Wirkung durch die chemischen Bindungen hindurch erheblich grösser als die elektrostatische Feldwirkung sein kann.

Es muss aber betont werden, dass die grosse Differenz im $K_1/2K_2$-Wert für die hier fraglichen festen Dinitritonitrate nicht nur von der Verschiedenartigkeit der Stellung der Dinitritogruppen, sondern auch von der andersartigen Natur der übrigen Liganden in der inneren Sphäre bedingt werden

kann. Deshalb wird eine Untersuchung über die Isomerisationsgeschwindigkeit des festen *trans*-Dinitritodiäthylendiaminkobalt(III)-nitrats geplant, deren Daten mit denjenigen der entsprechenden *Cis*verbindung dieser Arbeit zu vergleichen sind. Dabei wird nur die Unähnlichkeit im räumlichen Bau der Kationen mit hineinspielen.

Die Zuverlässigkeit der Konfigurationsbestimmung von den fraglichen Verbindungen ist natürlich eine unerlässliche Bedingung jeder Diskussion der obigen Art. Bei $\left[\begin{matrix}(1)\ ONO\\(2)\ ONO\end{matrix}\ Co\ en_2\right] NO_3$ ist die Konfiguration durch die Spaltbarkeit der entsprechenden Dinitroverbindung in zwei Spiegelbildisomere ermittelt worden. Bei $\left[\begin{matrix}(1)\ ONO\\(6)\ ONO\end{matrix}\ Co\ \begin{matrix}py_2\\(NH_3)_2\end{matrix}\right] NO_3, H_2O$ andrerseits gründet Werner [11] die Konfigurationsbestimmung auf eine empirisch gefundene Regel. Die entsprechende feste Dinitroverbindung, das Endprodukt der Isomerisation, gibt nämlich wie alle 1,6-Dinitrosalze, mit konzentrierter Salzsäure verrieben, das intensiv rote Chlorid $\left[\begin{matrix}(1)\ Cl\\(6)O_2N\end{matrix}\ Co\ \begin{matrix}py_2\\(NH_3)_2\end{matrix}\right] Cl_2, H_2O$. Durch einen besonderen Versuch habe ich bestätigt, dass auch das entwässerte Salz $\left[\begin{matrix}(1)\ ONO\\(6)\ ONO\end{matrix}\ Co\ \begin{matrix}py_2\\(NH_3)_2\end{matrix}\right] NO_3$, das in der vorigen Untersuchung [1] benutzt wurde, nach vollständiger Isomerisation die obige Reaktion zeigte. Der Schluss auf die Axial- oder Kantenstellung der Dinitritogruppen ist natürlich nur so lange bündig, als man annehmen darf, dass diese Gruppen während der Isomerisation ihre gegenseitige Stellung innerhalb des Komplexions nicht verändern, und für die Transverbindung ausserdem, dass die erwähnte empirische Regel ausnahmslos gilt.

ZUSAMMENFASSUNG

1. Für $\left[\begin{matrix}(1)\ O_2N\\(2)\ O_2N\end{matrix}\ Co\ en_2\right] NO_3$ und $\left[\begin{matrix}(1)\ ONO\\(2)\ ONO\end{matrix}\ Co\ en_2\right] NO_3$ werden Extinktionskurven wässeriger Lösungen bei 20,0° C und in den Wellenlängengebieten 380—560, bzw. 390—570 mμ mit einem Beckman-Quarz-Spektrophotometer (Modell DU) ermittelt. Für das Dinitritonitrat ergeben sich wegen der Isomerisation des komplexen Kations nur annähernde Extinktionswerte. Das Dinitronitrat hat bei 390 mμ ein Absorptionsminimum und bei 435 mμ ein Maximum; für das Dinitritonitrat liegt das Minimum bei 415 mμ und das Maximum bei 455 mμ. Im letzten Fall sind beide somit gegen grössere Wellenlängen verschoben. Das Maximum ist hier auch bedeutend kleiner als bei der Dinitroverbindung.

2. Es wird der Isomerisation von festem $\left[\begin{matrix}(1)\ \mathrm{ONO} \\ (2)\ \mathrm{ONO}\end{matrix}\ \mathrm{Co\ en_2}\right] \mathrm{NO_3}$ bei 20,0°, 30,0° und 40,0° C in etwa derselben Weise wie in einer früheren Arbeit [1] dadurch gefolgt, dass man von Zeit zu Zeit Proben der konstanten Zusammensetzung

$$0{,}004\ M\ \text{Komplexsalz} + 0{,}01\ M\ \mathrm{HOCOCH_3} + 0{,}01\ M\ \mathrm{NaOCOCH_3}$$

bereitet und deren Extinktion $(E_V)_T$ im Auflösungszeitpunkte T des festen Salzes bei der Wellenlänge $\lambda = 510$ mμ, der Schichtdicke 3,00 cm und der Temperatur 20,0° C bestimmt.

3. $(E_V)_T$ wird durch graphische Extrapolation aus 10 bis 12 innerhalb etwa 20 Minuten und t Minuten nach T gemessenen Extinktionswerten $(E_V)_{T+t}$ der fraglichen Versuchslösung ermittelt. Die $(E_V)_{T+t}$-Werte ergeben, gegen t eingezeichnet, Punkte, die sehr nahe an einer Gerade liegen.

4. Mit wachsender Zeit fällt $(E_V)_T$ (im folgenden E_V geschrieben) und nähert sich langsam als Grenzwert der Extinktion E_G einer Versuchslösung der Zusammensetzung 0,004 M Dinitronitrat + 0,01 M $\mathrm{HOCOCH_3}$ + 0,01 M $\mathrm{NaOCOCH_3}$ (die übrigen Versuchsbedingungen gleich den in 2 oben angegebenen).

5. Das Dinitronitrat wird durch Erhitzen des Dinitritoisomeren über Nacht auf 60° C hergestellt.

6. Die gefundenen $(E_V—E_G)$-Werte können sehr gut in Formeln vom Typ

$$E_V—E_G = C \cdot 10^{-\lambda_1 \cdot t} + D \cdot 10^{-\lambda_2 \cdot t}$$

zusammengefasst werden. Die Konstanten C, D, λ_1 und λ_2 werden graphisch wie in der vorigen Untersuchung [1] aus logarithmischen Kurven bestimmt.

7. Es wird gezeigt, dass die Gleichungen dieser Form sich durch die Annahme einer zweistufigen Isomerisation:

$$\left[\begin{matrix}\mathrm{ONO} \\ \mathrm{ONO}\end{matrix}\ \mathrm{Co\ en_2}\right] \mathrm{NO_3} \longrightarrow \left[\begin{matrix}\mathrm{O_2N} \\ \mathrm{ONO}\end{matrix}\ \mathrm{Co\ en_2}\right] \mathrm{NO_3} \longrightarrow \left[\begin{matrix}\mathrm{O_2N} \\ \mathrm{O_2N}\end{matrix}\ \mathrm{Co\ en_2}\right] \mathrm{NO_3}$$

verstehen lassen, die als zwei konsekutive Umwandlungen erster Ordnung verläuft. Wenn man die Geschwindigkeitskonstanten der ersten und zweiten Teilisomerisation mit K_1 und K_2 bezeichnet, dann muss K_1 gleich λ_1 und K_2 gleich λ_2 sein, wo λ_1 grösser als λ_2 ist.

8. Für K_1 resultieren bei 20,0°, 30,0° und 40,0° C die Werte 0,0195; 0,049 und 0,162 und für K_2 0,0025; 0,0093 und 0,039 (dekadische Logarithmen;

Zeit in Stunden angegeben). Daraus ergibt sich für die erste Isomerisationsstufe die Aktivierungsenergie $q' = 19{,}30$ und die Aktionskonstante $a' = 1{,}3 \cdot 10^9$ sec^{-1}; für die zweite entsprechend $q'' = 25{,}05$ kcal und $a'' = 3{,}2 \cdot 10^{12}$ sec^{-1}.

9. Der Quotient K_1/K_2 macht also bei 20,0°; 30,0° und 40,0° 7,8; 5,3 und 4,2 aus, während er für das früher[1] untersuchte Salz $\left[\begin{matrix}(1)\ ONO \\ (6)\ ONO\end{matrix}\ Co\ \begin{matrix}py_2 \\ (NH_3)_2\end{matrix}\right]NO_3$ bei bzw. 47,2, 37,1 und 26,9 und also erheblich höher liegt. Diese Tatsache wird diskutiert.

Meinem Assistenten Boo Frejrud verdanke ich eine intensive und interessierte Mitarbeit. Sein Anstellen wurde durch mir von *Statens Naturvetenskapliga Forskningsråd* bewilligte Mittel ermöglicht. Hierfür sage ich meinen besten Dank. Dem Leiter unseres Instituts, Herrn Professor Dr. S. Bodforss, danke ich für freundliches Entgegenkommen, das die Arbeit in mehreren Hinsichten erleichtert hat. Das in dieser Arbeit verwendete Äthylendiamin ist mir von A. B. Leo, Hälsingborg, durch Herrn Zivilingenieur Ove Fernö geliefert worden. Ich bin ihm für seine grosse Zuvorkommenheit sehr dankbar.

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On the Use of Rayleigh-Philpot-Cook Interference Fringes for the Measurement of Diffusion Coefficients

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It is known that certain types of interferometers in conjunction with an optical device for making an image of a certain object are capable of giving interferometric patterns which are to be regarded as topographic maps of the object, each interference fringe indicating a change of the optical thickness of one wave-length. This fact was made use of by Labhart and Staub [1] for electrophoresis measurements. They used a Jamin interferometer. Later, Lotmar [2] has presented a number of possible optical arrangements more or less based on the Michelson interferometer.

In the Rayleigh interferometer, it is necessary to produce an image of the light source slit. Hence the possibility of producing a topographic map of an object does not exist in this case because the introduction of a lens focused on the object would destroy the interference phenomenon altogether. However, using an astigmatic optical system, it is sufficient to produce an image of the slit in the plane perpendicular to it; in its own plane, it need not be in focus. Consequently, in the image of the slit, the dimension along the same is free for the production of an optical image of the object. Hence it is possible, using the Rayleigh interferometer, to obtain one-dimensional interferograms of an object whose optical thickness does not vary in the direction perpendicular to the slit. Since in diffusion cells, electrophoresis cells, etc., the optical thickness is constant along a horizontal line, it is evident that a Rayleigh interferometer in combination with an astigmatic optical system is capable of yielding exact information of the refractive index course along the vertical coordinate.

This fact was first understood and tested experimentally by Philpot and Cook [3] and, independently, by the present author [4*]. It has later been found

* Added in proof: Rögener describes in a recent article (*Kolloid-Z.* **118** (1950) 10) an optical system which was designed in the years 1943/44 and which, to judge from the interferograms published, works according to the same principles as the Philpot-Cook system. However, the

that the optical system widely used for more than 10 years in electrophoretic measurements, the diagonal slit method, serves the purpose as well (Svensson [5]). The integral fringe method, so called because it gives directly the refractive index function and not the derivative, has already been tested by Longsworth [6] in connection with an investigation of two new types of diffusion cells, and its importance in electrophoresis has also been stressed by him (Longsworth [7]). The present author has not until now had the opportunity of making methodical experiments with the new method. In this paper, the use of the integral fringe method for the determination of diffusion coefficients by the height-area method will be described. In addition, the method of computation used by Longsworth will be considered.

EXPERIMENTAL

The optical system of the diagonal slit method (see Svensson [5], Fig. 1) was used in this investigation with the modification that the light source slit was vertical and the diagonal slit removed. On adjusting this system, it was found that the angular orientation of the cylindrical lens was very critical, much more than in the diagonal slit method. By screening off the slit to essentially a point source, however, the fringes could always be easily found. Then the point source was gradually extended to a slit, the cylindrical lens being turned a little after each increase in length in order to retain the fringes. After the slit had been opened to its total length again, a final adjustment of the cylindrical lens gave fringes as bright and well-defined as in the optical system originally suggested by Philpot and Cook.

The diffusion cell was the flowing-junction cell which was described in an earlier publication (Svensson [4]). It is very similar to the stainless steel cell recently described by Longsworth [6], the main difference being that the suction slit of the latter can be closed. Unfortunately, no thermostat was available during these experiments, but they were carried out in an underground room without windows and with a remarkably constant temperature. Distilled water was constantly stored in this room, and the preparation to be studied was dissolved in this water an hour or two before the experiment was started.

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very essential feature of producing an optical image of the cell in elevation is not mentioned in the text, nor is it evident from the optical arrangement. Possibly Rögener's second slit-focusing lens serves this purpose also. Rögener's earlier arrangement (*Kolloid-Z.* **105** (1943) 110) works with horizontal slits and is more related to the Gouy interference method than to the Philpot-Cook method.

An attempt to use the Rayleigh interferometer for diffusion studies has also been made by Kroepelin (*Sitzungsber. physikal.-med. Soz. Erlangen* **58/59** (1926/27) 237), yet without any cell-focusing device.

Care was taken not to warm up the water or the diffusion cell by touching or by radiation from the body. A thermometer placed close to the cell showed, in general, a variation in temperature of less than $\pm$ 0.1° C during an experiment.

Cane sugar (Merck) of analytical purity was chosen as the test substance. Very accurate measurements of the diffusion of this preparation have recently been performed by Gosting and Morris [8].

As also recommended by Longsworth [6], the first fringe photograph was taken before starting, with both solutions still flowing through the exit slit. This photograph was subsequently used for measuring the fractional part of the total fringe displacement between top and bottom solutions. The time for closing the stop-cock in connection with the exit slit was noted as the experimental zero time of the diffusion. Five to eight exposures were taken during the diffusion process, the last one 14 000 to 16 000 seconds after the start.

The cross section of the diffusion channel was round 3 $\times$ 50 mm^2. The reference channel was filled with distilled water.

COMPUTATION OF THE DIFFUSION COEFFICIENT BY THE HEIGHT-AREA METHOD

This method makes use of the equation:

$$D = \frac{(n_1 - n_2)^2}{4\ \pi\ t\ n'^2_{\text{max.}}(x)} \tag{1}$$

where n_1 and n_2 are the refractive indices of the two solutions, t the time, and $n'_{\text{max.}}(x)$ is the maximum derivative of the refractive index function with respect to the position in the cell. Since a fringe is, in this case, as good a unit of refractive index as any other, and since n is present in the same power in numerator and denominator, the readings need not be recalculated to real refractive index units. Consequently the calculation can be carried out without knowledge of the thickness of the cell.

The integer part of the total fringe displacement was counted from any one of the later exposures, while the fractional part was measured from the first exposure in a comparator with a cross-motion arrangement for the table. The plate was aligned in the comparator so that the hair-cross in the microscope, on moving the table cross-wise, followed the middle of a fringe along the whole half-cell on one side of the boundary. The distance between the hair-cross and that fringe on the other side of the boundary which was last passed by it on bringing this side into view, was then measured by moving the

table in the direction of the micrometer screw (perpendicular to the fringes). Similarly, the distance between two consecutive fringes was measured. This measurement should be carried out across the original position of the hair-cross since the distance between fringes varies somewhat across the interferogram, especially if the number of fringes is small. This is explained by the fact that the outer fringes are situated on the sloping light intensity curve in the central diffraction band. The ratio between the two distances thus measured is the fractional part of the total fringe displacement. It could be measured with a reproducibility of 0.02 fringes.

The experimental determination of the maximum derivative is more difficult. Taking the differences between consecutive readings and inverting them gives rise to large accidental errors and too large a spreading in the resulting derivative curve. On the other hand, taking the differences between, say, every tenth fringe and dividing 10 by them, is likely to give little spreading but serious systematic deviations from the true derivative. Plotting the integral curve and using a mechanical differentiator gives an accuracy far behind that inherent in the interferogram.

In order to find the best way of computing the maximum derivative, we will study the systematic deviation from the true derivative resulting from taking too large differences in the numerical differentiation. In every numerical differentiation, the quantity $\Delta n/\Delta x$ is measured. By writing this quantity in the form:

$$\frac{\Delta n}{\Delta x} = \frac{n(x + \Delta x/2) - n(x - \Delta x/2)}{\Delta x} \tag{2}$$

and by development into powers of Δx, we get the following third-order approximation:

$$\frac{\Delta n}{\Delta x} = n'(x) + \frac{(\Delta x)^2}{24} n'''(x) \tag{3}$$

If we require that the third-order term be less than a certain fraction ϱ of the main term, we get the condition:

$$(\Delta x)^2 < \frac{24\, \varrho\, n'(x)}{n'''(x)} \tag{4}$$

At the top of the Gaussian curve this reduces to:

$$(\Delta x)^2 < 48\, \varrho\, Dt \tag{5}$$

Table 1. Determination of the maximum derivative.

Comparator reading mm	Δx for $\Delta n = 5$ mm
23.287	
23.729	
24.132	
24.546	
24.930	
25.312	3.799
25.673	3.709
26.051	3.643
26.380	3.576
26.748	3.513
27.086	3.469
27.438	3.462
27.775	3.439
28.122	3.435
28.443	3.433
28.781	3.420
29.135	3.435
29.490	3.449
29.815	3.497
30.181	3.522
30.506	3.585
30.873	3.643
31.224	3.707
31.619	3.793
31.965	
32.366	
32.778	
33.197	
33.608	

This is the condition in terms of the abscissa increment that has to be satisfied in a numerical differentiation with the precision ϱ. For the interferograms in question, however, it is more convenient to have a condition in terms of the ordinate increment since the ordinates are integers. Consequently, we introduce the value of Δx according to (5) into the numerator of (2) and get:

$$\Delta n = \Delta x \; n'(x) = (n_1 - n_2) \sqrt{\frac{12 \, \varrho}{\pi}} \tag{6}$$

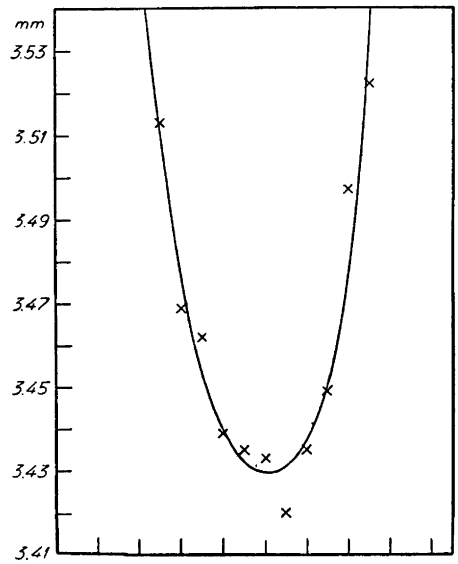

Fig. 1. The distance between every fifth fringe as a function of the fringe number.

For a precision of 1 part in 1 000, therefore, we get a permissible ordinate difference of 0.062 (n_1—n_2), *i. e.*, with 50 fringes between the two solutions, one can measure the distance between every third fringe without introducing a greater relative error than 0.1 per cent. However, the author prefers to use still greater ordinate differences and to apply the correction ϱ according to the equation:

$$\varrho = \frac{\pi}{12}\left(\frac{\Delta n}{n_1 - n_2}\right)^2 \tag{7}$$

If the correction is allowed to rise to one per cent, it is possible to compute the maximum derivative using ordinate differences of 20 per cent of the total refractive index change. The relative accidental errors are then extremely small. It should be noted that the correction (7) is independent of the time.

The procedure that has been followed in the determination of the maximum derivative is consequently the following. After the plate had been aligned on the comparator table, every fringe or half-fringe was measured throughout a region round the centre of the boundary comprising about 40 per cent of the total number of fringes. The readings were written down in a table, and the differences between every mth fringes were taken, the integer m being chosen in each case to give a correction ϱ of about one per cent. The differences were plotted against the fringe number in a diagram, and a smooth curve was drawn through the scattered points. The minimum of this curve was read. Division of the integer m by this minimum gave the approximate derivative

Table 2. The calculation of diffusion coefficients by the height-area method.

Time sec.	Maximum derivative fringes per cm	Inverted and squared maximum derivative	Time calculated sec.	Time discrepancy sec.
187	93.47	0.0001145	166	+ 21
480	60.15	0.0002764	469	+ 11
1080	40.44	0.0006115	1097	− 17
2220	28.68	0.0012157	2228	− 8
3180	24.02	0.0017332	3197	− 17
5640	18.18	0.0030256	5617	+ 23
8580	14.73	0.0046089	8582	− 2
Equation of the line: $n'^2(x)\ (t + 48) = 1.8725 \cdot 10^6$				

according to (2), and finally the correction (7) was computed and applied to give the true derivative.

Table 1 and Fig. 1 give a typical example of such a calculation. The uncertainty in the minimum value of Δx is round 0.003 mm, which is consistent with the sharpness of the fringes and their separation. The relative accidental error, therefore, becomes of the order of 0.001.

The data in Table 1 are taken from an experiment with an 0.2 per cent sugar solution diffusing against water. With the cell used, round 50 mm thick, this gave 26.43 fringes. In all exposures, the ordinate increments used in the differentiation were 5 fringes, and the correction applied was $\varrho = 0.0094$. In Table 2, the first column gives the experimental times from the start of the diffusion, whereas the second column gives the corrected maximum derivatives. In the third column, we have the squared and inverted values of the maximum derivatives, which, according to equation (1), should be proportional to the time. After having controlled in a plot that no point deviated seriously from a linear relationship between the data in the columns 1 and 3, the equation of the straight line that best satisfied the data was computed with the aid of the method of least squares. The constant term in this equation is the zero-time correction, which serves as a quantitative measure of the quality of the starting boundary and of the reliability of the experiment as a whole. The second parameter of the equation of the straight line is the slope, which was inserted into equation (1) to give the diffusion coefficient. The two last columns in Table 2 contain the times calculated from the equation of the straight line and the discrepancy between them and the experimental times. The latter is in no case greater than the time of exposure, 30 seconds.

Table 3. Results.

Sugar concentration per cent	Total fringe displacement	Mean temperature °C	Zero-time correction seconds	Diffusion constant $cm^2/sec. \cdot 10^6$	Remarks
0.2	26.43	20.6	46	5.231	Two calculations of
			48	5.221	the same material
0.5	66.33	21.1	21	5.250	
0.5	66.33	21.5	52	5.198	
0.5	67.80	21.65	8	5.201	
0.5	67.16	20.6	63	5.234	
0.75	98.46	20.5	59	5.246	
0.25	33.81	20.3	55	5.301	Large temperature variation
0.5	67.1	20.1	63	5.38	The same, and bad starting boundary

In all, eight experiments were carried out. In order to compare the diffusion constants obtained with the values given by Gosting and Morris [8], corrections were applied to zero concentration using Gordon's relation and to 25.0° C using Stokes-Einstein's relation (see Gosting's and Morris' paper). These data are given in Table 3. The first column gives the concentration of sugar, the second the total number of fringes, the third the temperature, the fourth the zero-time correction, and the fifth the diffusion constant corrected to zero concentration and to 25.0 °C.

DIRECT COMPUTATION OF THE DIFFUSION COEFFICIENT FROM THE POSITION OF THE INTEGRAL FRINGES

The numerical differentiation of the refractive index function in the cell in order to use equation (1) may seem to be a somewhat round-about way since the integral of the error function is well known and available in mathematical tables. By direct comparison between the coordinates of the fringes and a table of the integral function it is possible to derive a value of the diffusion constant for each fringe or half-fringe. This method, which was originally suggested by Gosting and described by Longsworth [6], is, like the former method, based on the assumption that the diffusion is ideal, but it is capable of showing up deviations from the ideal behaviour very clearly since calculations can be carried out from every part of the curve.

The procedure adopted by the author was the following. The interferogram in question was aligned in the comparator so that the fractional part of the

Table 4. Normal analysis of the boundary.
$(n_1-n_2 = 66.33)$

Fringe number (ν)	Comparator reading mm	$\frac{n_1-n_2-2\nu}{n_1-n_2}$	z	Plate coordinate calculated	Discrepancy μ
0.5	16.460	0.98492	1.7186	16.462	− 2
1	17.955	0.96985	1.5331	17.942	+ 7
2	19.579	0.93969	1.3283	19.577	+ 2
3	20.615	0.90954	1.1971	20.624	− 9
4	21.404	0.87939	1.0976	21.418	− 14
6	22.613	0.81908	0.9461	22.627	− 14
8	23.564	0.75878	0.8287	23.565	− 1
10	24.350	0.69848	0.7306	24.348	+ 2
12	25.028	0.63817	0.6448	25.032	− 4
15	25.938	0.54771	0.5315	25.937	+ 1
18	26.751	0.45726	0.4304	26.744	+ 7
21	27.489	0.36680	0.3375	27.485	+ 4
24	28.190	0.27634	0.2500	28.183	+ 7
27	28.853	0.18589	0.1663	28.851	+ 2
30	29.513	0.09543	0.0848	29.502	+ 11
35	30.577	− 0.05533	− 0.0491	30.571	+ 6
40	31.658	− 0.20609	− 0.1847	31.653	+ 5
43	32.325	− 0.29655	− 0.2692	32.327	− 2
46	33.036	− 0.38700	− 0.3577	33.034	+ 2
47	33.281	− 0.41716	− 0.3884	33.279	+ 2
50	34.054	− 0.50761	− 0.4855	34.054	0
53	34.904	− 0.59807	− 0.5927	34.909	− 5
55	35.540	− 0.65837	− 0.6724	35.546	− 6
57	36.255	− 0.71868	− 0.7618	36.259	− 4
59	37.076	− 0.77898	− 0.8654	37.086	− 10
61	38.080	− 0.83929	− 0.9919	38.096	− 16
63	39.447	− 0.89959	− 1.1617	39.451	− 4
65	41.782	− 0.95990	− 1.4515	41.764	+ 18
66 *	44.885	− 0.99005	− 1.8227	44.726	+ 159

* Excluded from the treatment by least squares.

fringe number became the same as that measured in the exposure of the flowing boundary. Then the position of a great number of fringes (the measurement and computation of every intensity maximum and minimum is generally too time-consuming) was measured throughout the entire interferogram. In Table 4, the first column gives the number of the fringe (number 0 being that fringe with which the hair-cross of the microscope coincided outside the bound-

ary), and the second column contains the comparator readings. The third column is headed $(n_1—n_2—2\nu)/(n_1—n_2)$ and is the fraction of the total area of the error curve enclosed between the comparator reading and the symmetrically situated coordinate on the other side of its centre. In a Table of the integral function, the values of the independent variable corresponding to these figures were found by interpolation, column 4. The figures in the columns 2 and 4 should now show a linear relationship, which was controlled in a plot. The readings of the first and last fringes, running almost parallel to the micrometer screw, are naturally very inaccurate and must often be discarded. The rest of the points were treated by the method of least squares to get the equation of the straight line. The plate coordinates corresponding to this line are presented in column 5, and the discrepancies between them and the observed values, column 2, are given in column 6. With few exceptions, they are smaller than 1/50 of the distance between consecutive fringes. This means that, in this particular case, the concentration distribution in the boundary was that required by the law of ideal diffusion.

From the slope of the line, the diffusion constant can be calculated with the aid of the equation:

$$D = \frac{x^2}{4\,t\,z^2} \tag{8}$$

and these values check reasonably well with those obtained by the height-area method. However, to get good results the zero-time correction has to be applied, which necessitates the complete evaluation of all exposures according to the above procedure or to use the zero-time correction derived from the height-area method. Since the former alternative would be too time-consuming, the author has restricted the use of that method to one exposure in each experiment in order to control the shape of the boundary. Even if the method of least squares is omitted, the interpolation of a great number of data in the table of the integral function remains and is very laborious. Although the method is more direct than the height-area method, the differentiation being omitted, it requires much more work.

DISCUSSION

If the two last experiments in Table 3 are excluded, the individual diffusion constants deviate from the mean value, $5.226 \cdot 10^{-6}$, by less than 0.5 per cent. The value given by Gosting and Morris for 24.95 °C is 5.224, which yields 5.231 at 25.0 °C. The latter figure is 0.1 per cent higher than the mean value from Table 3. Taking into consideration that these experiments were

carried out without thermostating and that great temperature corrections have been applied, the conclusion seems justified that the new method of observation is capable of a precision that can be compared with that of the Gouy method. Moreover, the results seem to indicate that accurate temperature regulation is not as essential in diffusion measurements as is generally believed.

From the normal analysis of the boundary carried out as suggested by Gosting and Longsworth directly from the integral fringes and as demonstrated in Table 4, two interesting conclusions can be drawn. First, the exact linear relationship between the comparator readings and the quantity z shows that Longsworth [6] was right when he blamed the cylindrical lens for the non-linear relationship found by him. In his optical system, which was identical with that originally described by Philpot and Cook, an uncorrected cylindrical lens was used at a comparatively high relative aperture (axis horizontal, point source). In the present investigation, the cylindrical element had its axis parallel to the light source slit, hence it was active at a very low relative aperture. In addition, it was spherically and chromatically corrected.

The possibility of using the diagonal slit method simultaneously with the integral fringe method, as discussed in reference 5, also speaks in favour of that optical system. However, if no use is made of the diagonal slit method, there is no point in using the longer optical system required by the two-fold image formation of the light source slit. In such cases, other ways of restricting the relative aperture of the cylindrical lens should be tried. One possibility is to use an astigmatic lens system composed of a spherical objective and a negative cylindrical lens with a vertical axis. The spherical objective, then, is focused on the cell and the compound objective on the light source slit. The conditions for interference are then again fulfilled.

The second conclusion that can be drawn from the normal analysis is that the diffusion cell is working satisfactorily. It is certainly free of leakage since there are no sliding parts. The optical precision is good; clamping of windows in general gives rise to less distortion than does cementing. The small volume of solution which is left in the exit slit and which could be feared to cause trouble by back-diffusion has been found to be unimportant. These conclusions are in agreement with Longsworth's tests of similar diffusion cells of the flowing junction type.

The method of direct computation of the diffusion constant by comparison with the tables of the integral of the error function renders good service as a method of normal analysis. As a method of calculating the diffusion constant, it involves an excessive amount of work if use is made of every fringe in every exposure. In Table 4, only 29 readings were taken out of 133 possible read-

ings. The number of readings can of course be reduced still more, with a corresponding reduction of the numerical calculations, if the diffusion is ideal.

The calculation with the aid of the height-area method is fairly rapid. It involves the measurement, under standard conditions, of some 20 fringes, taking and plotting of some 10 differences, and some numerical work with the minimum difference, everything in each exposure. The determination of the total fringe displacement is simple. The work is not in any way overwhelming, yet it would be very advantageous with a more convenient way of determining the maximum derivative. The differential interference refractometer described recently (Svensson [9]) will probably be valuable in this respect. It requires only four plano-parallell glass plates in addition to the optical equipment of the integral interferometer. From the differential interferogram, the maximum derivative (as well as the derivative in any point) can be directly measured. The use of this method, in combination with the integral fringe method, will be described in a forthcoming communication.

A comparison between this type of interferometer and those giving the optical path differences as functions of both dimensions in the cell mentioned in the beginning of this article reveals the following. In the case of optical distortions along horizontal lines in the cell, the Rayligh-Philpot-Cook interferogram is blurred, and it will be necessary to use only a narrow vertical strip of the cell for the optical analysis. With a Michelson or Jamin interferometer, direct information of the lateral distortions in the cell is gained, and no blurring of the fringes occurs. On the other hand, the Rayleigh-Philpot-Cook interferometer has two great advantages, that of permitting direct photographing of the refractive index function, and the possibility of measuring small fractions of a wave-length. The arrangement described by Labhart and Staub [1] has the former advantage, but not the latter, while that described by Antweiler [10] possesses the latter advantage, but not the former. Fractions of wave-lengths, however, can be measured in Labhart's and Staub's arrangement by visual observation during the experiment if Antweiler's device for that purpose is introduced. Correspondingly, Antweiler's arrangement permits photography if a monochromatic light source is applied. In no case, however, can fractions of wave-lengths be measured on the photographic plates.

SUMMARY

The use of the interference refractometer devised by Philpot and Cook in diffusion measurements has been submitted to experimental tests. Although a differentiation is necessary, it has been shown that the conventional height-area method of computing diffusion coefficients can be used successfully. A

detailed description of a method for the accurate determination of the maximum derivative of the refractive index in the cell has been presented.

The direct comparison between the fringe distribution with tables of the integral of the error function, as suggested by Gosting and Longsworth, has also been tested, and the results discussed.

The influence of too poor a correction of the cylindrical element of the optical system has been discussed in relation to the relative aperture of this lens. Optical systems have been proposed in which the cylindrical element is working at a very low relative aperture. In such systems, it should be possible to get satisfactory results even with simple cylindrical lenses.

This investigation is part of a research program for the development of improved methods of optical analysis of stationary and flowing liquids, which program is generously supported by the Swedish Technical Research Council. Laboratory facilities and additional financial aid has been given by *LKB-Produkter Fabriksaktiebolag*, Stockholm, which is also gratefully acknowledged. For valuable assistance the author is indepted to Mr. Karl Odengrim.

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Studies of the Constituents of Crassulacean Plants

I. Paper Chromatographic Investigation of the Free Sugars of some *Sedum*, *Sempervivum*, *Echeveria*, and *Crassula* Species

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The study of naturally-occuring sugars has been greatly facilitated by the introduction of paper partition chromatography [1], the substances being identified by their R_f-values with various solvent systems, and by the colours produced with various spraying reagents.

The plant family *Crassulaceae* is interesting from a biochemical point of view, among other reasons, because there has been found in certain species of this family a seven carbon ketose, *sedoheptulose* (D-altroheptulose, *cf. Advances in Carbohydrate Chemistry* I, p. 47) [2]. Identification of this sugar has previously been effected by the isolation of derivatives such as the dibenzylidene derivative of the anhydro sugar or the osazone. Sedoheptulose gives, like the other heptoses, a colour reaction with orcinol and hydrochloric acid (Bial's reagent); in this case the colour is bluish-green. The reaction is, however, not specific, as pentoses and uronic acids give approximately the same colour. As described elsewhere [3], we have found a spraying reagent which seems to give a specific colour reaction with ketoheptoses on paper chromatograms. As both the location of the spots on the chromatogram and the colour produced with this reagent are characteristic, we think that the method affords complete certainty for the detection of ketoheptoses. A few drops of fresh juice (the plants are succulents) are sufficient for the examination of the free sugars in the plants.

The objects of the investigations described here have been:

1. to study the distribution of sedoheptulose in the plant family *Crassulaceae* and possibly in closely related families,
and
2. to investigate the presence of other free sugars in these plants.

With respect to the last point there appears to be some disagreement in the relevant literature. According to Hudson (*Advances in Carbohydrate Chemistry* I, p. 14) [2] fermentable sugars are absent from the tissues in which heptuloses (ketoheptoses) occur, and our previous researches had supported this contention. This presupposes, however, a special form of carbohydrate metabolism, as the plants contain starch. In plantphysiological studies of Crassulacean metabolism investigators have made particular use of *Bryophyllum calycinum* Salisb. [e.g. 4, 5], a Crassulacean plant which contains sedoheptulose. In this case they have shown that at least a part of the sugar mixture is fermentable, and have calculated this part as glucose in the usual way. It has also been found that there exists a genetic connection between starch and fermentable sugars on the one hand and organic acids on the other [4,5].

Our investigations have shown that all the species examined contain sedoheptulose and in addition, fermentable sugars. The proportions of the individual sugars are, however, different. Sedoheptulose is the predominating sugar in most of the species we have examined. In the genus *Crassula* it seems, however, that the hexoses predominate. We have not carried out quantitative determinations of the sugars, but from the size of the spots and the intensity of the colour we have obtained some estimate of the relative proportions. Attention is drawn to the table in the experimental section.

It is our aim to investigate the free sugars of representatives of as many as possible of the genera belonging to the family *Crassulaceae*. Much remains to be done in this respect, however, as most of the genera have no representatives growing in the wild state in Norway. The species we have examined were practically all obtained from the Botanical Gardens of the University of Oslo, but even there only a minority of the genera are represented. Nevertheless it is our hope to be able to extend the investigations to cover more genera and species of this and closely related families.

EXPERIMENTAL

Juices pressed from fresh plants were used for the experiments, in some cases directly, in others after concentration on a water bath. About 0.01 ml of the juice was placed on the paper (Whatman No. 1). The solvent used was ethyl acetate-acetic acid-water (3 : 1 : 3) [6] and the chromatogram was run for 24—48 hours, during which time the solvent ran off the lower edge of the paper. R_f-values were therefore unobtainable and

instead we noted the distance which the individual sugars had advanced in the course of 24 hours. The following sugars were used for comparison: sedoheptulose, fructose, glucose and sucrose, all in approximately 1 % solution, using 0.005—0.01 ml of the solutions each time.

As spraying reagents we used:

1. Orcinol and trichloracetic acid in *n*-butanol, for the detection of ketoses [3].
2. Aniline hydrogen phthalate in *n*-butanol [7], and
3. *m*-Phenylenediamine in aqueous alcohol [8], the latter two for the detection of both aldoses and ketoses.

The results are given in Table 1.

The reagent which has been used most extensively is orcinol and trichloracetic acid, with which, however, glucose cannot be detected. General sugar reagents (nos. 2 and 3 above) were only used on chromatograms of 3 species, in order to demonstrate positively the presence of glucose. Although we have not demonstrated the presence of this in the other species, we consider it extremely probable that it occurs there also, together with fructose and sucrose.

Table 1. Free sugars of some Crassulacean plants, detected by means of paper partition chromatography. In cases where the presence of the same sugar has been demonstrated earlier, the reference is given. The sugars have not been determined quantitatively, but from the size of the spots and the intensity of the colours we have estimated the quantitative relationship. The predominating sugar is denoted by ++: very weak spots are denoted by (+).

Species	Sedo-heptulose	Fructose	Glucose	Sucrose
Sedum spectabile Bor.	++ [9]	+	+	
S. acre L.	++ [10, 11]	+		+
S. rupestre L.	++	+	+	+
S. spurium M. B.	++ [11]	+		+
S. Telephium L.	++ [11]	+	+	+
Sempervivum Verloti Lamotte	++	+		(+)
Echeveria secunda Booth var. *glauca* Hort.	++	+		+
Crassula lactea Soland	+	++		(+)
C. portulacea Lam.	+	++		(+)
Distance the substances had run in the course of 24 hours at ca. 18° C (in cm):	14.5—15.5	15—16	13—14	7—8

SUMMARY

The free sugars in the pressed out juices of some *Crassulacean* plants have been studied by means of paper partition chromatography. In this way sedoheptulose has been demonstrated to occur in some species in which it has not

been detected previously. Together with sedoheptulose, there was also found glucose, fructose and sucrose.

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On the Phosphorus Fractions and the Uptake of Phosphate by Low-phosphorus *Torulopsis utilis*

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Working with *Torulopsis utilis* yeast we have observed that through starvation in respect to phosphorus the total phosphorus content of yeast cells can be lowered to one seventh or more from the normal content. On the other hand, when this low-phosphorus yeast was again placed in a medium containing phosphate, the total phosphorus of the cells rapidly increased so that they could contain two times more phosphorus than the normal yeast and about ten times more than the low-phosphorus yeast. The question arises how these great changes are possible and which cell fractions are affected by them. Further are these changes in chemical composition followed by changes in the physiological properties.

In the present work the object was to investigate especially the various phosphorus fractions of low-phosphorus *Torulopsis utilis* yeast and the uptake of phosphate by it.

EXPERIMENTAL

Cultivation

All cultivation experiments were performed in Kluyver flask at 29 °C with powerful aeration (400 l/hour). The pH was kept within the range 4.5—5.0. The *normal yeast* was cultivated in a solution containing an excess of phosphate, ammonia nitrogen and glucose. The *low-phosphorus yeast* was cultivated by placing the normal yeast in a medium with normal composition but devoid of phosphate.

Analytical methods

The fractionation of yeast substances for analyses was performed with tricloracetic acid as follows: The first extraction was carried out with 10 per cent tricloracetic acid at room temperature (*cold TCA extract*). This fraction contained acid-soluble phosphorus and nitrogen compounds. The remaining cell mass was treated 15 min. with 5 per cent tricloracetic acid at 90 °C (*hot TCA extract*). In this hydrolysis nucleic acids and some

other phosphorus compounds were completely extracted (*cf. Di Carlo* and *Schulz* [1] and *Wiame* [2]). *The insoluble residue* after extraction with hot tricloracetic acid contained stabile phosphorus and nitrogen compounds, mainly protein.

Total nitrogen was determined by the Kjeldahl micro method. *Phosphate* determination was carried out according to the modification of *Berenblum and Chain* [3], in which the colour extraction with isobutyl alcohol was used. *Total phosphorus* was determined as phosphate after wet combustion with sulfuric acid and hydrogen peroxide. *Nucleic acid* content was determined from the hot tricloracetic acid extract with the Beckman spectrophotometer at 260 mμ. The amount of nucleic acid equivalent to the density readings was read from a standard curve constructed with ribonucleic acid preparation (Ribonucleic acid from yeast B.D.H.). As a relative measure of the amount of soluble nucleotides in the cold tricloracetic acid extract the transmission density at 260 mμ even of this fraction was determined.

RESULTS AND DISCUSSION

The growth of the normal yeast when placed in a medium devoid of phosphate was at the beginning very intensive. Only after some hours the rate of growth gradually diminished without a clear endpoint as the cells were exhausted in respect to phosphorus on account of their multiplication.

The analyses of yeast samples (Table 1) taken during the starvation process showed that dry matter content had a slight tendency to increase. In this experiment the total P of the cells gradually diminished to one seventh of the original normal value. The total N content also decreased to some extent.

Table 1. Dry matter, phosphorus and nitrogen content of the yeast cells during cultivation in the absence of phosphate.

	Cultivation hours			
	0	3	6	9
Dry matter % of fresh matter	17.8	18.0	19.4	21.0
Total P % of dry matter	2.25	1.12	0.52	0.33
» N » » » »	8.78	8.30	8.26	7.80

Table 2 shows the results of the more minute analyses of the different fractions of normal yeast and low-P yeast (starved 9 hours in respect to phosphorus). For the sake of comparison we have also presented corresponding values of low-N yeast (starved 7 hours in respect to nitrogen). The dry matter contents of both the low-P and low-N yeasts were clearly greater than that of the normal yeast. This is mainly due, as can be seen from the N content of the insoluble residue fraction, to the disappearance of the water binding proteins from the cells during the starvation processes (*cf.* even Roine [4]). Accordingly

Table 2. Different fractions of normal yeast, low-phosphorus yeast and low-nitrogen yeast.

	Normal yeast	low-P yeast	low-N yeast
Dry matter % of fresh matter	17.75	20.0	21.5
Total P % of dry matter	2.09	0.53	1.77
» N » » » »	8.95	7.93	4.78
Cold TCA extract			
P % of dry matter	0.80	0.11	0.80
PO_4—P » » » »	0.42	0.05	0.41
N » » » »	1.09	2.10	0.37
Transmission density at 260 mμ	0.118	0.055	0.109
Hot TCA extract			
P % of dry matter	1.12	0.29	0.82
N » » » »	1.46	0.63	0.65
Nucleic acids % of dry matter	7.46	1.88	2.02
Insoluble residue			
P % of dry matter	0.17	0.13	0.15
N » » » »	6.40	5.20	3.76

even the total N content of low-P yeast is lower than that of normal yeast. On the other hand the starvation in respect to nitrogen had a similar effect on the P content of low-N yeast. This proves that certain parts of nitrogen and phosphorus of the cells are so closely connected with each other that the changes in one of these elements cannot fail to influence the other. In both cases this parallel decrease of P and N was due to the disappearance of compounds which contain both of these elements viz. nucleic acids and hence proteins. Accordingly this reciprocal influence of P and N was greatest in the compounds of the hot TCA extract and in those of the insoluble residue. The amount of nucleic acids in low-P and low-N yeasts showed an equal and very considerable decrease, which in low-P yeast is quite parallel with the decrease of P content of the hot TCA extract. In low-N yeast the P content of the hot TCA extract has decreased noticeably less than the nucleic acid content. On the other hand the N content of this fraction of both low-P and low-N yeasts has decreased equally. The low nucleic acid content of low-N *Torulopsis* yeast has already been shown by Virtanen and Miettinen [5]. In the acid-soluble fraction (cold TCA extract) the P content of low-P yeast showed the greatest decrease, which is parallel with the decrease of the free PO_4-P of this fraction. This is very interesting because the P content of this fraction in low-N yeast has not changed from the normal value. Accordingly the N content of this soluble fraction seems to

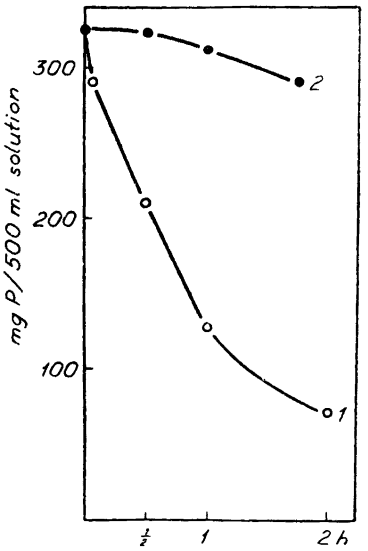

Fig. 1. Decrease of phosphate in culture solution during the uptake of phosphate by low-phosphorus and normal yeasts.

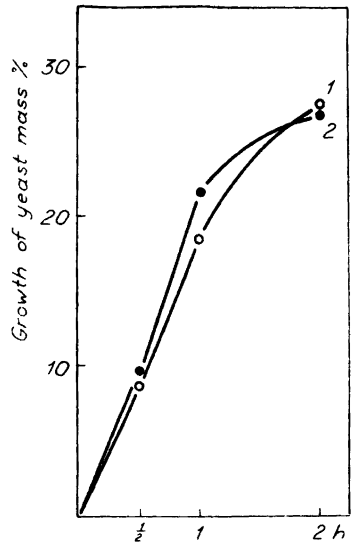

Fig. 2. Growth of low-phosphorus and normal yeasts during the uptake of phosphate.

Curve 1: Low-P yeast
Curve 2: Normal yeast

be quite independent of the corresponding P content and vice versa. The relative amount of soluble free nucleotides (transmission density at 260 mμ) decreased in low-P yeast to one half of the original, whereas in low-N yeast no remarkable drop of these substances occurred.

The first task when beginning to study the resynthesis of the compounds which had disappeared from the yeast cells during the phosphorus starvation was to follow the uptake of phosphate and the growth of the P-starved cells in a solution containing phosphate, ammonium, and glucose in ample amounts. For the sake of comparison the corresponding processes were followed even in normal yeast. As it can be seen from the curves in Fig. 1 and 2 the very vigorous uptake of phosphorus by low-P yeast begins immediately, and the amount of phosphorus taken up by it during the first few minutes is as great as taken up by the normal yeast in two hours. This enormous consumption lasts, however, only approximately two hours, whereafter it continues at the normal rate. On the other hand the growth curves of low-P and normal yeasts presented in Fig. 2 are practically identical. This proves that only a very small quantity of the consumed phosphorus in low-P yeast is necessary for the growth.

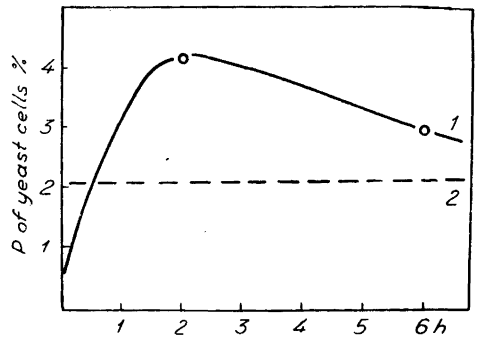

Fig. 3. Total phosphorus content of low-phosphorus yeast during the uptake of phosphate.

Curve 1: Low-P yeast
Curve 2: Level of normal yeast

The fate of the phosphorus taken up by the yeast cells was examined in another experiment of longer duration. As can be seen from Fig. 3 the total P content of the yeast cells increases very rapidly and reaches its temporary very high maximum in 2 hours, after which it begins to fall. The other facts of the analyses of this experiment are given in Table 3. The original values of normal yeast are even presented.

Table 3. Different fractions of low-phosphorus yeast during cultivation in the presence of phosphate, compared with the corresponding fractions of normal yeast.

	Low-P yeast Cultivation hours			Normal yeast
	0	2	6	
Dry matter % of fresh matter	20.0	19.25	19.20	17.75
Total P % of dry matter	0.53	4.19	2.94	2.09
» N » » » »	7.93	7.77	8.62	8.95
Cold TCA extract				
P % of dry matter	0.11	2.06	1.15	0.80
PO_4—P » » » »	0.05	0.65	0.52	0.42
N » » » »	2.10	1.76	1.48	1.09
Transmission density at 260 mμ	0.055	0.118	0.143	0.118
Hot TCA extract				
P % of dry matter	0.29	1.99	1.56	1.12
N » » » »	0.63	1.16	1.42	1.46
Nucleic acids % of dry matter	1.88	5.37	7.19	7.46
Insoluble residue				
P % of dry matter	0.13	0.14	0.23	0.17
N » » » »	5.20	4.80	5.72	6.40

Table 4. Different fractions of normal yeast during cultivation in the presence of great excess of phosphate.

	Cultivation hours		
	0	2	6
Dry matter % of fresh matter	17.8	17.9	19.4
Total P % of dry matter	2.09	2.32	2.14
» N » » » »	8.95	8.65	8.70
Cold TCA extract			
P % of dry matter	0.80	0.83	0.82
PO_4—P » » » »	0.42	0.47	0.41
N » » » »	1.09	1.32	1.28
Transmission density at 260 mμ	0.118	0.123	0.175
Hot TCA extract			
P % of dry matter	1.12	1.08	1.19
N » » » »	1.46	1.43	1.36
Nucleic acids % of dry matter	7.46	7.25	6.83
Insoluble residue			
P % of dry matter	0.17	0.41	0.13
N » » » »	6.40	5.90	6.06

Dry matter content of the starved yeast rises all the time towards the level of the normal yeast. During the very intensive accumulation of phosphorus (at 2 hours) the increase of total P is 8-fold, the increase of acid-soluble P (cold TCA extract) approximately 20-fold which is much more greater than the increase of free PO_4-P of this fraction. The increase of P of the fraction containing *inter alia* nucleic acids (hot TCA extract) is 6-fold. The simultaneous increase of nucleic acids is only 3-fold. Accordingly the accumulation of inorganic orthophosphate and the resynthesis of the nucleic acids shows no parallellism with this accumulation of phosphorus *per se*.

According to the very exhaustive work of Wiame [2] concerning the phosphorus uptake by low-P baker's yeast, the accumulation of phosphorus is mainly due to the formation of soluble and insoluble inorganic metaphosphates in the yeast cells. The results of our work are in this respect in good agreement with the facts reported by Wiame, and we can conclude that even in *Torulopsis* occurs an abundant accumulation of soluble and insoluble metaphosphates. In this work, however, no particular attention has been paid to their more minute chemical determination and characterization. The further synthesis of nucleic acids takes place after the accumulation period at the

expense of the metaphosphates. As a consequence of this nucleic acid synthesis even the N content of the hot TCA extract rises continually.

The amount of the soluble nucleotides (transmission density at 260 mμ of the cold TCA extract) have reached their normal level in 2 hours and have later even exceeded this average. The total N content shows hardly any increase during the first hours. This is mainly due to the fact that depending on experimental conditions the powerful resynthesis of the proteins starts not until 2—3 hours atferwards.

The most striking property of the P-starved yeast is its ability to accumulate phosphorus from the phosphate containing medium in a much greater degree than is needed for the synthesis of the organic phosphorus compounds in the cells. The question arises whether this ability is characteristic only of low-P yeast and could not even the normal yeast be able to increase its phosphorus content when placed in a medium of very heavy phosphate concentration. For this reason normal yeast was suspended in a culture solution containing three times as much phosphate as was used in its cultivation. Samples for the analyses of the yeast cells were taken at intervals of 2 hours. The results given in Table 4 show that no increase of the percentage content of total P or of its fractions occurred. The fact that no free PO_4-P was accumulated in the cells deserves special attention. Neither were the analyzed N fractions changed. Only the amount of the soluble nucleotides (transmission density at 260 mμ of the cold TCA extract) have a clear tendency to increase over the normal value. Accordingly the normal yeast cannot take an excess of phosphorus, and its P content is in wide limits independent of the in excess available phosphate in its environment.

SUMMARY

It has been shown that the starvation of *Torulopsis utilis* yeast in respect to phosphorus is followed by considerable decrease in various phosphorus fractions of the cells. Even the nitrogen content of such cells has clearly lowered owing to the disappearance of nitrogen compounds containing also phosphorus, *inter alia* nucleic acids.

The very considerable and rapid increase of the phosphorus content of phosphorus-starved yeast when placed in a medium containing phosphate is caused by the accumulation of phosphorus in the cells in much higher degree than needed for the growth. It is surprising that in consequence of this accumulation the total phosphorus content of the yeast cells exceeds even twice that of the normal yeast, which has been cultivated in the same phosphate

concentration. The resynthesis of the disappeared nucleic acids proceeds at a relatively slow rate at the expense of this accumulated phosphorus.

When cultivating yeast of normal phosphorus content in the presence of a high excess of phosphate no increase of the phosphorus content of the cells occurred.

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Effect of Glycine-Peptides on the Growth of *Leuconostoc mesenteroides*

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In general, the effect of peptides on the growth of microorganisms is relatively little known so far. Nevertheless, many proofs have been obtained showing that, at least in the microbiological determination of amino acids, peptides are noteworthy growth factors, for in certain cases they can replace the amino acid to be determined.

As early as 1943 Kuiken *et al.*[1], using *Lactobacillus arabinosus* 17—5 as a test organism, found that DL-leucylglycine and glycyl-L-leucine possess 80—100 % of the activity of leucine. Later, Schweigert [2] noted with the same organism that DL-leucylglycylglycine, glycyl-DL-leucylglycine and glycyl-glycyl-DL-leucine have a leucine-activity ranging from 30 to 90 % depending on peptide. On the contrary, Hegsted [3] failed to demonstrate any valine-activity with *L. arabinosus* in three different isomers of benzoylvalylvaline. The investigations of Stokes *et al.*[4] with *Streptococcus faecalis* and of Lewis and Olcott [5] with *L. arabinosus* 17—5 also suggest that the peptides are generally inactive if one component in them is an acid other than amino acid.

Apart from these few separate observations, only Ågren [6,7], Krehl and Fruton [8] have examined in detail the effect of synthetic peptides on the growth of lactic acid bacteria and Simmonds *et al.*[9–12] on the growth of *Escherichia coli*.

According to the studies of Ågren [6] with *Str. faecalis* R, *L. delbrückii* LD5, and *L. casei*, these test organisms are able to utilize dipeptide-valine and dipeptide or tripeptide-leucine. However, the activity of these peptides depends decidedly on the position of valine and leucine in the peptide. Krehl and Fruton [8] also arrived at similar results with *L. arabinosus* 17—5 and *Str. faecalis* R when examining the activity of di- and tri-peptides. Likewise, the later studies by Ågren [7] with 10 different strains of lactic acid bacteria and

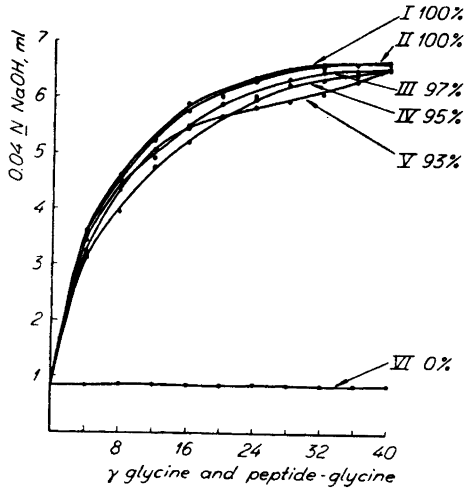

Fig. 1. The effect of glycine peptides on the growth of Leuconostoc mesenteroides P-60.

I glycyl-L-leucine
II glycine
III glycyl-glycine
IV DL-alanyl-glycine
V DL-leucyl-glycine
VI hippuryl-glycine and sarcosine

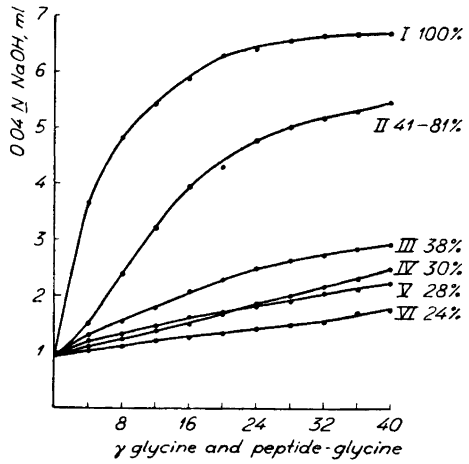

Fig. 2. The effect of glycine peptides on the growth of Leuconostoc mesenteroides P-60.

I glycine
II glycyl-L-tyrosine methylester
III triglycine ethylester
IV pentaglycine ethylester
V glycyl-DL-phenylalanine methylester
VI glutathione

8 leucine-peptides support the concept that the activity of peptides depends on the position of the indispensable amino acid in the peptide as well as on the order of other amino acids in respect to this amino acid. According to Ågren the activity of peptides may vary depending on the composition of the basal medium.

Simmonds *et al.* [9–12] report that mutants of *E. coli*, too, are able to utilize peptides in addition to amino acids. The activity of peptides in these bacteria, as well as in lactic acid bacteria, depends on the position of the indispensable amino acid in the peptide. Of special interest is the exceptional activity of proline-peptides which is greater than that of the equimolar free proline.

The present paper deals with the effect of 10 different glycine-peptides and sarcosine on the growth of *Leuconostoc mesenteroides* P-60 (obtained from Prof. E. E. Snell, University of Wisconsin, U. S. A.).

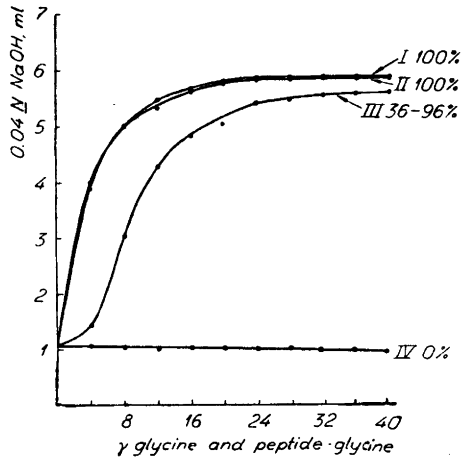

Fig. 3. The influence of glycine, benzoylglycine, benzoylglycylglycine and benzoic acid on the growth of Leuconostoc mesenteroides P-60.

Compound	Amounts of unbound and peptide-glycine in γ *											Glycine-activity %
	0	4	8	12	16	20	24	28	32	36	40	
	0.04 *N* NaOH in ml											
Benzoic acid + glycine	1.08	3.90 97.1	5.01 100.0	5.50 104.5	5.64 100.0	5.77 98.8	5.85 100.0	5.91 101.0	5.93 99.9	5.95 100.7	5.94 100.1	100
Glycine	1.08	4.02	5.01	5.30	5.64	5.84	5.85	5.85	5.94	5.90	5.93	100
Hippuric acid	1.09	1.43 35.7	3.03 60.5	4.30 81.2	4.84 85.8	5.03 86.2	5.46 93.3	5.54 94.7	5.60 94.2	5.65 95.8	5.67 95.6	36—96
Benzoic acid	1.08	1.07	1.04	1.00	1.03	1.01	1.01	1.04	1.00	1.01	1.00	0

* The amounts of benzoic acid were respectively in γ: 0, 5.5, 11.0, 16.5, 22.0, 27.5, 33.0, 38.5, 44.0, 49.5, 55.0.

EXPERIMENTAL

The experimental technique introduced by Henderson and Snell[13] was chiefly employed in the activity determinations. The quantity of DL-alanine was one fifth of the alanine concentration of the original basal medium. The investigation comprised 5 series of experiments grown at different times. Each series included standard solutions of glycine, the glycine concentration of which was increased by 4 gammas from 0 to 40 gammas, and different peptide solutions, in which the peptide-glycine concentration corresponded to the quantity of equimolar free standard glycine (the quantity of glycylglycine was, however, the same as that of glycine). The duration of

growth was 72 hours in each series and the growth temperature 37° C. Titrations were carried out with 0.04 *N* NaOH electrometrically by using quinhydrone electrode.

The results are presented by curves in Figs. 1—3.

RESULTS

It can be seen from the results that the glycine-activity of DL-alanylglycine, glycylglycine, glycyl-L-leucine, DL-leucylglycine, and glycylglycine has been 100 % or a little less. The position of glycine in the dipeptides has not much affected the activity. On the other hand, the glycine-activity of glycyl-DL-phenylalaninemethylester, glycylglycylglycine-ethylester and glycylglycyl-glycylglycylglycine-ethylester has only about one third of the activity of free equimolar glycine. Glycyl-L-tyrosinemethylester behaves in a very interesting way — its glycine activity rises from 41 to 81, while the peptide-glycine concentration rises from 4 to 40 gammas. A corresponding rise is detectable also with benzoylglycine (hippuric acid), thus — contrary to former findings — peptide can be active, although one component in it is an acid other than amino acid. With all other peptides, instead, the peptide-glycine activity is fairly independent of the concentration of peptide. The lowest activity, a little more than 20 %, was noted with glutathione glycine. Benzoylglycylglycine was entirely inactive and so was sarcosine, too. Benzoyl-glycylglycine and benzoic acid had no inhibitory action on the glycine activity.

SUMMARY

The suitability of different glycine peptides for the glycine nutrition of *Leuconostoc mesenteroides* P-60 has been examined. Most of the peptides studied possessed a distinct or a very strong glycine-activity. The mechanism of the growth effect of glycine-peptides will be discussed in another paper.

We wish to express our cordial thanks to Prof. Dr. E. E. Snell for kindly sending at our disposal the pure cultures of lactic acid bacteria and the necessary reagents for the basal medium.

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10-Aminoacylphenothiazines

I. Aminoacetyl and Aminopropionyl Derivatives

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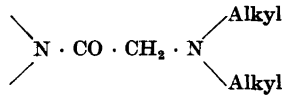
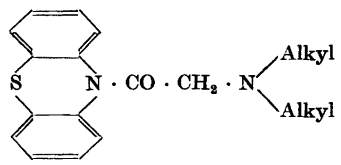
Aminoalkyl derivatives of phenothiazine have been made the object of exhaustive chemical [1-5] and pharmacological [6,7] investigations, which have shown that compounds of this type have some outstanding pharmacological properties. In particular, they are very potent antihistamine agents; but also their spasmolytic and local anesthetic activities are in some cases rather high.

According to the literature no corresponding aminoacylphenothiazines seem to have been prepared. Since compounds containing the general grouping

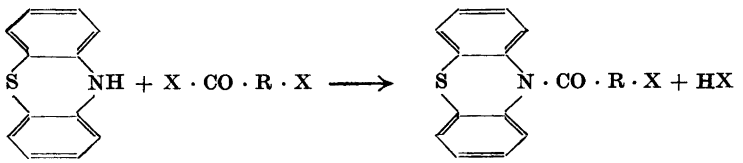


have shown very interesting properties [8,9] especially as local anesthetics (Xylocaine), we decided to synthesise the corresponding 10-phenothiazine derivatives, *viz.*



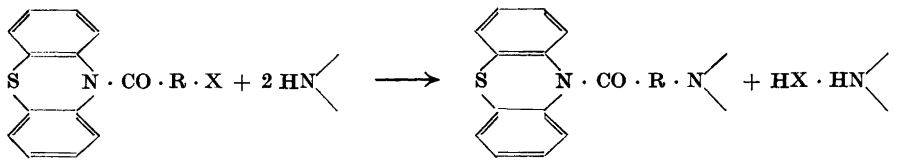
As even these simple compounds had rather promising properties and were easy to prepare, we considered it worth while making a more exhaustive investigation involving varying both the amino component and the intermediate chain. A preliminary communication on this work has already been published [10] and this paper gives a more detailed report on the chemical and pharmacological properties of the compounds we have prepared.

Phenothiazine reacted readily with haloacyl halides when heated under reflux in benzene or toluene until hydrogen halide was no longer evolved, forming the corresponding 10-haloacylphenothiazines in good yields.



In this way we have treated phenothiazine with chloroacetyl chloride, α-bromopropionyl bromide, β-chloropropionyl chloride and α,β-dibromopropionyl chloride.

The haloacylphenothiazines thus formed were treated with a number of different primary and secondary amines, giving the desired 10-aminoacylphenothiazines.



In a few cases some complications occurred. When, for example, 10-chloroacetylphenothiazine was treated with β-hydroxydiethylamine, the expected amount of amine hydrochloride separated but apparently the hydroxyaminoacyl compound initially formed was not stable and decomposed at about the same rate as it was produced, since only phenothiazine separated (in theoretical yield) from the reaction mixture. We also attempted to prepare the desired compound by the addition of ethylene oxide to 10-ethylaminoacetylphenothiazine, but even in this case only phenothiazine was formed. It should be added, that 10-chloroacetylphenothiazine, 10-ethylaminoacetylphenothiazine, and 10-diethylaminoacetylphenothiazine are all quite stable compounds. Phenothiazine, however, was formed even when we tried to prepare the hydroxy compound under very mild conditions (room temperature). Attempts to prepare other hydroxyaminoacylphenothiazines by the same two routes were also unsuccessful. No attempts were made to isolate the hydroxyaminoacyl moiety of the molecule, but since there is a possibility that it may form a heterocyclic compound, we shall investigate this reaction more fully at a later date.

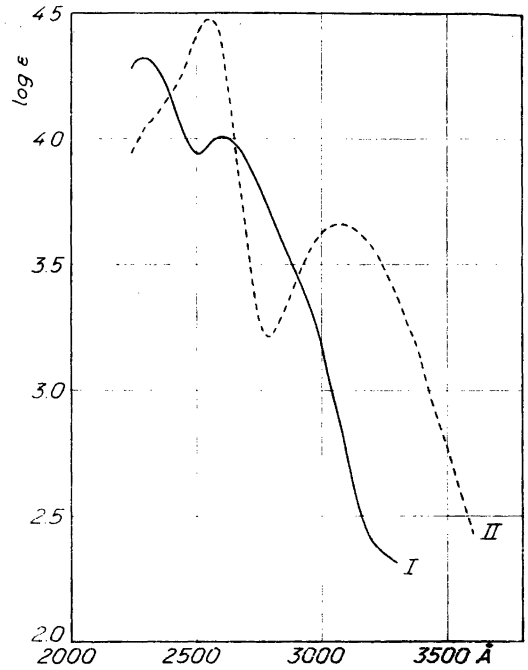


Fig. 1. The absorption spectra of 10-(α-diethylaminopropionyl)-phenothiazine (I) and 10-(β-piperidinopropyl)-phenothiazine (II) in abs. ethanol (concentrations ca. $5 \times 10^{-5} M$)

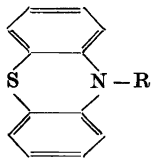
The reaction between α,β-dibromopropionylphenothiazine and amines invariably yielded the bisamino compounds, even when the halocompound was used in excess.

It is known that the CO-group in acid amides can be reduced to $—CH_2—$ by lithium aluminium hydride [11,12]. As the corresponding reduction of aminoacylphenothiazines might provide a rather easy route to the aminoalkylphenothiazines, we attempted reductions of this type. We found, however, that aminoacylphenothiazines when treated with lithium aluminium hydride in ether at room temperature were split instantaneously into phenothiazine and the corresponding aminoalcohol.

ULTRA-VIOLET ABSORPTION SPECTRA

We have not been able to find in the literature any data on the UV-absorption of 10-substituted phenothiazines. For control and comparison purposes we have measured the absorption of a number of 10-aminoalkyl- and 10-

Table 1. 10-Alkyl- and 10-acylphenothiazines.



R	E^1_{max}		E^2_{max}	
	λ Å	log ε	λ Å	log ε
$-C_2H_5$	2 560	4.55	3 100	3.64
$-C_3H_7$	2 550	4.53	3 080	3.66
$-CH_2 \cdot CH_2 \cdot N(CH_2 \cdot CH_2)_2O$	2 550	4.51	3 080	3.65
$-CH_2 \cdot CH(CH_3) \cdot N(CH_2)_3$	2 550	4.48	3 080	3.66
$-CO \cdot CH_3$	2 290	4.38	2 600	4.05
$-CO \cdot CH_2 \cdot N(CH_2)_4$	2 300	4.30	2 600	4.01
$-CO \cdot CH_2 \cdot N(C_2H_5)_2$	2 290	4.30	2 600	4.00
$-CO \cdot CH(CH_3) \cdot N(C_2H_5)_2$	2 290	4.32	2 600	4.01
$-CO \cdot CH(N(C_2H_5)_2) \cdot CH_2 \cdot N(C_2H_5)_2$	2 300	4.43	2 600	4.00

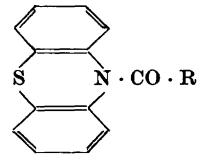
aminoacylphenothiazines. The measurements were made with a Beckman Model DU spectrophotometer using calibrated cells, and absolute ethanol as solvent throughout. The alkyl compounds were prepared by methods previously described [4,13]—. It was found, that all the alkyl compounds gave almost identical spectra, and the same was the case with the acyl compounds. However the two types were distinctly different as is shown in Fig. 1. The wavelengths of the maxima and the extinction coefficients are detailed in Table 1. As expected, the spectra of the alkyl compounds are very similar to the spectrum of unsubstituted phenothiazine [14].

PHARMACOLOGY

The compounds have been subjected to pharmacological tests *, for local anesthetic activity (rabbit cornea, Xylocaine-HCl being used as a standard). Antihistaminic activity and the effects on spasms induced by acetylcholine and $BaCl_2$ were determined on isolated guinea pig small intestine (standard, diphenhydramine-HCl). The results of these tests are summarised in Table 2.

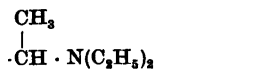
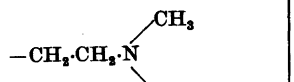
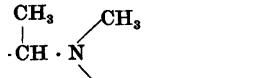
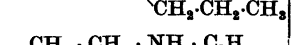
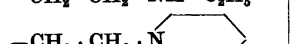
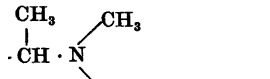
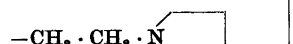
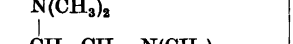
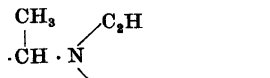
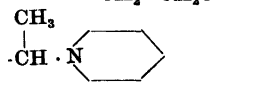
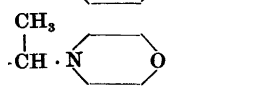
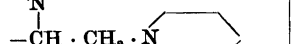
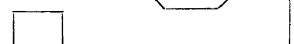
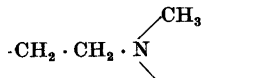
Local anesthetic effect. All the compounds possessed a marked activity, usually superior to that of Xylocaine. A variation of the intermediate chain in the order acetyl-, α-propionyl-, β-propionyl-increased the activity. The propionyl derivatives with two amino groups were even more active. Pyrrolidine appears to be the most favourable amino component. Unfortunately the α,β-bispyrrolidino-propionylphenothiazine was not soluble enough to be tested.

Table 2. Some pharmacological properties of 10-aminoacylphenothiazines.



R	Local anesthetic activity	Effect in reducing the spasm produced by		
		Acetylcholine	$BaCl_2$	Histamine
$-CH_2 \cdot N(CH_3)_2$	1.25	1.33		0.1
$-CH_2 \cdot N(C_2H_5)_2$	1.75	2.3	1.45	0.2
$-CH_2 \cdot N(CH_3)(C_2H_5)$	1.8	2.2	2	0.5
$-CH_2 \cdot N(CH_3)(CH_2 \cdot CH_2 \cdot CH_3)$	1.75	1.9	1.8	0.03
$-CH_2 \cdot N(C_2H_5)(CH_2 \cdot CH_2Cl)$		3.3	0.5	0.05
$-CH_2 \cdot NH \cdot C_2H_5$	2	2.0	0.15	0.08
$-CH_2 \cdot NH$ 		0.33	0.2	0.005
$-CH_2 \cdot N$ 	0.13	4	1	0.22
$-CH_2 \cdot N$ 		0.07		0.05
$-CH_2 \cdot N$ 	2.5	3	3	0.33
$-CH(CH_3) \cdot N(CH_3)_2$	3	1.6	1.1	0.06

* Acknowledgement is made to Dr. S. Wiedling of Astra's Biological Department for performing these tests. The pharmacological data will later be discussed by Dr. Wiedling in a wider context.

	3	12	6.6	0.1		3.1	9	1.3	0.07
	2	6.5	1.5	0.2		4	3.3	2.4	0.17
							7	2.6	0.25
	4	6	7	0.3		7	18	20	0.25
						8	1.0		0.0075
		11	7.7	0.06		9	1.0		0.027
	4	1.25	1.2	0.08					
		0.06		0.03			0.15		0.005
	2.5	2.2	3	0.11			0.3	0.8	0.007
$-CH_2 \cdot CH_2 \cdot N(CH_3)_2$	2.9	12	5	0.2	Atropine sulphate		30		
$-CH_2 \cdot CH_2 \cdot N(C_2H_5)_2$	5	7.5	1.2	0.1	Papaverine-HCl			0.33	
	2.5	14	1	0.18	Xylocaine-HCl	1.0			
					Diphenhydramine-HCl		1.0	1.0	1.0

Antihistaminic effect. All the compounds were inferior to diphenhydramine, the bisamino compounds having a substantially weaker effect.

Antispasmodic effect. Some of the compounds had outstanding spasmolytic properties. Against spasm induced by $BaCl_2$ some of the derivatives were comparable with, or superior to previously known antispasmodics. The propionyl compounds were somewhat superior to the acetyl compounds and the bisamino derivatives had a weak effect. The highest antispasmodic activity was exhibited by β-pyrrolidino-propionylphenothiazine.

EXPERIMENTAL

Halogenoacylphenothiazines

10-Chloroacetylphenothiazine (I). To a solution of phenothiazine (19.9 g, 0.1 mole) in boiling benzene (100 ml), chloroacetyl chloride (17.0 g, 0.15 mole) was added in por-

tions. The mixture was refluxed for two hours, most of the solvent was evaporated and the residue cooled in ice water. The precipitate which formed (21 g) was collected and recrystallised twice from ethanol. M. p. 115—116.5°.

$C_{14}H_{10}ClNOS$ (275.8)	Calc.	Cl 12.9	N 5.08	S 11.6
	Found	» 13.0	» 5.16	» 11.9

10-(α-Bromopropionyl)-phenothiazine (II). This compound was prepared in the same way as described above from phenothiazine (10.0 g) and α-bromopropionyl bromide (16.0 g) by refluxing in toluene (50 ml) for three hours. The crude product (12.8 g) was recrystallised twice from ethanol. M. p. 147.5—148.5°.

$C_{15}H_{12}BrNOS$ (334.2)	Calc.	Br 23.9
	Found	» 23.8

10-(β-Chloropropionyl)-phenothiazine (III), was prepared similarly from phenothiazine (10.0 g) and β-chloropropionyl chloride [15] (7.5 g) by refluxing for five hours in benzene (70 ml). The crude product (13.4 g) was recrystallised from methanol. M. p. 142—143°.

$C_{15}H_{12}ClNOS$ (289.8)	Calc.	Cl 12.2
	Found	» 12.2

10-(α,β-Dibromopropionyl)-phenothiazine (IV). From phenothiazine (26.0 g) and α,β-dibromopropionyl chloride [16] (48.0 g) by refluxing in toluene (260 ml) for three hours. Yield 49.0 g. M. p. 135—135.5° after recrystallisation from ether.

$C_{15}H_{11}Br_2NOS$ (413.1)	Calc.	C 43.6	H 2.68	Br 38.7
	Found	» 43.5	» 2.85	» 38.7

Aminoacylphenothiazines

The compounds described below were all prepared in essentially the same way. The halogenoacyl phenothiazine (1 mole) was dissolved in benzene or toluene and heated under reflux with excess of the appropriate amine (usually 2.6 mole). In some cases when the amine was rather volatile it was found advantageous to carry out the reaction in a sealed bottle. The hydrohalide of the amine was separated and the filtrate extracted with *N* hydrochloric acid. The extract was made alkaline with sodium carbonate solution and the precipitated base, which usually soon solidified, collected and recrystallised. In some cases when it was impossible to obtain the base in crystalline form it was converted to the hydrochloride by extracting the oil with ether and precipitating the hydrochloride by the addition of a dry ethereal solution of hydrogen chloride.

10-(Dimethylaminoacetyl)-phenothiazine. Dimethylamine (24.0 g) and 10-chloroacetylphenothiazine (I) (41.4 g) in benzene (100 ml) were shaken in a sealed bottle for eight hours. After isolation as described above the product (41.0 g) was recrystallised twice from ethanol, and then had m. p. 148.5—150° *.

* In the preliminary communication on this compound, the m. p. was erroneously given as 114.5 — 116.

$C_{16}H_{16}N_2OS$ (284.4)	Calc.	C 67.6	H 5.67	S 11.3
	Found	» 67.4	» 5.69	» 11.1

10-(Diethylaminoacetyl)-phenothiazine, prepared by boiling diethylamine (49.5 g) and I (69 g) in benzene (250 ml) for four hours. Yield 57.6 g. Recrystallised from light petroleum (40—60°), m. p. 58—59°.

$C_{18}H_{20}N_2OS$ (312.4)	Calc.	C 69.2	H 6.45	N 8.97
	Found	» 68.9	» 6.45	» 8.94

10-(Methylethylaminoacetyl)-phenothiazine. Methylethylamine [17] (1.5 g) and I (2.8 g) were heated at 100° in toluene (25 ml) for seven hours. The crude product (2.7 g) was recrystallised from light petroleum-ethanol (1 : 1). M. p. 113—115°.

$C_{17}H_{18}N_2OS$ (298.4)	Calc.	C 68.4	H 6.08
	Found	» 68.4	» 6.11

10-(Methylpropylaminoacetyl)-phenothiazine. From methylpropylamine [17] (1.9 g) and I (2.8 g) by refluxing in toluene (25 ml) for five hours. Yield 2.8 g. Recrystallised from light petroleum. M. p. 80—81°.

$C_{18}H_{20}N_2OS$ (312.4)	Calc.	C 69.2	H 6.45
	Found	» 69.1	» 6.34

10-(β-Chloroethyl-ethylaminoacetyl)-phenothiazine. β-Chloroethylethylamine hydrochloride [18] (7.2 g) was suspended in water (100 ml) and the mixture made alkaline with ammonia. The precipitated base was extracted with toluene (3 × 15 ml), the toluene solution dried, I (5.5 g) added and the mixture was heated at 50° for five hours. As the crude product did not crystallise it was converted to the hydrochloride. Yield 1.8 g. Recrystallised from light petroleum-ethanol (1 : 1). M. p. 190—191° (dec.). The low yield in this synthesis may be due to the instability of the chloroalkylamine.

$C_{18}H_{19}ClN_2OS \cdot HCl$ (383.3)	Calc.	Cl 18.5	N 7.31
	Found	» 18.5	» 7.28

10-(Ethylaminoacetyl)-phenothiazine, was prepared from ethylamine (5.4 g) and I (13.8 g) in benzene (50 ml) by heating in a sealed bottle to 80° for two hours. The crude base (12.2 g) was crystallised from light petroleum. M. p. 95—96°.

$C_{16}H_{16}N_2OS$ (284.4)	Calc.	C 67.6	H 5.67
	Found	» 67.9	» 5.85

10-(Cyclohexylaminoacetyl)-phenothiazine. Cyclohexylamine (40 ml) and I (27.6 g) in benzene (200 ml) were shaken in a sealed bottle at room temperature for ten hours. In this case the base could not be extracted with hydrochloric acid owing to the low solubility of its hydrochloride. The reaction mixture was filtered and evaporated to

dryness, yielding 39.5 g of product which was recrystallised from light petroleum-ethanol (1 : 1). M. p. 124—126°.

$C_{20}H_{22}N_2OS$ (338.5)	Calc.	C	71.0	H	6.55
	Found	»	70.3	»	6.71

10-(Piperidylacetyl)-phenothiazine. Piperidine (14.5 g) and I (17.7 g) were refluxed in benzene (75 ml) for three hours, and the product (17.1 g), recrystallised from ethanol, had m. p. 164—165°.

$C_{19}H_{20}N_2OS$ (324.4)	Calc.	C	70.3	H	6.21
	Found	»	70.3	»	6.23

10-(Morpholinoacetyl)-phenothiazine. Morpholine (11.3 ml) and I (13.8 g) in benzene (30 ml) were refluxed for four hours. The product (14.9 g) was recrystallised from light petroleum-acetone (1 : 1), m. p. 141—142°.

$C_{18}H_{18}N_2O_2S$ (326.4)	Calc.	C	66.2	H	5.56	N	8.58
	Found	»	66.2	»	5.50	»	8.68

10-(Pyrrolidinoacetyl)-phenothiazine. Pyrrolidine (1.85 g) and I (2.76 g) were refluxed in toluene (25 ml) for four hours, and the product (1.7 g) recrystallised from light petroleum-ethanol (5 : 1), m. p. 142—142.5°.

$C_{18}H_{18}N_2OS$ (310.4)	Calc.	C	69.6	H	5.84	N	9.03
	Found	»	69.5	»	5.83	»	9.10

10-(α-Dimethylaminopropionyl)-phenothiazine. α-Bromopropionylphenothiazine, II, (33.4 g) and dimethylamine (17.0 g) were heated in benzene (100 ml) at 85° over night, and the product (24.3 g) recrystallised from acetone — light petroleum (1 : 1.5). M. p. 98.5—100°.

$C_{17}H_{18}N_2OS$ (298.4)	Calc.	C	68.4	H	6.08	N	9.39
	Found	»	68.0	»	6.15	»	9.30

10-(α-Diethylaminopropionyl)-phenothiazine. Diethylamine (19.0 g) and II (33.4 g) were refluxed for five hours in toluene (100 ml). Yield 32.8 g. Recrystallised from ethanol. M. p. 100.5—101.5°.

$C_{19}H_{22}N_2OS$ (326.5)	Calc.	C	69.9	H	6.79
	Found	»	69.8	»	6.70

From this compound the hydrobromide (m. p. 200—201°, dec.) and hydrochloride (m. p. 200—201°, dec.) have also been prepared.

10-(α-Methylethylaminopropionyl)-phenothiazine. Methylethylamine (1.5 g) and II (3.3 g) were heated in toluene (25 ml) at 80° for seven hours, giving 3.0 g of product of m. p. 67—69° after recrystallisation from light petroleum.

$C_{18}H_{20}N_2OS$ (312.4)	Calc.	C	69.2	H	6.45
	Found	»	69.3	»	6.33

10-(α-Methylpropylaminopropionyl)-phenothiazine. Methylpropylamine (1.9 g) and II (3.4 g) were refluxed in toluene (25 ml) for five hours. The oily base was converted to the hydrochloride (0.8 g) which crystallised from acetone. M. p. 188—190°.

$C_{19}H_{22}N_2OS \cdot HCl$ (362.9)	Calc.	C	62.9	H	6.39
	Found	»	62.9	»	6.52

10-[α-(β'-Chloroethylethylamino)-propionyl]-phenothiazine. This compound was prepared from the free base from β-chloroethylethylamine hydrochloride (7.2 g) and II (6.7 g) by refluxing for five hours in toluene (45 ml). The hydrochloride (1.0 g) was recrystallised from ethanol, m. p. 188—189°.

$C_{19}H_{21}ClN_2OS \cdot HCl$ (397.4)	Calc.	C	57.4	H	5.58	N	7.05
	Found	»	56.8	»	5.75	»	7.06

10-(α-Piperidinopropionyl)-phenothiazine. Piperidine (30 ml) and II (33.4 g) in toluene (100 ml) were heated over night to 85° in a sealed bottle. The crude base (35.0 g) was crystallised from 60 % ethanol. M. p. 110—111°.

$C_{20}H_{22}N_2OS$ (338.5)	Calc.	C	71.0	H	6.57
	Found	»	70.9	»	6.57

10-(α-Morpholinopropionyl)-phenothiazine. Morpholine (11.3 ml) and II (16.7 g) were refluxed in benzene for two hours and the product (17.0 g) had m. p. 123—124° after recrystallisation from ether.

$C_{19}H_{20}N_2O_2S$ (340.4)	Calc.	C	67.0	H	5.92	N	8.23
	Found	»	67.2	»	5.85	»	8.35

10-(α-Pyrrolidinopropionyl)-phenothiazine. Pyrrolidine (1.85 g) and II (3.34 g) were refluxed in toluene (25 ml) for four hours. The crude base (2.7 g) was recrystallised from light petroleum. M. p. 94.5—95.5°.

$C_{19}H_{20}N_2OS$ (324.4)	Calc.	C	70.3	H	6.21	N	8.64
	Found	»	70.0	»	6.03	»	8.69

10-(β-Dimethylaminopropionyl)-phenothiazine. Dimethylamine (2.3 g) and β-chloropropionylphenothiazine, III, (5.8 g) were heated in toluene (100 ml) at 100° over night. The product (5.8 g) after recrystallisation from light petroleum had m. p. 90.5—91.5°.

$C_{17}H_{18}N_2OS$ (298.4)	Calc.	C	68.4	H	6.08	N	9.39
	Found	»	67.8	»	6.14	»	9.24

10-(β-Diethylaminopropionyl)-phenothiazine, prepared from diethylamine (9.5 g) and III (14.5 g) by refluxing in toluene (100 ml) for three hours. After recrystallisation from light petroleum m. p. 44.5—45.5°. Yield 15.0 g.

$C_{19}H_{22}N_2OS$ (326.5)	Calc.	C 69.9	H 6.79	N 8.58
	Found	» 70.2	» 6.86	» 8.45

10-(β-Methylethylaminopropionyl)-phenothiazine. Methylethylamine (1.5 g) and III (2.9 g) were heated in toluene (25 ml) to 100° for seven hours. The product (2.7 g) after recrystallisation from light petroleum had m. p. 58—60°.

$C_{18}H_{20}N_2OS$ (312.4)	Calc.	C 69.2	H 6.45	N 8.97
	Found	» 69.5	» 6.38	» 9.07

10-(β-Methylpropylaminopropionyl)-phenothiazine. From methylpropylamine (1.9 g) and III (2.9 g) by refluxing in toluene (25 ml) for five hours. As the base did not solidify and the hydrochloride also was oily, in this case the oxalate was prepared. Yield 2.7 g. M. p., after recrystallisation from acetone, 145—146°.

$C_{19}H_{22}N_2OS \cdot (COOH)_2$ (416.5)	Calc.	C 60.6	H 5.81	N 6.73
	Found	» 60.5	» 5.76	» 6.79

10-(β-Ethylaminopropionyl)-phenothiazine. Ethylamine (15.4 ml) and III (29.1 g) were heated in benzene (100 ml) to 100° for three hours. The crude base (29.8 g) was recrystallised from acetone. M. p. 108—110°.

$C_{17}H_{18}N_2OS$ (298.4)	Calc.	C 68.4	H 6.08	
	Found	» 68.5	» 6.15	

10-(β-Piperidinopropionyl)-phenothiazine. Piperidine (4.4 g) and III (5.8 g) were refluxed for four hours in toluene (50 ml). The product, (6.5 g) after recrystallisation from 50 % ethanol, had m. p. 97—99°.

$C_{20}H_{22}N_2OS$ (338.5)	Calc.	C 71.0	H 6.57	N 8.28
	Found	» 70.9	» 6.49	» 8.21

10-(β-Pyrrolidinopropionyl)-phenothiazine. Pyrrolidine (2.78 g) and III (4.3 g) were refluxed in toluene (25 ml) for four hours. The crude base (4.5 g) was recrystallised from light petroleum-ethanol (5 : 1). M. p. 108—109°.

$C_{19}H_{20}N_2OS$ (324.4)	Calc.	C 70.3	H 6.21	N 8.64
	Found	» 70.5	» 6.33	» 8.66

10-[α,β-Bis(dimethylamino)-propionyl]-phenothiazine. Dimethylamine (4.5 g) and 10-(α,β-dibromopropionyl)-phenothiazine, IV, (10.2 g) were heated with toluene (100 ml)

in a sealed bottle to 110° for three hours. The product (8.1 g) recrystallised from light petroleum had m. p. 80—81°.

$C_{19}H_{23}N_3OS$ (341.5)	Calc.	C 66.8	H 6.79	N 12.3
	Found	» 67.0	» 6.89	» 12.1

10-[α,β-Bis(diethylamino)-propionyl]-phenothiazine was prepared from diethylamine (36.5 g) and IV (41.3 g) by refluxing in toluene (400 ml) for three hours. The free base (36.7 g) was recrystallised from ethanol. M. p. 107—108°.

$C_{23}H_{31}N_3OS$ (397.6)	Calc.	C 69.5	H 7.86	N 10.6
	Found	» 69.6	» 7.98	» 10.5

10-[α,β-Bis(piperidino)-propionyl]-phenothiazine was prepared from piperidine (6.4 g) and IV (6.2 g) by refluxing in toluene (60 ml) for two hours. The crude base (7.7 g) was recrystallised from acetone. M. p. 136.5—137.5°.

$C_{25}H_{31}N_3OS$ (421.6)	Calc.	C 71.2	H 7.41	N 9.97
	Found	» 71.5	» 7.53	» 9.91

10-[α,β-Bis(pyrrolidino)-propionyl]-phenothiazine. Pyrrolidine (3.7 g) and IV (4.1 g) were refluxed in toluene (40 ml) for three hours. The base (2.5 g) was recrystallised from light petroleum. M. p. 102—104°.

$C_{23}H_{27}N_3OS$ (393.5)	Calc.	C 70.2	H 6.93	N 10.7
	Found	» 70.3	» 7.15	» 10.2

SUMMARY

The synthesis of 29 10-aminoacetyl- and 10-aminopropionyl-phenothiazines is described. Most of the compounds are powerful local anesthetics and some of them show outstanding spasmolytic properties. The absorption spectra of some 10-aminoacyl- and 10-aminoalkyl-phenothiazines have been determined.

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Methanethiosulphonates of Divalent Sulphur, Selenium and Tellurium

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These compounds, *viz.*:

$$S(S_2O_2CH_3)_2;\quad Se(S_2O_2CH_3)_2;\quad Te(S_2O_2CH_3)_2$$

are described in this article for the first time. They are polythionic compounds of the pentathionic type, and, supposedly, structural analoga of the pentathionate, selenopentathionate and telluropentathionate ions, *viz.*, the thiosulphates:

$$S(S_2O_3)_2^{--};\qquad Se(S_2O_3)_2^{--};\qquad Te(S_2O_3)_2^{--}$$

Thiosulphonates of divalent selenium and tellurium are new types of compounds. Sulphur thiosulphonates, *i. e.*, aromatic ones, are well known and have been the subject of much study *. Thus, the *p*-toluene derivative was isolated by Blomstrand [2] in 1870. The only aliphatic representative known hitherto, the ethane compound, dates from 1945 [1].

SYNTHESIS AND CHEMICAL PROPERTIES

Sulphur and selenium di(methanethiosulphonate) were obtained from sodium methanethiosulphonate, suspended in ether, and sulphur dichloride and diselenium dichloride, respectively:

$$2\ CH_3SO_2SNa + SCl_2 = S(S_2O_2CH_3)_2 + 2\ NaCl$$

$$2\ CH_3SO_2SNa + Se_2Cl_2 = Se(S_2O_2CH_3)_2 + Se + 2\ NaCl$$

* For literature, see Ref. 1.

The first of these reactions is generally used [1] for the synthesis of thio derivatives of S^{++}. The only example of the second type seems to be that of Baroni [3], who prepared selenium di(phenylmercaptide) from diselenium dichloride and benzenethiol.

Tellurium di(methanethiosulphonate) resulted when aqueous sodium methanethiosulphonate was added to tellurium dioxide dissolved in hydrochloric acid:

$$TeOOH^+ + 4\ CH_3SO_2S^- + 3\ H^+ = Te(S_2O_2CH_3)_2 + (CH_3SO_2S)_2 + 2\ H_2O$$

Tellurium dioxide reacts in an analogous way with sodium thiosulphate [4] and with thiolactic acid [5].

Sulphur di(methanethiosulphonate) forms colourless crystals which melt at 70—70.5° C, and are readily soluble in ethylacetate, benzene and chloroform, sparingly soluble in ether and carbon disulphide.

Selenium di(methanethiosulphonate) appears as pale greenish crystals which are only moderately soluble in cold chloroform and ethylacetate, and almost insoluble in ether, carbon disulphide and benzene. The crystals of tellurium di(methanethiosulphonate) are yellow, and so are dilute solutions, whereas more concentrated solutions have an orange tinge. The solubility is very low, the best solvents being chloroform and, especially, bromoform. The melting points of selenium and tellurium di(methanethiosulphonate) depend on the rate of heating. The selenium compound starts to decompose (become red) at about 75° C and liquifies at 85—90° C; the tellurium compound becomes black at about 120° C and appears to melt at about 135° C.

The crystals, when pure, are relatively stable; thus, they have been kept unchanged for months. They are insoluble in water. With alkalies, they rapidly liberate sulphur, selenium or tellurium.

The following nucleophilic displacement reactions serve to characterize the compounds as derivatives of divalent electropositive sulphur, selenium and tellurium. The reactions are rapid and, seemingly, quantitative.

Sulphur di(methanethiosulphonate) reacts with piperidine to give sulphur dipiperidide, as do other derivatives [1] of S^{++}:

$$S(S_2O_2CH_3)_2 + 4\ C_5H_{10}NH = S(NC_5H_{10})_2 + 2\ C_5H_{10}NH_2^+ + 2\ CH_3SO_2S^-$$

Selenium and tellurium di(methanethiosulphonate) react with sodium dimethyldithiocarbamate to give selenium and tellurium *bis*(dimethyldithiocarbamate), as do the selenopentathionate and telluropentathionate ions [4, 6, 7]:

$$Se(S_2O_2CH_3)_2 + 2\ (CH_3)_2NCS_2^- = Se(S_2CN(CH_3)_2)_2 + 2\ CH_3SO_2S^-$$

$$Te(S_2O_2CH_3)_2 + 2\ (CH_3)_2NCS_2^- = Te(S_2CN(CH_3)_2)_2 + 2\ CH_3SO_2S^-$$

Sodium thiosulphate reacts to displace the methanethiosulphonate groups, thus forming pentathionate, selenopentathionate and telluropentathionate:

$$S(S_2O_2CH_3)_2 + 2\ S_2O_3^{--} = S(S_2O_3)_2^{--} + 2\ CH_3SO_2S^-$$

$$Se(S_2O_2CH_3)_2 + 2\ S_2O_3^{--} = Se(S_2O_3)_2^{--} + 2\ CH_3SO_2S^-$$

$$Te(S_2O_2CH_3)_2 + 2\ S_2O_3^{--} = Te(S_2O_3)_2^{--} + 2\ CH_3SO_2S^-$$

The reactions serve to emphasize the structural relationship of the thiosulphonates and the thiosulphates of divalent sulphur, selenium and tellurium. It appears, furthermore, that the nucleophilic reactivity of the methanethiosulphonate ion, in displacements on these divalent elements, is lower than that of the thiosulphate ion, as has been demonstrated earlier [1, 8] in the case of displacements of ethane- and *p*-toluenethiosulphonate by thiosulphate, on divalent sulphur.

From a chemical point of view, the reactions with piperidine and with dimethyldithiocarbamate and thiosulphate ions demonstrate the structure of the compounds as methanethiosulphonates of divalent sulphur, selenium and tellurium. The reactions strongly indicate that in each compound a divalent sulphur, selenium or tellurium atom forms a bridge between two methanethiosulphonate groups, through the thio sulphur atom of these groups, *cf.* the analogous argument for the presence of unbranched chains in the pentathionate, selenopentathionate and telluropentathionate ions [1, 4, 6, 9].

Experimental

Sulphur and selenium di(methanethiosulphonate). About 20 % excess, with respect to sulphur dichloride and diselenium dichloride, of finely powdered, dry sodium methanethiosulphonate was employed, and 4 ml of dry ether per g of thiosulphonate. The suspensions were cooled in ice-salt, and the chloride slowly added from a pipet. On treatment of the solid particles with a glass rod the sulphur dichloride colour vanished rapidly; in the case of diselenium dichloride, selenium was liberated. The mixtures, while cold, were filtered, drained well, and the solid residues on the filters were dried *in vacuo* over sulphuric acid, while the filtrates were discarded. The residues were subsequently treated several times with water, drained, and dried again. The sulphur di(methanethiosulphonate) was recrystallized by dissolving 2 g of the crude product in 8 ml of warm chloroform, and adding an equal volume of carbon disulphide. The crude selenium compound, containing an equimolar amount of selenium, was treated with ethylacetate at about 60° C (10 ml per g of sodium methanethiosulphonate employed). The mixture was filtered, and the pale green filtrate cooled in ice-salt. The selenium di(methanethiosulphonate) thus obtained was recrystallized from ethylacetate (1 g dissolved in 15—20 ml at 60° C, and cooled in ice-salt) or from chloroform (1 g dissolved in 25 ml at boiling temperature, and 25 ml of carbon disulphide added).

0.1007 g substance: 0.4616 g $BaSO_4$.
$S(S_2O_2CH_3)_2$ (254.4) Calc. S 63.01 Found S 62.96
0.1138 g substance: 15.05 ml of 0.1002 *N* iodine (Norris & Fay).
$Se(S_2O_2CH_3)_2$ (301.3) Calc. Se 26.20 Found Se 26.15

Tellurium di(methanethiosulphonate). To 4 g of tellurium dioxide dissolved in 15 ml of concentrated hydrochloric acid and 5 ml of water were added dropwise, over a period of 10—15 minutes, under stirring and cooling in ice-salt, 15 g (about 10 % excess) of sodium methanethiosulphonate dissolved in 15 ml of water. A semi-solid oil separated out, which, on continued stirring and treatment with a glass rod, changed to a yellow solid. It was filtered off, washed with 10 ml of 6 *N* hydrochloric acid, and treated on the filter with portions of methanol (3 times 10 ml), ether (3 times 10 ml), and warm chloroform (5 times 20 ml), and finally washed with ether and dried *in vacuo* over sulphuric acid. The crude products (5—6 g) thus obtained, in various preparations, contained about 32 % of tellurium, or 85—90 % of tellurium di(methanethiosulphonate). It was dissolved in boiling chloroform (100—150 ml per g) and the solution filtered. About half of the chloroform was subsequently boiled off, until crystals began to separate out, and the solution was then allowed to cool to room temperature. The crystals were filtered off, washed with ether and dried *in vacuo* over sulphuric acid.

0.3063 g substance: 0.1114 g Te.
$Te(S_2O_2CH_3)_2$ (349.9) Calc. Te 36.47 Found Te 36.37

The reactions with piperidine and with sodium dimethyldithiocarbamate. (1) To 2.067 millimole (0.5260 g) of sulphur di(methanethiosulphonate) dissolved in 20 ml of chloroform were added 10 millimole (1 ml) of piperidine. After a few minutes the chloroform was evaporated off *in vacuo* and the excess of piperidine removed *in vacuo* over sulphuric acid. The colourless crystalline residue was then treated with water, and the sulphur dipiperidide filtered off, dried *in vacuo* over sulphuric acid, and weighed: 0.4092 g, *i. e.*, 2.042 millimole of $S(NC_5H_{10})_2$. It was recrystallized from methanol [1] and thus melted at 75° C, not depressed in mixture with a sample obtained from other sources. The aqueous filtrate, containing piperidinium methanethiosulphonate, was made up to 500 ml in a volumetric flask, and 25 ml were pipetted out and analyzed iodometrically as described earlier [1] in the case of analogous experiments with sulphur ethanethiosulphonates and dimethylthiophosphates: 16.16 ml of 0.1002 *N* iodine, corresponding to 4.048 millimole of piperidinium methanethiosulphonate.

(2) 1.004 millimole (0.3025 g) of selenium di(methanethiosulphonate) dissolved in 30 ml of ethylacetate were added rapidly, with stirring, to 100 % excess (4 millimole) of sodium dimethyldithiocarbamate in 15 ml of methanol. A yellowish green solid immediately separated out. It was filtered off, washed with methanol and ether, and dried: 0.3032 g, *i. e.*, 0.9491 millimole of selenium *bis*(dimethyldithiocarbamate). It melted at 180—182° C and was found to contain 24.78 % Se; calc. [7] for $Se(S_2CN(CH_3)_2)_2$: 24.72 %.

(3) To 0.9936 millimole (0.3476 g) of tellurium di(methanethiosulphonate) dissolved in 10 ml of bromoform were added 2.5 millimole of sodium dimethyldithiocarbamate in 5 ml of methanol. A reddish solid immediately separated out, which was filtered off and treated as under (2): 0.3430 g, *i. e.*, 0.9322 millimole of tellurium *bis*(dimethyldithiocarbamate). It was found to contain 34.56 % Te; calc.[7] for $Te(S_2CN(CH_3)_2)_2$: 34.67 %.

The reactions with sodium thiosulphate. About 0.4 millimole of sulphur, selenium or tellurium di(methanethiosulphonate) was employed in each experiment, and about 1 millimole (25 % excess) of sodium thiosulphate.

(4) 0.1080 g of $S(S_2O_2CH_3)_2$ was dissolved in 5 ml of ethylacetate and 20 ml of ethanol in a 100 ml volumetric flask. 10 ml of 0.09977 *N* sodium thiosulphate were added, and the solution was made up to 100 ml by means of water. 25 ml were pipetted out, and, after addition of 5 ml of 10 % acetic acid and 1 g of potassium iodide, the excess of thiosulphate was back-titrated with 0.01096 *N* iodine: 3.71 ml (theoretically, 3.40 ml). The original reaction mixture became faintly opaque after about 10 minutes. On addition of sodium hydroxide, a distinct turbidity developed immediately (sulphur being liberated from the formed pentathionate).

The addition of potassium iodide serves to depress the oxidation of methanethiosulphonate ions by iodine. Although thiosulphonate ions are indifferent to iodine in presence of iodide, in neutral and acid solutions, their oxidation by iodine is to a slight degree induced by the simultaneous oxidation of thiosulphate by iodine [1].

(5) 0.1234 g of $Se(S_2O_2CH_3)_2$ were dissolved in 10 ml of chloroform in a 100 ml volumetric flask, and 25 ml of 0.09977 *N* sodium thiosulphate were added. On shaking, the chloroform layer became rapidly colourless, its greenish colour reappearing in the aqueous layer. The mixture was made up to 100 ml, and 25 ml of the aqueous layer (total volume, 90 ml) were pipetted out and titrated with 0.01096 *N* iodine as described under (4): 2.43 ml (theoretically, 2.00 ml).

The pale greenish aqueous layer showed the typical reactions of selenopentathionate; thus, selenium was liberated when alkalies were added, and, on addition of sodium dimethyldithiocarbamate, greenish selenium *bis*(dimethyldithiocarbamate) separated out.

(6) 0.1392 g of $Te(S_2O_2CH_3)_2$ was dissolved in 10 ml of bromoform, and 10 ml of 0.09977 *N* sodium thiosulphate were added. On shaking, the yellow colour of the bromoform layer became rapidly transferred to the aqueous layer. In this case, the excess of thiosulphate cannot be back-titrated with iodine, since the product, telluropentathionate, is not indifferent to iodine [4]. However, since tellurium di(methanethiosulphonate) is insoluble in water, the shifting of the yellow colour from the bromoform layer to the aqueous layer provides definite evidence that telluropentathionate has been formed. Furthermore, the aqueous layer liberated tellurium with alkalies and gave tellurium *bis*(dimethyldithiocarbamate) with sodium dimethyldithiocarbamate.

PRELIMINARY X-RAY DATA

The crystals of sulphur, selenium and tellurium di(methanethiosulphonate) are monoclinic prismatic. They appear as needles or prisms elongated along the *b* axis, and in most cases flattened along the *a* axis. The most frequent forms are $\{001\}$ and $\{102\}$ and, less developed, $\{10\bar{1}\}$.

The dimensions of the unit cells * were obtained from oscillation and Weissenberg photographs, using FeK_α radiation, $\lambda = 1.934$ Å. Density determinations were made by flotation in suitable solvents.

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* The data reported at *Det 7de Nordiska Kemistmøtet*, Helsingfors, Aug. 21–25, 1950, correspond to a different choise of *c* axis (space group symbol, $P2_1/c$).

Table 1. Unit cells of sulphur, selenium and tellurium di(methanethiosulphonate).

	a ± 0.02 Å	*b* ± 0.02 Å	*c* ± 0.04 Å	β	Density calc.	Density found
$S(S_2O_2CH_3)_2$	11.33	5.21	16.14	91°	1.78	1.77
$Se(S_2O_2CH_3)_2$	11.38	5.23	16.23	91°	2.07	2.05
$Te(S_2O_2CH_3)_2$	11.43	5.29	16.32	91°	2.36	2.35

There are four molecules per unit cell. Absent spectra, $(h0l)$ when $h + l$ is odd, and $(0k0)$ when k is odd. Space group, for all three compounds, C_{2h}^5 — $P2_1/n$.

SUMMARY

Methanethiosulphonates of divalent sulphur, selenium and tellurium are described for the first time. X-ray data show that the crystals are is isomorphous, and, thus, that the compounds have analogous structures.

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Constituents of Pine Heartwood

XXV. * Investigation of Forty-Eight *Pinus* Species by Paper Partition Chromatography

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Previous parts of this series have dealt with the isolation of heartwood substances from a number of pines by conventional methods. In Part XX [1] the general methods for analysing pine heartwood extracts by paper chromatography were described, and it was there mentioned that a large number of pines had been investigated by this new technique.

The present paper describes the results of these investigations, which have been carried out on a total of forty-eight *Pinus* species including those which were studied by the earlier methods. It has been found that the use of paper chromatography generally reveals the presence of additional substances which could not be isolated by these methods; in one case only, that of strobochrysin [2] in *P. strobus*, was it not possible to identify on the chromatogram a phenol which had been isolated from the heartwood by other means.

The results are summarised in Table 1 (see below), in which the pine species are listed in the order given in Shaw's monograph [3]. A few pines which were regarded by Shaw to be subspecies or varieties of other pines have been listed as separate species according to more recent literature [4,5]. The pines that have not been investigated are omitted from the table.

SPOTS BELONGING TO UNKNOWN SUBSTANCES

As described in previous papers, some of the new substances discovered by the use of paper chromatography have later been actually isolated from the heartwood extracts. There are, however, several such substances which are visible on the chromatogram but have not yet been isolated. These are:

* XXIV. *Acta Chem. Scand.* 5 (1951) 1.

Table 1. Heartwood constituents of the genus P i n u s.

+ = isolated from the heartwood
− = not found by paper chromatography
no sign = not investigated
+ = identified by paper chromatography
? = identification uncertain

PSM = Pinosylvin monomethyl ether
DHPS = Dihydropinosylvin
DHPSM = Dihydropinosylvin monomethyl ether

		Species	Pinosylvin	PSM	DHPS	DHPSM	Pinocembrin	Chrysin	Pinostrobin	Tectochrysin	Pinobanksin	Strobobanksin	Strobopinin	Cryptostrobin	Unknown compounds (see text) D	E	F	G
A. Haploxylon																		
Subsection *Cembra*	*Cembrae*	*P. koraiensis* Sieb. & Zucc.	+	+	+	+	+	+	+	+	+	−	−	−	+	−	−	−
		P. cembra L.	+	+	+	+	+	+	+	+	?	−	−	−	+	−	−	−
		P. albicaulis Engelm.	+	+	+	+	+	+	+	+	+	−	−	−	+	−	−	−
	Flexiles	*P. flexilis* James	?	+	+	+	+	+	+	+	+	−	−	−	+	−	−	−
	Strobi	*P. ayacahuite* Ehrenb.	+	+	+	+	?	+	?	+	+	−	−	−	−	−	−	−
		P. Lambertiana Dougl.	−	−	−	−	+	?	+	+	+	+	+	?	+	−	−	−
		P. parviflora Sieb. & Zucc.	+	+	+	+	+	+	−	−	+	−	+	+	+	−	−	−
		P. peuce Griseb.	−	−	−	−	+	+	+	+	?	−	+	+	+	−	−	−
		P. Griffithii M'Clelland (= *P. excelsa* Wall.)	+	+	−	+	+	+	+	+	+	−	−	−	−	−	−	−
		P. monticola Dougl.	+	+	−	+	+	+	+	+	?	−	+	+	+	−	−	−
		P. strobus L.	+	+	−	+	+	+	+	+	+	+	+	+	−	−	−	−
	Cembroides	*P. cembroides* Zucc.	−	+	−	+	+	+	+	?	?	−	−	−	−	+	+	−
Subsection *Paracembra*	*Gerardianae*	Not investigated																
	Balfourianae	*P. Balfouriana* A. Murr.	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	−
		P. aristata Engelm.	?	+	−	+	+	+	+	+	?	−	−	−	+	+	−	−
B. Diploxylon																		
Subsection *Parapinaster*	*Leiophyllae*	*P. leiophylla* Schlecht. & Cham.	+	+	−	−	+	−	−	−	+	−	−	−	+	−	−	−
		P. Lumholtzii Robins. & Fern.	+	+	?	+	+	−	−	−	+	−	−	−	−	−	−	−
	Longifoliae	*P. canariensis* Smith	+	+	−	−	+	−	−	−	+	−	−	−	−	+	−	−
	Pineae	*P. pinea* L.	+	+	−	−	+	−	−	−	+	−	−	−	−	+	−	−

		Species	Pinosylvin	PSM	DHPS	DHPSM	Pinocembrin	Chrysin	Pinostrobin	Tectochrysin	Pinobanksin	Strobobanksin	Strobopinin	Cryptostrobin	Unknown compounds (see text) D	E	F	G
Subsection *Pinaster*	*Laricines*	*P. resinosa* Ait.	+	+	–	–	?	–	–	–	?	–	–	–	–	+	+	–
		P. massoniana Lamb.	+	+	–	–	+	–	–	–	+	–	–	–	+	+	+	+
		P. densiflora Sieb. & Zucc.	+	+	–	–	+	–	–	–	+	–	–	–	+	+	+	–
		P. sylvestris L.	+	+	–	–	+	–	–	–	+	–	–	–	–	+	–	–
		P. mugo Turra (= *P. montana* Mill.)	+	+	–	–	+	–	–	–	?	–	–	–	+	+	+	?
		P. nigra var. *Poiretiana* (= *calabrica*) Schneid.	+	+	–	–	+	–	–	–	–	–	–	–	–	+	+	?
		P. nigra var. *austriaca* (Hoess) Badoux	+	+	–	–	+	–	–	–	–	–	–	–	+	+	+	–
	Australes	*P. Montezumae* Lamb.	+	+	–	–	+	–	–	–	+	–	–	–	–	+	+	–
		P. ponderosa Dougl.	+	+	–	–	+	–	–	–	+	–	–	–	+	+	+	–
		P. Jeffreyi Balf.	+	+	–	–	+	–	–	–	+	–	–	–	+	+	+	+
		P. occidentalis Swartz	+	+	–	–	+	–	–	–	+	–	–	–	–	+	+	+
		P. palustris Mill.	?	+	–	–	+	–	–	–	+	–	–	–	+	+	+	+
		P. caribaea Morelet	–	+	–	–	+	–	–	–	+	–	–	–	+	+	+	+
		P. taeda L.	+	+	–	–	+	–	–	–	+	–	–	–	+	–	–	–
		P. glabra Walt.	+	+	–	–	+	–	–	–	+	–	–	–	+	+	+	+
		P. echinata Mill.	+	+	–	–	+	–	–	–	+	–	–	–	+	+	?	+
	Insignes	*P. halepensis* Mill.	+	+	–	–	+	–	–	–	+	–	–	–	+	+	+	+
		P. pinaster Ait.	+	+	–	–	+	–	–	–	+	–	–	–				
		P. virginiana Mill.	?	+	–	–	+	–	–	–	+	–	–	–	?	+	+	–
		P. clausa Vasey*	+	+	–	–	+	–	–	–	+	–	–	–	–	–	–	–
		P. rigida Mill.	+	+	–	–	+	–	–	–	+	–	–	–				
		P. Pungens Lamb.	+	+	–	–	+	–	–	–	+	–	–	–	+	+	+	+
		P. Banksiana Lamb.	+	+	–	–	+	–	–	–	+	–	–	–	+	+	?	–
		P. contorta var. *latifolia* Engelm.	+	+	–	–	+	–	–	–	+	–	–	–	+	+	–	+
		P. muricata D. Don	+	+	–	–	+	–	–	–	+	–	–	–				
		P. attenuata Lemm.	?	+	–	–	+	–	–	–	+	–	–	–	+	+	–	?
		P. radiata D. Don	+	+	–	–	+	–	–	–	+	–	–	–	+	+	+	–
		P. radiata var. *insignis* Dougl.	+	+	–	–	+	–	–	–	+	–	–	–				
	Macrocarpae	*P. sabiniana* Dougl.	+	+	–	–	+	–	–	–	+	–	–	–	+	+	+	+
		P. Coulteri D. Don	+	+	–	–	+	–	–	–	+	–	–	–	+	+	+	+

* One sample of *P. clausa* also contained pinostrobin and 3,5-dihydroxy-7-methoxyflavanone, see Part XXII[6].

1. *Dihydropinosylvin?* A spot with $R_F \sim 0.1$ giving a red colour at the first moment of spraying with benzidine reagent has been observed on the chromatograms of some pines from the subgenus *Haploxylon*. Since an authentic specimen of dihydropinosylvin gives a spot with identical properties [1], it seems very probable that the spot should be due to this substance, especially as its monomethyl ether has been found in several pines. (See Table 1 and Part XXIII [7]). To judge from the colour intensity of the spot, the content of dihydropinosylvin is always very low, and no efforts have been made to isolate the substance.

2. *Compound A.* A spot with $R_F \sim 0.1$ which is stained yellow by benzidine reagent. This compound has been found in *P. Massoniana*, *P. densiflora*, *P. Montezumae* and *P. occidentalis*, all from the subgenus *Diploxylon*.

3. *Compound B.* A pale red spot appearing just below the pinobanksin spot ($R_F \sim 0.2$). It is not due to chrysin and has been observed in a few *Diploxylon* pines.

4. *Compound C.* A spot with $R_F \sim 0.4$ giving a red colour with benzidine reagent. This spot has only been observed on chromatograms from *P. koraiensis* and *P. Griffithii*.

5. *Compound D.* A rather sharp spot with $R_F \sim 0.45$ in standard solvent, giving a bluish-green fluorescence when the unsprayed paper is exposed to ultraviolet light but giving no distinct colour with benzidine reagent. This spot has been observed in most pines from both subgenera (see Table 1). It is always strongest in the sodium carbonate fraction.

6. *Compound E.* A large and trailing spot with $R_F \sim 0.55$ in standard solvent. It is not visible after spraying with benzidine reagent but gives a faint bluish fluorescence in ultraviolet light which changes to very intense blue after spraying with sodium carbonate. This compound has been found in most pines from the subgenus *Diploxylon* but only in three *Haploxylon* pines, all of which belonging to the subsection *Paracembra*. (See Table 1.)

7. *Compound F.* A small crescent-shaped spot with the same fluorescence as E, which appears just below the spot of pinosylvin monomethyl ether ($R_F \sim 0.73$). It is found in roughly the same pines as E. (See Table 1.) Both these compounds accumulate in the strong alkali fraction.

8. *Compound G.* A spot having $R_F \sim 0.85$ in ligroin-ether (5 : 1), giving a yellowish fluorescence in ultraviolet light after spraying with sodium carbonate. It accumulates in the strong alkali fraction and has only been found in the subgenus *Diploxylon* (see Table 1).

The chromatograms of *P. palustris* contain one spot which seems to be unique for this species. It is stained pale red by benzidine reagent and has $R_F \sim 0.9$ but does not fluoresce under the quartz lamp. It accumulates in

the sodium carbonate and 0.2 % sodium hydroxide fractions. This compound may possibly be identical with "pinopalustrin", a phenolic lactone of the lignan type which has recently been isolated from stumps of *P. palustris* by Harris. (Private communication from Dr. C. G. Harris to Prof. H. Erdtman.)

Finally, in the strong alkali fraction from *P. Coulteri*, there is a compound which gives a reddish-brown spot with benzidine reagent, having $R_F \sim 0.8$. In contrast to dihydropinosylvin monomethyl ether, this spot requires some time to become visible. Traces of a similar spot can be observed on the chromatogram of *P. sabiniana*.

WATER-SOLUBLE COMPOUNDS

The isolation of *l*-arabinose from the heartwood extract of *P. sylvestris* was described by Erdtman in Part IV of this series [8]. Subsequently, the same sugar has been isolated from several other pines. In the present investigation, the water-soluble fraction of the acetone extract from each pine was investigated for sugars by paper chromatography. The presence of arabinose could be demonstrated for all pines investigated except *P. koraiensis* and *P. parviflora*. In addition to the arabinose spot, the chromatograms often showed a second spot, belonging to glucose.

Pinitol, which has been isolated from some pines belonging to the subgenus *Haploxylon*, is more difficult to identify on the paper chromatogram. Potassium permanganate in sodium carbonate solution [9] and ammoniacal silver nitrate have been tried as spraying reagents, but neither of them gives a sufficiently sure identification of pinitol, especially as its R_F value does not differ very much from that of glucose.

PINOSYLVIN DIMETHYL ETHER

A small quantity of pinosylvin dimethyl ether was once isolated from *P. nigra* by Erdtman [10]. In ligroin, this compound has $R_F \sim 0.9$ and can be identified with the aid of its fluorescence in ultraviolet light. The neutral fractions of the pines available here have been investigated in that way. In most cases, a spot similar to that of pinosylvin dimethyl ether was found on the chromatograms, both from *Haploxylon* and *Diploxylon*. The identification is, however, very uncertain, the fluorescence of pinosylvin dimethyl ether being much weaker than that of other spots in the neighbourhood. (The neutral fractions often fluoresce even in daylight.) Therefore, no list of the pines giving a positive test will be published here.

INVESTIGATION OF SAPWOOD

When stained with diazotised benzidine reagent, the sapwood is only slightly yellow coloured, thus indicating that its content of phenols is very low. The heartwood always acquires a more or less strongly red colour by the same treatment [11]. Traces of the heartwood phenols can, however, be found by paper chromatography even in sapwood extracts. Table 2 shows the result of an investigation of the sapwood of nine pine species.

Table 2. Investigation of sapwood by paper chromatography.

Species	Pinosylvin	PSM	DHPSM	Pinocembrin	Chrysin	Pinostrobin	Pinobanksin	Arabinose	Glucose	Unknown compounds	
										D	E
A. Haploxylon											
P. albicaulis	−	+	+	−	−	−	−	+	+	+	−
P. Griffithii	−	+	+	−	−	−	+	+	+	−	+
P. monticola	−	+	−	+	−	+	−	+	+	+	−
P. strobus	−	+	+	+	+	+	+	?	+	−	−
P. aristata	−	+	+	−	−	−	−	+	+	+	+
B. Diploxylon											
P. sylvestris	+	+	−	−	−	−	−	+	+	+	−
P. mugo	+	+	−	+	−	−	−	+	?	+	+
P. Banksiana	+	+	−	+	−	−	+	+	+	+	+
P. contorta	−	−	−	+	−	−	−	+	?	+	−

The content of phenols in the sapwood is always very low, but the content of sugars and compounds D and E seems to be comparable with that in the heartwood, to judge from the colour intensity of the spots. These substances, therefore, cannot be regarded as true heartwood constituents.

INVESTIGATION OF BARK

The bark of three species, *P. strobus*, *P. sylvestris* and *P. Banksiana*, has also been investigated. In no case could any traces of the heartwood phenols be discovered on the chromatograms. The initial spot usually gives a strong red or yellowish-brown colour with diazotised benzidine, indicating the presence of phenolic products with $R_F = 0$ in standard solvent.

EXPERIMENTAL

The amount of heartwood from each pine used for the investigation was 20—30 g, if such a quantity was available. In some cases, however, only 0.5—1 g were used. The finely-ground heartwood was extracted with ether in a Soxhlet apparatus for four hours, then dried in the air and extracted with acetone for another four hours. Both extracts were tested on the paper chromatogram. If the ether extract contained any appreciable amounts of heartwood phenols, it was concentrated to a small volume and precipitated with light petroleum. The insoluble part was then separated, dissolved in ether and combined with the ether solution of the acetone extract.

The acetone extract was concentrated to a small volume and the residue treated with a few ml of water. The water solution (W) was used for the sugar test (see below). The water-insoluble part was dissolved in ether, filtered and divided into fractions by shaking with sodium bicarbonate, sodium carbonate, 0.2 % sodium hydroxide and 4 % sodium hydroxide. The bicarbonate extraction was sometimes omitted. Each extract was acidified and taken up in ether. These ether solutions were used for the paper chromatograms; their concentrations often had to be adjusted by dilution or evaporation of the solvent in order to get good chromatograms. Two chromatograms with standard solvent [1] were run for each fraction. One of these was stained with benzidine reagent [1], and the other observed under the quartz lamp before and after spraying with 5 % sodium carbonate [12]. The two-dimensional technique and the chromatograms for identification of tectochrysin and pinostrobin described in Part XX [1] were also used. The neutral fractions were chromatographed in ligroin for the detection of pinosylvin dimethyl ether (see above).

The W fraction was concentrated in a vacuum to a small volume, and a paper chromatogram of this solution was run for 16 hours, using ethyl acetate-acetic acid-water (3 : 1 : 3) as the solvent [13]. Eight chromatograms were run simultaneously on a 25 × 40 cm sheet (Munktell OB). One of these contained a reference mixture of arabinose and glucose. The solvent was allowed to drip off the lower edge of the paper, which had been cut like the teeth of a saw. The spraying reagent used was aniline hydrogen phtalate [14], which gives reddish-brown spots for pentoses and greyish spots for aldohexoses after drying at 105°.

SUMMARY

The heartwood constituents of forty-eight *Pinus* species have been investigated by the aid of paper chromatography.

The results will be discussed in a succeeding paper.

We are indebted to a large number of persons, in Sweden and abroad, for the generous supply of wood samples. In addition to those mentioned in earlier papers, samples have been received from Mr. P. G. Arne, Böda; Prof. H. Burström, Lund; Mr. K. E. Johansson, Alvastra; Count H. von Schwerin, Esperöd, Kivik; Dr. P. Greguss, Szeged, Hungary; Dr. B. H. Paul, Madison, Wisc., USA; Dr. T. Walsh, Dublin, Eire; and Dr. G. N. Wolcott, Puerto Rico.

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Constituents of Pine Heartwood

XXVI. * A General Discussion

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The investigation of natural products has in the past been mainly concentrated on substances which are of interest either because of their colour or because they have potential technical, pharmacological or biochemical value. Taxonomic considerations have seldom guided the choice of the substances investigated. In recent years a considerable amount of research has been carried out on the biochemical and organic chemical aspects of genetics, and this work is a necessary condition for future developments in the chemistry of the evolution of species. At the present stage useful contributions in this field can be made by systematic studies of the chemistry of suitable genera of plants and animals. For this purpose genera should be chosen in which the number of species is neither too small nor too large, and it must also be decided which organs of the plant or animal and which of the substances found in them are of the greatest taxonomic significance, which is often difficult. Those organs which were evolved far back in geological time provide the best material for systematic studies, specialised organs of more recent evolution being of less value. The best "taxonomic tracer substances" should occur in organs of the former type and have little-specialised functions. Such substances ought to be essentially indifferent to external influences and hence "conservative".

The conifers constitute a group of plants which ought to be well suited for studies on chemical taxonomy. They are not too numerous (about 560 species), and they are comparatively well investigated from a taxonomic point of view. Some genera are of suitable size, as for instance *Pinus* (80—90 species). This genus is well distinguished from other conifers; botanists divide it into two subgenera (sections), *Haploxylon* (Soft or White Pines) and *Diploxylon*

* XXV. *Acta Chem. Scand.* **5** (1951) 121.

(Hard Pines). (Recently, the names *Strobus* and *Eupitys* have been proposed for these subgenera by Rehder[1].) Both subgenera can be traced back to the early cretaceous period and have thus existed for about 125 million years[2].

Different botanists have subdivided the genus *Pinus* into smaller units in different ways. Shaw, in his monograph of 1914, divided it into four subsections and thirteen groups [3]. More recent handbooks contain systems of subdivision which are somewhat different from that of Shaw [1, 4, 5, 6]. The main differences between these systems, however, belong to the subgenus *Diploxylon* and are of little interest for this investigation, as will be seen below. Shaw's system has been employed in Table 1, Part XXV [7].

An attempt to classify the pines from a chemical standpoint has been made by Mirov. In a recent paper [8], he has collected all information available about the composition of turpentines from different *Pinus* species. He also reviews previous investigations on some other genera. In many cases, pines which according to botanists are closely related also give turpentines of similar composition, but sometimes two very intimately related pines may yield turpentines of quite different composition, as for instance *P. ponderosa* and *P. Jeffreyi*. There does not seem to be any fundamental difference between the turpentines of pines from the two subgenera, *Haploxylon* and *Diploxylon*.

It was considered to be of great interest to compare the chemistry of pine heartwood constituents with either of the systems of classification based on botanical evidence. The investigations described in this series have dealt mainly with the phenolic constituents of the heartwood, but the water-soluble constituents (sugars and pinitol) have also been investigated to some extent. Other extractable heartwood products, such as the resin acids, have been left entirely outside the investigation, because they do not seem to be characteristic for the heartwood and, furthermore, they are more difficult to isolate in a pure state.

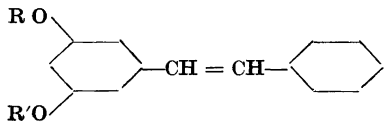
The non-digestibility of the heartwood of *P. sylvestris* in the normal sulphite process was investigated among others by Hägglund and his co-workers [9]. They arrived at the conclusion that it must be due to the occurrence of crystalline compounds in the heartwood. These compounds could be extracted from the wood with acetone, but not with ether, although they were soluble in ether when isolated. The acetone extract of pine heartwood contained ether insoluble compounds ("membrane substances"), later investigated by Erdtman [10], which were supposed to protect the active compounds mechanically against ether extraction from the wood. From the acetone extract of *P. sylvestris*, Erdtman isolated two phenols, pinosylvin and its monomethyl ether (Part II [11]), which are responsible not only for the non-digestibility of pine heartwood but also for its high resistance to attack by wood-destroying

fungi [12, 13]. Further investigations revealed the presence of pinosylvin phenols in other pine species, and in addition hydroxylated flavones and flavanones were isolated. (Parts V—VII [14].) These early investigations have now been continued and extended to include a large proportion of the recent pine species (about 25 %). (Parts IX—XVII [15, 16] and XIX [17].) The general methods of extraction and fractionation used in these investigations are described in Part IX [15]. In one case, a new compound was isolated by these methods from a pine previously investigated (Part XVIII [18]).

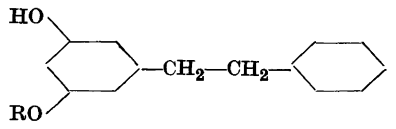
The introduction of paper chromatography for investigating heartwood extracts rendered a rapid and sensitive qualitative analysis of a large number of pines possible. (Part XX [19].) A total of forty-eight species were investigated, including those which had been studied by the earlier methods (Part XXV [7]). Several new compounds were now discovered, and some of them subsequently isolated from the heartwood extracts and their structures elucidated. (Parts XXII [20], XXIII [21], and XXIV [22].) Other substances of incompletely elucidated structure isolated during the earlier period of this series of investigations have been subjected to renewed studies. (Parts XXI [23] and XXIV [22].)

BIOCHEMISTRY OF HEARTWOOD PHENOLS

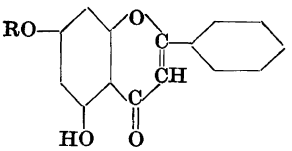
The following scheme gives the structures of all pine heartwood phenols at present known:



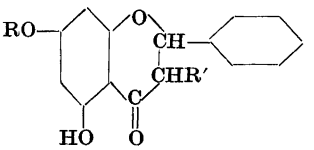
R = H, R′ = H: Pinosylvin
R = H, R′ = CH_3: Pinosylvin monomethyl ether
R = CH_3, R′ = CH_3: Pinosylvin dimethyl ether



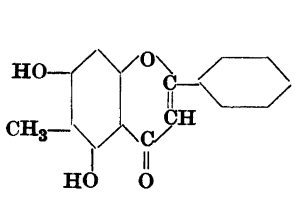
R = H: Dihydropinosylvin
R = CH_3: Dihydropinosylvin monomethyl ether



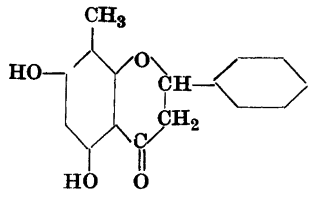
R = H: Chrysin
R = CH_3: Tectochrysin



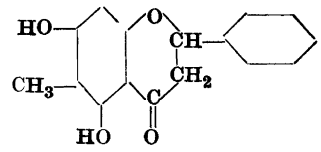
R = H, R′ = H: Pinocembrin
R = CH_3, R′ = H: Pinostrobin
R = H, R′ = OH: Pinobanksin
R = CH_3, R′ = OH: Pinobanksin monomethyl ether



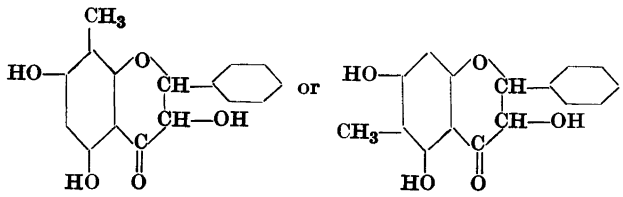
Strobochrysin?



Stropopinin?



Cryptostrobin?



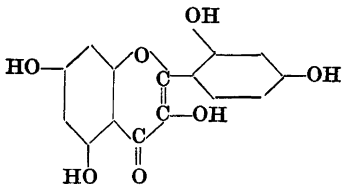
Strobobanksin

One of these fifteen substances, dihydropinosylvin, has not yet been isolated from any pine, but the paper chromatogram clearly shows that it is present in those pines which contain dihydropinosylvin monomethyl ether [7]. There are five pairs of compounds in which one (a flavanone or a dibenzyl) is the dihydro derivative of the other (a flavone or a stilbene). As will be seen below, this is of the greatest taxonomic importance. Three of the compounds listed above are 3-hydroxyflavanones (pinobanksin, its monomethyl ether and strobobanksin). The flavonols which can be formed by dehydrogenation of these substances have not so far been found in the heartwood of any pine. All the flavones or flavanones contain either a phloroglucinol or a methylphloroglucinol nucleus. The flavanones are all optically active.

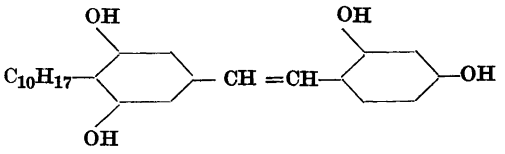
Only two of these compounds have been found in plants other than pines, namely chrysin and tectochrysin, which occur in poplar buds [24].

All pine heartwood phenols isolated up to the present have one group in common, the unsubstituted phenyl group. Flavones and flavanones (C_{15} compounds) are widely distributed in the vegetable kingdom, but only a few stilbenes (C_{14} compounds) have been discovered so far. As far as the writer knows, flavones and stilbenes have never been isolated from one and the same plant except in the genus *Pinus*. In one case, however, such compounds have been found in two species belonging to the same genus. The wood of *Chlorophora tinctoria* Gaud., a South American tree, contains morin (3,5,7,2′,4′-pentahydroxyflavone) [25]. Recently, a compound called chlorophorin has been isolated from *Chlorophora excelsa* Benth. and Hook f., an African tree with

highly durable wood. This compound has been shown to be a stilbene derivative [26]:



Morin



Chlorophorin

Like the unsubstituted phenyl group in pine heartwood phenols, the 2′,4′-dihydroxyphenyl group is repeated both in morin and in chlorophorin. This group is rare in natural flavones, the 3′,4′ or 5′-positions generally being substituted. The occurence of the same grouping in both types of compounds may possibly indicate some biochemical inter-relationship.

The occurrence of flavones and flavanones derived from methylphloroglucinol (C_{16} compounds) may be compared with the facile C-methylation of phloroglucinol. Perkin has reported C-methylation of quercetin by the action of methyl iodide in alkaline solution [27]. Thus, the C_{16} compounds may perhaps be regarded as the result of a biochemical methylation of a normal C_{15} compound.

As to the biological significance of the heartwood phenols, little is known except that the pinosylvin phenols serve as a defence against attacks from fungi, and possibly also from insects. The C_{15} compounds have no great fungicidal activity, and their insecticidal activity has not yet been tested. They may possibly serve as anti-oxidising agents.

HEARTWOOD PHENOLS AND BOTANICAL RELATIONSHIP

The distribution of the heartwood phenols in the genus *Pinus* is summarised in Tab. 1, Part XXV [7]. The great difference between pines from the two subgenera, *Haploxylon* and *Diploxylon*, is clearly demonstrated. Four substances, pinosylvin and its monomethyl ether, pinocembrin and pinobanksin are very common in both subgenera. Only two pines, *P. Lambertiana* and *P. peuce*, contain no pinosylvin phenols, but this may of course be due to individual variations. Pinocembrin is found in all pines investigated except perhaps *P. resinosa*. Pinobanksin is also widely distributed. The pines belonging to *Diploxylon* very seldom contain any phenols other than these four, the only exceptions being some of the unidentified compounds mentioned in Part XXV [7]. Pinostrobin and pinobanksin monomethyl ether were isolated from a

single (perhaps anomalous) specimen of *P. clausa* (Part XXII [20]) and are therefore not considered.

The subgenus *Haploxylon* contains four types of substances, which are not found in *Diploxylon*. The first two are flavones (chrysin, tectochrysin) and dibenzyls (dihydropinosylvin or its monomethyl ether). The latter were totally absent only in two species, *P. Lambertiana* and *P. peuce*, which also did not contain any pinosylvin phenols (see above). Thus, apparently the subgenus *Haploxylon* is able to dehydrogenate flavanones to flavones and to hydrogenate stilbenes to dibenzyls. Probably these two reactions are in some way biochemically connected. Since the stilbenes and the flavanones are distributed throughout the genus they are probably the primary products of biosynthesis.

The third group of heartwood constituents specific to *Haploxylon* consists of flavones and flavanones containing a methylphloroglucinol nucleus (strobopinin, cryptostrobin, strobochrysin, strobobanksin, see Part XXIV [22]). These substances have only been found in five pines, *P. Lambertiana* and *P. monticola* from the Pacific coast of North America, *P. strobus* from North-eastern America, *P. parviflora* from Japan and *P. peuce* from the Balkan Mountains. All these pines belong to the group *Strobi* in Shaw's system [3]. (See Table 1, Part XXV [7].) The same group is also contained in more recent systems [1,5,6]. Two other species from the same group, *P. Griffithii* (= *P. excelsa*) from the Himalayas and *P. ayacahuite* from Mexico, did not contain any C_{16} compounds. Thus, the geographical distribution of the species seems to have no connection with the occurence of C_{16} compounds.

The fourth class of compounds specific to *Haploxylon* comprises flavones or flavanones methylated in the 7-position (tectochrysin, pinostrobin). With the exception of the anomalous specimen of *P. clausa* mentioned above, such compounds have not been found in *Diploxylon*.

P. Lumholtzii, a Mexican pine belonging to the subgenus *Diploxylon*, may constitute an interesting exception to the rules stated above, since it seems to contain dihydropinosylvin monomethyl ether and possibly also dihydropinosylvin. The other heartwood constituents are those ordinarily found in *Diploxylon*. (See Tab. 1, Part XXV [7].) *P. Lumholtzii*, in Shaw's system [3], belongs to the subsection *Parapinaster*, containing five species, which have some features in common with *Haploxylon*. In Pilger's system [5], *P. Lumholtzii* belongs to the section *Pseudostrobus* containing among other species *P. Montezumae*, *P. ponderosa* and *P. Jeffreyi*. If these dibenzyls actually did prove to be present in *P. Lumholtzii*, that species could be regarded as an interesting intermediate between *Haploxylon* and *Diploxylon*, but an investigation of many specimens of this somewhat inaccessible species is necessary to settle the point.

Of the unidentified compounds which have been found on the chromatograms, some give a colour reaction with the benzidine reagent [19], whilst others can only be traced by their colour in ultraviolet light. The former compounds seem to occur more or less at random in a few species, but the fluorescent substances are more widely distributed. Compound D (Part XXV [7]) has been found in most pines from both subgenera, compounds E and F in *Diploxylon* and in a few pines from *Haploxylon*, but compound G only in *Diploxylon*. At present, nothing can be said about the chemical nature of these compounds, except that they must be weak acids, since they are extracted from the ether solution by sodium carbonate or hydroxide. Furthermore, the fluorescence of three of them (E, F, G) is increased by the addition of sodium carbonate.

WATER-SOLUBLE HEARTWOOD CONSTITUENTS

The occurrence of free *l*-arabinose in the acetone extract of pine heartwood [10, 16, 17] is remarkable, because apart from its discovery in the heartwood of another conifer, *Thuja plicata* D.Don [28], this sugar does not appear to have been previously found in nature in the free state [29]. Of course, the possibility remains that the arabinose occurs in the form of a poly- or oligosaccharide, which is hydrolysed even under the very mild conditions of extraction (acetone at a temperature below 50°). Free glucose also seems to occur in the acetone extracts from most pines [7].

Due to the difficulty of identifying pinitol on the chromatogram, it has not been possible to give an answer to the question whether this cyclitol is characteristic for the subgenus *Haploxylon* only. Considering however that pinitol has been isolated from six different *Haploxylon* pines but never from any *Diploxylon* pine, all evidence points in that direction.

The sugars and the fluorescent compounds D and E have also been found in sapwood (Part XXV [7]), in which their concentrations seem to be comparable with those in the heartwood. Pinitol is possibly a true heartwood substance. Erdtman was unable to isolate it from the sapwood of *P. strobus* [14].

PHENOLS IN SAPWOOD AND BARK

As seen from Tab. 2, Part XXV [7], traces of the most common heartwood phenols have also been found in the sapwood. This observation indicates that these compounds are generated in the cambium, from where they are transported centripetally through the sapwood via the medullary rays and finally accumulated in the heartwood. If the cambium is injured, the formation of heartwood phenols stops behind that injury [13]. The heartwood phenols are

apparently not distributed centrifugally into the bark. In the three species, the bark of which was investigated, not even traces of the heartwood phenols were detected. The bark is, however, rich in phenolic products of other kinds. From other conifers, an example is known of the same substance occurring both in the bark and the heartwood, namely the 3,5,7,3′,4′-pentahydroxyflavanone in *Pseudotsuga taxifolia* Britt.[30, 31]. Recently, a flavonol derivative of unknown structure has been isolated from the bark of *P. ponderosa*[32]. This compound, which is responsible for the yellow colour of the bark, is not identical with any of the heartwood phenols.

VARIATIONS WITHIN THE SAME SPECIES

Generally, only one wood sample from each species has been investigated, and this, of course, limits the possibility of drawing general conclusions. In the cases where two or more samples from one species have been investigated, however, the coincidence between the different samples was generally good as regards the number of substances found on the chromatogram. For instance, two samples of *P. Griffithii* (= *P. excelsa*), one from Himalaya and one from a pine cultivated in England, gave identical chromatograms. An exception was *P. clausa*, one sample of which contained pinostrobin and pinobanksin monomethyl ether, which were not found in other samples of the same species (Part XXII[20]). The percentage of each constituent in the heartwood may, however, vary within rather wide limits for different samples of the same species. Therefore, the yields of heartwood phenols reported from the preparative investigations[15–17] must not be regarded as average contents for the species investigated. An investigation of several hundred pines from different parts of Sweden (*P. sylvestris*) shows that the content of pinosylvin phenols in the heartwood varies between 0.4 % and 1.3 %, the average being 0.9 %. (Erdtman, Frank, and Lindstedt, to be published.)

MEMBRANE SUBSTANCES AND ETHER-EXTRACTABILITY OF THE PHENOLS

As mentioned earlier, the ether extract of *P. sylvestris* does not contain any appreciable amounts of phenols. The small quantities found have been ascribed to mechanical destruction of the "membranes" during the milling process[9, 10]. Some of the heartwood phenols are nearly always visible on the paper chromatogram of the ether extract, but the amount which is extracted with ether certainly varies very much for different species. In *P. Griffithii* (= *P. excelsa*) and *P. aristata*, a very large fraction of the total yield of heartwood phenols came from the ether extracts. (Parts XV and XVII[16].) The

content of "membrane substances" in these pines was a little above 0.1 % of the heartwood, which is equal to the yield generally obtained from *P. sylvestris* (Part IV [10]). Thus, it seems as if the quantity of »membrane substances» in the heartwood, contrary to the assumption of Erdtman [14], should have no great influence on the ether-extractability of the phenols, when different species are compared. However, as a general rule, the heartwood phenols can be extracted with ether to a higher extent in the subgenus *Haploxylon* than in the subgenus *Diploxylon*.

CONCLUSION

The present series of investigations shows that the heartwood phenols can be employed for a "chemical classification" of the genus *Pinus* and that there is a general agreement between botanical and chemical evidence. The two subgenera are clearly distinguished from each other. A further subdivision based on the heartwood phenols is possible only in a few cases. Considering that the investigation has included more than half of all recent *Pinus* species known, and that all groups except one are represented, it is hard to believe that any new phenolic constituents of fundamental significance will be found in the species which have not been investigated. "Trace compounds" may still remain to be discovered.

The systematic investigation of other chemical constituents of the pines, such as resin acids and their derivatives, would be an interesting complement to the present work. Another problem for the future would be to attempt hybridisation of different pines, especially between the two subgenera, and to investigate the heartwood phenols of the hybrids. The effect on the heartwood constituents of grafting a shoot of, for example, a *Haploxylon* pine on to a *Diploxylon* pine could also be studied.

SUMMARY

The investigation of the constituents of pine heartwood, as presented in previous parts of this series, has been reviewed and discussed. The following substances are characteristic for the two subgenera of the genus *Pinus:*

1) Subgenus *Haploxylon:* Stilbenes and dibenzyls; flavones and flavanones derived from phloroglucinol; and for the group *Strobi*, in addition, flavones and flavanones derived from methylphloroglucinol.

2) Subgenus *Diploxylon:* Stilbenes but not the corresponding dibenzyls; flavanones but no flavones.

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A Potentiometric and Extinctiometric Study of the Cupric Nitrite Complex System

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In the literature several investigations of cupric nitrite solutions are to be found, but the purpose has chiefly been to study the decomposition of the solutions. References to such investigations can be found in a paper by Gallais and Vives [1]. These authors have studied cupric nitrite solutions by several methods in order to establish which complexes are formed. The investigation is essentially of a qualitative nature and gives no information about the strength of the complexity. The only quantitative investigation, found in the literature, is an extinctiometric investigation by Kossiakoff and Sickman [2]. At the calculation of the complexity constants, however, certain assumptions have been made that render the estimation of the results rather difficult.

Previously no potentiometric investigation of this complex system seems to have been carried out, presumably due to the fact that copper and copper amalgam cannot be used to determine the cupric ion concentration in cupric nitrite solutions. The present author found that such solutions are rapidly reduced by copper amalgam with the formation of nitrite oxide.

The investigation methods chosen

The method of ligand displacement, developed by the present author in a previous paper [3], was used in this investigation for a potentiometric determination of the complexity constants of the mononuclear complexes. In this case ammonia was chosen as a displacing ligand. Then the measurements could be performed at such values of $p[H^+]$ * that the decomposition of the nitrite ion proceeded slowly and did not disturb the measurements. Besides ammonia forms so strong complexes with the cupric ion that the nitrite ion can be completely displaced as ligand even at rather low values

* $p[H^+] = -\log [H^+]$.

of the concentration of free ammonia, and this is essential for the applicability of the method (see Fronæus [3, p. 75]).

As in the earlier investigations of the present author the measurements were carried out at a constant ionic strength I = 1 C. In order to prevent the formation of hydroxo complexes the ionic medium consisted of ammonium perchlorate (0.500 C) and sodium perchlorate. Then, as the complex solutions contained an ammonia buffer, the concentration of free ammonia could be determined by emf measurements with the use of a glass electrode.

As a different method of investigating the cupric nitrite system the extinctiometric method was selected. Also these measurements were carried out at the ionic strength I = 1 C with sodium perchlorate as the neutral salt. In this case it was not possible to let ammonium perchlorate form part of the ionic medium, for in the absence of ammonia the p[H^+] in the solutions then would have become too low. Naturally such a difference in the composition of the ionic medium may cause some discrepancy between the potentiometrically and the extinctiometrically determined complexity constants.

CHEMICALS USED

Sodium nitrite, p. a. Stock solutions were prepared from calculated amounts of the dried preparation. The concentration was checked by oxidation with ceric sulphate in excess, strongly acidified with sulphuric acid. During stirring the nitrite solution was added from a pipette, put down into the ceric sulphate solution. Then the surplus of ceric sulphate was titrated with ferrous sulphate, the *o*-phenanthroline ferrous complex being used as oxidation-reduction indicator.

Ammonium perchlorate, p. a. The concentration of the stock solutions was checked by Kjeldahl distillation and titration of the ammonia obtained. From ammonium perchlorate and carbon dioxide-free ammonia a buffer was prepared.

The *other chemicals* were the same as before [4, p. 31].

THE POTENTIOMETRIC INVESTIGATION

Summary of notation and equations used

As in a previous paper (cf. Fronæus [3]) the following notation is used:

C_M, C_A, C_B = total concentrations of the central group M and the ligands A and B.

a = a quantity, defined by eq. (1) below.

$\beta_{j,k}$ = the complexity constant of the complex MA_jB_k ($j, k \geqq 0$)

$$X([A],[B]) = 1 + \sum_{j+k=1}^{N} \beta_{j,k} [A]^j [B]^k$$

$\bar{n}$ = the ligand number with respect to the ligand B.

$\Delta\bar{n}$ = the difference between the ligand numbers of two solutions, the first of which has [A] = 0, while [B] and a are the same in both solutions.

At the measurements described below C_M was a function of C_B:

$$C_M = a \cdot f(C_B) \tag{1}$$

Here the value of the parameter a was kept constant in a series of measurements. If we allow $a \to 0$ (that is $C_M \to 0$), the limiting function $(\Delta\bar{n})_{C_M=0}$ will be a function of $[A] = C_A$ and $[B]$. Corresponding values of $[A]$ and $X([A],0)$ can be calculated from eq. (14) in the previous paper [3, p. 75]. When the integration is performed graphically, it is often advantageous to use the following equivalent eq:

$$\log X([A],0) = 0.4343 \cdot \int_0^{b'} \left(\frac{\Delta\bar{n}}{[B]}\right)_{C_M=0} \cdot d[B] + \int_{\log b'}^{\log b} (\Delta\bar{n})_{C_M=0} \cdot d \log[B] \tag{2}$$

The upper limit of integration b must be selected in such way that $(\Delta\bar{n})_{C_M=0} = 0$, when $[B] \geqq b$ [3, p. 75]. Then the complexity constants $\beta_{j,0}$ of the mononuclear complexes MA_j can be computed from corresponding values of $[A]$ and $X([A],0)$ according to the methods described before [4, p. 28].

For the calculation of the complexity constants $\beta_{j,1}$ of the mixed complexes MA_jB we have the eq: [3, p. 76]

$$\lim_{\substack{C_M \to 0 \\ [B] \to 0}} \left(\frac{\bar{n}}{[B]}\right) = \frac{\sum_{j=0}^{N-1} \beta_{j,1} [A]^j}{X([A],0)} \tag{3}$$

The measurements and calculations

The emf measurements that tended to a determination of $[NH_3] = [B]$ in the complex solutions, were performed, as already mentioned, with a glass electrode (Radiometer, type G 100). The cells measured were of the following type:

$$+ \text{ RE} \;\|\; \begin{matrix}\text{complex} \\ \text{solution}\end{matrix} \;|\; \begin{matrix}\text{glass} \\ \text{electrode}\end{matrix} \; -$$

The reference electrode RE and the salt bridge had the same composition as before [4, p. 34]. In a measurement series the solution of the right half-cell was obtained by mixing two solutions S_1 and S_2 of the composition:

$$S_1 = \begin{cases} a & \text{mC } Cu(ClO_4)_2 \\ C_A & \text{mC } NaNO_2 \\ 500 & \text{mC } NH_4ClO_4 \\ (500 - 3\,a - C_A) & \text{mC } NaClO_4 \end{cases} \qquad S_2 = \begin{cases} C_A & \text{mC } NaNO_2 \\ 252 & \text{mC } NH_3 \\ 500 & \text{mC } NH_4ClO_4 \\ (500 - C_A) & \text{mC } NaClO_4 \end{cases}$$

Then the relation (1) between C_M and C_A in mC takes the following form:

$$C_M = a \cdot \left(1 - \frac{C_B}{252}\right)$$

To a known volume of the solution S_1 in the electrode vessel portions of the solution S_2 were added from a burette. Stirring in the cell solution was arranged by a stream of nitrogen gas. A valve potentiometer (Radiometer, type PHM 3 h) was used and the measurements were carried out in an air thermostat at 20.0° C. Concerning experimental details, the reader is referred to a previous treatise [4, p. 40]. The reproducibility of the emf was 0.3—0.4 mV. All measurements were repeated at least once.

After the last emf measurement in each series the nitrite concentration in the measuring solution and in S_1 and S_2 was determined. The determination was performed by oxidation with ceric sulphate in the manner described above (p. 140). In the solution S_1 the decrease in C_A was < 1 % (at $a = 20$ mC) after about one hour. The solution S_2 and the last measuring solutions in each series, which had higher p[H^+] -values, were rather stable, so that no decrease in C_A could be detected after twenty-four hours. Thus, as a measurement series could be performed fairly rapidly, the instability of the solutions did not affect the emf measurements.

The emf E in mV of the cell above is given by

$$E = E_k - s \cdot log\,[H^+] \tag{4}$$

Here E_k is a constant, as long as the asymmetri potential of the glass electrode, the liquid junction potential and the activity coefficient of H^+ has not changed, and s is the slope of the glass electrode.

At $C_M = 0$ but at the same values of C_A and C_B as in (4) we use the notations E_0 and $[H^+]_0$. Then, as the ionic medium is approximately unchanged, we have:

$$E_0 = E_k - s \cdot log\,[H^+]_0 \tag{4 a}$$

For the concentration $[H^+]_0$ it holds:

$$\frac{[H^+]_0 \cdot C_B}{[NH_4^+]} = K_c \tag{5}$$

K_c is the dissociation constant of the ammonium ion in the salt medium used and $[NH_4^+] = 500$ mC. At C_B varying between 3 and 125 mC E_0 was measured and graphically represented as a function of $log\ C_B$. The result was

a practically stright line with the slope $s = 57.5$ mV. The variation of E_0 with C_A is quite slight at $C_A \leqq 300$ mC which seems to justify the assumption that the partial exchange of sodium perchlorate for sodium nitrite does not affect the activity coefficients very much.

Combining eq. (4) and (4a) we obtain the expression of $E_B = E_0 - E$ in mV

$$E_B = 57.5 \; log \frac{[H^+]}{[H^+]_0} \tag{6}$$

A measurement series (with constant values of a and C_A) was started with the determination of the E_0-values, and then the emf:s E were measured. The reproducibility of the E_B-values was used as a control that the asymmetri potential had not changed perceptibly during the measurements of E_0 and E.

For the dissociation of the ammonium ion it holds:

$$\frac{[H^+] \cdot [B]}{500 - \vartheta} = \frac{[H^+]_0 \cdot C_B}{500} \tag{7}$$

Here $\vartheta = [HNO_2]$ is a quantity quite negligible.

Thus we have:

$$\frac{[H^+]}{[H^+]_0} = \frac{C_B}{[B]} \tag{7a}$$

The expression for the ligand number in respect of B is:

$$\bar{n} = \frac{C_B + \vartheta - [B]}{C_M} \tag{8}$$

At a constant value of $[A] \simeq C_A$ the correction term ϑ is fixed by the value of $[H^+]$ only. Therefore ϑ could be determined in an acetate buffer with a low value of C_{HAC}, which had the same value of $[H^+]$ (measured with the glass electrode) as the complex solution. The solutions I and II below with known values of C_{HAC} and C_{AC} were measured.

I $\begin{cases} C_{HAC} \text{ mC HAc} \\ C_{AC} \text{ mC NaAc} \\ C_A \text{ mC } NaNO_2 \\ (1\,000 - C_{AC} - C_A) \text{ mC } NaClO_4 \end{cases}$ II $\begin{cases} C_{HAC} \text{ mC HAc} \\ C_{AC} \text{ mC NaAc} \\ (1\,000 - C_{AC}) \text{ mC } NaClO_4 \end{cases}$

From the difference ΔE between the emf:s ϑ could be computed:

$$\Delta E = 57.5 \; log \frac{(C_{AC} + \vartheta) \cdot C_{HAC}}{(C_{HAC} - \vartheta) \cdot C_{AC}} \tag{9}$$

Table 1. Emf measurements on the three component complex system $Cu^{2+} - NO_2^- - NH_3$.

No.	C_M mC	C_B mC	$C_A = 0$ mC	$C_A = 100$ mC	$C_A = 200$ mC	$C_A = 300$ mC
			E_B mV			
1	19.48	6.56	137.6	131.5	127.7	123.0
2	19.24	9.71	136.1	130.3	126.3	122.1
3	18.76	15.81	131.2	125.3	121.8	117.6
4	18.16	23.0	123.5	117.2	114.3	109.4
5	17.64	29.7	114.4	108.2	104.7	99.5
6	16.68	42.1	93.3	87.4	83.2	79.3
7	15.00	63.1	49.7	47.1	45.7	42.9
8	13.64	80.3	27.7	27.2	27.0	26.0
9	12.00	101.0	15.6	15.9	15.8	16.0
10	10.00	126.3	8.9	8.5	8.8	8.9
11	9.74	6.56	117.5	111.5	107.3	103.0
12	9.62	9.71	112.6	106.3	102.5	97.9
13	9.38	15.81	100.0	93.0	89.4	85.4
14	9.08	23.0	81.0	73.8	70.7	66.9
15	8.82	29.7	60.9	55.8	53.2	50.4
16	8.34	42.1	33.9	31.8	30.7	30.5
17	7.50	63.1	15.9	15.4	15.2	16.0
18	6.82	80.3	10.4	10.1	9.8	10.3
19	6.00	101.0	6.6	6.6	6.8	7.0

The values of E_B, obtained at the emf measurements on the complex system are shown in Table 1. For the solutions with nos. 1—10 the parameter a in eq. (1) has a value of 20 mC and for the solutions nos. 11—19 a value of 10 mC.

In Table 2, [B] and $\bar{n}/[B]$ or $\bar{n}$ of the solutions measured have been calculated. At greater values of C_B the correction term ϑ is quite negligible. Though the relative random error in $\bar{n}$ is rather great for the solutions with nos. 10, 18 and 19, it is evident that at $[B] \simeq 50$ mC $\bar{n}$ has attained the "characteristic" co-ordination number $N = 4$ in every measurement series. Then A is completely displaced as ligand.

In Figs- 1 and 2, the measurements are graphically represented. As the measurements could be performed at so small values of the parameter a, owing to the fact that ammonia forms rather strong complexes, the difference C_A—[A] is comparatively small. The consequence of this is that the curves, corresponding to the same value of C_A but different values of a, coincide within the

Table 2. Determination of corresponding values of [B] and $\bar{n}/[B]$ or $\bar{n}$ at different C_A and a.

No.	$C_A = 0$		$C_A = 100$ mC			$C_A = 200$ mC			$C_A = 300$ mC		
	[B] mC	$\frac{\bar{n}}{[B]}$ C^{-1}	ϑ mC	[B] mC	$\frac{\bar{n}}{[B]}$ C^{-1}	ϑ mC	[B] mC	$\frac{\bar{n}}{[B]}$ C^{-1}	ϑ mC	[B] mC	$\frac{\bar{n}}{[B]}$ C^{-1}
1	$2.65 \cdot 10^{-2}$	12 650	0.16	$3.39 \cdot 10^{-2}$	10 100	0.29	$3.94 \cdot 10^{-2}$	8 900	0.37	$4.76 \cdot 10^{-2}$	7 400
2	$4.17 \cdot 10^{-2}$	12 050	0.09	$5.26 \cdot 10^{-2}$	9 650	0.17	$6.13 \cdot 10^{-2}$	8 300	0.24	$7.31 \cdot 10^{-2}$	7 050
3	$8.26 \cdot 10^{-2}$	10 150	0.03	0.105	8 000	0.05	0.120	6 700	0.07	0.142	5 900
4	0.164	7 650		0.211	5 950		0.237	5 300		0.288	4 340
		$\bar{n}$			$\bar{n}$			$\bar{n}$			$\bar{n}$
5	0.304	1.667		0.390	1.661		0.449	1.661		0.553	1.650
6	1.00	2.46		1.27	2.45		1.50	2.43		1.76	2.42
7	8.62	3.63		9.57	3.57		10.1	3.53		11.3	3.45
8	26.5	3.94		27.0	3.91		27.2	3.89		28.3	3.81
9	54.1	3.91		53.4	3.97		53.6	3.95		53.2	3.98
10	88.4	3.80		89.9	3.64		88.8	3.75		88.4	3.80
		$\frac{\bar{n}}{[B]}$ C^{-1}			$\frac{\bar{n}}{[B]}$ C^{-1}			$\frac{\bar{n}}{[B]}$ C^{-1}			$\frac{\bar{n}}{[B]}$ C^{-1}
11	$5.94 \cdot 10^{-2}$	11 250	0.07	$7.55 \cdot 10^{-2}$	8 900	0.10	$8.93 \cdot 10^{-2}$	7 550	0.13	0.106	6 400
12	0.107	9 350	0.02	0.138	7 200	0.02	0.160	6 200	0.02	0.193	5 150
		$\bar{n}$			$\bar{n}$			$\bar{n}$			$\bar{n}$
13	0.288	1.655		0.382	1.645		0.441	1.639		0.517	1.630
14	0.897	2.43		1.20	2.40		1.36	2.38		1.58	2.36
15	2.59	3.07		3.18	3.00		3.53	2.97		3.95	2.91
16	10.8	3.75		11.8	3.63		12.3	3.57		12.4	3.56
17	33.4	3.96		34.1	3.87		34.3	3.84		33.2	3.99
18	52.9	4.02		53.6	3.92		54.3	3.81		53.2	3.97
19	77.5	3.92		77.5	3.92		76.9	4.02		76.3	4.11

limits of experimental errors. Thus the limiting curves at $a = 0$ (that is $C_M = 0$) are obtained directly.

In Table 3 the functions $(\Delta\bar{n}/[B])_{C_M=0}$ and $(\Delta\bar{n})_{C_M=0}$ have been calculated at different [B] and [A] = 100, 200, and 300 mC. The values of the functions have been obtained from the curves in Fig. 1 and Fig. 2.

From Table 3 it is obvious that $b = 50.0$ mC can be taken as an upper limit of integration in eq. (2). The values of *log X* ([A],0), given in Table 4, have been computed by graphical integration and with $b' = 0.20$ mC.

The complexity constants $\beta_{j,0}$ of the mononuclear nitrite complexes MA_j have been calculated from corresponding values of [A] and X([A],0) in Table 4.

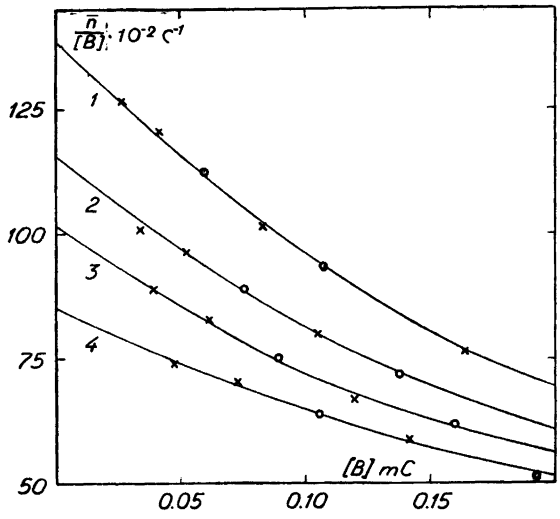

Fig. 1. $\bar{n}/[B]$ as a function of $[B]$. 1. $C_A = 0$; 2. $C_A = 100$ mC; 3. $C_A = 200$ mC; 4. $C_A = 300$ mC. ——— × : $a = 20$ mC; ○: $a = 10$ mC.

The determination was performed graphically (*cf.* Fronæus [4, p. 28—29]). The estimation of the maximum random errors was carried out in a similar way as before [3, pp. 77 and 84]. But in this case we neglect the error that the finite

Table 3. Determination of $(\Delta\bar{n}/[B])_{C_M=0}$ and $(\Delta\bar{n})_{C_M=0}$ as functions of $[B]$ at different values of the parameter $C_A = [A]$.

[B] mC	$C_A = 100$ mC	$C_A = 200$ mC	$C_A = 300$ mC	$C_A = 100$ mC	$C_A = 200$ mC	$C_A = 300$ mC
	$(\Delta\bar{n}/[B])_{C_M=0}$ C^{-1}			$(\Delta\bar{n})_{C_M=0}$		
0	2 200	3 650	5 300			
0.02	2 150	3 450	4 900			
0.05	1 900	3 050	4 200			
0.10	1 450	2 400	3 100	0.145	0.240	0.310
0.15	1 100	1 750	2 300	0.165	0.260	0.345
0.25	700	1 100	1 500	0.180	0.275	0.380
0.50				0.190	0.285	0.420
1.00				0.19	0.31	0.42
2.50				0.18	0.29	0.41
5.00				0.16	0.25	0.36
10.0				0.12	0.20	0.27
25.0				0.05	0.10	0.15
50.0				0	0	0
75.0				0	0	0

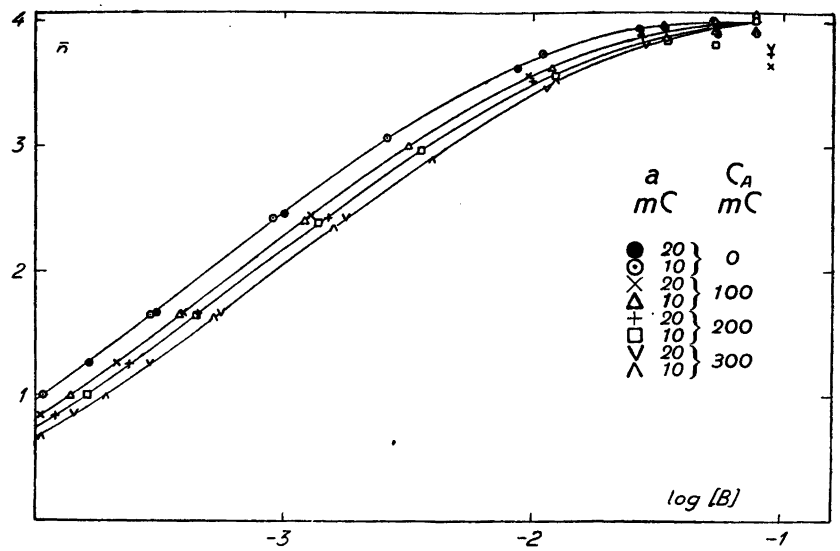

Fig. 2. $\bar{n}$ as a function of log [B] at different values of a and C_A.

range of integration in eq. (2) instead of an infinite may cause, as the ligand number with respect to ammonia attains the limiting value 4 within the concentration range of [B] used. The values of the constants obtained are:

$$\beta_{1,0} = 17 \pm 2\ \mathrm{C}^{-1} \quad ; \quad \beta_{2,0} = 30 \pm 8\ \mathrm{C}^{-2}$$

The $X([A],0)$ -value at [A] = 300 mC indicates that also the complex MA_3 is formed to some extent, but it seems too delicate to determine the constant $\beta_{3,0}$ from these measurements.

Table 4. Corresponding values of [A], $X([A], 0)$ and $(\bar{n}/[B])_{\substack{C_M=0 \\ [B]=0}}$.

[A] mC	*log* $X([A]), 0)$	$X([A], 0)$	$(\bar{n}/[B])_{\substack{C_M=0 \\ [B]=0}}$ C^{-1}	$\sum_{j=0}^{N-1} \beta_{j,1}\,[A]^j$ C^{-1}
0		1.00	13 900	13 900
100	0.473	2.97	11 600	34 500
200	0.759	5.74	10 200	58 500
300	1.053	11.3	8 500	96 000

With the constants obtained, the ligand number and the composition of the two component system $Cu^{2+} - NO^{2-}$ has been computed in Table 5. For the calculation the eq. (39—41) of a previous treatise [4, p. 29] were used.

The values in the fourth column of Table 4 have been obtained by extrapolation to [B] = 0 of the curves in Fig. 1. From corresponding values of [A] and $\sum_{j=0}^{N-1} \beta_{j,1}\,[A]^j$ in the same table the complexity constants of the complexes MB, MAB and MA_2B have been calculated.

$$\beta_{0,1} = (1.40 \pm 0.05) \cdot 10^4\ C^{-1}, \quad \beta_{1,1} = (1.9 \pm 0.2) \cdot 10^5\ C^{-2};$$
$$\beta_{2,1} = (1.6 \pm 0.5) \cdot 10^5\ C^{-3}$$

From the investigation of J. Bjerrum [5, p. 127] one gets $\beta_{0,1} = 1.6 \cdot 10^4\ C^{-1}$ at 20° C and in 1 C ammonium nitrate.

Assuming that we may disregard the mutual forces between the ammonia molecule and the nitrite ions taken up as ligands, we should by purely statistical reasons expect the following relation to hold for the thermodynamical complexity constants:

$$\beta_{j,1} = \frac{N-j}{N} \cdot \beta_{j,0} \cdot \beta_{0,1} \tag{10}$$

If we further assume that the taking up of an ammonia molecule does not change the activity coefficient of the complex ion very much, that is $f_{MA_jB} \simeq f_{MA_j}$ and $f_{MB} \simeq f_M$, eq. (10) can be used also at the ionic strength I = 1 C. With the values of $\beta_{1,0}$, $\beta_{2,0}$, and $\beta_{0,1}$ obtained and with N = 4 eq. (10) gives $\beta_{1,1} \simeq \beta_{2,1} \simeq 2.0 \cdot 10^5\ C^{-2}\ (C^{-3})$ in good agreement with the values calculated above.

Table 5. The ligand number $\bar{n}_A$ with respect to A and the composition of the two component system $Cu^{2+} - NO_2^-$ as calculated at different [A] with the constants obtained.

$\beta_1 = 17 \pm 2\ C^{-1}$ $\beta_2 = 30 \pm 8\ C^{-2}$

[A] mC	$\bar{n}_A$	100 α_0	100 α_1	100 α_2
10	0.155	85.0	14.5	0.5
50	0.520	52.0	44.0	4.0
100	0.765	33.5	56.5	10.0
200	1.035	18.0	60.5	21.5
300	1.190	11.5	58.0	30.5

THE EXTINCTIOMETRIC INVESTIGATION

The equations for the calculation of the complexity constants

At the calculations in this section the following notation is used (*cf.* Fronæus [4]):

E = the extinction of the complex solution.
d = the thickness of the absorbing layer.
$e = E/d$.
ε_M^0, ε_A^0, ε_j = the molar extinctions of M, A and the complex MA_j.
$\varepsilon_M = (e - \varepsilon_A^0 \cdot C_A)/C_M$.
β_i and $X([A])$ = the quantities denoted $\beta_{i,0}$ and $X([A],0)$ at the potentiometric measurements.
$X_1 = (X-1)/[A]$, $X_2 = (X_1-\beta_1)/[A]$.
$\bar{n}$ = the ligand number with respect to A.

The determination of the ligand number extinctiometrically is possible, only if mononuclear complexes are formed exclusively (*cf.* Olerup [6, p. 70] Fronæus [4, p. 90]). The calculation method has been described in a previous treatise [4, p. 87], so we shall only give a summary of the main points here.

Assuming that Beer's law can be applied to the complex solutions, we obtain the exprssion for e:

$$e = \varepsilon_M^0 [M] + \sum_{j=1}^{N} \varepsilon_j [MA_j] + \varepsilon_A^0 [A] \tag{11}$$

Introducing the complexity constants, we get:

$$\varepsilon_M = \frac{\varepsilon_M^0 + \sum_{j=1}^{N} (\varepsilon_j - j \cdot \varepsilon_A^0)\, \beta_j [A]^j}{X\ ([A])} \tag{12}$$

In every measurement series (with a constant value of d) we have a relation between C_M and C_A of the kind, given in eq. (1). Then ε_M can be graphically represented as a function of C_A and with a as a parameter. From eq. (12) it is obvious that a constant value of ε_M corresponds to a constant value of [A] and thus of $\bar{n}$. From the relation $C_A = [A] + \bar{n} \cdot C_M$ we consequently obtain:

$$\left(\frac{\partial C_A}{\partial C_M}\right)_{\varepsilon_M} = \bar{n} \tag{13}$$

Then, if C_A is plotted against C_M at a constant value of ε_M, a straight line with the intercept [A] on the C_A-axis and with the slope $\bar{n}$ should be obtained. The values of C_A are taken from the (ε_M, C_A)-diagram, mentioned above, and the values of C_M are calculated from the relations (1). When the ($\bar{n}$/[A], [A])-curve has been determined in this way, pairs of values (X([A]), [A]) are calculated in the manner, described before (see Fronæus [4, pp. 14 and 110]).

In the previous treatise [4, p. 90] it has been shown that extinctiometric measurements give a false "ligand number", when also dinuclear complexes are formed. The expression for $(\partial C_A/\partial C_M)_{\varepsilon_M}$ in this case contains [M] and the molar extinctions of the different complexes. So the derivative may depend on C_M and at a constant value of the intercept on the C_A-axis the calculated value of the "ligand number" may depend on the wave length λ used. At $C_M = 0$ the derivative is made up of the real ligand number $\bar{n}$ and a term depending on the wave length.

If possible, extinctiometric measurements should be carried out at different wave lengths in different absorption bands of the extinction curve. Of course the dependence on C_M and λ sometimes may be so small that it falls within the limits of the experimental random errors, and yet the calculated values f $\bar{n}$ may be wrong.

The measurements and calculations

The extinctiometric investigation was carried out with a Beckman Quartz Spectrophotometer (Model DU). In order to make measuring at a constant temperature (20.0° C) possible the apparatus had been altered in the manner described by Adell [7, p. 3]. Concerning other experimental details and the arranging of the measurements the reader is referred to a previous treatise [4, p. 93].

In order to select convenient wave lengths, at which the measurements could be carried through, the extinction curve *log* (e/C_M) of a solution of the composition 25 mC $Cu(ClO_4)_2$ + 925 mC $NaNO_2$ was determined and is represented in Fig. 3. At 725 mμ the curve has a relative maximum and differs considerably from the extinction curve of Cu^{2+}. As the systematic error in the extinction E, that not strictly monochromatic light may cause, vanishes, where the curve has a horizontal tangent, it was advisable to select this wave length. The other wave lengths chosen were 465 and 430 mμ. From Fig. 3, curve 3 it is evident that *log* ε_A^0 is very small at these wave lengths.

In a measurement series, corresponding to a constant value of d, the measuring solution was obtained by mixing two solutions of the composition a mC $Cu(ClO_4)_2$ + (1000—3 a) mC $NaClO_4$ and 1000 mC $NaNO_2$. In the manner,

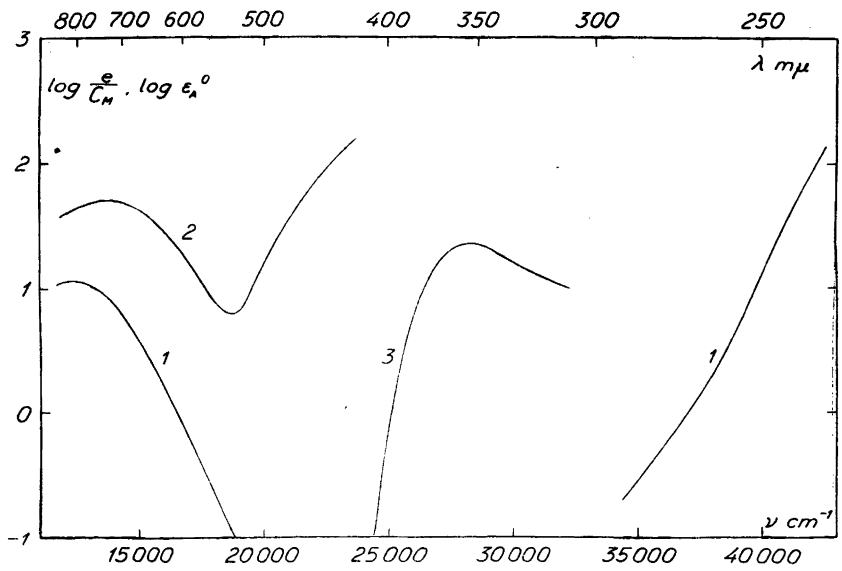

Fig. 3. Extinction curves of: 1. the cupric ion; 2. a complex solution with C_M = 25 mC and C_A = 925 mC; 3. the nitrite ion.

described above, it was checked that the nitrite concentration had not altered during the measurement. For the relation between C_M and C_A in mC we have:

$$C_M = a\left(1 - \frac{C_A}{1000}\right) \tag{14}$$

In the different measurement series the product $d \cdot a$ was kept constant and equal to 22.5 cm.mC. Thus at a constant concentration C_A the product $d \cdot C_M$ had the same value in different series. In order to avoid systematic errors in $\bar{n}$, caused by imperfect monochromaticity of the light and its partial reflection at the end-plates of the absorption cell, solutions with the same value of ε_M should be measured at approximately the same value of E that is of $d \cdot C_M$ (see Fronæus [4, p. 98], Olerup [6, p. 50]). From Table 7 it is evident that this condition is fulfilled.

Table 6 contains the values obtained of the function ε_M. The reproducibility was about 0.5 %. In Fig. 4 the fulldrawn curves represent the measurements at 725 mμ. The quotient $\varepsilon_M(430)/\varepsilon_M(465)$ is practically constant for all the solutions measured and the mean value is 2.42 ± 0.02. Thus $\varepsilon_{j+1}/\varepsilon_j$ (j = 1,2,...) is independent of the wave length within this wave length range. From the

Table 6. Extinction measurements on the complex system $Cu^{+2}-NO_2^-$.

d cm	C_M mC	C_A mC	$\lambda = 725$ mμ	$\lambda = 465$ mμ	$\lambda = 430$ mμ	$\lambda = 725$ mμ	$\lambda = 465$ mμ	$\lambda = 430$ mμ
			$(e-\varepsilon_A^0 \cdot C_A)$ cm^{-1}			ε_M $cm^{-1} \cdot C^{-1}$		
3	7.40	13.2	0.0945	0.0247	0.0603	12.75	3.34	8.15
3	7.35	19.6	0.106	0.0353	0.0867	14.4	4.80	11.80
3	7.26	32.3	0.125	0.0537	0.136	17.2	7.40	18.0
3	7.16	44.6	0.139	0.0693	0.168	19.4	9.68	23.5
3	7.03	62.5	0.158	0.0883	0.211	22.5	12.55	30.0
3	6.82	90.5	0.177	0.110	0.266	26.0	16.1	39.0
3	6.62	117.6	0.188	0.127	0.306	28.4	19.2	46.2
3	6.25	166.7	0.200	0.149	0.355	32.0	23.8	56.8
3	5.92	211	0.206	0.161	0.384	34.8	27.2	64.9
3	5.63	250	0.207	0.167	0.400	36.8	29.7	71.0
3	5.12	318	0.203	0.172	0.413	39.6	33.6	80.7
3	4.50	400	0.190	0.169	0.406	42.2	37.6	90.2
1	22.2	13.2	0.267	0.062	0.150	12.05	2.80	6.75
1	22.1	19.6	0.297	0.090	0.218	13.45	4.07	9.86
1	21.8	32.3	0.346	0.137	0.338	15.9	6.28	15.5
1	21.5	44.6	0.391	0.186	0.440	18.2	8.65	20.5
1	21.1	62.5	0.442	0.233	0.567	20.9	11.05	26.9
1	20.5	90.9	0.497	0.302	0.731	24.2	14.7	35.7
1	19.8	117.6	0.547	0.355	0.863	27.6	17.9	43.6
1	18.8	166.7	0.585	0.423	1.02	31.1	22.5	54.3
1	17.8	211	0.605	0.464	1.13	34.0	26.1	63.5
1	16.9	250	0.608	0.485	1.18	36.0	28.7	69.8
1	15.3	318	0.600	0.505	1.22	39.2	33.0	79.7
1	13.5	400	0.570	0.499	1.20	42.2	37.0	88.9
0.3	74.0	13.2	0.790	0.123	0.307	10.70	1.66	4.15
0.3	73.5	19.6	0.867	0.187	0.460	11.80	2.54	6.26
0.3	72.6	32.3	0.990	0.300	0.743	13.65	4.13	10.25
0.3	71.6	44.6	1.09	0.403	0.997	15.2	5.63	13.90
0.3	70.3	62.5	1.24	0.543	1.34	17.6	7.72	19.1
0.3	68.2	90.9	1.44	0.759	1.85	21.1	11.15	27.1
0.3	66.2	117.6	1.58	0.926	2.25	23.9	14.0	34.0
0.3	62.5	166.7	1.76	1.18	2.82	28.2	18.9	45.1
0.3	59.2	211	1.84	1.33	3.23	31.1	22.5	54.6
0.3	56.3	250	1.86	1.45	3.50	33.0	25.8	62.2
0.3	51.2	318	1.90	1.55	3.73	37.1	30.3	72.9
0.3	45.0	400	1.81	1.57	3.77	40.2	34.9	83.8

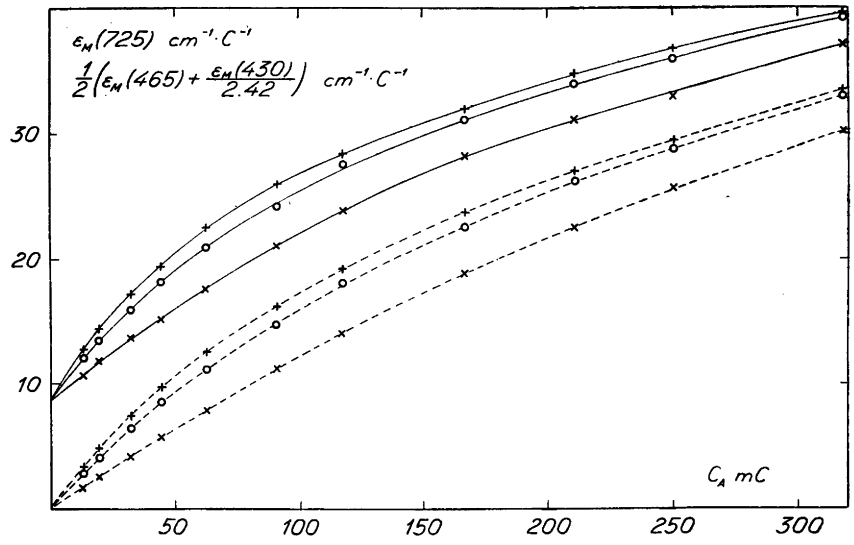

Fig. 4. Fulldrawn curves: $\varepsilon_M(725)$ as a function of C_A at different a. Dashed curves: 0.5 $\{\varepsilon_M(465) + \varepsilon_M(430)/2.42\}$ as a function of C_A at different a. —— +: *a = 7.5 mC;* ○: *a = 22.5 mC;* ×: *a = 75 mC (see eq. (14)).*

calculation method it is evident that the measurements at 430 mμ give the same ($\bar{n}$/[A], [A])-curve as the measurements at 465 mμ. In order to reduce the calculations but make use of all ε_M-values obtained, the mean values 0.5 (ε_M (465) + ε_M (430)/2.42) are represented in Fig. 4, the dashed curves.

Table 7 contains a number of selected ε_M-values. The corresponding concentrations C_A and C_M are taken from Fig. 4 and eq. (14). The connection between C_M and C_A at a constant ε_M proves to be linear and, as already mentioned, this is a necessary condition for the computation of $\bar{n}$.

In Fig. 5 the function $\bar{n}$/[A] is represented graphically. It is obvious that the measurements at 725 mμ within the limits of experimental random errors give the same $\bar{n}$/[A]-curve as the measurements at 465 and 430 mμ. This indicates that only mononuclear complexes are formed.

In Table 8 values of the functions X, X_1, and X_2 have been calculated at different [A]. By extrapolation of X_1 and X_2 to [A] = 0 we get the following values of the complexity constants β_1 and β_2:

$$\beta_1 = 20 \pm 1 \text{ C}^{-1}; \quad \beta_2 = 45 \pm 5 \text{ C}^{-2}$$

At the higher nitrite concentrations there is some indication that also the complex MA_3 is formed, but the tendency is so weak that β_3 cannot be determined.

Table 7. Determination of corresponding values of [A] and $\bar{n}/[A]$ from the extinction measurements.

ε_M (725) $cm^{-1} \cdot C^{-1}$	d = 0.3 cm		d = 1 cm		d = 3 cm		$C_M = 0$	$\bar{n}$	$\frac{\bar{n}}{[A]}$ C^{-1}
	C_M mC	C_A mC	C_M mC	C_A mC	C_M mC	C_A mC	$C_A = [A]$ mC		
12.0	73.4	21.7	22.2	13.2	7.43	10.4	9.3	0.169	18.2
14.0	72.3	35.6	22.0	22.6	7.37	18.0	16.4	0.267	16.3
17.0	70.7	57.5	21.6	38.0	7.27	31.0	28.6	0.410	14.3
20.0	68.9	82.0	21.2	56.5	7.15	48.0	44.5	0.545	12.2
23.0	66.9	108.0	20.7	77.5	7.00	67.0	63.0	0.675	10.7
26.0	64.5	140	20.1	105.0	6.82	91.0	87	0.82	9.4
29.0	61.6	179	19.4	139	6.57	125	120	0.96	8.0
32.0	58.0	227	18.5	180	6.25	167	159	1.17	7.4
35.0	54.0	280	17.3	230	5.89	215	207	1.35	6.5
37.0	51.2	317	16.4	270	5.59	255	247	1.37	5.55
$\frac{1}{2}\{\varepsilon_M(465) + \frac{\varepsilon_M(430)}{2.42}\}$									
3.00	73.3	23.2	22.2	14.3	7.41	11.7	10.4	0.175	16.8
5.00	72.1	39.2	21.9	24.6	7.34	20.5	18.2	0.291	16.0
8.00	70.2	64.4	21.6	41.5	7.24	35.3	31.5	0.470	14.9
11.0	68.3	89.5	21.1	62.0	7.10	52.5	49.0	0.595	12.1
14.0	66.2	117.5	20.6	84.5	6.95	73.5	69.0	0.730	10.6
17.0	63.9	148	20.0	110	6.77	98.0	92.0	0.875	9.5
21.0	60.6	192	19.1	149	6.48	136	130	1.02	7.8
25.0	56.9	242	18.1	195	6.13	183	174	1.20	6.9
28.0	53.6	286	17.1	238	5.81	226	217	1.29	5.9
30.0	51.4	315	16.4	269	5.56	259	250	1.26	5.0

Table 8. The functions $X([A])$, $X_1([A])$, and $X_2([A])$ at different [A].

[A] mC	$X([A])$	$X_1([A])$ C^{-1}	$X_2([A])$ C^{-2}
10	1.20	20	
25	1.53	21.2	
50	2.13	22.6	
75	2.79	23.9	52
100	3.53	25.3	53
150	5.26	28.4	56
200	7.43	32.2	61
250	9.98	35.9	64

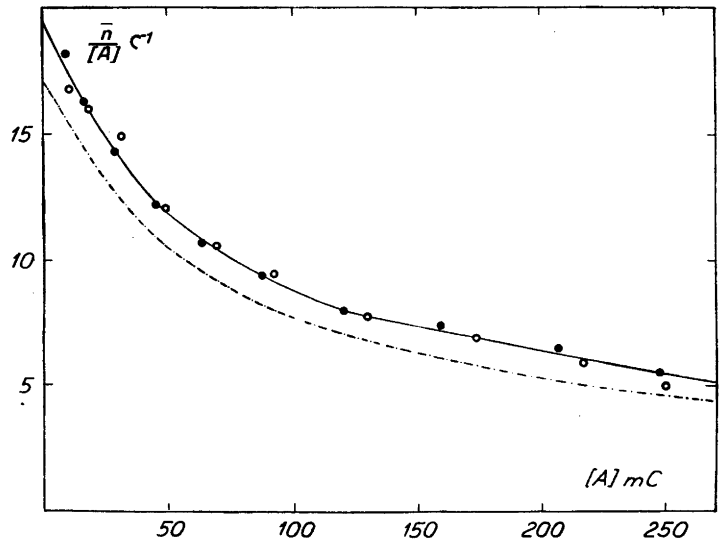

Fig. 5. Fulldrawn curve: the extinctiometrically determined $\bar{n}/[A]$ as a function of $[A]$. ——— ●: λ = 725 mμ; ○; λ = 465 and 430 mμ. Dashed curve: $\bar{n}/[A]$ calculated from the potentiometrically determined complexity constants.

Thus there is a good agreement between the results from the potentiometric and the extinctiometric investigations, and the constants obtained may be considered as true complexity constants. The small divergence between the two sets of constants may be caused, as mentioned before, by the difference in the composition of the ionic mediums in the two cases.

SUMMARY

The complexity of the cupric nitrite system has been investigated potentiometrically and extinctiometrically.

The method of ligand displacement was used, as it is the only potentiometric method, applicable on this system. Ammonia was the displacing ligand and the measurements were carried out with a glass electrode.

The extinctiometric investigation was performed in two different absorption bands at the wave lengths 725, 465, and 430 mμ. Consistent results have been obtained at the different wave lengths, showing that only mononuclear complexes are formed.

According to both methods the complexity constants β_j ($j = 1,2$) of the complexes $Cu(NO_2)_j^{2-}$ have been determined at 20° C and at the ionic strength 1 and found to be:

	β_1 C^{-1}	β_2 C^{-2}
pot. method	17 ± 2	30 ± 8
ext. method	20 ± 1	45 ± 5

The complex $Cu(NO_2)_3^-$ is formed in so small amounts within the nitrite concentration range used that β_3 cannot be computed.

From the potentiometric investigation the complexity constants $\beta_{j,1}$ ($j = 1,2$) of the mixed complexes $Cu(NO_2)_j\ NH_3^{2-j}$ have been calculated:

$$\beta_{1,1} = (1.9 \pm 0.2) \cdot 10^5\ C^{-2};\quad \beta_{2,1} = (1.6 \pm 0.5) \cdot 10^5\ C^{-3}$$

The author wishes to thank Professor S. Bodforss for the facilities, put at my disposal.

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On the Isolation and Structure of Hydroxylysine

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In 1921 van Slyke and Hiller [1] reported that an unidentified base occurred in gelatine. Later Schryver, Buston and Mukherjee [2] suggested that a basic amino acid occurring in gelatine which they isolated as a tribenzoate was an "oxylysine", α, ε-diamino-β-hydroxy-*n*-hexanoic acid. In 1938 van Slyke, Hiller, Dillon and MacFadyen [3] reported the isolation of a monopicrate of hydroxylysine melting at 225°. Elementary analysis, amino-N and amino acid carboxyl content corresponded to a diamino acid $C_6H_{14}N_2O_3$. van Slyke *et al.* [4, 5] later showed that alkaline periodate yielded one mole each of ammonia and of formaldehyde with the consumption of one atom of oxygen. As no indication of lactone formation was observed they suggested that the possible formulas were limited to I and II (Table 1). Martin and Synge [6] also isolated hydroxylysine from gelatine and isinglass as the picrate which decomposed at 226—27°. They used the acetylation-benzoylation method of Synge [7] on the base fraction isolated by precipitation with phosphotungstic acid.

Recently Heathcote [8] isolated the basic amino acids from gelatine hydrolysates by electrodialysis and after removal of arginine and histidine with flavianic acid studied the fractionation of the picrate of the remaining "lysine fraction". He also isolated small amounts of a picrate melting at 225°, but found this method of separation unsuitable, because the picrates like the phosphotungstates [5] of lysine and hydroxylysine apparently form solid solutions.

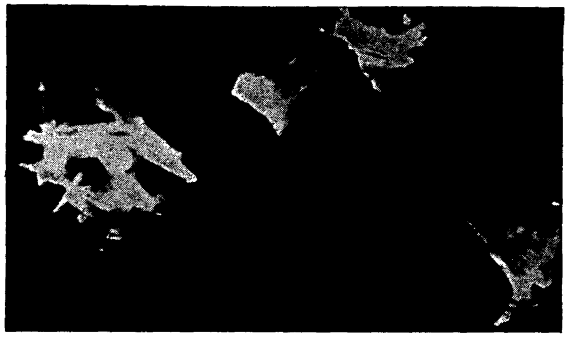
A number of workers [5, 9–15] have investigated the occurrence of hydroxylysine in various proteins with modifications of the periodate method. Collagen and gelatine have been found to contain about one per cent hydroxylysine, whereas only smaller amounts seem to be present in other proteins analyzed. Using paper chromatography, Gordon [16] recently made the interesting observation that a phosphate ester of hydroxylysine occurred in dialysate of calf embryo muscle juice.

As already mentioned in a preliminary note [17] we have utilized ion exchangers and partition chromatography to isolate pure hydroxylysine from gelatine or a preparation of dried fish skin.

After hydrolysis in 6 *N* hydrochloric acid and evaporation to dryness, the residue was dissolved in water and the remaining hydrochloric acid together with the acidic amino acids were removed with the anion exchanger Amberlite IR—4B. The basic amino acids in the effluent were then isolated by passing the solution through the carboxylic type cation exchanger IRC-50 essentially as described by Kunin and Winters [18]. However, instead of using the resin as the sodium salt we used it as the ammonium salt and eluted the basic amino acids with ammonia instead of with mineral acid. In this way the basic amino acids arginine, hydroxylysine, lysine are obtained after evaporation of the ammonia without contamination with much inorganic salts. Lysine and hydroxylysine were then absorbed on a column of the strong base anion-exchanger Amberlite IRA-400 that does not adsorb arginine [18] and eluted with hydrochloric acid. In this way a fraction was obtained consisting of a mixture of lysine and hydroxylysine in the proportion 7 to 1 contaminated with a small amount of arginine.

The separation of these amino acids was effected by partition chromatography with 0.1 *N* hydrochloric acid saturated with phenol supported on "Hyflo Super-Cel" as the stationary phase and phenol saturated with 0.1 *N* hydrochloric acid as the moving phase. With the latter medium on paper chromatograms lysine moves about twice as fast as hydroxylysine while arginine is still faster. In accordance with this all arginine and practically all lysine had left the aforementioned column before hydroxylysine appeared in the effluent. The fractions containing only hydroxylysine were combined, dilute hydrochloric acid was added and the phenol extracted with ether. Hydroxylysine can be crystallized in low yield directly from the residue but a good yield was obtained by benzoylation of the whole fraction in alkaline solution when a N,N′-dibenzoate of hydroxylysine was obtained (m. p. 166—67°) with the composition $C_{20}H_{22}O_5N_2$. The methylester (m. p. 138—39°) was prepared with diazomethane. Benzoylation of this ester with benzoylchloride in pyridine yielded a tribenzoate (m. p. 171—73°). The composition of these derivatives all corresponded to the one expected for a monohydroxydiaminohexanoic acid. $C_6H_{14}O_3N_2$. Fifty per cent of the nitrogen was liberated with periodate [4, 5]. Hydrolysis yielded the crystalline monohydrochloride of hydroxylysine, (Fig. 1).

In order to determine the configuration of the grouping split by periodate we oxidized the dibenzoate (III or IV) with an excess of chromic trioxide in 97.5 per cent acetic acid at 50° and determined the excess of oxidant left after

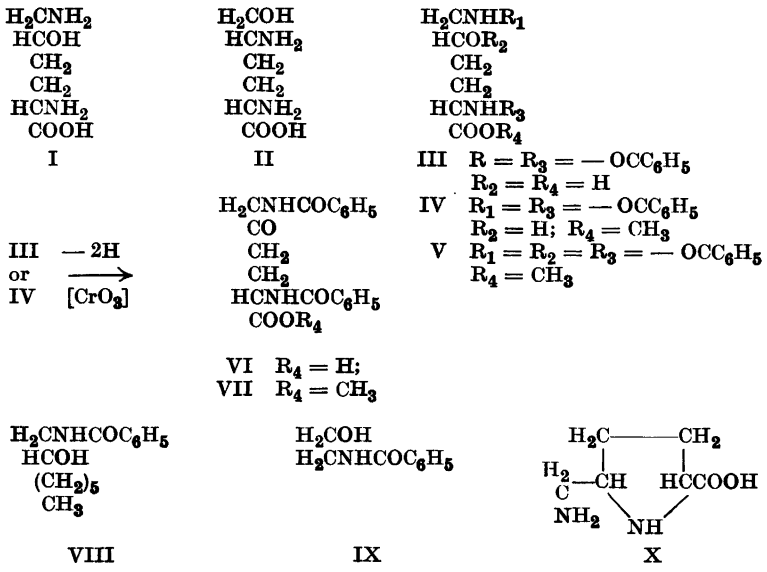


Hydroxylysine hydrochloride from aqueous ethanol (30 ×).

different times. The dibenzoate rapidly consumed two equivalents corresponding to the dehydrogenation of a secondary alcohol to a ketone (VI or VII) as shown in figure 2. 1-Benzamido-2-octanol (VIII) also consumed 2 equivalents whereas 2-benzamido-1-ethanol (IX) consumed 4 equivalents with the formation of a carboxyl group. The group reacting with periodate thus clearly has the configuration corresponding to VIII and hydroxylysine should have the configuration I of the two formulas proposed by van Slyke *et al.*[4, 5]. The reaction product from the oxidation of IV was isolated (m. p. 134—35°) and characterized. However, the configuration of the carbon chain of hydroxylysine had not been proved by van Slyke *et al.* We subjected a small sample of hydroxylysine to reduction with hydroiodic acid and red phosphorous at 160° for 4 hours. With paper chromatography it was found that no hydroxylysine could be found in the reaction mixture but instead a compound behaving like lysine had been formed together with another compound giving a strong yellow spot with ninhydrin. The yellow spot that represents the main reaction product is possibly caused by the cyclic derivative 5-aminomethyl proline (X) that is formed in analogy with the proline synthesis from α-amino-γ-hydroxy or γ-halogene pentanoic acid [19–21]. That the reaction mixture contained L-lysine was further strengthened by microbiological assay with *Leuconostoc mesenteroides* P-60. According to this analysis 5—10 per cent of the expected amount of L-lysine had been formed. Hydroxylysine does not support the growth of this organism.

The optical activity of the N,N′-dibenzoate and corresponding methyl esters of L-lysine and hydroxylysine are collected in Table 2 together with the δ-keto lysine derivative (VII) prepared from natural hydroxylysine in which the asymmetry of the δ-carbon atom has been destroyed. From these data it appears highly probable that hydroxylysine has the same configuration

Table 1.



as L-lysine. This is also supported by the recent work of Weisiger [29] with the enzymatic synthesis of the anilide from natural hydroxylysine.

Since our preliminary report of this work appeared [17], the isolation of pure hydroxylysine has also been announced from three different laboratories [27-29]. Sheehan and Bolhofer [27] have furthermore described the conversion of hydroxylysine to an optically inactive derivative, methyl-α,ε-diphthalimido-δ-keto-D,L-hexanoate which they also prepared from glutamic acid by an unambigous synthesis. Weisiger [29] has synthesized hydroxylysine and separated it into the L and D isomeres with aid of the enzymatic anilid formation.

Table 2.

R	R HCNHCOC$_6$H$_5$ COOCH$_3$	R HCNHCOC$_6$H$_5$ COOH
— $CH_2CH_2CH_2CH_2NHCOC_6H_5$	— 120	— 101
— CH_2CH_2CO, $CH_2NHCOC_6H_5$	— 84	
— CH_2CH_2CHOH, $CH_2NHCOC_6H_6$	— 156	— 119

M_D in pyridine

The configuration of the second asymmetric carbon atom in hydroxylysine is not yet known. The question remains if partial or total racemisation might have taken place during the preparation of gelatine or during the isolation procedure that would explain the differences in the reported specific rotations of hydroxylysine [27, 29].

EXPERIMENTAL

Isolation of the "lysine" fraction from gelatine hydrolysates

As a source of hydroxylysine Difco Bacteriological gelatine was first used. It was found, however, that this material contained some ornithine, probably originating by partial destruction of arginine in the alkali treatment during the manufacturing of the gelatine. As ornithine was difficult to separate from hydroxylysine in the chromatographic procedure, a commercial preparation of dried fish skin was used for some isolation experiments, because it was found to be free from ornithine. The material was hydrolyzed with about fifty times its weight of boiling 6 *N* hydrochloric acid for 20 hours. Excess hydrochloric acid was removed by repeated evaporation *in vacuo* with water. The "lysine" fraction was isolated with the aid of a series of ion exchange resins, essentially according to the method of Winters and Kunin [18]. The amino acids were dissolved in water and passed through a column of the weak base anion exchange resin Amberlite IR—4B which had been pretreated with one normal sodium hydroxide and then washed with distilled water until the effluent was neutral. This removed the hydrochloric acid left and most of the dicarboxylic amino acids. The effluent was then directly passed through a column with the carboxylic type cation exchange resin Amberlite IRC-50. This column had been converted to salt form by treatment with *N* ammonia instead of sodium hydroxide as recommended by Winters and Kunin. The column was then treated with ammonia-ammonium chloride buffer of pH 7.5 and finally water until the washings were free from chloride. With this treatment only amino acids with an isoelectric point higher than that of histidine were adsorbed, *i. e.* arginine, lysine, hydroxylysine, ornithine.

The column was then eluted with *N* ammonia in fractions. By using ammonia as the regenerating and eluting reagent, the formation of salts in the eluate was avoided. Attempts to obtain a complete fractionation of the basic amino acids by using ammonia of increasing strength were not successful, though hydroxylysine was enriched in the first fractions. The fractions containing hydroxylysine were combined and evaporated to dryness *in vacuo*, redissolved in water and passed through a column of the strong base anion-exchange resin Amberlite IRA-400 which had been converted to hydroxyl form by treatment with sodium hydroxide and washed to neutral reaction. As Winters and Kunin have shown, arginine passes through while amino acids that are weaker bases are adsorbed. After washing with water until the washings showed a negative Sakaguchi reaction, the column was eluted with *N* HCl and the eluate taken to dryness repeatedly with water to remove excess hydrochloric acid.

In some preparations the hydrolysis was performed with 6N sulfuric acid which was removed with barium hydroxide or barium carbonate. In these cases the first column with Amberlite IRA-4B was not used.

The Amberlite columns used in the following examples were pretreated as follows:

1. Amberlite IR-4B (28 × 500 mm). The column was washed with one liter of *N* hydrochloric acid and then with water until the effluent was chloride free.

2. Amberlite IRC-50 (28 × 300 mm) was converted to free acid with one liter *N* hydrochloric acid and washed chloride free with water. The resin was then converted to the ammonium salt with one liter of N ammonia and then buffered with 750 ml ammonia-ammonium chloride buffer of pH 7.5 and subsequently washed with 750 ml water.

3. Amberlite IRA-400 (28 × 400 mm) was treated with 2 liters of *N* sodium hydroxide followed by one liter of water.

Example 1. 50 g Difco Gelatine was hydrolyzed with 3.5 l boiling 6 *N* hydrochloric acid for 24 hours and then repeatedly evaporated to dryness *in vacuo* after addition of water. The residue was dissolved in 750 ml water and passed through the IR – 4B column followed by 750 ml water. The effluents were combined and directly passed through the IRC-50 column. The column was then washed with about one liter of water. The adsorbed basic amino acids were displaced by 0.1 *N* ammonia. The effluent was collected in fractions of 250 ml and the fractions giving a positive ninhydrin (fraction 2–5) were combined and evaporated to dryness *in vacuo*. The residue was dissolved in 750 ml and passed through the IRA-400 column that was then washed with one liter of water. The adsorbed lysine and hydroxylysine were then eluted with 500 ml *N* hydrochloric acid. The residue from the hydrochloric acid eluate weighed 2.72 g and contained[4,5] 0.34 g hydroxylysine calculated as the free base corresponding to a content of 0.68 per cent hydroxylysine of the starting material. Paper chromatography showed that the fraction consisted of lysine and hydroxylysine contaminated with some arginine and a little ornithine.

Example 2. 70 g of a commercial fish skin preparation was hydrolyzed with 3 l boiling 6 *N* hydrochloric acid for 24 hours and the hydroxylysine isolated using the same columns.

The isolated fraction weighed 2.0 g and analyses showed the presence of 0.145 g hydroxylysine. Hydroxylysine nitrogen constituted 12.6 per cent of the total nitrogen. Paper chromatography indicated that a little arginine was the only ninhydrin positive compound present except lysine and hydroxylysine.

Partition chromatography of the "lysine" fraction

Freshly distilled phenol was mixed with enough 0.1 N hydrochloric acid to yield a small aqueous phase over the phenolic phase. These solutions are stable and can be kept at room temperature for weeks. 30 ml of the aqueous phase was added to 60 g Hyflo Super-Cel. The mixture was worked with a spatula until homogenous. Enough of the phenolic phase was then added to give a suspension that could be poured into the chromatographic tube made by annealing a glass tubing (i. d. 35 mm, length 350 m) to a fritted glass funnel with the same inner diameter (Corning medium). The column was filled under a slight vacuum. When the upper surface was almost dry, the suction was discontinued, enough dry sand poured on top of the column to form a horizontal layer of about 10 mm depth. The solution containing the amino acids, generally 5–15 ml, was immediately carefully added with a pipette without disturbing the surface of the sand. The solution was washed into the column with several 5 ml portions of the phenolic phase.

When the "lysine" fractions were dissolved in the phenolic phase, an aqueous phase separated and an emulsion was formed by shaking the sample immediately before adding it to the column. The layer of dry sand helps in keeping the aqueous phase dispersed in small droplets. The column was then fastened over an automatic fraction collector [22] that changed the receiving vessel every half hour. A layer of fresh phenolic phase about 10 cm high was maintained by hanging a 1 liter separatory funnel with the tip of the stem at this height over the surface of the sand. The phenolic phase was then poured into the funnel, the stopper was inserted and the stop cock carefully opened. Each half hour fraction was 16—20 ml. The amino acids present in the fractions were ascertained by running one dimensional paper chromatograms with the same acid phenolic phase as moving phase.

Any arginine present first appeared in the effluent followed by lysine. When almost all lysine had left the column, hydroxylysine appeared in the effluent. When the fractions only contained hydroxylysine the addition of phenol was stopped and the column washed with water to collect the hydroxylysine left on the column. If ornithine was present, it appeared in the later lysine and earlier hydroxylysine fractions.

The fractions containing only hydroxylysine and the final water fraction were combined, a little hydrochloric acid was added and the phenol was extracted with ether. The aqueous phase was then taken to dryness *in vacuo*.

Benzoylation of the hydroxylysine-fraction in alkaline solution

The residue from a chromatogram was dissolved in a minimum of water (hydroxylysine content calculated from periodate-ammonia: 180 mg) and after addition of 5 ml 2 *N* sodium hydroxide it was benzoylated at room temperature by the method of Carter and Stevens [23] with 1.8 ml benzoyl chloride and 18 ml 2 *N* sodium hydroxide added in 9 equal portions during 3 hours. The reaction mixture was then acidified and filtered. After the dried precipitate had been exhaustively extracted with hot petroleum ether the residue weighed 470 mg. Crystallization from hot absolute ethanol yielded 252 mg of colorless needles, m. p. 165—67°. After recrystallization from ethanol or acetone the *N,N'-dibenzoate of hydroxylysine* melted at 166—67°.

$C_{20}H_{22}O_5N_2$ (370.4)
Calc. C 64.85 H 5.99 N 7.56
Found » 64.61, 65.00 » 5.98, 5.88 » 7.37, 7.35
Equiv. wt. found 376.9 (microtitration).
Rotation: $[\alpha]_D^{20°} = -31° \pm 2°$ (C = 0.05; pyridine; microtube).

The *methyl ester of the N,N'-dibenzoate of hydroxylysine* was prepared by treating the acid in methanol with an ethereal solution of diazomethane. The methyl ester was recrystallized from methanol-ether; m. p. 138—39°.

$C_{21}H_{24}O_5N_2$ (384.4) Calc. C 65.61 H 6.29 N 7.29
Found » 65.59 » 6.36 » 7.24
Rotation: $[\alpha]_D^{24°} = -40.7° \pm 1.9°$ ($C = 0.355$; pyridine; microtube).

Methyl ester of tribenzoate of hydroxylysine (V)

32 mg of the dibenzoylmethyl ester (IV) was dissolved in 2 ml pyridine. 0.1 ml benzoylchloride was added and the solution left for 20 hours at 30°. Two drops of water were added and after 2 hours the reaction mixture was evaporated to dryness. Dilute hydrochloric acid and ether were added and the ether phase was washed with dilute hydrochloric acid, sodium carbonate, water, dried over sodium sulphate and evaporated to dryness. The residue was recrystallized from methanol-water yielding clusters of needles m. p. 171—73°.

$C_{28}H_{28}O_6N_2$ (488.5)	Calc.	C 68.84	H 5.78	N 5.74
	Found	» 69.14	» 6.25	» 5.77

Hydroxylysine monohydrochloride

350 mg of the hydroxylysinedibenzoate was hydrolyzed by refluxing 20 hours in 40 ml 5 normal hydrochloric acid. The solution was evaporated to dryness and redissolved in hot water, the benzoic acid was extracted with ether and the aqueous phase taken to dryness. The residual sirup that consisted of the dihydrochloride crystallized partly. It was dissolved in a few drops of water and one tenth ml of pyridine was added. Ethanol precipitated an oil that rapidly crystallized. Recrystallization was effected by dissolving in water and carefully adding ethanol when the monohydrochloride of hydroxylysine rapidly crystallized in clusters of needles. 159 mg was obtained decomposing at 220°.

$C_6H_{15}N_2O_3Cl$ (198.66)	Calc.	C 36.27	H 7.61	N 14.10	Cl 17.85
	Found	» 36.07	» 7.47	» 14.23	» 18.25

Rotation: $[\alpha]_D^{22} = +7.5° \pm 1.0$ (C = 0.4; N HCl microtube).

Oxidation of N,N′-dibenzoyl hydroxylysine, 1-benzamido-octanol and 2-benzamido-1-ethanol with chromic trioxide

One to 2 mg samples of the substance were weighed into test tubes (18 × 200 mm) with a weighing tube. The samples were then dissolved in 1 ml glacial acetic acid purified by distillation from chromic trioxide. Exactly 1 ml of a stock solution containing approximately 50 mg chromic trioxide per 100 ml of 95 per cent acetic acid was then added. These tubes together with an equal number of blanks without any added substance were then placed in a water thermostat at 50°. A sample and a blank were taken out after times indicated, 1 ml 10 per cent potassium iodide solution was added and the liberated iodine titrated with 0.01 *N* sodium thiosulphate. The results are plotted in Fig. 2. *N*-benzoyl-ethanolamine rapidly consumed two atoms of oxygen whereas N benzoyl-1-amino-2-octanol only consumed one atom. The N,N′-dibenzoate of hydroxylysine also consumed only one atom oxygen per mole.

N-Benzoyl-1-amino-2-octanol

960 mg 1-nitro-2-octanol, prepared according to Spray and Degering [24], was hydrogenated with W 6-Raney nickel [25] in ethanol at room temperature. After 1.5 hours 410 ml hydrogen had been adsorbed, the catalyst was filtered off and the solution was taken

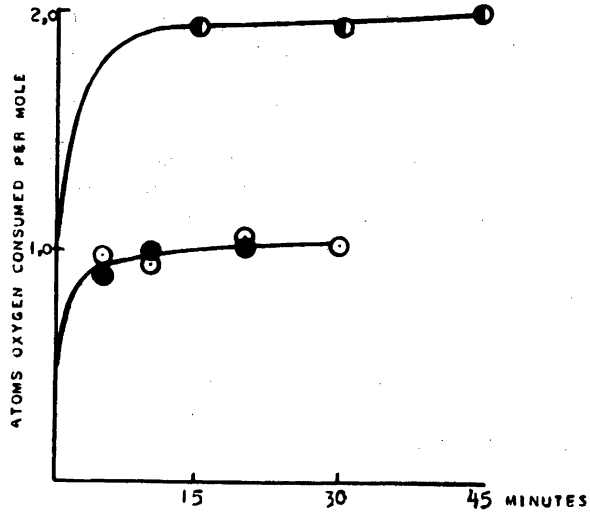


Fig. 2. Oxidation with CrO_3 in 97.5 per cent acetic acid at 50°

O-O *NN'-Dibenzoylhydroxylysine*
●-● *1-Benzamido-2-octanol*
◐-◐ *2-Benzamido-ethanol*

to dryness. The residue was dissolved in 10 ml dry pyridine and 2 ml benzoyl chloride was added. After 16 hours at room temperature a few drops of water were added and the solution was taken to dryness *in vacuo*. Ether and water were added and the ether phase was washed with dilute hydrochloric acid, water, dilute alkali, water, and dried over sodium sulfate. The residual oil obtained after evaporating the ether weighed 1.96 g.

The oil was dissolved in 25 ml ethanol and 15 ml 0.4 N barium hydroxide was added and the solution left over night at room temperature. The solution was then neutralized with 0.2 ml *N* HCl and concentrated somewhat *in vacuo* when the *N*-benzoate crystallized.

The crystals weighed 550 mg and melted at 97—98°. Recrystallization from aqueous ethanol raised the m. p. to 99—101°.

$C_{15}H_{23}O_2N$ (249.3)	Calc.	C	72.25	H	9.30	N	5.62
	Found	»	72.12	»	9.31	»	5.55

N-benzoyl-amino-2-ethanol IX[26]

This compound was prepared from amino ethanol (Schering, A. G.) by benzoylation and partial saponification as described above. The product melted at 60°.

$C_9H_{11}O_2N$ (165)	Calc.	N	8.5	Found	N 8.63

Preparative oxidation of the methylester of N,N'-dibenzoyl-hydroxylysine (IV) to ketone (VII)

115 mg of the dibenzoylmethyl ester (IV) was dissolved in 30 ml glacial acetic acid (distilled from chromic trioxide). 30 ml of a solution of 70 mg CrO_3 in 50 ml 95 per cent acetic acid was added and the mixture was placed in a water bath at 38° C. After 50 minutes the calculated amount of oxidant had been consumed. A little methanol was added and the solution evaporated to a syrup *in vacuo*. The residue was dissolved in 25 ml methanol, water, and ether was added and the aqueous phase was extracted with about 500 ml ether in six portions. The residue after evaporation of the ether was diluted with a few drops of methanol and left at − 15°. 85 mg of colorless needles were obtained. Recrystallization from methanol-ether yielded 68 mg of needles m. p. 134—135°.

$C_{21}H_{22}O_5N_2$ (382.4)	Calc.	C	65.95	H	5.80	N	7.33
	Found	»	65.70	»	5.80	»	7.44

$[\alpha]_D^{24} = -21.9 \pm 1.8$ (pyridine; C = 0.0223; microtube).

SUMMARY

Hydroxylysine has been isolated from gelatine and fish skin and a number of derivatives have been prepared.

The structure has been shown to be α,ε-diamino-δ-hydroxy-*n*-hexanoic acid.

Hydroxylysine probably belongs to the L-series.

The microanalyses have been made at the microanalytical laboratory of the Department of Medical Chemistry at Upsala.

We are greatly indebted to professor G. Ågren, Upsala, for the microbiological determinations.

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The Importance of Bivalent Ions for the Aggregate Molecular Weight of Sodium Thymonucleate in Aqueous Solution

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Bivalent ions have in several respects unique effects on sodium thymonucleate (DNA). Amongst them is gelation on dialysis against $MgSO_4$ in relatively strong solutions (0.7 per cent) of DNA [1]. It has now been found that, when determining the dielectric molecular weight, very large molecular aggregates can be obtained with bivalent ions. The results of a number of experiments and studies are reported in the following. On the investigation of the occurrence of metals in DNA made on the basis of this observation a relatively high concentration of Mg could be demonstrated [2]. Special attention will therefore be directed to the effect of Mg^{++}.

As in other studies of the dielectric properties of DNA made in this laboratory, the ellipsoid method originated by Fürth was used. The method was adapted to the determination of the anomalous dielectric dispersion by measurement of the dielectric constant (DC) at high frequencies within the radio frequency range. The computation of molecular weight, M, was done on the basis of the formulae that can be deduced for stiff thread molecules with transverse dipole moments, as follows:

$$M = \frac{N \cdot k \cdot T \cdot \lambda_c}{3 \cdot v \cdot \eta \cdot 2\pi c \cdot \psi_2} \cdot \frac{\varepsilon_h + 2}{\varepsilon_l + 2} = 3.2 \cdot \frac{\lambda_c}{v} \cdot \frac{\varepsilon_h + 2}{\varepsilon_l + 2}$$

The usual designations for known constants were used (k = Boltzmann's constant: 1.38×10^{-16} ergs per molecule; N = Avogadro's number; 6.02×10^{23}; T = the absolute temperature). ψ_2 = Perrin's form factor which can be given as 4/3 for thread molecules having a large axial ratio and rotating about the long axis; η is the viscosity of the solvent. In addition to these constant factors in a given solution, we have λ_c which is the wavelength at the centre

of the curve of dispersion, *i. e.*, the critical wavelength, and v which is the specific volume. ε_h and ε_l are the dielectric constants at considerable distances from the actual dispersion range, *i. e.*, at higher and lower frequencies respectively.

This formula is not identical with that earlier used and reported [3], as Perrin's form factor was then incorrectly applied. The erroneous results thereby obtained have been corrected [4]. Although later studies have shown that the first determinations were not correct, it is nevertheless a fact that the dielectric method gives a lower order of magnitude for the molecular weights of certain thread molecules with considerable transverse dipole moments than do the methods commonly used.

Reference is made to a detailed report from the laboratory [5] respecting the method employed and for the improvements introduced.

EXPERIMENTAL

I. The formation of high-molecular aggregates of DNA under the influence of Mg^{++}, Ca^{++} and Zn^{++}

It is difficult to obtain reproducible values in the dielectric determination of the molecular weight of DNA in extremely dilute aqueous solutions. This variability is strongly accentuated under certain conditions in the presence of a number of ions, of which Mg^{++}, Ca^{++} and Zn^{++} have been studied in this connexion. The following experiment may serve as an example of the way in which the "molecular weight" can be very greatly increased.

DNA, in a concentration of approximately 2.0 per cent, is dialyzed in a cellophane bag against re-distilled water until it is free from chloride and then for a further 24 hours. $MgCl_2$ is added to approximately 0.05 N and the DNA precipitated with 5 volumes of ethanol. The precipitate is dissolved in water and once more precipitated to reduce the excess of salt. When this procedure is used for a DNA preparation made according to Hammarsten [1] the effect shown in Fig. 1 is obtained. It is seen that the anomalous dielectric dispersion is displaced to longer wavelengths corresponding to an increase in the dielectric molecular weight from approximately 120 000 (I) to over 500 000 (II). If, after the dialysis, DNA is instead precipitated with alcohol without the presence of Mg^{++} or similar ions (which requires the addition of, for example, NaCl) the molecular weight is found to be the same as the original one.

The polarity of the molecular aggregate formed is not as high as that of DNA. It is true that measured absolutely the dipole moment increases considerably, whereas the "specific", polarity, *i. e.*, the dipole moment per average mononucleotide, is appreciably lower. The aggregate is labile in that the specific dipole moment falls, particularly during the first few hours.

Similar effects to those of Mg^{++} are obtained with Ca^{++} and Zn^{++}. The effects of the two last-mentioned are approximately equally pronounced but the course of the change in the DC with the frequency is not the same as with Mg^{++}. Unlike Mg^{++} in the form of the chloride, which results in a mainly theoretical course for the anomalous dispersion of

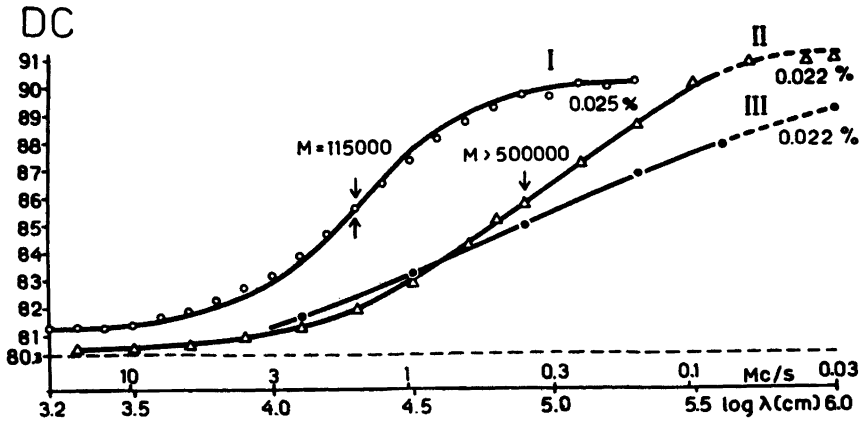

Fig. 1. Dielectric dispersion of aggregates of thymonucleate (obtained by precipitation with ethanol in the presence of bivalent ions of sodium thymonucleate prepared according to Hammarsten [5]).

I Original preparation
II Precipitated from 0.05 N $MgCl_2$
III Precipitated from 0.05 N $CaCl_2$
Temp. 20.0° C. DC = dielectric constant.

a largely monodisperse substance, Ca^{++} and Zn^{++} give a somewhat different appearance, with a slow rise at increasing wavelengths, approximately proportional to the logarithm of the wavelength. Fig. 1 shows this difference for Mg^{++} and Ca^{++} (II and III respectively). It is possible that some galvanic polarisation may influence the values at the longest wavelengths in the region of 10 000 metres but this cannot alter the course to any great extent. The appearance of the curves for Ca^{++} and Zn^{++} is an expression of a pronounced dielectric polydispersion, possibly due to a gel- like structure in the solution.

The effect of Ca^{++} and Zn^{++} is more dominating than that of $MgCl_2$ in the same concentration. On the other hand, it may be mentioned that $MgSO_4$ gives the same type of dielectric dispersion as $CaCl_2$ and $ZnCl_2$.

II. The effect of Mg^{++} on the preparation of DNA

A specimen with an extremely high aggregate molecular weight is obtained by preparing DNA from calf thymus in the presence of Mg^{++}. With the methods of Hammarsten [1] and Gulland, Jordan and Taylor [6] use is made, *inter alia*, of treatment with NaCl in a strong concentration. Since this could be expected to expel the Mg^{++} it was considered advisable to investigate the matter *.

* The results of further experiments have meanwhile been published by I. Jungner [5]. In the present report there will only be given some basic results that are of importance in this connexion.

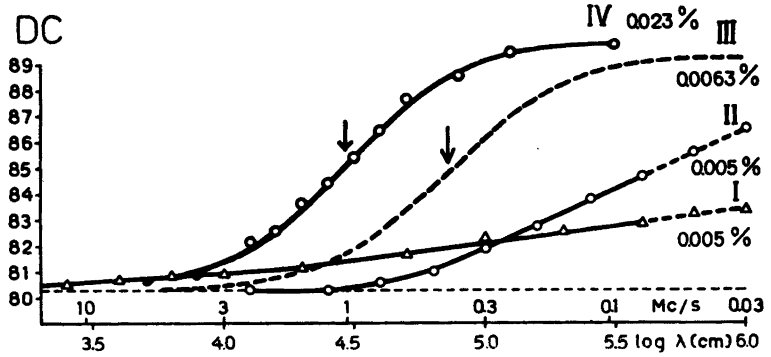

Fig. 2. Dielectric dispersion of thymonucleate prepared according to Gulland et al.[6] *but in the presence of* $MgCl_2$.

I Specimen prepared with 2 N $MgCl_2$ *(only)*
II Specimen prepared with 0.05 N $MgCl_2$ *in 10 % NaCl*
III Original preparation (without $MgCl_2$*)*
IV Specimen I after treatment with half-saturated NaCl for 5 days.

Fig. 2 shows the results of some experiments on the production of DNA from calf thymus with the method of Gulland *et al.* but in the presence of $MgCl_2$. Experiments were made both with the addition of 0.05 *N* $MgCl_2$ (curve II) to the prescribed concentration of NaCl (10 %) and with the substitution of 2 *N* $MgCl_2$ for the NaCl (curve I). It is seen from the figure that there is then an extremely pronounced increase in the dielectric aggregate weight from that usually obtained of about 600 000 (III) but that the preparations are pronouncedly polydisperse and hard to define. With the apparatus available it was only possible to study the beginning of the anomalous dielectric dispersion. The lowest molecular weights that could be estimated from these measurements were of the magnitude of several millions. The preparations were still more labile than those obtained on precipitation in the presence of Mg^{++} and similar ions.

III. The influence of the Mg^{++} concentration on the formation of high-molecular aggregates

Some experiments have been made to investigate the minimum Mg^{++} concentration necessary to get formation of the aggregates. Dialysed specimens of DNA (prepared according to Hammarsten[1]) were precipitated by ethanol in the presence of various Mg^{++} concentrations. The reproducibility is not, however, always good. This is presumably due to the great sensitivity of the precipitation mechanism. A low NaCl content is necessary to get precipitation by ethanol. Fig. 3 shows the results in diagram. The dielectric dispersion for the original preparation is not included since this is impossible to differentiate significantly from the curve obtained with 10^{-4} *N* $MgCl_2$. The effect of 10^{-3} *N* $MgCl_2$ is obvious (but badly reproducible) and is still more pronounced in 10^{-2} *N* $MgCl_2$. At higher concentrations no further tendency to give larger aggregates is obtained (cf., curve II in Fig. 1, obtained with 0.05 *N* $MgCl_2$).

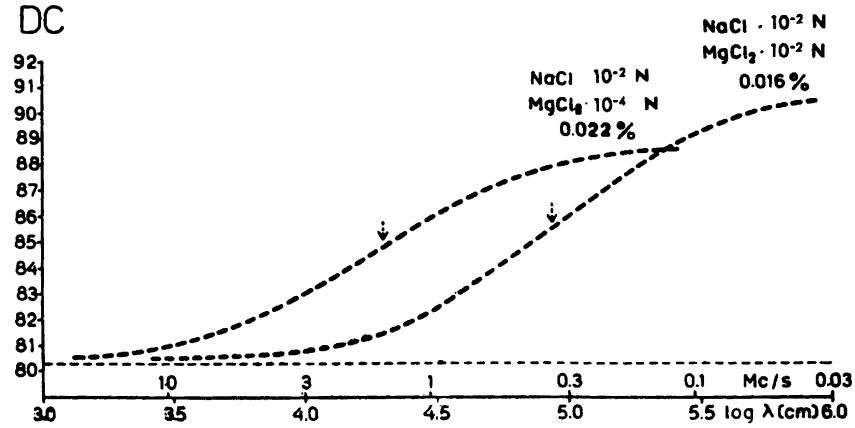

Fig. 3. Dielectric dispersion of DNA, precipitated in the presence of small concentrations of $MgCl_2$. (See text.)

IV. On the presence of Mg in DNA

It is found that the greater part of the original Mg content in DNA (about 0.1 per cent [2]) can be eliminated by dialysis. A further quantity of Mg is removed on treatment with NaCl, *e. g.*, 0.5 *N*. The residual Mg content (< 0.01 per cent) is difficult to deal with. It can, however, be decreased by acid hydrolysis and then only occurs in traces as do metals of other kinds. The magnesium contents were estimated in the experiments by means of spectral analyses and spot tests (*e. g.*, Titan Yellow).

V. Reversibility of the action of Mg

DNA prepared according to Gulland et al [6], but with 2 *N* $MgCl_2$ instead of 10 per cent NaCl (curve 1, Fig. 2), was treated with half-saturated NaCl for 5 days. After repeated precipitations with ethanol it was reinvestigated dielectrically. As shown in fig. 2 (curve IV), there is now obtained a well-defined dispersion curve with a high increment at considerably shorter wave-lengths. In fact, the aggregate weight — about 200 000 — is considerably smaller than that found for an original preparation according to Gulland *et al.* without addition of Mg (curve III, Fig. 2, page 171).

DISCUSSION

Bivalent ions have evidently a considerable effect on aggregate conditions of DNA. Mg is of particular interest in this connexion and various stages of its effect on DNA can be distinguished. First, there is a non-specific electrolyte effect similar to that obtained with other cations [1,5]. Under these conditions,

when Mg^{++} is added to dilute aqueous solutions of DNA, *disaggregation* results[5]. The inter-molecular distances are presumably too large to permit an aggregating effect but in stronger concentrations of DNA, as in Hammarsten's experiments [1] (with 0.7 per cent) or on precipitation with alcohol, conditions for the formation of aggregates are present.

Secondly, Mg^{++} can enter the phosphoric acid groups instead of Na^{+} but can possibly also enter less acid radicals. Such an exchange of ions must certainly take place in such a polybasic acid as thymonucleic acid. It can be assumed that the bivalent ions studied, on precipitation with alcohol, bring about the large aggregates and possibly a gel formation owing to linking various molecules. The aggregate formation is presumably non-specific. Its reversibility by means of NaCl implies that we are dealing with an exchange of ions.

Thirdly, there is a more resistant Mg content of DNA, and this may indicate that Mg also is bound in a non-ionic way.

From the experiments with preparations of DNA it appeares possible to neutralize in the presence of Mg^{++} a possible effect on the aggregate molecular weight by treatment with NaCl.

The experiments indicate that the molecular aggregates obtained are to be regarded as mainly artificial products. Nevertheless, these aggregates presumably also contain such polarly associated components as may have been built up in an analogous way to the original molecules. Thus, it may be considered that Mg^{++} and similar metals may be of importance for the formation of the DNA macromolecule.

SUMMARY

Large molecular aggregates are obtained on precipitation of sodium thymonucleate (DNA) from aqueous solution with ethanol in the presence of a number of bivalent ions (Mg^{++}, Ca^{++} and Zn^{++}). In analogy herewith, preparations with very high molecular weights (several millions) are obtained on the production of sodium thymonucleate in the presence of Mg^{++}.

The aggregates produced seem not to have the same high polarity as that characterizing sodium thymonucleate in the original state. The bivalent ions can be expelled to some extent by means of treatment with strong sodium chloride solution. Smaller molecular weights are then obtained, down to about 200 000.

It should be noted that all molecular weights mentioned are determined by a dielectric method which gives relatively lower values for DNA than those obtained by other methods.

The results are discussed in view of the possibility that the bivalent ions- and in particular Mg — can be of importance for the formation of the DNA macromolecule.

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Further Investigations on the Solubilization of Carcinogenic Hydrocarbons by Association Colloids

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In a previous paper[1] it has been shown that the fat-soluble, but water-insoluble, polycyclic hydrocarbons with carcinogenic properties can be brought into aqueous solutions with the aid of association colloids. These solutions are homogeneous and stable. The investigation has been continued on different lines. The previously published solubility values have been verified and the investigation has been extended to include several new association colloids and some new polycyclic hydrocarbons. The properties of the prepared aqueous solutions of these substances have been investigated. The ability of these solutions to penetrate in the skin and other tissues and to carry the carcinogen with them, as well as the carcinogenic activity of the hydrocarbons in these solutions have been studied.

SOLUBILIZATION STUDIES

We have determined the solubilities of five hydrocarbons: 1,2-benzanthracene (BA), 9,10-dimethyl-1,2-benzanthracene (DMBA), 1,2,5,6-dibenzanthracene (DBA), 20-methylchloantrene (MC), and 3,4-benzpyrene (BP), in aqueous solutions of the following association colloids: potassium myristate, sodium oleate, sodium lauryl and sodium myristyl sulphate, sodium cholate, sodium deoxycholate, sodium taurocholate, a cationic association colloid mixture ("Quatrogan"), the alkyl aryl polyether alcohol "Triton N 100", and the polyoxyethylene sorbitan monopalmitate "Tween 40". In addition, we have found that solutions of a number of commercial products, *viz.* "Aerosol IB", "Aerosol AY", "Aerosol MA", "Aerosol OT", "Cetavlon", "Nacconol NR", "Tween 20", "Tween 60", "Tween 80", "Atlas G 2149", "Atlas G 2152", "Atlas G 2153", and "Triton WR 1339", are able to solubilize polycyclic hydrocarbons.

The 3,4-benzpyrene was a product of Hoffmann-La Roche & Co, Basel, while the other hydrocarbons were from Eastman-Kodak Co, Rochester, N. Y. Potassium myristate, sodium oleate, sodium cholate and sodium deoxycholate were prepared from the corresponding acids by dissolving the latter in absolute ethanol and adding an equivalent amount of sodium or potassium alcoholate to the warm solutions. The acids were of the following qualities: myristic acid: Eastman-Kodak Co, Rochester, N. Y.; oleic acid: May & Baker Ltd, Dagenham; cholic acid: Hoffmann-La Roche & Co, Basel (recrystallized from ethanol); deoxycholic acid: Hoffmann-La Roche & Co, Basel (recrystallized from acetic acid). Sodium taurocholate was obtained by purification of a product of Merck, Darmstadt. Sodium lauryl sulphate and sodium myristyl sulphate were prepared from the commercial products "Duponol PC" and "Duponol ME" (Du Pont de Nemours Wilmington, Del.), respectively, by ethanol extraction and repeated recrystallization from ethanol. They may have contained slight amounts of homologues. All these substances were dried in vacuo over phosphorous pentoxide before use. "Quatrogan" (Ab. Recip, Stockholm) was used as the commercial product containing 10 per cent cetyl pyridinium chloride and 90 per cent alkyl dimethyl benzyl ammonium chlorides with alkyl groups containing from 8 to 18 carbon atoms. "Triton N 100" was the product of Rohm & Haas Company, Philadelphia, Pa. It consists of an alkyl aryl polyether alcohol with the average molecular weight 710. Presumably the preparation is a mixture of different homologues. "Triton NE", which we have used earlier, is a 32 per cent aqueous solution of "Triton N 100". "Tween 40" was the commercial product of Atlas Powder Company, Wilmington, Del. It consists of polyoxyethylene sorbitan monopalmitate.

As all the hydrocarbons studied are strongly fluorescent in solution their solubilities in the association colloid solutions were determined by measuring the fluorescence intensities. The procedure was the following: The colloid solution was saturated with the hydrocarbon by shaking it with an excess of the latter in a ground-glass stoppered test-tube in a thermostat at 40° C. When equilibrium was reached after 40 to 70 hours, the excess of hydrocarbon was removed by filtration. The intensity of the fluorescence of the saturated solution was compared with that of a solution of the same colloid concentration containing a known amount of the hydrocarbon in question. The latter solution was prepared in the following manner: A definite volume (0.01—0.18 ml) of a standard solution of the hydrocarbon in benzene was measured into a test-tube by means of an "Agla" micrometer syringe. The benzene was then evaporated in vacuo at room temperature in the dark. A known amount of the colloid solution was added and the hydrocarbon was brought into solution by shaking under the same conditions as in the preparation of the saturated solution. It was necessary to adjust the hydrocarbon concentration of both solutions to within the range in which a linear relation exists between the fluorescence intensity and the hydrocarbon concentration. In many cases the saturated solutions were diluted with the original colloid solution before measurement. The concentrations of both solutions were adjusted to be relatively near each other. As in our previous work, the intensity of the fluorescence

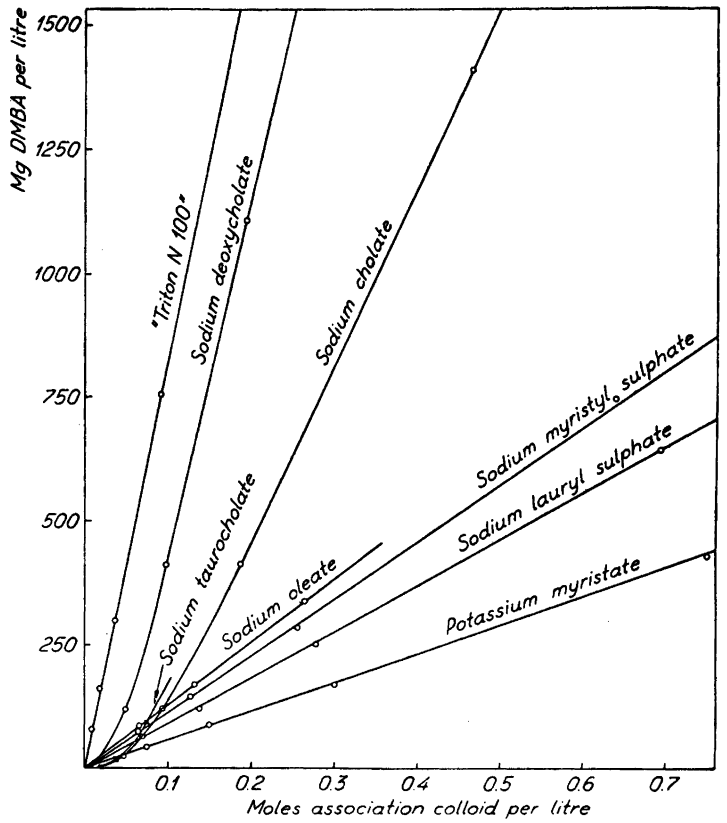

Fig. 1. The solubility of 9,10-dimethyl-1,2-benzanthracene in various association colloid solutions.

was measured with a Pulfrich Step-photometer provided with an ultraviolet mercury lamp and with a thermostat arrangement which made measurements possible at 40° C.*

The solubility values obtained in this manner are given in Table 1 and Figs. 1—5.

The previously published solubility values [1] have been confirmed on the whole. These reveal again that the solubilities of the hydrocarbons increase

* Quite recently H. B. Klevens (*J. Phys. & Colloid Chem.* **54** (1950) 283) has reported on the solubilities of a number of polycyclic hydrocarbons, some carcinogenic (methylcholantrene, 1,2,5,6-dibenzanthracene) in potassium laurate solutions at 25° C. He determined the solubilities by measuring the absorption in the ultra violet.

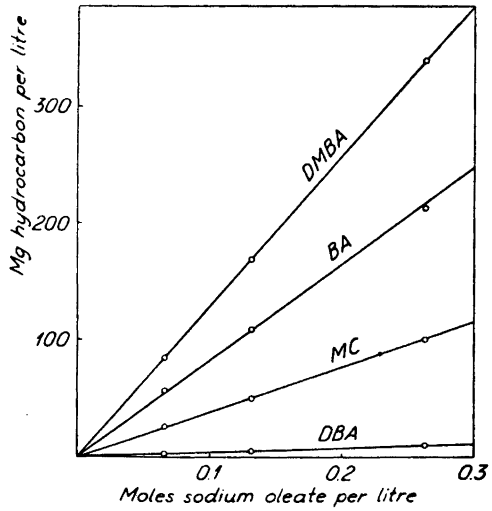

Fig. 2. The solubilities of various polycyclic hydrocarbons in aqueous sodium oleate solutions.

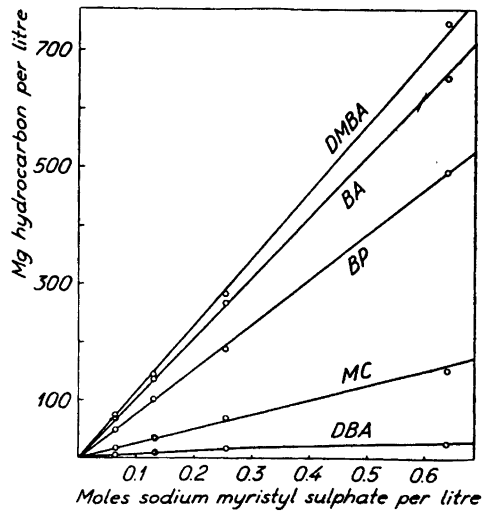

Fig. 3. The solubilities of various polycyclic hydrocarbons in aqueous sodium myristyl sulphate solutions.

linearly with the concentration of the colloid in the case of the paraffin-chain salts investigated (potassium myristate, sodium oleate, sodium lauryl sulphate and sodium myristyl sulphate). The same is the case also in solutions of the non-ionic alkyl aryl polyether alcohol "Triton N", of the polyoxyethylene sorbitan monopalmitate "Tween 40", and of the cationic colloid mixture "Quatrogan". In the solutions of the bile acid salts, on the other hand, the solubilities increase slowly in the beginning, but gradually the solubility increases more rapidly until at the higher colloid concentrations they tend to approach the linear dependence observed in other solutions. Within the range in which a linear relation between the solubility and the colloid concentration exists, all the solutions may be diluted without any precipitation of the carcinogen.

Different colloids differ considerably in their ability to solubilize the same hydrocarbon. In the case of the anionic paraffin-chain salts this ability increases on the whole with the length of the carbon chain. However, the nature of the ionic end group also seems to have an influence, as shown by the fact that sodium lauryl sulphate has a greater solubilizing power than potassium myristate. The alkyl aryl polyether alcohol "Triton N" is considerably superior in its solubilizing power to these anionic association colloids. For the

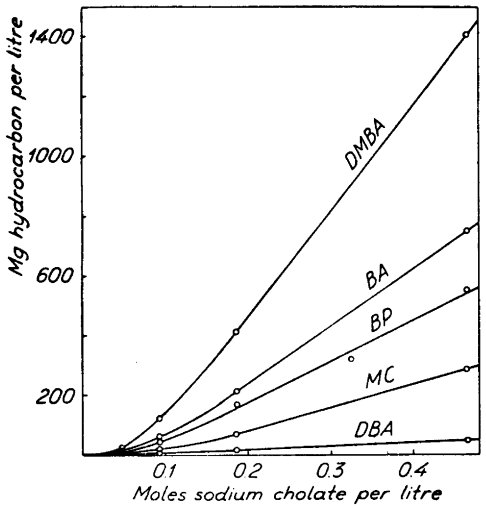

Fig. 4. The solubilities of various polycyclic hydrocarbons in aqueous sodium cholate solutions.

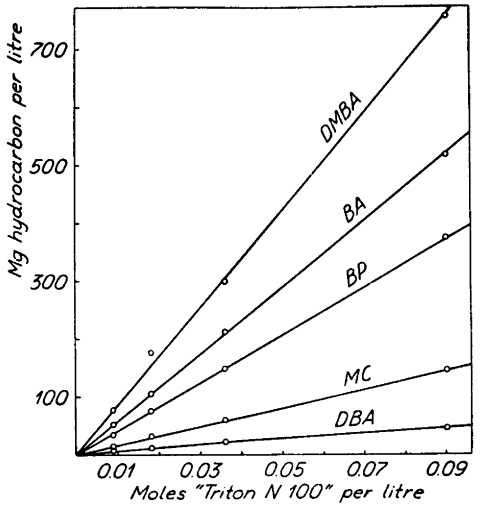

Fig. 5. The solubilities of various polycyclic hydrocarbons in aqueous "Triton N" solutions.

present we do not know the size of the alkyl group in this colloid. Its average molecular weight 710, however, points to a long hydrocarbon chain. At higher concentrations also the bile acid salts surpass the paraffin-chain salts in their solubilizing power and deoxycholate has even a greater solubilizing power than "Triton N", when solutions containing the same weight per cent of colloid are compared. Deoxycholate solubilizes considerably more than cholate. This is partly due to the fact that micelle formation in deoxycholate begins at a much lower concentration than in cholate solutions (the respective critical concentrations are 0.006 *M* and 0.018 *M* [2]). But as will be seen later, the deoxycholate micelles themselves are able to solubilize more carcinogen per mole than the cholate micelles. The power to solubilize carcinogenic hydrocarbons thus increases with decreasing number of OH-groups in the bile acid molecule. It seems therefore probable that the salts of lithocholic acid and the cholanic acids have a still greater solubilizing power. The conjugation of taurine to cholic acid to give taurocholic acid does not seem, as far as our study shows, to have an appreciable influence on the solubilizing power.

A comparison of the ability of the association colloids to solubilize polycyclic aromatic hydrocarbons and their ability to solubilize low molecular

Table 1. The solubilities of polycyclic hydrocarbons in aqueous solutions of different association colloids in mg hydrocarbon per litre association colloid solution. Colloid concentrations in weight per cent.

Aqueous colloid solution	DBA	MC	BP	BA	DMBA
Potassium myristate 20 %	23.7	137			428
» » 8 »	10.1	54.0	118		169
» » 4 »	5.2	25.0	59.9	83.8	86.9
» » 2 »	2.0	11.2			41.7
Sodium oleate 8 %	10.0	101	—	213	338
» » 4 »	5.1	50.1	—	109	169
» » 2 »	2.7	26.3	—	56.5	84.9
Sodium lauryl sulphate 20 %				721	644
» » » 8 »				287	250
» » » 4 »		42.4	48.0	144	119
» » » 2 »		18.2		72.1	63.3
Sodium myristyl sulphate 20 %	24.5	149	492	653	749
» » » 8 »	15.1	70.0	188	268	283
» » » 4 »	8.0	33.6	101	138	144
» » » 2 »	3.5	17.7	48.8	68.2	73.1
Sodium cholate 20 %	48.3	286	553	764	1 409
» » 8 »	14.3	67.9	167	212	413
» » 4 »	3.8	17.6	43.0	63.0	121
» » 2 »	0.9	4.2	10.0	13.5	23.7
Sodium deoxycholate 20 %		677			2 082
» » 8 »		269			1 106
» » 4 »		113			410
» » 2 »		33.0	63.1	73.5	119
Sodium taurocholate 4 %		13.1	33.7		94.4
» » 2 »		3.4			18.7
» » 1 »		0.7		2.6	2.7
"Quatrogan" 10 %					1 150
» 5 »					620
» 2 »					255
"Triton N 100" 20 %			1 143		
» » » 6.4 % *	44.8	145	373	516	755
» » » 2.56 »	22.2	59.8	148	201	299
» » » 1.28 »	10.3	30.8	74.8	105	160
» » » 0.64 »	5.8	13.4	34.1	51.0	77.4
"Tween 40" 20 %		290			
» » 15 »		208			
» » 10 »		128			
» » 5 »		61.2			
» » 2.5 %		27.3			

* 6.4 per cent "Triton N 100" corresponds to 20 per cent "Triton NE".

hydrocarbons, *e. g.* benzene, xylene, hexane, *etc.*[3] *, reveals the following similarities and differences: Independant of the nature of the hydrocarbon the solubilization begins at a definite concentration of the colloid-forming substance, *viz.* at its critical concentration for micelle formation. Above this concentration, the solubilization of benzene, xylene, *etc.*, passes first through a transition range where the solubility of hydrocarbon per mole association colloid increases [3]. This range is relatively narrow for the paraffin-chain salts, but wider in the case of the bile acid salts [2]. Then there follows a range in which the solubility of the hydrocarbon increases linearly with the colloid concentration, the increase in solubility per added mole colloid remaining constant [3, 2]. We have found the mentioned transition range of the solubilization of polycyclic hydrocarbons to occur only in solutions of the bile acid salts. In the other colloid solutions, the solubilities of these hydrocarbons increase linearly from the critical concentration. It may further be pointed out that in the solubilization of the low molecular hydrocarbons by concentrated solutions, the solubility of the hydrocarbon per mole colloid begins to increase when a certain limiting concentration, "the second critical concentration", is exceeded, at least in solutions of paraffin-chain salts [3, 4]. Contrary to this, no increase in the solubility of the polycyclic hydrocarbons per mole colloid was observed at higher concentrations, although the measurements were extended over a fairly broad concentration range. The linear course of the solubility curves predominates, especially with the paraffin-chain salts.

From the slope of the linear part of the solubility curve, it is possible to calculate the maximum solubilizing power ("the saturation capacity") of the micellar substance for the hydrocarbon in question [3]. These values are given in Table 2. From these values we have calculated the number of molecules micellar substance required to solubilize one molecule hydrocarbon.

Table 2 shows that the micelles of "Triton N" have the greatest solubilizing power, followed by the deoxycholate and cholate micelles.

The number of molecules of the micellar substance required for the solubilization of one molecule of a polycyclic hydrocarbon varies considerably for different colloids and for different hydrocarbons. As a rule more than 200 molecules of the paraffin-chain salts are required. This value, however, increases to 8 000—9 000 molecules of the micellar substance per molecule of

* Previously one of us (Ekwall) has quantitatively investigated the solubilization of benzene, toluene, *p*-xylene, *p*-cymene, naphtalene, phenantrene, diphenyl, cyclohexane, and hexane in sodium oleate solutions at 20° C and *p*-xylene in sodium caprinate and sodium laurate solutions at 20° C and in sodium laurate, myristate, oleate, myristyl sulphate, cholate, and deoxycholate solutions at 40° C. The solubilities have been determined with a Zeiss-Löwe interferometer. Some of the results are mentioned in references (3) and (2).

Table 2. The maximum solubilizing power of micellar substances for polycyclic hydrocarbons.

Association colloid	Mg hydrocarbon per mole micellar substance					Moles micellar substance per mole solubilized hydrocarbon				
	DMBA	BA	BP	MC	DBA	DMBA	BA	BP	MC	DBA
Potassium myristate	578	564	398	182	33	445	405	629	1 460	8 470
Sodium oleate	1 320	825	—	379	32	191	278	—	710	8 700
Sodium lauryl sulphate	930	1 046	350	300		308	218	725	890	
Sodium myristyl sulphate	1 142	1 095	780	278	60	225	208	324	960	4 630
"Triton N"	8 340	5 830	4 200	1 650	630	31	39	61	163	440
Sodium cholate	4 057	1 860	1 800	735		63	123	140	385	
Sodium deoxycholate	7 200					36				

the hydrocarbon in the case of the poorly soluble 1,2,5,6-dibenzanthracene. Various methods have shown that in the range just above the critical concentration the micelles of the colloids in question are composed of about 50—200 molecules, and that the number of molecules somewhat increases when hydrocarbons are solubilized by the micelles [3–5]. Our results thus indicate that there can hardly be more than one molecule of polycyclic hydrocarbon per micelle in these solutions *. — In the case of bile acid salts and "Triton N", a much smaller number of molecules in the micellar state is sufficient to solubilize one molecule of the hydrocarbon. Whether there is more than one molecule (from one to four molecules) of the hydrocarbon present per micelle can not yet be decided because the sizes of the micelles of these substances are not known.

* In this connection we do not wish to discuss how to account for the large number of moles micellar substance per mole solubilized carcinogen, *e. g.* in solutions of DBA. It may, however, be suggested that it is scarcely possible that in presence of DBA micelles containing from 4 000 to 8 000 molecules are formed at the critical concentration in solutions of paraffin-chain salts. We are rather inclined to the interpretation that an equilibrium exists between micelles of moderate size containing the polycyclic hydrocarbon and normal micelles which are as such unable to solubilize the large hydrocarbon molecules. This hypothesis is at the present the subject of further examination.

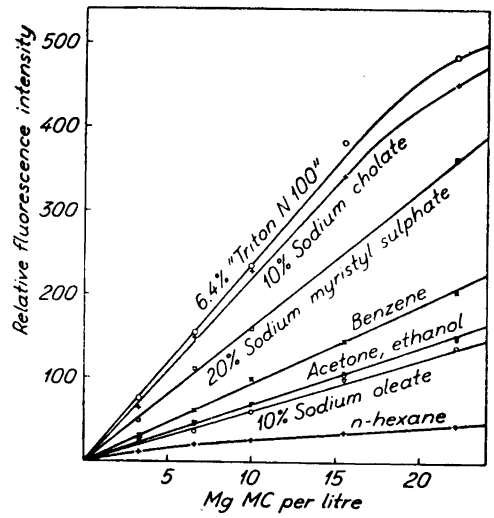

Fig. 6. The relative fluorescence intensity of 20-methylcholantrene in various solvents.

The preceding data indicate that the carcinogenic hydrocarbons are present in a highly-dispersed state in the solutions in question, each molecule being separated from the others and embedded within the hydrocarbon parts of the micelles. These solutions can thus not be compared with the previously used suspensions of the carcinogens which contain small suspended hydrocarbon crystals and which are unstable systems.

The solubilities of the different hydrocarbons in the same colloid differ greatly. For the four hydrocarbons which possess carcinogenic properties, the solubilities increases in the following order: 1,2,5,6-dibenzanthracene < 20-methylcholantrene < 3,4-benzpyrene < 9,10-dimethyl-1,2-benzanthracene. The carcinogenic activity increases on the whole in the same order. In Berenblum's scale[6] concerning painting on the skin, these four hydrocarbons have been given the following ciphers: VI, VIII, VIII, X. 1,2-benzanthracene, which has no carcinogenic properties, is approximately as soluble as the most active hydrocarbon, 9,10-dimethyl-1,2-benzanthracene. Although an immediate connection between the solubilities of the hydrocarbons in the micelles and their carcinogenic activity scarcely can be expected, it seems that a greater solubility assists the penetration of the carcinogen into the tissues and cells of the living organism and thus increases the possibility of the carcinogen for exerting its effect.

The hydrocarbons exhibit a stronger fluorescence in most of the colloid solutions than in the usual solvents. This is seen from Fig. 6, which shows the relative intensity of the fluorescence of 20-methylcholantrene in different

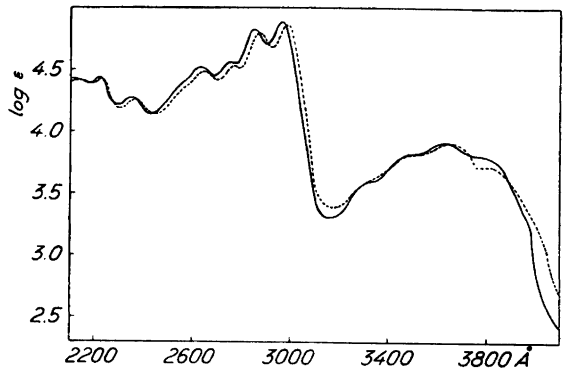

Fig. 7. The absorption spectra of 9,10-dimethyl-1,2-benzanthracene in absolute ethanol (————) and in a 10 per cent aqueous sodium lauryl sulphate solution (– – – – –).

solvents. A benzene solution containing 10 mg 20-methylcholantrene per litre has been used as a reference solution (relative fluorescence = 100). In solutions of "Triton N" and sodium cholate the fluorescence of 20-methylcholantrene is about four times stronger than in acetone and ethanol. Of the colloid solutions studied only in the oleate solutions is a weaker fluorescence (approximately as strong as in ethanol) observed. It may also be pointed out that oleate solutions of 3,4-benzpyrene become brownish and the fluorescence has a greenish shade, which points to a chemical change undergone by the hydrocarbon. An investigation of whether this observation and the weaker fluorescence of the other hydrocarbons in oleate solutions is possibly due to oxidation induced by the oleate is in progress.

An investigation of the absorption of the polycyclic hydrocarbons dissolved in solutions of association colloids indicated that their absorption spectra remain almost unchanged. In all cases, however, a slight shift towards the longer wavelengths has been observed (Fig. 7)*.

BIOLOGICAL EXPERIMENTS

We have conducted biological experiments to determine whether these aqueous association colloid solutions of carcinogenic hydrocarbons can be employed in the study of chemical carcinogenesis. The main results of this work are outlined below.**

* The absorption spectra of these hydrocarbons in different association colloid solutions, as well as the strong fluorescence of these solutions, is the subject of a detailed investigation which has not yet been completed.

** These experiments are described in detail elsewhere [7-12, 19, 20, 22, 23].

The aqueous solutions of association colloids have a low surface tension and make a very small contact angle with lipid surfaces. They therefore easily wet and penetrate such surfaces. By painting the skin of mice with solutions of carcinogenic hydrocarbons in these solvents, we have found that the solutions rapidly penetrate into the skin and transport the solubilized hydrocarbon into the tissues. A short time after the application of the solution the fluorescent carcinogen may be observed with a fluorescence microscope in the keratinized layers of the epidermis, in the sebaceous elements, and in the fat deposits of the skin [1, 9, 12].

Furthermore, we have found that these solutions, when introduced into the stomachs of mice, penetrate into the cells of the walls of the forestomach [11] and into a kind of fine network in the outer muscle layer of the stomach [14]. In this respect the aqueous carcinogen-association colloid solutions are similar to the entirely lipid solvents (*e. g.* paraffin oil), and lipophilic-hydrophilic solvents such as polyethylene glycols ("Carbowaxes") *etc.*[13, 14].

Until quite recently it has not been definitely observed that the carcinogenic hydrocarbon penetrates into the cells of the glandular stomach. We have, however, shown that such a penetration takes place when the carcinogen is dissolved in anhydrous polyethylene glycols ("Carbowaxes") [13], and in other solvents possessing both marked lipophilic and hydrophilic properties, such as anhydrous non-ionic association colloids (alkyl aryl polyether alcohols *etc.* [14, 19, 22]). Also when the carcinogen is dissolved in aqueous solutions of association colloids, *e. g.* a 70 per cent sodium taurocholate solution *, 90—20 per cent "Triton N" solutions *etc.*, a penetration into the wall of the glandular stomach can be observed [14]. In some cases a very faint and shortly disappearing fluorescence in the superficial layer has been observed also when more dilute colloid solutions were used. Whether this is to be interpreted as a sign of penetration of the carcinogen has not as yet been cleared up.

Experiments with mice have revealed that the polycyclic hydrocarbons retain their carcinogenic activity when solubilized in the aqueous solutions of association colloids of different types [7–12, 19, 20, 22, 23]. Representatives of widely different association colloids such as the non-ionic association colloids with neutral micelles (alkyl aryl polyether alcohol), anionic association colloids with negatively charged micelles (fatty acid salts, alkyl sulphates, bile acid salts), and cationic association colloids with positively charged micelles (cetyl trimethyl ammonium salts, alkyl dimethyl benzyl ammonium salts, cetyl

* When BP is solubilized in a synthetic bile containing taurocholate, oleate, cholesterol, and lecithin, it penetrates into the superficial layers of the wall of the glandular stomach. The same result is obtained with an emulsion of arachidic oil and natural bile containing BP (14).

pyridinium salts) have been used. Malignant cutaneous tumours have been produced in mice by carcinogens solubilized in all these colloid solutions when painted on the skin. Malignant tumours have also been produced in the forestomach of mice by feeding carcinogens solubilized in solutions of the first two types of association colloids. Cutaneous tumours have been caused by a concentration of 9,10-dimethyl-1,2-benzanthracene as low as 0.0045 per cent in aqueous lauryl sulphate solutions. Tumours in the forestomach have developed after the administration of only 0.6—1.0 mg carcinogen in different colloid solutions. This and other observations [11] may be considered to indicate that these solutions facilitate the action of the carcinogen.

The type and concentration of the association colloid seem to have a certain effect on the carcinogenesis [9,11,12]. A systematic investigation of the significance of this "solvent effect" is in progress.

DISCUSSION

In the experimental study of the chemical carcinogenesis induced by polycyclic hydrocarbons one has to introduce the fat-soluble, but practically water-insoluble, carcinogens into the tissues and cells with their aqueous systems. The introduction of carcinogens in aqueous association colloid solutions may therefore in certain cases be more advantageous than the use of their solutions in purely lipophilic fats and oils, or in organic solvents such as benzene and acetone which are foreign to the living organism, and the use of their aqueous suspensions. Owing to their aqueous character and since they, because of the presence of micelles, also possess lipophilic properties, these solutions approach in some respects the conditions prevailing in the living cells. As known, there are in the living organism many substances possessing properties more or less typical of the association colloids: fatty acid and bile acid salts, some bile acids themselves, phosphatides and cerebrosides etc. The solubilization mechanism studied thus provides a model solubilization and transport mechanism for water-insoluble carcinogens which may also apply in principle within the living organism.

From a methodical point of view, the use of aqueous association colloid solutions of carcinogens offers many advantages in the experimental study of the chemical carcinogenesis [9, 18, 19, 21, 22] *. As we have seen, these solutions are homogeneous and stable and the carcinogen is dispersed in them to the highest possible degree. In many cases they permit a high dilution without any precipi-

* In many respects these aqueous solutions offer advantages similar to solutions of the hydrocarbons in the anhydrous water-soluble polyethylene glycols ("Carbowaxes") [13–17, 19, 22].

tation of the carcinogen, which offers possibilities for the carcinogen to remain in solution also in the tissues provided that the micelles are stable there. With the aid of these colloid-forming substances it is possible to vary the chemical and physico-chemical character of the solution within wide limits. The carcinogen can be administered solubilized in neutral, in positively, or negatively charged micelles. On the other hand, the carcinogen can be solubilized in micelles which are stable at the hydrogen ion concentration of the biological environment or in micelles which are broken up at a given pH so that the carcinogen is precipitated; these possibilities can be of interest especially in the study of the behaviour of the carcinogens in the alimentary canal. Because these aqueous solutions are not appreciably volatile, the carcinogen concentrations of the solutions remain constant. This fact and the simplicity of dosing these solutions facilitate quantitative experiments. The exceptionally strong fluorescence of the carcinogens in these solutions may also be advantageous in the study of the first stages of their penetration. Because the solutions possess both lipophilic and hydrophilic properties it is possible to introduce at the same time other fat-soluble or water-soluble substances and to study their co- or anti-carcinogenic action [17].

The biological experiments have shown that these solutions are appropriate for application by painting on the skin and that in feeding experiments they offer considerably greater possibilities than the solutions employed earlier for varying and defining the experimental conditions. For subcutaneous [24] and intravenous injection the possibilities are more limited: at the present it seems that most colloids can be used only in relatively dilute solutions. A few of them can, however, be injected intravenously in fairly high concentrations [25].

As mentioned above, the surface chemical and colloid chemical properties of the association colloids assist the penetration of carcinogens into the tissues and cells, and they perhaps also promote their spreading in the organism. We are paying particular attention to the role of the solvent in the stages which precede the carcinogenesis, but we are also studying the significance of the solvent for the actual carcinogenic process.

An investigation with such a purpose provides a greater interest through the already mentioned fact that the living organism itself contains substances that are in principle of the same type as the solvents we have used. The bile, for example, is an association colloid solution which is known to solubilize carcinogens. Although we have not as yet been able to show that carcinogens in natural bile penetrate into the wall of the glandular stomach, one can not dismiss the possibility that bile which accidentally enters the stomach can, at least under certain conditions, assist the penetration of a carcinogen which has entered the stomach, *e. g.* along with food. The study of the

role of the association colloids in the first stages of the chemical carcinogenesis is of interest also because substances of this type are coming into use nowadays in ever increasing amounts.

SUMMARY

The solubilities of five polycyclic hydrocarbons in aqueous solutions of several association colloids of different types have been determined. In all these colloid solutions, except those of bile acid salts, the solubilities increase linearly with the colloid concentration from the critical concentration up to high concentrations. The maximum solubilizing power ("the saturation capacity") of the micellar substance for the different hydrocarbons has been calculated. The values obtained indicate that the hydrocarbons in these solutions are dispersed to a very high degree, apparently with only one molecule per micelle. The intensity of the fluorescence of the hydrocarbons is considerably greater in these solutions than in the usual solvents. Except for a slight shift towards longer wavelengths, their absorption spectra remain almost unchanged.

Animal experiments have revealed that a carcinogenic hydrocarbon solubilized in these solutions easily penetrates through the skin of mice. It also penetrates into the wall of the forestomach of mice and at least when the colloid concentration is not too low, also into the wall of the glandular stomach. The hydrocarbons maintain their carcinogenic activity in these solutions independent of the chemical structure and the physico-chemical properties of the colloid. Malignant tumours have been produced in the skin and in the forestomach of mice. Tumours have been caused by minute amounts and very dilute solutions of the carcinogen.

The advantages of the aqueous association colloid solutions in the study of chemical carcinogenesis are discussed.

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Short Communications

Synthesis of Polynucleotides in Slices from Regenerating Liver

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During the course of an investigation on the extent to which orotic acid is a natural intermediate for the synthesis of pyrimidines it became desirable to inquire into the possibilities of using an in vitro system capable of synthesizing the bases of polynucleotides. Greenberg [1] has shown that a homogenate prepared from pigeon liver can synthesize hypoxanthine from $C^{14}O_2$ or $HC^{14}OOH$, and Schulman *et al.*[2] have shown that the same system can synthesize hypoxanthine from C^{14}-labeled-4-amino-5-imidazole-carboxamide. Hypoxanthine however, is not a natural constituent of polynucleotides, nor can it be used as a precursor for polynucleotide purines by the rat [3]. After the start of the present investigation Weed et al [4] reported the occurence of C^{14} in the pyrimidine-mononucleotides from ribonucleic acid (PNA) after incubation of liver slices with C^{14}-orotic acid. These authors, however, did not investigate the distribution of the isotope between the pyrimidines and ribose.

The present communication reports studies on the synthesis in slices of regenerating liver of PNA and DNA (desoxyribonucleic acid) purines and pyrimidines with N^{15}-glycine [5] or N^{15}-orotic acid [6] as precursors. In each experiment 8 rats were subjected to partial hepatectomy. The animals were killed 24 hours after the operation; slices were prepared from their livers (ca 10 g) and suspended in 100 ml of solution. Two different media were used for this incubation of the slices: one medium which was synthesizing ATP [7], and a Krebs-Henseleit medium [8] where $CaCl_2$ had been omitted. Different amounts of either N^{15}-glycine or N^{15}-orotic acid were added to the medium in each experiment. The incubations were carried out for varying periods between 2 and 8 hours. At the end of each incubation the suspension was cooled in ice water, 10 ml of 30 % trichloro acetic acid were added and the slices were filtered off. After homogenization in a Waring Blendor with 100 ml ice cold 3 % trichloroacetic acid the slices were filtered again and dried with alcohol and ether. PNA and DNA were extracted and separated from the dry material [9]; purines and pyrimidines were prepared from the polynucleotides[10] and analyzed for N^{15}. The results are summarized in Table 1.

The differences in values for the two media are not thought to be significant. The N^{15} incorporation into the purines in the experiments with orotic acid is low enough to be probably explained by degradation of orotic acid. Specific contribution of glycine N^{15} to nucleic acid pyrimidines might also be questioned. The values, however, do indicate a considerable synthesis of both PNA and DNA pyrimidines from orotic acid and a synthesis of polynucleotide purines from glycine. In considering the relatively low N^{15} incorporation into the purines one should keep

Table 1. Incubation of slices from regenerating liver with N^{15}-glycine (excess N^{15} = 32 atom per cent) and N^{15}-orotic acid (excess N^{15} = 16.2 atom per cent). AO = ATP medium + 5 mg orotic acid; KO1 = Krebs-Henseleit medium + 5 mg orotic acid; KO2 = Krebs-Henseleit medium + 25 mg orotic acid; KG = Krebs-Henseleit medium + 50 mg glycine.

	Time of incubation	Excess N^{15}							
		PNA				DNA			
		Adenine	Guanine	Cytidine	Uridine	Adenine	Guanine	Thymine	Cytosine
AO	4 h			0.038	0.098			0.035	0.021
AO	8 h	0.008	0.006	0.102	0.281	0.007	0.003	0.096	0.073
KO1	4 h	0.017	0.009	0.057	0.140				
KO1	8 h	0.026	0.019	0.156	0.390	0.005	0.002	0.099	0.068
KO2	4 h	0.000	0.001	0.093	0.209				
KO2	8 h	0.015	0.011	0.227	0.504	0.006	0.009	0.160	0.139
KG	2 h	0.041	0.013						
KG	4 h	0.066	0.030	0.007	0.011				
KG	8 h	0.105	0.067	0.022	0.031	0.029	0.025	0.016	0.014

in mind that probably most of the isotope is located in only one nitrogen atom [5] out of five.

Corresponding experiments with homogenates from regenerating liver in both types of media did not show a significant incorporation of N^{15} into the bases; apparently this system does not synthesize polynucleotides from these precursors.

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On Carbon Balance and Carbon Dioxide Fixation in Thermophilic Cellulose Fermentation

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In experiments with six different pure cultures of thermophilic bacteria on the carbon balance in fermentation of cellulose McBee [1] was able to obtain a satisfactory recovery of the carbon from the fermented cellulose in only one case. Similarly Imsenecki [2] in his experiments with a possibly pure culture * obtained a recovery of only c. 75 % of the carbon added. It should be noticed, however, that both these authors neglected succinic acid which appeared only as a trace among the products.

It seems probable that small changes in the general condition of the fermentation

* In spite of the doubtful isolation method it seems with regard to more recent results of McBee [1, 3] and the author [4, 5] that the culture of Imsenecki really was pure.

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		Adenine	Guanine	Cytidine	Uridine	Adenine	Guanine	Thymine	Cytosine
AO	4 h			0.038	0.098			0.035	0.021
AO	8 h	0.008	0.006	0.102	0.281	0.007	0.003	0.096	0.073
KO1	4 h	0.017	0.009	0.057	0.140				
KO1	8 h	0.026	0.019	0.156	0.390	0.005	0.002	0.099	0.068
KO2	4 h	0.000	0.001	0.093	0.209				
KO2	8 h	0.015	0.011	0.227	0.504	0.006	0.009	0.160	0.139
KG	2 h	0.041	0.013						
KG	4 h	0.066	0.030	0.007	0.011				
KG	8 h	0.105	0.067	0.022	0.031	0.029	0.025	0.016	0.014

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It seems probable that small changes in the general condition of the fermentation

* In spite of the doubtful isolation method it seems with regard to more recent results of McBee [1, 3] and the author [4, 5] that the culture of Imsenecki really was pure.

have a rather important influence on the composition of the products. Thus the author has obtained a better carbon recovery when working with more concentrated cellulose suspensions than McBee. The culture was isolated from the same mixed stock culture from which McBee isolated his strain EB [1].

The basal medium was a suspension of 10 g/l cellulose wadding and 10 g/l $CaCO_3$ in a solution of the following composition:

$(NH_4)_2SO_4$	2 g/l
KH_2PO_4	1 »
$MgSO_4$, 7 H_2O	0.5 »
NaCl	0.5 »
Biotin	2 µg/l

In experiments A also 2 g/l Bacto tryptone (Difco) and 2 g/l yeast autolyzate of bakers' yeast were added.

The fermentations were carried out anaerobically with 0.5 l medium in all

Table 1. Experiments A. Products obtained in fermentation of cellulose with a pure culture of cellulose thermophiles. Medium containing tryptone and yeast autolyzate. Incubation for 10 days at 55° C.

products per g of cellulose consumed:

Experiment no.	1		2		3		4		5		6		7	
	g	mM	g	mM	g	mM	g	mM	g	mM	g	mM	g	mM
Products of fermentation:														
Carbon dioxide	0.072	1.64	0.072	1.64	0.072	1.64	0.072	1.64	0.065	1.48	0.069	1.57	0.081	1.84
Ethanol	0.056	1.22	0.056	1.22	0.044	0.96	0.051	1.11	0.040	0.87	0.039	0.85	0.048	1.04
Formic acid	0.017	0.37	0.041	0.89	0.015	0.33	0.014	0.30	0.022	0.48	0.017	0.37	0.013	0.28
Acetic acid	0.160	2.67	0.069	1.15	0.151	2.52	0.106	1.77	0.169	2.82	0.125	2.08	0.103	1.72
Lactic acid	0.253	2.81	0.160	1.78	0.145	1.61	0.252	2.80	0.304	3.38	0.151	1.68	0.289	3.21
Succinic acid	0.055	0.47	0.140	1.19	0.113	0.96	0.098	0.83	0.132	1.12	0.128	1.09	0.060	0.51
Glucose	0.437	2.43	0.576	3.20	0.565	3.14	0.473	2.63	0.408	2.27	0.586	3.26	0.509	2.83

Table 1 a. Carbon recovery in experiments A.

g carbon obtained per g of cellulose consumed:

Experiment no.	1	2	3	4	5	6	7
Products of fermentation:							
Carbon dioxide	0.020	0.020	0.020	0.020	0.018	0.019	0.022
Ethanol	0.030	0.030	0.023	0.027	0.021	0.020	0.025
Formic acid	0.005	0.011	0.004	0.004	0.006	0.005	0.004
Acetic acid	0.067	0.028	0.060	0.042	0.068	0.050	0.041
Lactic acid	0.101	0.064	0.058	0.101	0.123	0.060	0.116
Succinic acid	0.024	0.057	0.046	0.040	0.054	0.052	0.024
Glucose	0.186	0.230	0.226	0.189	0.163	0.234	0.203
Total carbon recovery, g	0.433	0.440	0.437	0.423	0.453	0.440	0.435
Total carbon recovery in per cent of theoretical value	97.3	98.9	98.2	95.1	101.8	98.9	97.8

Table 2. Experiments B. Results of fermentation of cellulose in an inorganic salt solution with a pure culture of cellulose thermophiles. Incubation for 10 days at 55° C.

Experiment no.	1			2			3		
	Ferm. products per g cellulose consumed		g C per g cellulose consumed	Ferm. products per g cellulose consumed		g C per g cellulose consumed	Ferm. products per g cellulose consumed		g C per g cellulose consumed
	Mm	g		Mm	g		Mm	g	
Products of fermentation:									
Hydrogen	5.45	0.0109	—	6.35	0.0127	—	6.70	0.0134	—
Carbon dioxide	3.39	0.149	0.041	3.64	0.160	0.044	3.98	0.175	0.048
Ethanol	1.00	0.046	0.024	1.13	0.052	0.027	0.93	0.043	0.022
Formic acid	0.26	0.012	0.003	0.33	0.015	0.004	0.30	0.014	0.004
Acetic acid	3.28	0.197	0.079	3.22	0.193	0.077	3.07	0.184	0.074
Lactic acid	2.71	0.244	0.098	1.92	0.173	0.069	1.60	0.144	0.058
Succinic acid	0.71	0.084	0.034	1.14	0.135	0.055	0.97	0.114	0.046
Glucose	2.31	0.416	0.166	2.46	0.442	0.177	2.72	0.490	0.196
Total carbon recovery, g			0.445			0.453			0.448
Total carbon recovery in per cent of theoretical value			100.0			101.8			100.7
Oxidation-reduction index		1.04			1.02			1.08	

glass apparatus. As reducing agent 0.03 per cent thioglycollic acid was added.

In both cases a satisfactory recovery was obtained. It is probable that tryptone and yeast autolyzate in experiments A to a small extent have served as a source of carbon, but experiments B clearly show that the carbon recovery can also be as high in the absence of these organic nutrients.

The maximum influence of the thioglycollic acid on the oxidation-reduction index in the present case would have been c. + 0.03 if oxidation had been complete during the fermentation. This, however, was not the case.

The carbon recovery is very high, in spite of the fact that the carbon content of the bacterial cells formed during the fermentation was omitted in the calculation. A certain utilization of CO_2 for the synthesis of other products may therefore be assumed. A preliminary attempt to demonstrate CO_2-fixation was carried out.

Fermentations were made with 100 ml each of the medium mentioned above, but omitting tryptone and yeast autolyzate, and with addition of a small amount of $CaC^{14}O_3$ to the buffer.

Table 3 shows that the CO_2-fixation is very minute and possibly limited only to the formation of succinic acid. 1.15 per cent of the carbon dioxide added has taken part in the formation of fermentation products. Calculated on basis of carbon in the products and assuming 1.15 per cent of fixed carbon dioxide, the influence of CO_2-fixation in the carbon balance is about

Table 3. Results of a pure culture thermophilic cellulose fermentation with addition of $CaC^{14}O_3$.

Products	mg	mg carbon	counts per mg and minute	total counts per minute
Ethanol	61	32	23	1.400
Formic acid	10	3	81	810
Acetic acid	87	35	4.5	390
Lactic acid	67	27	88	5.900
Succinic acid	69	28	808	56.000
Total	294	125		64.500
Carbon dioxide, added	102	38	54.700	5.580.000
Cellulose, consumed	473	210		

2 pro mille. The succinic acid formed by means of CO_2-fixation corresponds to 1.6 per cent of the total succinic acid formation. Experiments are being planned in order to establish if more CO_2 would be fixed when the fermentation is carried out under higher CO_2-pressure than in the present case.

It should be noticed that Virtanen [6] and Koistinen [7] have demonstrated CO_2-fixation in cellulose fermentation with mixed cultures, but it was not proved that the fixation was not performed by the symbionts.

The author wishes to express his gratitude to Miss Elisabeth Johnsson and to Mr. H. Guthenberg for their valuable help. I am also indebted to Mr. T. Westermark for advice and to Professor H. Lundin for placing his Geiger-Müller counter at my disposal.

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7. Koistinen, O. A. *Suomen Kemistilehti* **19** (1946) 122.

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Chemistry of the Histochemical Phosphatase Reaction

H. HOLTER, S. LØVTRUP and INGER RUBIN

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Gomori's [2] and Takamatsu's [3] reaction for the histochemical detection of phosphatase in tissues is based on the following principle: The tissue is fixed in alcohol or acetone, dehydrated, embedded in paraffin and sectioned on the microtome. The deparaffinized sections are then incubated at pH > 9 with the phosphate ester (usually glycerophosphate) in the presence of calcium ions. Upon liberation of inorganic phosphate by enzymatic cleavage a precipitate of hydroxy-apatite is formed. Then the tissue is treated with cobalt nitrate in order to convert the precipitate to cobalt phosphate, and finally with ammonium sulfide in order to convert the cobalt phosphate into cobalt sulfide. The latter precipitate is black and reveals upon inspection in the microscope the presumed sites of phosphatase activity.

The technique is widely used and various variants of the method have been developed employing other combinations of substrates, salts and pH. In all of these, however, the principle is the same, since a colourless product of the enzymatic reaction is by suitable treatment converted into a colored substance which can be observed in the microscope. A number of critical investigations of the technique have been carried out by various authors, in order to clear up certain points which are vital for its validity, such as inactivation of the enzyme during the preliminary histological treatment, secondary effects of diffusion and re-adsorption, *etc.* As far as we are aware, however, the question of the conversion of the initial precipitate into the stained end-product has not received similar attention. In order to test this

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point, the following experiment has been carried out:

A calcium phosphate precipitate was prepared from Sørensen's secondary phosphate and a slight excess of calcium nitrate. After standing overnight the supernatant was decanted and an excess of cobalt nitrate added. The mixture was stirred over night, the supernatant was decanted, the precipitate resuspended in water and divided into two parts.

One part was treated with sodium sulfide and the resulting black precipitate filtered, washed and dried. The other part was washed and dried and then suspended in a 5 % agar solution. After gelation a cube of 2 cm side length was cut from the agar and immersed in sodium sulfide solution. Within 30 minutes the black coloration of cobalt sulfide had extended to the core of the cube. Accordingly, the main portion of the agar suspension was sliced in 2—3 mm thick discs and treated with sodium sulfide for 3—4 hours. Then the blackened precipitate was recovered by melting, diluting and decanting. Both preparations were then analyzed with the following result (values in % of water-free precipitate).

	Water	Agar
Sulfide-S	3.5	2.4
Co	22.0	26.5
PO_4	21.5	18.4

Besides, there was a very heavy qualitative reaction for SO_4^{--} and a somewhat slighter one for Ca^{++}. Calculated for CoS: Co 64.8 %, S 35.2 %, for Co_2S_3 : Co 55.1 %, S 44.9 %.

From the above experiments we have to conclude that the conversion of calcium phosphate into cobalt sulfide is by no means quantitative. Moreover, a substantial part of the cobalt sulfide, even if formed originally, seems to be oxidized to sulfate. Since this is the case even in model experiments it is to be assumed that in tissues the process of conversion will be still more complicated. As long as cobalt sulfide is formed at all, the value of the reaction as a qualitative method of phosphatase detection will scarcely be influenced by the results reported here; but in all cases where the intensity of blackening is taken as a quantitative indicator of the amount of phosphatase present, the greatest caution seems to be necessary. Numerous such attempts have been reported in the literature.

The only case that we know of in which the formation of sulfide has been evaluated quantitatively is an investigation by Doyle [1], where the sulfur is extracted and incorporated into methylene blue which is measured colorimetrically. The detailed communication about this method has not yet appeared, but Dr. Doyle has informed us that in this case, too, the conversion into sulfide has been found to be incomplete.

Dr. E. J. Krugelis has participated in some of the preliminary experiments of this study and we greatfully acknowledge her assistance. We also thank Professor Jannik Bjerrum in whose laboratory the analyses were carried out.

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Note on the Crystallisation of Chondrosamine

SVEN GARDELL

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In working with mucopolysaccharides it is often necessary to use chondrosamine (galactosamine) as a reference substance. However this substance is very difficult to obtain pure in any reasonable yield. The best method is that originally described by Levene [1], in which the lead salt

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or the barium salt of chondroitinsulfuric acid is hydrolysed with strong hydrochloric acid in the presence of stannous chloride. After removal of the heavy metals with H_2S the filtrate is evaporated to a syrup. This is taken up either in ethyl alcohol from which the chondrosamine hydrochloride crystallises on the addition of ether or in methyl alcohol to which aceton is cautiously added. Working with this method we very often found that a sticky mass appeared instead of crystals when ether or aceton was added or, when the mass did not appear, the yield was very poor. The frequent appearence of this sticky mass was sometimes due to incomplete hydrolysis. It was however found that when the aceton was sufficiently cooled satisfactory crystallisation was sometimes obtained in batches where a sticky mass had at first appeared. Improvements in the technique therefore seemed necessary. Several modifications having been tried we found the following technique the most suitable.

50 g chondroitinsulfuric acid, prepared from bovine tracheal cartilage according to the method of Strandberg [2], were dissolved in about 50 ml of water. Hydrochloric acid was added until the solution turned acid to Congo. The chondroitinsulfuric acid was precipitated with 5 volumes of alcohol, the precipitate washed with alcohol, dissolved in 500 ml of water and the solution neutralized with $Ba(OH)_2$ to faintly alkaline reaction. The solution was clarified by centrifugation and made neutral or faintly acid (pH 6.5) with dilute hydrochloric acid and the barium salt of the chondroitinsulfuric acid precipitated with 2 volumes of alcohol.

The barium salt from 50 g of chondroitinsulfuric acid thus obtained was hydrolysed over free flame under reflux for 7—8 hours in 500 ml of 20 % hydrochloric acid in the presence of 10 g stannous chloride. After a few minutes the solution turned brown and at the end of the hydrolysis the reaction mixture was black. It was poured into 2 liters of hot water and treated with H_2S. The solution was filtered and the clear faintly yellow solution aerated to remove the excess of H_2S. The filtrate was then evaporated under reduced pressure to dryness. The residue was then extracted with about 100 ml of methyl alcohol to which 5 ml of 1—N hydrochloric acid had been added. The insoluble part, consisting to 85 % of ash, was removed by filtration. The filtrate was placed in the icebox at —10° C for one hour. Cold (—5° to —10° C) aceton was then added very cautiously through a pipett under vigorous mixing until a faint opalescence appeared. The walls of the vessel were scratched and the solution left in the icebox over night. The following day the crystals formed were scraped down from the walls, aceton added again and the solution left in the icebox. This treatment was continued for 4—5 days until no more crystals appeared. The crystals formed were removed by filtration, washed first with methyl alcohol containing 10 % of aceton and 1 % normal HCl, and finally with pure aceton, and then dried over P_2O_5. Yield 9.8 g of chondrosamine hydrochloride (52 % of theoretical). This preparation is sufficiently pure for most purposes. It analysed as follows:

	Found	Theoretical
Moisture	0.42 %	—
Ash	< 0.05 »	—
Nitrogen:		
(Micro Kjeldahl)	6.51 »	6.5 %
Chlorine	16.1 »	16.5 »
Melting point:	179° C	182° C (Levene)

a_D^{20} (2 % solution) initial + 62°, final 92.5° (Levene + 96.4°).

After evaporation of the mother liquor to dryness and treatment as above another lot of 2 g of the hydrochloride was obtained. Total yield 63.7 %.

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New Books

Vitamin Methods. Volume I. Edited by Paul György. Academic Press Inc., New York, 1950. 569 pp. $ 10.

Eric T. Stiller: Physical methods of vitamin Assay.

Paul György and Saul H. Rubin: Chemical methods of vitamin assay.

Otto A. Bessey: Microchemical methods.

Esmond E. Snell: Microbiological methods in vitamin research.

Erich Hirschberg: Instrumentation in vitamin analyses.

Literature concerning vitamin determination is comprehensive, and it is often difficult for less experienced to choose the most suitable method among the many modifications which have been proposed. It is therefore of great value that vitamin methods are assembled and critically reviewed by acknowledged experts in their subjects.

The book *Vitamin methods*, volume one, deals with the physical, chemical, microchemical and microbiological methods of vitamin assay. A seccond volume to appear, will treat biological methods of vitamin assay.

The material is presented not around the various vitamins, but around the assay methods employed. Consequently the volume is devided into the chapters mentioned above. Even if this way of presentation makes it possible to treat the assay technique separately, it is questionable whether this arrangement has made the subject more surveyable than if built around the various vitamins.

In many cases the determination of a vitamin in different substances will differ quite considerably, whereas the final method of measurement is simple, at least as far as physical and chemical procedures are concerned.

The physical methods of vitamin determination comprise the application of ultraviolet absorption spectrophotometry, fluorimetry and some special physical methods. Much of this chapter deals with the spectrophotometric determination of vitamin A. There has been and still is much confusion regarding the conversion factors when the vitamin A concentration is to be expressed in the different units still in use. A survey of conversion factors and vitamin A units is given, and this shows clearly that a revision of the standard fixed by the Health Organisation of the Ligue of Nations (1934) is highly necessary.

The different methods for the U. V. spectrophotometric determination of vitamin A in different sources are well reviewed, and prosedures to avoid errors due to irrevalent absorption are also included. The application of spectrophotometry to other vitamins, such as vitamin E and K *etc.*, which may in special cases be of interest when working with pure or nearly pure consentrates is also dealt with in this chapter.

The fluorimetric method has long been used in thiamine research and has now also found wide application for riboflavin assay, since it is far more sensitive than the colorimetric measurement. The most reliable procedures for thiamine and riboflavin assay in different substances are described.

The chapter "Chemical methods of vitamin assay" deals with the colorimetric

determination of vitamins. It is a well known fact to all experienced in vitamin research that colorimetric methods must be applied with great criticism, as most of them suffer from lack of specificity. It is therefore of importance that the author considers the objections raised by research workers familiar with the different methods. The wide field of colorimetry in vitamin research is covered in this chapter. It may be mentioned that the colorimetric determination of riboflavin has been used by many investigators and is in many cases a suitable method. It is, however, not mentioned in the book.

Microchemical methods are chiefly described as assay methods for body fluids. In this chapter determination of other nutritive essentials of special interest for clinical laboratories is also included.

It is a matter of course that the microbiological assay methods have occupied a great space as these methods have found extensive use in vitamin B research during the latest years. Detailed description of microbiological methods available for assay of vitamin of the B-complex is given.

It is repetedly pointed out that, when extracts of food stuffs are tested, unknown factors may often have an influence on the growth response and the metabolism of the microorganisms, causing erronous results. The compensation method developed by Nielsen, Hartelius and Johansen accounts for such unknown factors (*Naturwissenschaften* 1943) and has been used in studies on pantothenic acid (Schmidt, Copenhagen, 1949). In the reviewers opinion, this procedure should have been considered in this chapter.

In the last chapter of this volume a description of different optical instruments used in vitamin analyses is given.

The numerous references are conveniently arranged at the end of each chapter.

As a conclusion it should be stated that the *Vitamin methods* is a valuable handbook for all laboratories working on vitamin analyses.

Hans Kringstad

ACTA CHEMICA SCANDINAVICA 5 (1951) 199–219

On the Complex Chemistry of the Uranyl Ion

IV.* The Complexity of Uranyl Acetate

STEN AHRLAND

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The hydrolysis of the uranyl ion is getting perceptible at as low a pH as 3. By this, investigations of the complexity of uranyl salts are rendered rather difficult for in all solutions of pH > 3 the complex equilibria searched for are always contaminated with hydrolytic equilibria (I p. 379). Certainly, not only the uranyl ion is hydrolysed, but also its complexes. For two reasons, however, the hydrolysis of the complexes must be less pronounced than that of the uranyl ion itself: they have less ionic charge, and fewer places of coordination ready for hydrolytic reactions. In a solution of a strongly complex uranyl salt, therefore, it is very probable that the hydrolysis may be neglected even if pH > 3. If that is the case, the complexity of the salt can be determined even by measurements at a pH, where the uranyl ion is hydrolysed in a fatal degree. But, of course, it is in every case necessary to test if the supposition holds. According to the method of measurement used, this test is performed in different ways, as will be seen below.

SELECTION OF URANYL ACETATE AS A SALT, SUITABLE FOR THE MEASUREMENTS

For several reasons, uranyl acetate has been selected as a salt, suitable to test the applicability of the principle outlined above.

Firstly, it is certainly rather strongly complex. This is clearly proved by Dittrich [4] by means of conductometric, cryoscopic and migration measurements. The last method gives the especially interesting information that the

* The preceding papers of this series (Ahrland [1–3]) are in the following referred to as I, II and III.

uranyl group forms part of an anion in solutions of high C_A.* The existence of such an anion is also reported by Brintzinger and Jahn [5]. By dialysis measurements they consider to have proved the ion $UO_2(CH_3 \cdot COO)_3^-$. Indeed, this view is strongly supported by the existence of numerous "double" salts of the type $NaUO_2(CH_3COO)_3$.

Secondly, the acetate permits both emf and extinction measurements according to the methods described in II, and the results can be compared as it was done there. The emf measurement forms, however, the principal base of the calculations. As will be seen below, it is namely impossible to extend the extinction measurements over the whole desirable C_A-range.

Thirdly, as the acetate ligand is always added to the uranyl solutions in the form of a buffer, the pH:s of the complex solutions do not become too high which, of course, increases the chance of success. Further, the acetate is, fortunately, very little sensible to light which is contrary to most other organic uranyl salts (*cf.* II pp. 784 and 804).

Last but not least, the acetate may pretend to have a certain general interest as a very common uranyl salt.

To make the present measurements comparable to those of I—III, the same ionic strength $I = 1$ has been chosen, and sodium perchlorate used as the supplementary neutral salt. But this proves to be somewhat disadvantageous here, on account of the rather slight solubility of $NaUO_2(CH_3 \cdot COO)_3$. At the high $[Na^+]$, ≈ 1, which prevails in the present solution, C_A cannot generally exceed 150 mC at the highest C'_M used, 50 mC, before precipitation occurs. A ionic medium of lower $[Na^+]$ or no $[Na^+]$ at all would have been more advantageous, but in order not to sacrifice the comparability, no change has been made

The temperature has been kept at 20° C, as in I—III.

CHEMICALS USED

The *glacial acetic acid*, Schering-Kahlbaum *pro analysi*, has been freezed out once.

The *sodium acetate*, Bakers analysed, has been used without further purification.

By weighing, three different buffers are prepared, with the $C'_{HA} : C'_A$ ratios 1/2 : 1, 2 : 1 and 5 : 1. Then the exact concentrations of acid are determined alkalimetrically and those of acetate acidimetrically. In the latter case, potentiometric indication of the endpoint is applied. By that, a quinhydrone electrode is used which is measured towards the quinhydrone reference electrode RE (*cf.* I p. 383).

The *other chemicals* used are of the same quality as before (I p. 382).

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* The meaning of symbols, see I—III.

THE EMF MEASUREMENTS

a. The complex formation function when hydrolysis occur in the solutions

The potentiometric method results in a determination of [A] and $\bar{n}$, *i.e.* the complex formation function (*cf.* II pp. 788—794). Therefore, we will deduce this function in the present case, where the complex equilibria of the solution are contaminated with hydrolytic ones.

In the case of a complex system with only one sort of ligand, it has been shown in I (p. 379) that $\bar{n}$ is a function of [A] only if only mononuclear complexes exist in the solution, but $\bar{n}$ is a function of both [A] and [M], if polynuclear complexes exist, too. As the essential difference between these two possibilities turns up as soon as dinuclear complexes exist, we may, for our purpose of differentiating between them, limit ourselves to consider mono- and dinuclear complexes. This considerably simplifies our future formulas. In such a case (8) of I (p. 379) takes the form:

$$\frac{C_A - [A]}{C_M} = \bar{n} = \frac{\sum_{n=1}^{N} n\beta'_n [A]^n + [M] \sum_{n=1}^{Q} n\beta''_n [A]^n}{1 + \sum_{n=1}^{N} \beta'_n [A]^n + 2[M] \sum_{n=1}^{Q} \beta''_n [A]^n} \qquad (1)$$

If only mononuclear complexes exist, this equation is further simplified by omission of the terms containing the factor [M] (= (2) of II, p. 785).

If now hydrolysis occurs besides the complex formation between M and A, this means that two sorts of ligand are operating in the same solution. A series of hydrolytic complexes with 1,2, ... OH^- may then be formed for every uranyl species present before. If we substitute $[H^+]$ for $[OH^-]$ according to $[OH^-] = K_w/[H^+]$ (*cf.* I p. 381), we can write the equilibrium equations of the new hydrolytic complexes thus:

$$\gamma'_{n,1} = [MA_nOH][M]^{-1}[A]^{-n}[H^+] \quad (2\,a) \qquad \gamma''_{n,1} = [M_2A_nOH][M]^{-2}[A]^{-n}[H^+] \quad (3\,a)$$

$$\gamma'_{n,2} = [MA_n(OH)_2][M]^{-1}[A]^{-n}[H^+]^2 \quad (2\,b) \qquad \gamma''_{n,2} = [M_2A_n(OH)_2][M]^{-2}[A]^{-n}[H^+]^2 \quad (3\,b)$$

If we now reform $(C_A - [A])/C_M$, including these hydrolytic complexes, we obtain:

$$\bar{n} = \frac{\sum_{n=1}^{N} n\beta'_n [A]^n + [M] \sum_{n=1}^{Q} n\beta''_n [A]^n + 1/[H^+] \left[\sum_{n=1}^{R} n\gamma'_{n,1} [A]^n + [M] \sum_{n=1}^{S} n\gamma''_{n,1} [A]^n \right] + \cdots}{1 + \sum_{n=1}^{N} \beta'_n [A]^n + 2[M] \sum_{n=1}^{Q} \beta''_n [A]^n + 1/[H^+] \left[\gamma'_{0,1} + \sum_{n=1}^{R} \gamma'_{n,1} [A]^n + 2[M] (\gamma''_{0,1} + \sum_{n=1}^{S} \gamma''_{n,1} [A]^n) \right] + \cdots}$$

$$\frac{+ 1/[H^+]^2 \left[\sum_{n=1}^{T} n\gamma_{n,2} [A]^n + [M] \sum_{n=1}^{U} n\gamma''_{n,2} [A]^n \right] + \cdots}{+ 1/[H^+]^2 \left[\gamma'_{0,2} + \sum_{n=1}^{T} \gamma'_{n,2} [A]^n + 2[M] (\gamma''_{0,2} + \sum_{n=1}^{U} \gamma''_{n,2} [A]^n) \right] + \cdots} \qquad (4)$$

Thus, besides being a function of [A] and [M], $\bar{n}$ is a function of $[H^+]$, too, if hydrolytic reactions occur. Inversely, if the formation function is found to be independent of $[H^+]$, no hydrolysis takes place in the solution, and the old formulas of $\bar{n}$ holds. By determining [A] and $\bar{n}$ at varying pH, we can thus observe if the hydrolysis has any influence and so test our introductory supposition. If slight deviations are found between the formation functions of different pH, the nonhydrolytic function may be found by an extrapolation to strongly acid solution, *i. e.* to $1/[H^+] = 0$.

At every pH, measurements are performed at different C_M, *i. e.* different [M], in order to make the usual decision between mono- and polynuclear complex formation.

b. The calculation of $\bar{n}$ and [A] from experimental data

The experiments are determinations of $[H^+]$ by quinhydrone electrode, carried out quite analogous to those of II (p. 788). From $[H^+]$, [A] and $\bar{n}$ will be calculated in the same manner as there, with some necessary modifications.

If C'_{HA} is the stoichiometric concentration of acetic acid, it is valid, in a buffer solution free from uranyl:

$$\frac{[H^+]' (C'_A + [H^+]')}{C'_{HA} - [H^+]'} = K_c \qquad (5)$$

For a solution, which has the uranyl concentration C_M and the same stoichiometric buffer concentration as above, [A] is the quantity searched for and for [HA] it is valid

$$[HA] = C'_{HA} + C^0_H - [H^+] - C_s \qquad (6)$$

Thus

$$\frac{[H^+]\,[A]}{C'_{HA} + C^0_H - [H^+] - C_s} = K_c \tag{7}$$

If we put $C'_{HA}/C'_A = \delta$, (5) and (7) give

$$[A] = \frac{[H^+]'}{[H^+]} \cdot \frac{(C'_A + [H^+])\ (\delta \cdot C'_A + C^0_H - [H^+] - C_s)}{\delta \cdot C'_A - [H^+]'}$$

or, approximately,

$$[A] = \frac{[H^+]'}{[H^+]} \cdot \left(C'_A + \frac{C^0_H - [H^+] - C_s + (1+\delta)[H^+]'}{\delta} \right) \tag{8}$$

In the calculation of $\bar{n}$ according to (1), C_A is the total concentration of species containing A except the acetic acid, and C_M is the total uranium concentration, hydrolysed as non-hydrolysed. Hence, with (14) of II:

$$\bar{n} = \frac{C'_A + [H^+] - C^0_H + C_s - [A]}{C_M} \tag{9}$$

In these equations of $\bar{n}$ and [A], $[H^+]'$, $[H^+]$, C^0_H and $[H^+]'/[H^+]$ are measured according to (16), (17) and (18) of II, and C'_A and δ are known by the analysis of the buffer stock solutions. The only unknown quantity is C_s, the term that originates from those hydrogen ions which are taken up by or set free from the uranyl species when hydrolytic reactions occur by the addition of buffer. *

Thus, in case of a hydrolysis, $\bar{n}$ is affected in two different ways: firstly, $\bar{n}$ becomes a function of $[H^+]$ (according to (4)) and, secondly, the very calculation of $\bar{n}$ according to its definition (1) becomes impossible.

On the other hand, if the hydrolysis can be neglected, $\bar{n}$ is independent of $[H^+]$ ((1) holds), and, moreover, its calculation is easily performed according to (9), as, of course, $C_s \approx 0$ in this case. Thus, if we calculate $\bar{n}$ according to (9), putting $C_s = 0$, and find a complex formation curve which is independent of pH, we are certainly allowed to conclude that no hydrolysis occurs. **

* In II, it was possible to correct approximately for this C_s, as the hydrolysis of uranyl is known to be very slight at the p $[H^+]$ of the chloroacetate buffer. The course of the hydrolysis in acetate buffers is, however, a priori unknown.

** The only conceivable alternative would be that the two factors influencing $\bar{n}$ just balance each other throughout the whole complex formation curve. This assumption is, however, extremely unlikely.

Table 1. Determination of E' as a function of C'_A, when perchlorate is exchanged for the different acetate buffers in solutions of $C_M = 0$. — Calculation of K_c in the medium used.

Buffer →	5 : 1		2 : 1		1/2 : 1	
C'_A mC	E' mV	(5) $K_c \cdot 10^5$ C	E' mV	(5) $K_c \cdot 10^5$ C	E' mV	(5) $K_c \cdot 10^5$ C
12.97	110.6	2.58	133.4	2.57	168.9	2.54
25.6	110.3	2.59	133.2	2.58	168.7	2.55
37.9	110.1	2.60	133.1	2.59	168.6	2.56
49.9	110.0	2.60	133.1	2.59	168.6	2.56
61.7	110.0	2.60	133.1	2.59	168.5	2.57
89.6	110.0	2.60	133.0	2.60	168.5	2.57
116.0	110.0	2.60	133.1	2.59	168.5	2.57
164.3	110.0	2.60	133.2	2.58	168.6	2.56
207.5	110.1	2.60	133.2	2.58	168.6	2.56
246.5	110.2	2.59	133.2	2.58	168.6	2.56
281.8	110.2		133.3		168.7	
343.0	110.5		133.5		168.7	
394	110.7		133.6		168.7	

c. Experimental data

The measurements are performed with the three different buffers mentioned above, which have the exact concentrations: $C'_A = 986$ mC and $C'_{HA} = 493$, 1983 and 4910 mC, respectively. For every buffer, series are made with $C'_M =$ 10, 25 and 50 mC, besides the titrations with $C_M = 0$. RE has in the present case $[H^+]_0 = 10.14$ mC. In all cases, the potentials measured are stable and reproduce themselves within ≈ 0.2 mV. No sign of any sensibility to light has been detected.

The series with $C_M = 0$ (Table 1) show that the ionic medium is not as seriously changed by acetate as by chloroacetate (II p. 789). C'_A can reach > 200 mC even at the buffer of the highest C'_{HA} before any change is perceptible. Up to that C'_A, we find (according to (16) of II and (5)) a constant $K_c = 2.58 \cdot 10^{-5}$, the same for all buffers if the random errors are considered.*

The results of the main titrations are collected in Tables 2—4, and shown in Figs. 1 and 2. At the two most acid buffers, the formation functions coincide, if one excepts a small deviation in their lowest parts, when $\bar{n} < 0.9$. At the

* Fronæus[6] finds $2.46 \cdot 10^{-5}$

Table 2. Determination of corresponding values of $\bar{n}$ and [A] at the pH of the buffer 5 : 1.

Table 2 A. C'_M = 10 mC. ● *

C_M mC	C'_A mC	E mV	(18) of II E_A mV	(17) of II C_H^0–[H⁺] mC	(16) of II [H⁺]′ mC	(8) [A] mC	(9) $\bar{n}$	$\bar{n}$/[A] C⁻¹
10.00	0	71.0						
9.87	12.97	81.8	28.8	0.20	0.13	4.16	0.875	210
9.74	25.60	84.4	25.9	0.23	0.13	9.18	1.66	181
9.62	37.9	88.8	21.3	0.3	0.1	16.37	2.20	134
9.50	49.9	93.3	16.7	0.3	0.1	25.8	2.51	97.5
9.38	61.7	96.5	13.5	0.3	0.1	36.2	2.67	74
9.27	73.0	99.1	10.0	0.4	0.1	47.6	2.70	56.5
9.09	89.6	101.3	8.7	0.4	0.1	63.6	2.82	44.5
8.82	116.0	103.7	6.3	0.4	0.1	90.6	2.83	31
8.57	140.9	105.0	5.0	0.4	0.1	115.6	2.91	25
8.33	164.3	105.9	4.1	0.4	0.1	139.6	2.90	21
8.11	186.5	106.5	3.5	0.4	0.1	162.6	2.90	18
7.90	207.5	107.0	3.1	0.3	0.1	183.6	2.99	16.5
7.70	227.5	107.4	2.7	0.3	0.1	204.7	2.93	14.5
7.50	246.5	107.7	2.5	0.3	0.1	223.5	3.02	13.5
7.14	281.8	108.3	1.9	0.3	0.1	261.2	2.85	11
6.82	313.7	108.7	1.6	0.3	0.1	294.4	2.79	9.5
6.52	343.0	109.1	1.4	0.3	0.1	324.3	2.81	8.5

least acid buffer, on the other hand, the formation function does not coincide with the other ones before $\bar{n}$ has reached 2.3. The deviations grow very large at low $\bar{n}$. Moreover, the curves of different C_M do not coincide here, as is the case at the other buffers. All the curves ultimately bend towards $\bar{n} = 3$.

Thus the hydrolysis is completely forced out by the acetate complex formation at the pH of the buffers 5 : 1 and 2 : 1, except at very low [A]. Moreover, the complex formation is mononuclear. At the buffer ½ : 1, on the other hand, the hydrolysis is very pronounced even at rather high [A]. At very high [A], however, the acetate complexes become predominant even here. As a difference of C_M means a difference of pH, the spreading of the curves of different C_M is at once clear. The existence of polynuclear hydrolytic complexes may, of course, also have influence, here, as well as the fact that the very calculation of $\bar{n}$ and [A] is erronous if a strong hydrolysis occurs (p. 203).

* In the Tables 2—4, these signs indicate the respective series in the Figs. 1 and 2.

Table 2 B. $C'_M = 25$ *mC.* ▲

C_M mC	C'_A mC	E mV	(18) of II E_A mV	(17) of II $C_H^0-[H^+]$ mC	(16) of II $[H^+]'$ mC	(8) [A] mC	(9) $\bar{n}$	$\bar{n}/[A]$ C^{-1}
25.0	0	52.3						
24.65	12.97	62.3	48.3	0.40	0.13	1.93	0.432	224
24.35	25.60	63.7	46.6	0.43	0.13	4.07	0.865	213
24.05	37.9	65.2	44.9	0.5	0.1	6.43	1.29	200
23.75	49.9	67.7	42.7	0.5	0.1	9.23	1.69	183
23.45	61.7	71.1	38.9	0.6	0.1	13.28	2.04	154
23.2	73.0	75.5	34.5	0.7	0.1	18.7	2.32	124
22.7	89.6	82.3	27.7	0.8	0.1	29.9	2.59	86.5
22.05	116.0	90.7	19.3	0.9	0.1	54.1	2.77	51
21.4	140.9	95.4	14.6	0.9	0.1	79.1	2.85	36
20.8	164.3	98.6	11.4	0.9	0.1	104.7	2.82	27
20.25	186.5	100.2	9.8	0.9	0.1	126.5	2.92	23
19.75	207.5	101.7	8.4	0.8	0.1	148.9	2.93	19.5
19.25	227.5	102.9	7.2	0.8	0.1	171.0	2.90	17
18.75	246.5	103.7	6.5	0.8	0.1	190.5	2.95	15.5
17.85	281.8	104.9	5.3	0.8	0.1	228.6	2.94	13
17.05	313.7	105.8	4.5	0.7	0.1	263.0	2.93	11
16.3	343.0	106.5	4.0	0.7	0.1	292.4	3.06	10.5

Table 2 C. $C'_M = 50$ *mC.* ■

50.0	0	38.0						
49.3	12.97	45.6	65.0	0.57	0.13	1.00	0.231	231
48.7	25.60	47.0	63.3	0.63	0.13	2.10	0.47	224
48.1	37.9	47.8	62.3	0.7	0.1	3.22	0.705	219
47.5	49.9	48.6	61.4	0.7	0.1	4.38	0.945	216
46.9	61.7	49.8	60.2	0.7	0.1	5.69	1.18	208
46.3	73.0	50.9	59.1	0.7	0.1	7.07	1.41	199
45.4	89.6	53.7	56.3	0.8	0.1	9.63	1.74	181
44.1	116.0	60.6	49.4	1.1	0.1	16.41	2.24	137
42.8	140.9	70.1	39.9	1.3	0.1	29.1	2.58	89

Above $\bar{n} = 0.9$ the coinciding curves of the buffers 5 : 1 and 2 : 1 are thus the non-hydrolytic complex formation curve. Below this $\bar{n}$, an extrapolation of $\bar{n}$ from $1/[H^+]$ of the three buffers used to $1/[H^+] = 0$ is to give the right function. By this extrapolation, however, it is seen that the $\bar{n}$ of $1/[H^+] = 0$ and the $\bar{n}$ of the $1/[H^+]$ of the buffer 5 : 1 coincide within the experimental

Table 3. Determination of corresponding values of $\bar{n}$ and [A] at the pH of the buffer 2 : 1

Table 3 A. $C'_M = 10$ mC. ○

C_M mC	C'_A mC	E mV	(18) of II E_A mV	(17) of II $C_H^0 - [H^+]$ mC	(16) of II $[H^+]'$	(8) [A] mC	(9) $\bar{n}$	$\bar{n}/[A]$ C^{-1}
10.0	0	70.7						
9.87	12.97	102.3	31.1	0.42	0.05	3.86	0.88	228
9.74	25.60	107.2	26.0	0.44	0.05	9.23	1.63	177
9.62	37.9	112.0	21.1	0.5		16.52	2.17	131
9.50	49.9	116.5	16.6	0.5		25.9	2.48	96
9.38	61.7	119.7	13.4	0.5		36.5	2.63	72
9.27	73.0	122.1	11.0	0.5		47.3	2.72	57.5
9.09	89.6	124.5	8.5	0.5		64.3	2.73	42.5
8.82	116.0	126.8	6.3	0.5		90.6	2.82	31
8.57	140.9	128.1	5.0	0.5		115.8	2.87	25
8.33	164.3	129.0	4.2	0.5		139.3	2.94	21
8.11	186.5	129.8	3.4	0.4		163.3	2.81	17
7.90	207.5	130.2	3.0	0.4		184.5	2.86	15.5
7.70	227.5	130.6	2.6	0.4		205.5	2.81	13.5
7.50	246.5	130.9	2.3	0.4		224.8	2.84	12.5
7.14	281.8	131.4	1.9	0.4		261.2	2.83	11
6.82	313.7	131.8	1.6	0.4		294.4	2.78	9.5
6.52	343.0	132.1	1.4	0.4		324.3	2.82	8.5

error. This is not surprising, as the uranyl solutions of low C'_A by the buffer 5 : 1 actually have a $p[H^+] \approx 3$ (Table 2), so their hydrolysis must be insignificant (*cf.* I). Thus the complex formation curve of the buffer 5 : 1 is, in its whole course, equal to the non-hydrolytic formation curve of the uranyl acetate system, and the complexity constants of the system can be calculated from it, according to II p. 785—787. Before that, however, we will look at that formation curve which is obtained by the extinction measurement.

THE EXTINCTION MEASUREMENTS

a. The ε_M-function when hydrolysis occurs in the solutions

The extinctiometric method implies the determination of ε_M of a number of solutions with different C_M and C_A. In the case of a mononuclear complex formation with one ligand, [A] and $\bar{n}$, *i.e.* the complex formation function,

Table 3 B. $C'_M = 25$ *mC.* △

C_M mC	C'_A mC	E mV	(18) of II E_A mV	(17) of II $C_H^0 - [H^+]$ mC	(16) of II $[H^+]'$ mC	(8) [A] mC	(9) $\bar{n}$	$\bar{n}/[A]$ C^{-1}
25.0	0	52.4						
24.65	12.97	79.0	54.4	0.82	0.05	1.56	0.435	278
24.35	25.60	84.5	48.7	0.89	0.05	3.79	0.86	227
24.05	37.9	87.6	45.5	0.9		6.34	1.27	200
23.75	49.9	90.6	42.5	0.9		9.38	1.67	178
23.45	61.7	94.4	38.7	1.0		13.46	2.02	150
23.15	73.0	98.9	34.2	1.0		18.96	2.29	121
22.7	89.6	105.6	27.4	1.0		30.5	2.56	84
22.05	116.0	114.0	19.1	1.0		54.7	2.74	50
21.4	140.9	118.7	14.4	1.0		80.0	2.80	35
20.8	164.3	121.5	11.7	1.0		103.7	2.86	27.5
20.25	186.5	123.5	9.7	1.0		127.3	2.88	22.5
19.75	207.5	124.9	8.3	0.9		149.6	2.89	19.5
19.25	227.5	125.9	7.3	0.9		170.6	2.91	17
18.75	246.5	126.7	6.5	0.9		191.2	2.90	15
17.85	281.8	128.0	5.3	0.9		228.5	2.94	13
17.05	313.7	128.9	4.5	0.8		263.0	2.93	11
16.3	343.0	129.6	3.9	0.8		293.8	2.97	10

Table 3 C. $C'_M = 50$ *mC.* □

50.0	0	37.9						
49.3	12.97	59.5	73.9	1.33	0.05	0.73	0.222	304
48.7	25.60	65.1	68.1	1.44	0.05	1.77	0.46	260
48.1	37.9	68.1	65.0	1.5		2.95	0.715	242
47.5	49.9	70.2	62.9	1.5		4.20	0.93	221
46.9	61.7	72.0	61.1	1.5		5.58	1.16	206
46.3	73.0	73.9	59.2	1.6		7.10	1.39	196
45.4	89.6	76.7	56.3	1.6		9.73	1.73	178
44.1	116.0	84.1	49.0	1.6		16.83	2.21	131
42.8	140.9	93.8	39.4	1.7		29.9	2.56	85.5

can be obtained from ε_M, and thus the complexity constants of the system calculated (see II and III).

When the complex formation involves hydrolytic equilibria, too, ε_M is modified in a manner analogous to that of the $\bar{n}$-function. In the ε_M-function,

Table 4. Determination of corresponding values of $\bar{n}$ and [A] at the pH of the buffer 1/2 : 1.

Table 4 A. C'_M = 10 mC.

C_M mC	C'_A mC	E mV	(18) of II E_A mV	(17) of II $C_H^0 - [H^+]$ mC	(16) of II $[H^+]'$ mC	(8) [A] mC	(9) $\bar{n}$	$\bar{n}/[A]$ C^{-1}
10.00	0	70.7						
9.87	12.97	120.8	48.1	0.51	0.01	2.08	1.05	505
9.74	25.60	135.8	32.9	0.54	0.01	7.24	1.83	252
9.62	37.9	145.0	23.6	0.6		15.3	2.29	150
9.50	49.9	151.0	17.6	0.6		25.4	2.52	99
9.38	61.7	154.8	13.7	0.6		36.5	2.62	72
9.27	73.0	157.4	11.1	0.5		47.7	2.68	56
9.09	89.6	159.8	8.7	0.5		64.3	2.73	42.5
8.82	116.0	162.2	6.3	0.5		91.3	2.75	30
8.57	140.9	163.5	5.0	0.5		116.4	2.80	24
8.33	164.3	164.5	4.1	0.5		140.6	2.79	20
8.11	186.5	165.1	3.5	0.5		163.3	2.80	17
7.90	207.5	165.5	3.1	0.5		184.5	2.85	15.5
7.70	227.5	165.9	2.7	0.5		205.6	2.78	13.5
7.50	246.5	166.2	2.4	0.5		225.4	2.76	12.5
7.14	281.8	166.7	2.0	0.4		261.2	2.83	11
6.82	313.7	167.0	1.7	0.4		294.4	2.78	9.5
6.52	343.0	167.3	1.4	0.4		325.8	2.58	8

however, we need not consider any non-hydrolytic polynuclear complexes.* The emf measurements indicate that they are not likely to exist, and this is confirmed, when the usual extinctiometric criterion (II p. 803) of polynuclear complexes is applied here (p. 216). If the molar extinctions of the new complexes MA_nOH, $MA_n(OH)_2$, ..., M_2A_nOH, $M_2A_n(OH)_2$, .. are indicated by $\varepsilon'_{n,1}$, $\varepsilon'_{n,2}$, ..., $\varepsilon''_{n,1}$, $\varepsilon''_{n,2}$, ... respectively, we thus obtain

$$\varepsilon_M = \frac{\varepsilon'_o + \sum_{n=1}^{N} \varepsilon'_n \beta'_n [A]^n + 1/[H^+] \left(\sum_{n=0}^{R} \varepsilon'_{n,1} \gamma'_{n,1} [A]^n + [M] \sum_{n=0}^{S} \varepsilon''_{n,1} \gamma''_{n,1} [A]^n \right) + \cdots}{1 + \sum_{n=1}^{N} \beta'_n [A]^n + 1/[H^+] \left(\sum_{n=0}^{R} \gamma'_{n,1} [A]^n + 2 [M] \sum_{n=0}^{S} \gamma''_{n,2} [A]^n \right) + \cdots} \quad (10)$$

* If such exist, no complexity constants can be calculated from ε_M. (II p. 799).

Table 4 B. $C'_M = 25$ *mC.*

C_M mC	C'_A mC	E mV	(18) of II E_A mV	(17) of II $C_H^0 - [H^+]$ mC	(16) of II $[H^+]'$ mC	(8) [A] mC	(9) $\bar{n}$	$\bar{n}/[A]$ C^{-1}
25.0	0	52.1						
24.65	12.97	90.8	78.1	0.99	0.01	0.68	0.46	677
24.35	25.60	103.7	65.0	1.08	0.01	2.11	0.92	437
24.05	37.9	112.4	56.2	1.1		4.33	1.35	312
23.75	49.9	119.8	48.8	1.1		7.55	1.74	230
23.45	61.7	126.3	42.2	1.1		12.02	2.07	172
23.15	73.0	132.5	36.0	1.1		18.15	2.32	128
22.7	89.6	140.1	28.4	1.1		29.9	2.58	86
22.05	116.0	148.8	19.7	1.1		54.2	2.76	51
21.4	140.9	153.7	14.9	1.1		79.5	2.82	35.5
20.8	164.3	156.8	11.8	1.1		104.2	2.84	27
20.25	186.5	158.7	9.9	1.0		127.4	2.87	22.5
19.75	207.5	160.0	8.6	1.0		148.9	2.92	19.5
19.25	227.5	161.0	7.6	1.0		169.8	2.95	17.5
18.75	246.5	161.8	6.8	1.0		189.7	2.98	15.5
17.85	281.8	162.9	5.8	0.9		225.4	3.11	14
17.05	313.7	163.9	4.8	0.9		261.2	3.03	11.5
16.3	343.0	164.7	4.0	0.8		294.4	2.94	10

Table 4 C. $C'_M = 50$ *mC.*

50.0	0	37.8						
49.3	12.97	68.6	100.3	1.57	0.01	0.304	0.225	740
48.7	25.60	79.4	89.3	1.77	0.01	0.847	0.47	555
48.1	37.9	86.7	81.9	1.9		1.63	0.715	440
47.5	49.9	92.4	76.2	1.9		2.63	0.96	365
46.9	61.7	97.2	71.3	1.9		3.89	1.19	306
45.4	89.6	107.9	60.6	1.9		8.47	1.75	206
44.1	116.0	118.2	50.3	1.9		16.37	2.22	136
42.8	140.9	128.3	40.2	1.9		29.5	2.56	87

Thus, if hydrolytic reactions occur, ε_M is a function of $[H^+]$. Inversely, if ε_M is independent of $[H^+]$ no such reactions take place, and (21) of II (p. 800) is valid. By determining ε_M at different pH, we are thus able to decide, whether the hydrolysis has any influence or not. It a small deviation is found between the pH:s, an extrapolation of ε_M to $1/[H^+] = 0$ will give the non-hydrolytic value.

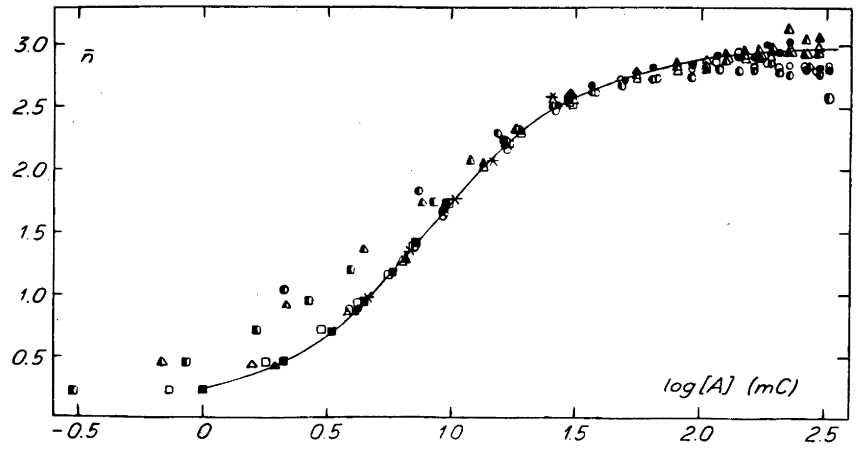

Fig. 1. The complex formation curves. — Circles, triangles and squares relate to the emf measurements at C'_M = 10, 25 and 50 mC respectively. Filled, open and half-filled signs relate to the buffers 5 : 1, 2 : 1 and 1|2 : 1 respectively. — The stars indicate the extinctiometrically determined values. — The fulldrawn curve is obtained from the complexity constants finally calculated.

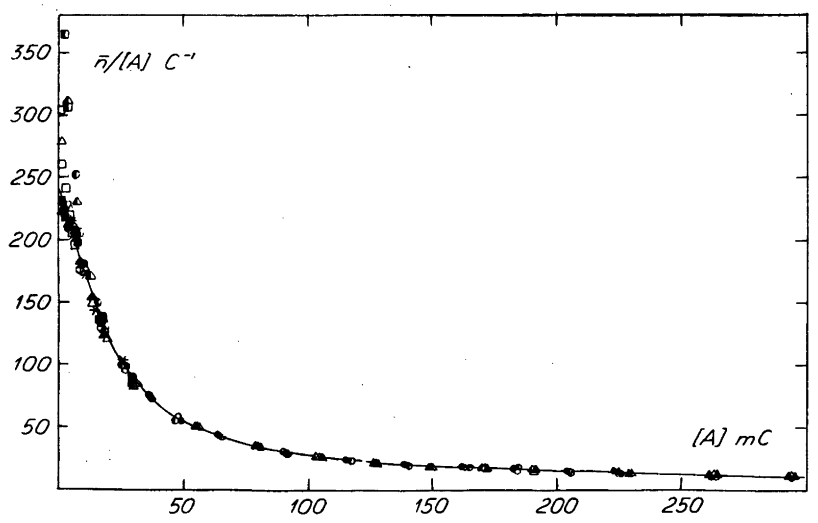

Fig. 2. $\bar{n}/[A]$ as a function of $[A]$. — The signs relate to the same measurements as in Fig. 1. — The fulldrawn curve represents the non-hydrolytic function the integration of which gives the X-functions (Table 8).

b. Apparatus and method of measurement

The present measurements are carried out with a Beckman Quartz Spectrophotometer, model DU, which had been inproved as described by Adell [7]. The absorption cells used are from B. Halle, Berlin. They are made from glass, with windows of silica, which are held water-tight by adhesion forces. The cells are mounted on a slide, which gives them a very reproducible position in the beam of light. The reproducibility of E is $\approx 5\,‰$, which is, it is true, somewhat lower than that obtained with the apparatus used before. But, on the other hand, the new apparatus is much more handy than the old one.

Another advantage is that its construction enables us, contrary to that of the old one, to correct separately for the differences of transparancy and thickness which may exist between the absorption cells (*cf.* II p. 798). At first we can establish, at every wave-length λ desired, the differences of transparancy between the two cells of every pair. This is done by measuring when both the cells contain solvent. Then those cells which are to be used to the uranyl solutions are compared by means of picrate solutions the concentrations of which are chosen so that E is always approximately constant (in the neighbourhood of 0.7). If these E:s are corrected for the differences of transparancy, they give the true ratios of thickness between the cells used, and not, as the proceeding of II, an "effective" ratio in which the differences of transparancy are involved. Contrary to the effective ratios, the true ones are, of course, independent of λ and E.

But then it is no more necessary to keep E constant throughout all the series of measurement. Only the condition remains that E has to be a constant for all solutions which are directly brought together for the calculation of a certain [A], *i. e.* for solutions of a constant ε_M. E may vary within a series if only those solutions of *different* series have the same E which are to be compared. But then we may simply keep a constant C_M throughout the series, allowing E to increase with C_A. This simplifies the measurements very much as now no especial function between ε_M and C_M has to be found, as was the case before (II p. 800). Here we only have to determine the right ratio of C_M between the series of different d, so that E becomes the same in all series for a given ε_M. If$^{(1)}$ and $^{(2)}$ indicate two different series which are to be compared at a constant ε_M it is valid: $\varepsilon_M = E^{(1)}/C_M^{(1)} \cdot d^{(1)} = E^{(2)}/C_M^{(2)} \cdot d^{(2)}$ *i. e.* $E^{(1)} = E^{(2)}$ if $C_M^{(1)} \cdot d^{(1)} = C_M^{(2} \cdot d^{(2)}$. That is, the C_M of the series ought to be inversely proportional to their d.

c. Selection of a wave-length suitable for measurement

To select a suitable λ, the extinction curves of the following solutions have been determined in the visible and UV regions down to $\lambda = 2500$ Å, also by the aid of the Beckman Spectrophotometer:

1. $C_M = 10$ mC, $C'_A = 30$ mC, by the buffers ½: 1, 2 : 1 and 5 : 1
2. $C_M = 10$ mC, $C'_A = 60$ mC, by the buffer 2 : 1.

The curves are found in Fig. 3, together with those of UO_2^{2+} and the solution $C_M = 33$ mC, p[H$^+$] = 4.1 (I p. 377). The extinction ε_A of the acetate buffers ½ : 1 and 5 : 1 have been measured, too, (*cf.* II p. 799), and are drawn in the same Fig.

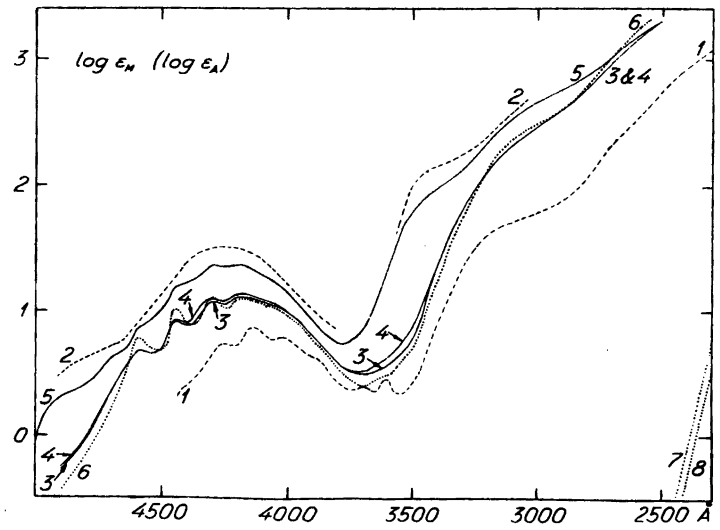

Fig. 3. Extinction curves of a) uranyl ion (dashed, 1), b) hydrolysed solution with C_M = 33 mC, p[H⁺] = 4.1 (dashed, 2), c) complex solutions with C_M = 10 mC, C'_A = 30 mC by the buffers 5 : 1, 2 : 1 and 1/2 : 1 (fulldrawn; 3,4 and 5 respectively), d) complex solution with C_M = 10 mC, C'_A = 60 mC by the buffer 2 : 1 (dotted, 6) and e) buffers 5 : 1 and 1/2 : 1 (dotted low to the right, 7 and 8 respectively).

As is seen from the curves of different buffers at the same C'_A = 30 mC, the influence of hydrolysis on ε_M is very pronounced at the long wave-lengths, but grows smaller at the short ones, to cease at $\lambda \approx 2600$ Å. Moreover, the curve of C'_A = 60 mC at the buffer 2 : 1 is situated below the one of C'_A = 30 mC at the same buffer at most long wave-lengths. This may be explained so that the third complex has an ε which is smaller than that of the second one, but it is more probable that it is due to the circumstance that the hydrolysis is more pronounced at the low C'_A. This is also suggested by the course of the curves at short λ: when the influence of hydrolysis vanishes, the ε_M:s of C'_A = 60 mC grow higher than those of C'_A = 30 mC. — The influence of ε_A can be neglected at all $\lambda > 2500$ Å.

In view of these facts, we select λ = 2600 Å for measurement. Here the influence of hydrolysis is small, and ε_M seems to show a steady increase with C_A, while the influence of ε_A has not yeat been perceptible.

It has also been established, that the ε_M:s are not changed during the measurements or by an exposition of the solutions to diffuse daylight lasting a whole hour (*cf.* II p. 804). This is in accordance with previous statements (*cf.* II p. 784).

Table 5. ε_M as a function of C_A at the different C_M and buffers used.

$C_M \rightarrow$ mC	7.5				2.5				0.75			
$d \rightarrow$ cm	0.1				0.3				1			
$\delta \rightarrow$	2 : 1 ○*		5 : 1 ●		2 : 1 ▽		5 : 1 ▼		2 : 1 ◇		5 : 1 ◆	
C'_A mC	ε_M**	C_A mC	ε_M	C_A mC	ε_M	C_A mC	ε_M	C_A mC	ε_M	C_A mC	ε_M	C_A mC
5					916	4.86	881	4.97	1 008	4.97	995	5.05
10	960	9.66	940	9.85	1 222	9.86	1 207	9.97	1 337	9.97	1 339	10.05
15	1 156	14.64	1 142	14.84	1 414	14.85	1 413	14.96	1 507	14.97	1 509	15.05
20	1 316	19.63	1 313	19.83	1 527	19.85	1 530	19.96	1 591	19.97	1 596	20.05
30	1 525	29.6	1 526	29.8	1 637	29.8	1 638	29.9	1 669	30.0	1 668	30.0
45	1 651	44.6	1 651	44.8	1 698	44.8	1 688	44.9	1 707	45.0	1 703	45.0

* These signs indicate the respective series in Fig. 4.

** The unit of ε_M is $C^{-1} \cdot cm^{-1}$.

Table 6. Determination of the non-hydrolytic functions $\varepsilon_M = f(C_A)$ by the extrapolation to $1/[H^+]^* = 0$.

	$C_M \rightarrow$ mC	7.5			2.5			0.75		
C_A mC	$\delta \rightarrow$	2 : 1	5 : 1		2 : 1	5 : 1		2 : 1	5 : 1	
5	$\varepsilon_M \rightarrow$				923	880	847	1 007	991	980
	$1/[H^+] \rightarrow$				11.9	5.2	0	16.7	6.9	0
10	$\varepsilon_M \rightarrow$	973	945	923	1 228	1 207	1 193			
	$1/[H^+] \rightarrow$	6.7	3.0	0	13.0	5.2	0			
15	$\varepsilon_M \rightarrow$	1 165	1 147	1 134	1 417	1 412	1 409			
	$1/[H^+] \rightarrow$	7.4	3.1	0	13.9	5.3	0			
20	$\varepsilon_M \rightarrow$	1 325	1 314	1 307						
	$1/[H^+] \rightarrow$	8.0	3.2	0						

* The unit of $1/[H^+]$ is mC^{-1}.

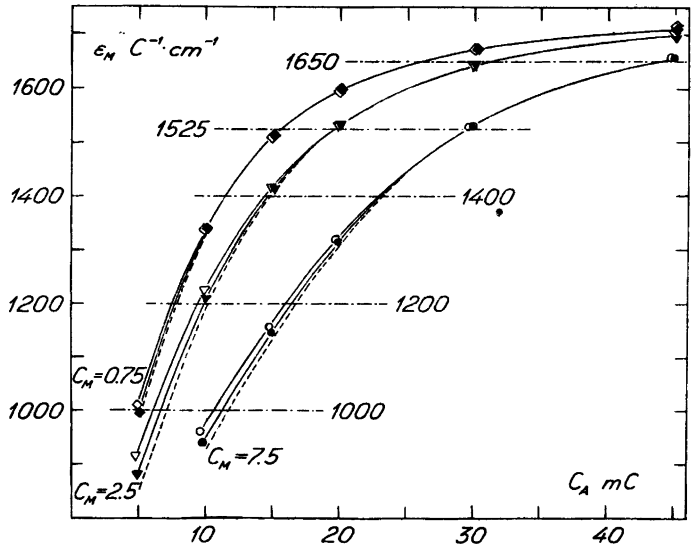

Fig. 4. ε_M as a function of C_A · Squares, triangles and circles relate to the measurements at C_M = 0.75, 2.5 and 7.5 mC respectively. Filled signs relate to the buffer 5 : 1 and open ones to the buffer 2 : 1. — Dashed curves represent the nonhydrolytic functions as obtained by extrapolation to $1/[H^+] = 0$. — The curves are cut at five ε_M, each of them representing a certain constant value of $[A]$ (and $\bar{n}$).

Table 7. C_A as a function of C_M at the five selected values of ε_M and the corresponding values of $[A]$, $\bar{n}$ and $\bar{n}/[A]$ hence obtained.

$C_M \rightarrow$ mC	7.5	2.5	0.75	0		
ε_M $C^{-1}\cdot cm^{-1}$	C_A mC			$C_A = [A]$ mC	$\bar{n}$	$\bar{n}/[A]$ C^{-1}
1 000	11.7	7.0	5.2	4.5	0.96	213
1 200	16.8	10.1	7.7	6.7	1.35	201
1 400	23.3	14.7	11.4	10.2	1.76	172
1 525	29.7	19.7	15.8	14.4	2.06	143
1 650	44.6	32.0	26.7	25.3	2.58	102

d. The measurements at $\lambda = 2600$ Å

Only the two buffers 5 : 1 and 2 : 1 have been used, on account of the strong influence of hydrolysis at the buffer ½: 1, demonstrated in Fig. 3. At 2600 Å, ε_M is high, so the low $C_M = 0.75$, 2.5 and 7.5 mC have to be chosen to fit $d = 1$, 0.3 and 0.1 cm respectively. For every C_M, C'_A has been varied between 5 and 45 mC. It is no use to increase C'_A any further, as the simultaneous increase of ε_M is very small. That is, at C'_A higher than those applied here, ε_M is almost a constant, independent of changes in the composition of the complex system.

The function searched for is $\varepsilon_M = f(C_A)$. In order to find C_A, (14) of II is applied as above, (p. 203) *i.e.* with the modification $C_s = 0$. Thus we have to know C_H^0 and $[H^+]$ of the solutions as correction terms (*cf.* II p. 802). They are determined by separate titrations performed quite analogous to the ones above.

In Table 5, the results are collected, and the $\varepsilon_M = f(C_A)$ found are shown as fulldrawn curves in Fig. 4. It is plain that the curves of both the buffers coincide in their upper parts, where the complex formation of the acetate predominates, but differ in their lower parts where the complex formation is still undeveloped, so allowing a measurable hydrolysis to exist. The $[H^+]$ of the solutions were, however, just measured above, thus we can perform the extrapolations to $1/[H^+] = 0$, which give the non-hydrolytic $\varepsilon_M = f(C_A)$ for every C_M (Table 6 and dashed curves of Fig. 4) *.

These functions are then cut at five constant ε_M, without being transformed before according to II (p. 802). On account of the rather strong complexity, they themselves allow quite well the graphical determination of the C_A:s belonging to a certain ε_M and so the transformation is unnecessary. By the cuts, C_A is found to be a linear function of C_M for every ε_M which indicates a mononuclear complex formation (*cf.* II p. 803). So it is allowed to calculate $\bar{n}$ and [A] from the functions. The figures are in Table 7, and the corresponding values of [A], $\bar{n}$ and $\bar{n}/[A]$ are also plotted in Figs 1 and 2.

It is at once observed, that it is necessary to correct for the hydrolysis in the extinction measurements also at such values of [A], where the emf measurements with the same buffers are quite independent of $[H^+]$. Thus it is possible to measure lower [A] by the potentiometric method than by the extinctiometric one.

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* As distinct from the way of proceeding in the emf measurement, the extropolation must be performed linearly here, as only two buffers are used.

CALCULATION OF THE COMPLEXITY CONSTANTS FROM THE CONCORDANT RESULTS OF THE TWO METHODS USED

According to both the methods applied, uranyl acetate is a mononuclear complex system. In solutions of not too low an [A], different for the different methods, the influence of hydrolysis is not larger than to permit the non-hydrolytic complex formation curve to be obtained without difficulty. As far as the measuring range can be covered by extinction measurements, identical curves are obtained with both methods. Above $\bar{n} = 2.6$, however, as well as below $\bar{n} = 0.9$, the formation curve is entirely founded on the emf measurements (*cf.* Table 7).

From the bend of the formation curve towards $\bar{n} = 3$, it is at once concluded that the saturated complex of the system is MA_3, in agreement with the views cited above (p. 200). So we have to calculate on three complexes. Their constants are obtained via the $X_n([A])$-functions, according to II (p. 785). The figures are in Table 8.

With the constants obtained, $\bar{n}$ is calcnlated ((2) of II) for some round [A] (Table 9). The values are introduced in Fig. 1 as a fulldrawn curve, which is seen to fit the experimental points very well. For the same [A], the composition of the system is also calculated ((8) of II) and given in Table 9.

Of these results, the statement of MA_3 as a saturated complex rests entirely on the part of the formation curve, which is determined only by emf measurements.

SUMMARY

Provided the complexity is sufficiently strong, complex solutions are not very much influenced by hydrolysis even at such pH:s where the central group is strongly hydrolysed. This opens a way to determine the complexity of such systems which cannot be measured at very low pH. It is, however, necessary to test the applicability of the method in every case by measuring at various pH. This proceeding also enables an extrapolation to strongly acid solution in the case of a slight influence of pH.

The method is applied to the system of uranyl acetate, where it functions well. Both emf and extinction measurements are performed, with consistent results. Three complexes are proved, *viz.* MA_1, MA_2 and MA_3, the last one being the saturated complex of the system. Their constants are, at the existing conditions:

$$\beta_1 = 240\ C^{-1} \qquad \beta_2 = 2.3 \cdot 10^4\ C^{-2} \qquad \beta_3 = 2.2 \cdot 10^6\ C^{-3}$$

The statement that MA_3 is a saturated complex is, however, founded merely on the emf measurements.

Table 8. $X([A])$, $X_1([A])$, $X_2([A])$ and $X_3([A])$ for given $[A]$, as obtained by graphical integration of the $\bar{n}/[A]$-function of Fig. 2.

[A] mC	(5a) of II ln $X([A])$	$X([A])$	(7a) of II $X_1([A])$ C^{-1}	(7b) of II $X_2([A]) \cdot 10^{-4}$ C^{-2}	$X_3([A]) \cdot 10^{-6}$ C^{-3}
0			*241*	*2.30*	*2.2*
2	0.4660	1.594	297		
4	0.9070	2.477	370	3.23	
6	1.323	3.76	460	3.65	2.25
8	1.712	5.53	566	4.07	2.21
10	2.075	7.96	696	4.55	2.25
15	2.868	17.62	1 107	5.78	2.32
20	3.520	33.7	1 635	6.97	2.34
30	4.526	92.3	3 040	9.33	2.34
40	5.278	195.9	4 870	11.54	2.31
50	5.875	357	7 120	13.77	2.29
75	6.987	1 084	14 450	18.95	2.22
100	7.792	2 420	24 190	23.95	2.17
150	8.957	7 760	51 800	34.4	2.14
200	9.781	17 760	88 300	44.0	2.08
250	10.435	34 000	136 000	54.4	2.08
300	10.966	57 800	192 700	64.2	2.07

Table 9. The ligand number and the composition of the system as calculated for some round $[A]$ with the constants obtained.

$\beta_1 = 240 \pm 10\ C^{-1}$ $\beta_2 = (2.3 \pm 0.2) \cdot 10^4\ C^{-2}$ $\beta_3 = (2.2 \pm 0.3) \cdot 10^6\ C^{-3}$

[A] mC	(2) of II $\bar{n}$	(8a) of II a_0 %	(8b) of II a_1 %	(8b) of II a_2 %	(8b) of II a_3 %
1	0.232	79	19	2	0
4	0.86	40.5	39	15	5.5
10	1.73	12.5	30	29.5	28
30	2.58	1	8	23.5	67.5
50	2.75	0.5	3.5	16.5	79.5
100	2.89	0	1	9	90
200	2.94	0	0.5	5	94.5
300	2.97	0	0	3.5	96.5

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On the Formation of Bound Hydroxylamine in *Azotobacter*

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The formation of hydroxylamine as an intermediate in *Azotobacter* cultures is very plausible both when molecular nitrogen and nitrate nitrogen serve as the source of nitrogen. A compound is then regularly formed in which an NOH-group is linked with a carbon atom. By hydrolyzing such compounds with sulphuric acid and by oxidizing then with iodine, nitrite is produced[1,2]. Consequently, the intermediate would contain either oxime, $>C=NOH$-group, or hydroxamic acid, $-C\overset{O}{\underset{NHOH}{<}}$-group. We have regarded the former as likely but the latter, too, must undoubtedly be considered, especially since Speck[3] and Elliot[4] discovered that an enzyme system acting in the synthesis of glutamine from glutamic acid and ammonia in animal tissues also causes formation of hydroxamic acid from glutamic acid and hydroxylamine. Recently Waelsch *et al.*[5] and Grossowicz *et al.*[6] have noted in bacteria and Stumpf and Loomis[7] in plants another interesting enzymatic reaction which also leads to hydroxamic acid:

$$\text{glutamine} + NH_2OH \rightarrow \text{glutamohydroxamic acid} + NH_3$$

In addition to this system hydroxylamine also reacts with asparagine forming the corresponding hydroxamic acid.

The present investigation which was carried out more than a year ago (preliminary report[8]) does not deal more closely with the question whether the hydroxylamine, which is set free in the hydrolysis of Azotobacter, originates from oxime or hydroxamic acid or from both of them. This question is now under investigation in this laboratory. From the point of view of nitrogen cycle it is most important that the formation of either compound is probably preceded by formation of hydroxylamine which thus would be a common intermediate both in nitrate reduction and nitrogen fixation[9].

Since, however, bound hydroxylamine is also formed when ammonium salts serve as the source of nitrogen for *Azotobacter* it is not impossible that hydroxylamine may be produced by oxidation from ammonia, and hence, its formation in the nitrogen fixation and nitrate reduction should not be a proof of its intermediary nature.

In order to obtain elucidation to this important theoretical question we examined the formation velocity of bound hydroxylamine in *Azotobacter* cultures by using in parallel experiments N_2, NO_3^- and NH_4^+ for the source of nitrogen. In order to speed up assimilation of nitrogen and thus to enable analytical determination of oxime nitrogen in experiments of short duration we used so heavy inoculation of *Azotobacter* in most experiments that nitrogen fixation as well as assimilation of other nitrogen sources was analytically detectable within 1—2 hours. In the following some characteristic experiments are recorded both from experiments of long duration (normal inoculation) and experiments of short duration (heavy inoculation).

EXPERIMENTAL

Azotobacter vinelandii (strain Lipmann, original culture obtained from Prof. A. J. Kluyver in Delft some years ago) was cultivated in a nutrient solution containing K_2HPO_4 1.6 g, KH_2PO_4 0.4 g, $MgSO_4$ 0.2 g, NaCl 0.2 g, $CaSO_4$ 0.1 g, $Fe_2(SO_4)_3$ 0.01 g, $FeSO_4$ 0.015 g, glucose 5 g, saccharose 20 g, Na_2MoO_4 0.003 g, tap water 1 000 g. pH of the nutrient solution was 7.2.

Nitrogen liberated in the hydrolysis as hydroxylamine (*bound hydroxylamine*) was determined according to the principle of Endres by hydrolyzing for 6 h with 3 *N* sulphuric acid, by oxidizing the hydroxylamine set free with iodine to nitrite and by determining nitrite (Blom [1]). In details the procedure of Csáky [10] was followed.

Total N was determined by the micro method of Miller [11]. The number of bacteria was counted by the Skar ocular (Virtanen [12]).

Experiments of long duration by using small inoculation

In the first experiments bound hydroxylamine-N was determined when the above mentioned nutrient solution either without combined nitrogen or with NH_4^+ or NO_3^- was inoculated with a small amount of Azotobacter culture. 5 ml of 2 days old bacterial culture without combined nitogen was inoculated into 200 ml of nutrient solution in 1 litre flat flasks, one without combined nitrogen, one containing 40 mg % ammonium nitrogen and one 40 mg % nitrate nitrogen. Temperature of growth was 30° C. Bound hydroxylamine was determined at the start of the experiment and, after the growth had started, at different intervals. Before the samples were taken the flasks were given a good shake. Samples of 1 ml were taken with sterile pipets from the growth solution and placed immediately into test tubes containing 0.5 ml 12 *N* H_2SO_4 and 0.5 ml sulphanilic acid (1 g sulphanilic acid in 100 ml of 30 % acetic acid). The tubes were kept for 6 h in a boiling water bath. Table 1 gives the data of two experiments performed according to this method.

Table 1.

Nitrogen nutrition	Period of growth, h	pH	Bound hydroxylamine (extinction)	Nitrogen nutrition	Period of growth, h	pH	Bound hydroxylamine (extinction)
	Experiment 1				Experiment 2		
N_2	0	7.2	0	N_2	0	7.2	0
	30	7.0	0		20	6.9	18
	42	6.5	10		40	6.4	26
40 mg % NH_4^+	0	7.2	0	40 mg % NH_4^+	0	7.2	0
	30	7.1	0		20	6.6	5
	42	6.2	5		40	6.4	26
40 mg % NO_3^-	0	7.2	0	40 mg % NO_3^-	0	7.2	0
	30	6.8	2		20	6.7	8
	42	6.4	63		40	6.5	49

Controls subtracted

In general, determination of hydroxylamine in the bacterial cultures was uncertain because the sugar-containing solution turns brownish during the hydrolysis.

The results show, however, that while the Azotobacter grows in the nutrient solution with a small inoculation when many hours are required before the cells have multiplied sufficiently to enable detection of nitrogen fixation and assimilation by ordinary methods of analysis, no clear difference due to the nature of nitrogen nutrition can be distinguished in the formation velocity of bound hydroxylamine. Yet after 40 h the amount of bound hydroxylamine was much greater in the cultures on nitrate nitrogen than in those on molecular or ammonium nitrogen.

Experiments of short duration by using heavy inoculation

Azotobacter was cultivated in 200 ml of nitrogen-free nutrient solution in 1 litre flat flasks. The flasks were placed on their broad sides in a cupboard the temperature of which was maintained by means of an electric heater at about 30° C during the day but fell during the night (in about 14 h) to about 18° C. The period of growth was 48 h. Bacteria were separated by centrifugation in the morning when after the low night temperature the amount of bound hydroxylamine in the cells was only about one third of that contained in the bacteria grown at an even temperature of 30° C. The bacteria isolated from 200 ml of culture were suspended in 600 ml of nitrogen-free nutrient solution, the suspension was shaken to as homogeneous a mass as possible and divided in 3 portions, 200 ml each, in 1 litre flat flasks. The flasks contained:

1. No combined nitrogen
2. $(NH_4)_2HPO_4$
3. KNO_3

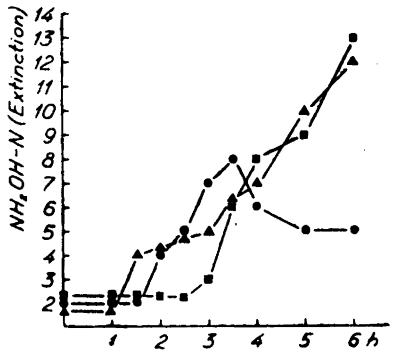

Fig. 1. Formation of bound hydroxylamine in parallel experiments with molecular nitrogen, nitrate nitrogen (40 mg %) and ammonium nitrogen (40 mg %).

●—● N_2
■—■ NH_4^+
▲—▲ NO_3^-

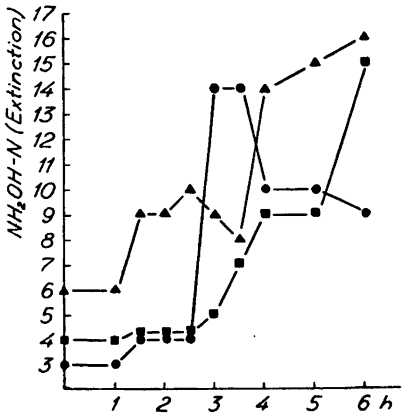

Fig. 2. Formation of bound hydroxylamine in parallel experiments with molecular nitrogen, nitrate nitrogen (40 mg %) and ammonium nitrogen (40 mg %).

●—● N_2
■—■ NH_4^+
▲—▲ NO_3^-

Total N of the bacterial mass after extraction with trichloracetic acid was as follows:

N-nutrition	N_2	NH_4	NO_3
Time min.	*Total N mg/ml*	*Total N mg/ml*	*Total N mg/ml*
0	*0.058*	*0.054*	*0.058*
60	*0.059*	*0.058*	
120		*0.067*	
180	*0.068*	*0.069*	*0.068*
210	*0.070*	*0.070*	
360	*0.070*	*0.070*	*0.069*

Temperature was 30° C. pH was in the beginning of each experiment 6.8. It fell during the experiment on N_2 and NH_4^+ nutrition to pH 6.6—6.5, on NO_3^- to 6.7. The amount of nitrogen added to different flasks are mentioned below in connection with the results.

From each flask a sample was taken with sterile pipet in the beginning of the experiment for determination of pH, hydroxylamine and total N. In the course of the experiment samples were taken in the same way at intervals of 30 mins. Total N of the bacterial mass was determined after extraction with trichloracetic acid (see below). A

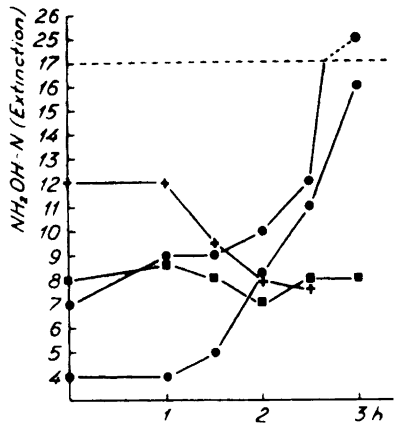

Fig. 3. Formation of bound hydroxylamine in parallel experiments with molecular nitrogen and ammonium nitrogen (2 mg % and 4 mg %).

●—● N_2
■—■ NH_4—N (2 mg)
+—+ NH_4—N (4 mg)

number of low-molecular substances had by then dissolved from the cells but the results are, however, comparable with each other to a great extent.

For determination of bound hydroxylamine, 5 ml of the sample were put in a centrifuge tube kept on ice. After centrifugation for 9 min the clear solution was poured away (in some experiments 1 ml of it was used for determination of bound hydroxylamine in the solution), 5 ml of ice-cold distilled water were added, shaken with the bacterial mass and re-centrifuged for 9 min. The washing water was poured off and replaced with 5 ml of 8 % trichloracetic acid. The bacterial mass was mixed well with the solution and the suspension was kept for 24 h in the ice-box. The procedure from taking the sample up to placing the bacterial mass with trichloracetic acid solution in the ice-box was carried out in 20—22 min. After 24 h, the suspension with trichloracetic acid was centrifuged for 15 min, a 1 ml sample was taken from the clear solution and bound hydroxylamine was determined in it in the manner described above.

Results of four experiments have been illustrated by curves in Figs. 1, 2, 3 and in Table 2.

Determination of bound hydroxylamine in nutrient solutions from which bacteria were separated by centrifugation, gave high extinction values right from the start of the experiment. The cause of this may be the heating of the sugar-containing solution at a strongly acid reaction as mentioned above. Determination of hydroxylamine is then uncertain. However, the values of hydroxylamine seem to rise in the nutrient solution somewhat parallel with the increase of the cell mass.

DISCUSSION

The results recorded above show that in the experiments of short duration, in which great amounts of bacterial mass were used for inoculation and therefore fixation of nitrogen and assimilation of ammonium nitrogen could be noted within 1—1 ½ h, bound hydroxylamine was formed more rapidly in the bacteria on nitrate and molecular nitrogen than on ammonium nitrogen. In the latter case bound hydroxylamine could not be noted until after 3 h, whereas

Table 2.

No	Nitrogen nutrition	Duration of growth min	pH	Ammonia N mg/ml	Bound hydroxylamine (extinction) in separated bacterial mass
1	N_2	0	6.8		0 (7)
		60			2 (9)
		90			2 (9)
		120			3 (10)
		150			5 (12)
		180	6.5		18 (25)
2	N_2	0	6.8		0 (4)
		60			0 (4)
		90			1 (5)
		120			4 (8)
		150			7 (11)
		180	6.5		12 (16)
3	NH_4^+—N, 2 mg %	0	6.8	0.019	0 (8)
		60		0.011	1 (9)
		90			0 (8)
		120		0.003	0 (7)
		150			0 (8)
		180	6.5		0 (8)
4	NH_4^+—N, 4 mg %	0	6.8		—
		60			0 (9)
		90			0 (8)
		120			0 (7)
		150	6.5		0 (8)

The numbers in parentheses indicate the readings.

in the former case it was regularly found after 1 ½—2 h. There is naturally the possibility that the ammonium content, which is always much higher in experiments with ammonium sulphate than when ammonium is possibly formed as an intermediate at the N_2-fixation, should have a retarding effect on the formation of bound hydroxylamine. However, in our experiments, in which the concentration of ammonium nitrogen varied from 2 to 40 mg % no formation of bound hydroxylamine could be noted even at the lowest concentration after 3 h. At that time ammonium nitrogen was completely used up. Our experimental material therefore speaks for the concept that in nitrogen fixation and nitrate reduction hydroxylamine is formed before the complete

reduction of nitrogen to ammonia. Accordingly, the results favour the intermediary nature of hydroxylamine.

On the other hand, it appears from the results that ammonium or amino nitrogen is oxidized to some extent. Otherwise it is not possible to explain the formation of bound hydroxylamine after 3 h in nutrient solution with 40 mg % ammonium nitrogen since after 6 h about 90 % of ammonium nitrogen were still left in the solution. Since the increase of bound hydroxylamine does not begin until the assimilation of nitrogen has well started it is possible that either amide or amino group is oxidized. However, no definite conclusions can be drawn from this.

SUMMARY

In cultures of *Azotobacter vinelandii* in which heavy inoculation was used and accordingly, nitrogen fixation and assimilation of nitrogen compounds were detectable by ordinary chemical analyses within 1—2 h, bound hydroxylamine could be found in 1 ½—2 h when molecular nitrogen and nitrate nitrogen served as the source of nitrogen. When ammonium nitrogen provided the nitrogen nutrition, bound hydroxylamine was not found during the first 3 h, later it appeared in increasing amounts.

The results are considered to support the concept that hydroxylamine is formed in nitrate reduction and in nitrogen fixation as an intermediate before ammonia. On the other hand, oxidation of ammonia to hydroxylamine also appears from the results obtained.

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Die Hydrolyse von Triarylmethylsalze

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Die Triphenylmethanderivate treten oft in zwei Formen auf: eine farblose und eine farbige Reihe, die, nach den spektralen Beobachtungen zu urteilen, diskontinuierlich im einander übergehen [1]. Die gefärbten Verbindungen, die auch in manchen »indifferenten» Lösungsmitteln Elektrolyte sind, z. B. Triphenylmethylperchlorat, werden nach Hantzsch [1] als Carboniumsalze bezeichnet. Die farblosen Formen sind als echte Methanderivate, homöopolare Verbindungen, aufzufassen, z. B. Triphenylcarbinol, Triphenylmethylcyanid usw.

Die beiden Formen können angeblich mitunter in Lösungsgleichgewicht miteinander stehen, das durch „indifferente" Zusätze, durch Temperaturveränderung oder durch Lichtstrahlung verschoben werden kann. So werden die gelben Lösungen von Tritylchlorid durch Äthyläther gebleicht oder entfärbt, durch Amyläther violett gefärbt [1]. Tritylbromid wird bei Temperatursteigerung in $C_2H_2Cl_2$ dunkler, in $C_2H_2Cl_4$ heller gefärbt. Krystallviolettcyanidlösung wird im Licht intensiv violett [2].

Gegen diese angenommenen Gleichgewichtsänderungen kann man anführen: Farbänderungen mit der Temperatur und den Lösungsmitteln sind bei vielen anderen Stoffen beobachtet worden, ohne dass man Veranlassung gehabt hat, stoffliche Gleichgewichtsänderungen anzunehmen. So sind z. B. die Azokörper für Lösungsmitteländerung sehr empfindlich.

Die Angabe [3], dass Triphenylmethylchlorid in Azetylentetrachlorid beim Erhitzen reversibel gelb gefärbt werde, haben wir nicht bestätigen können. Das gilt zwar für HCl-saure unreine Lösungsmittel. Wird die Flüssigkeit aber mit K_2CO_3 getrocknet und destilliert, so wird die Tritylchloridlösung in der Hitze nicht merklich dunkler, sondern im Gegenteil vielleicht ein wenig heller.

Ob die Farbänderungen, die im Licht eintreten, wirklich immer reversibel sind, dürfte fraglich sein. Vorläufige Versuche mit alkoholischer Krystallviolettcyanidlösung haben nach Bestrahlung keine Reversibilität gezeigt.

Eine durch Überschuss von Alkali fast ausgebleichte Lösung von Phenolphtalein wird, wie wir kontrolliert haben, in Quecksilberlicht reversibel rot. Die Stoffe, die hier in Lichtgleichgewicht miteinander stehen, sind Phenolphtalein und sein Hydrolysenprodukt Dioxytriphenylcarbinolcarbonsäure.

Die heteropolaren Formen entstehen nach Lifschitz[4] aus den homöopolaren durch Einlagerung von Lösungsmittel oder anderen Molekülen. Die Verbindungsreihen sind nach ihm optisch verschieden, obgleich gewöhnlich nur im Ultraviolett. Ist der Ligand stark polarisierbar oder besteht er aus einem zweiten Molekül des Elektrolyten (Autokomplexbildung), so ist die Farbe besonders ausgesprochen. Die Molgewichtsbestimmungen[5] von Tritylderivaten in verschiedenen Lösungsmitteln zeigen z. B., dass die stark gefärbte Lösung von Trianisylmethylperchlorat in $CHCl_3$ assoziiert ist. Dieselbe Verbindung ist aber in Azeton und Nitrobenzol, subjektiv geschätzt, ebenso stark gefärbt, die ebullioskopischen Versuche zeigen aber Dissoziation an. Irgend eine Stütze für seine Postulate kann aus den Versuchen von Lifschitz wahrscheinlich nicht erhalten werden. In einem Lösungsmittel von niedriger Dielektrizitätskonstante ist bei einem Salz immer eine Assoziation zu erwarten.

Das Triphenylmethylrhodanid zeigt nach Lifschitz und Girbes bei der Leitfähigkeitsprüfung in Alkohol ein Zeitphänomen. Die Leitfähigkeit ist momentan wahrscheinlich gleich null und erreicht in 24 Stunden einen Endwert, der nur etwa 17 % von dem des »Normalelektrolyten», Tetraäthylammoniumjodid, beträgt. Das Di-*p*-anisylphenylmethylrhodanid zeigt keinen merklichen Zeiteffekt. Lifschitz und Girbes deuten das Zeitphänomen als eine langsame Einlagerung des Lösungsmittels. Nun haben Hantzsch und Burawoy[6] gefunden, dass die Methylalkohollösung von Tritylrhodanid weniger hydrolysiert ist als die des Chlorids, Bromids und Perchlorats. Die drei letzteren Lösungen gaben etwa dieselben Werte. Der Befund wird dadurch erklärt, dass die Rhodanwasserstoffsäure in dem Alkohol eine schwache Säure wäre. Das ist eine ad hoc aufgestellte und wenig wahrscheinliche Hypothese.

Die Angaben von Lifschitz,[5] dass Tritylrhodanid aus siedendem Alkohol unverändert krystallisierbar sei, ist nach unseren Beobachtungen nicht richtig. Der Schmelzpunkt wird immer niedriger, es tritt eine langsame Zersetzung ein. Die ebullioskopischen Molgewichtsbestimmungen in Alkohol sind also wertlos.

Die Zersetzung in Alkohol ist sicher nur eine partielle Alkoholyse. Sie lässt sich leicht qualitativ demonstrieren. Eine frische Lösung von Tritylrhodanid, mit einer Spur Eisenchlorid versetzt, ist farblos, wird aber bald unter Bildung von Eisenrhodanid rot. Wie wir gefunden haben, lässt sich Tritylrhodanid ganz einfach durch Umsetzung von Tritol mit Kaliumrhodanid in

Eisessig herstellen. Die Reaktion $R_3C \cdot OH + HSCN \rightleftharpoons R_3C \cdot SCN + H_2O$ ist also reversibel.

Das Tritylrhodanid ist in festem Zustande, und in allen untersuchten indifferenten Lösungsmitteln farblos, und die Lösungen zeigen keine Leitfähigkeit. Es ist demnach als eine homöopolare Verbindung zu formulieren. Die Hydrolyse (Alkoholyse) kann auf zwei verschiedenen Wegen verlaufen: 1. Eine direkte Verseifung unter Wasseraddition und Spaltung in Analogie mit der Verseifung der Carbonsäureestern (deren Mechanismus noch unsicher ist), 2. Eine primäre Bildung von echtem Salz

$$2\,a: R_3C \cdot SCN \rightarrow [R_3C]^+ SCN',$$

gefolgt von einer Reaktion zwischen Carboniumjon und Wasser (Alkohol)

$$2\,b: [R_3C]^+ + H_2O \rightarrow R_3C \cdot OH + H^+$$

Da die Reaktion farblos erfolgt, muss die Konzentration der Carboniumjonen jedenfalls sehr niedrig sein.

SAURE HYDROLYSE VON TRIANISYLMETHYLPERCHLORAT

Nach Baeyer[7] u. a. werden die Triarylmethylcarboniumsalze, z. B. Trihalogentritylsulfate und Trianisylmetylsulfat, durch wässrigen Alkohol sofort entfärbt.

Giesst man eine Azetonlösung von Trianisylmethylperchlorat (die Lösung ist stark bichromatrot gefärbt) in verdünnten Alkohol oder Azeton, so wird sie ebenfalls momentan entfärbt. Enthält aber das Verdünnungsmittel im voraus etwas Natriumhydroxyd oder einen zweckmässigen Acetatpuffer, so ist die Entfärbung, wie wir beobachtet haben, nicht mehr momentan und kann reaktionskinetisch als Funktion der Wasserstoffjonenkonzentration verfolgt werden.

Die ersten Versuche wurden folgendermassen gemacht. Eine Reihe kleiner Glaskolben wurden mit je 15 ml K-acetat-Essigsäurepuffer oder NaOH-Lösungen, die aus 50 %igem Alkohol (resp. 50 %igem Azeton) bereitet waren, beschickt. Jeder Kolben wurde dann mit 0,5 ml 0,046 C Azetonlösung von Trianisylmethylperchlorat versetzt und die Entfärbungszeit gemessen. Da dieser Zeitpunkt ziemlich subjektiv ist, war es besser, die Entfärbung bis zu einer bestimmten schwachen Vergleichsfarbe zu verfolgen. Im diffusen Sonnenlicht bei konstanter Zimmertemperatur gelang es auf diese primitive Weise verwendbare Resultate zu bekommen (Tabelle 1). Für eine pseudo-

monomolekulare Reaktion ist die auf diese Weise bestimmte Entfärbungszeit (t) der Geschwindigkeitskonstanten umgekehrt proportional.

Die Versuche zeigen, dass der Ausdruck

$$\frac{t\ [\mathrm{HAc}]}{[\mathrm{KAc}]} \text{ konstant ist (in der Tabelle} = K_0).$$

Das bedeutet, dass in »sauren» Pufferlösungen die Hydrolysengeschwindigkeit des Perchlorats proportional der Wasserstoffjonenkonzentration ist, aber fast unabhängig von der absoluten Konzentration des Salzes (also von der Jonenstärke) verläuft. Letzteres scheint nur für Acetatgemische, nicht für Neutralsalze, siehe unten, zu gelten.

Tabelle 1. Entfärbungszeit von Trianisylmethylperchlorat in Acetatpuffer.

I. 50 % Alkohol, II. 50 % Azeton.
KAc-Lösung 0,25 C, HAc-Lösung 1,0 C. Zu jeder Probe 0,5 ml 0,046 C Trianisylmethylperchloratlösung in Azeton.
Gesamtvolymen 15,5 ml.

I				II			
ml KAc	ml HAc	Zeit Min	K_0	ml KAc	ml HAc	Zeit Min	K_0
0,5	4	0,42	13	1	1	4,66	19
1	4	0,83	13	2	1	10	20
1	2	1,18	10	3	1	14,5	19
1,3	1	3,84	12	4	1	19	19
1,6	1	4,66	12	5	1	25	20
2,0	1	5,5	11	10	1	50	20
3	1	8,0	11	1	2	2,5	20
3,5	1	9,5	11	1	3	1,66	20
4,1	1	13	13	1	5	0,92	18
5,0	1	15	12	0,5	5	0,42	17
10	1	24	10			K_0 im Mittel	19,2
10	0,5	50	10				
		K_0 im Mittel	11,6				

Die kinetischen Versuche in alkalischen Lösungen können nur ein enges $[OH^-]$-Gebiet umfassen. Bei grösserem $[OH^-]$ verläuft die Reaktion zu schnell, bei zu geringer Alkalität treten Störungen auf.

Die Versuche in Azeton sind in Fig. 1 wiedergegeben, wo das pH so berechnet ist, als ob das Lösungsmittel reines Wasser wäre ($pK_s = 5$). Wie aus

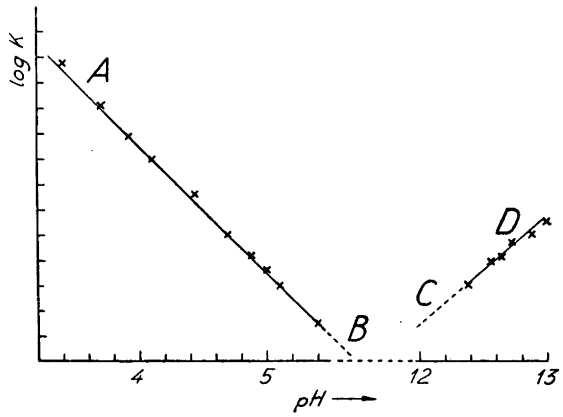

Fig. 1. Verseifungsgeschwindigkeit von Trianisylmethylperchlorat in 50 %igem Azeton. pH-Skala relativ.

der Figur deutlich hervorgeht, ist log K (= log $1/t$) eine geradlinige Funktion des relativen pH der Lösungen. Da die R. G. mit abnehmendem [OH^-] immer kleiner wird (D—C), aber in reinem verdünnten Lösungsmittel unmessbar gross ist, muss in diesem Falle ein Katalysator vorhanden sein, der von OH-Jonen vernichtet wird. Das kann nichts anderes als die durch die Hydrolyse freigemachte Überchlorsäure sein, da wir gesehen haben, dass in saurem Gebiet (A—B) die R. G. von H-Jonen katalysiert wird.

Die Hydrolyse des Trianisylmethylperchlorats durchläuft also eine *katalytische Kettenkurve*, etwa wie man es bei der Hydrolyse von Estern, Säureamiden, Lactonen gefunden hat. Wir haben also bei einem Tritylderivat einen Reaktionsverlauf beobachtet, der auch bei einfachen aliphatischen Verbindungen gefunden worden ist. Diese formale Ähnlichkeit dürfte ein Ausgangspunkt für ein besseres Verständnis der Konstitution der Tritylverbindungen bilden können.

Die Reaktion ist wahrscheinlich eine Hydrolyse unter Bildung von Trianisylcarbinol (Formel oben 2 b). Da das Carbinol sehr schlecht krystallisiert, konnte das Reaktionsprodukt nicht isoliert werden. Nun ist bekannt, dass viele Carbinole oder Carbinolsalze mit Alkohol leicht unter Ätherbildung reagieren. Eine solche Reaktion ist in verdünntem Spiritus nicht sehr wahrscheinlich, um aber diese denkbare Komplikation zu vermeiden, sind die meisten Versuche in wässrigem Azeton gemacht.

Die genaueren quantitativen Versuche wurden mit einem Beckmannschen Spektralphotometer gemacht. Die Lampe war in etwa 0,5 Meter Entfernung aufgestellt, und das Licht mittels einer grossen Quarzlinse in den Apparat

geworfen. Das wassertemperierte Küvettenhaus und das ebenfalls thermostatregulierte Zimmer gewährten eine genügend konstante Temperatur in den Küvetten. Die Extinktion wurde bei 4000 Å gemessen. Die Orginalküvetten von Beckman sind für flüchtige Lösungsmittel nicht zu empfehlen; die Küvettenführung ist ausserdem etwas primitiv. Es wurden 2 geschlossene Küvetten benutzt, die auf einem besonderen Schlitten mit Schwalbenschwanzführung montiert waren.

In Tabelle 2 ist als Beispiel ein beliebiger Versuch angeführt. E ist die bei der Zeit t gemessene Extinktion (Schichtdicke 3 cm), E_∞ die wahrscheinlich von Verunreinigungen herrührende, nach etwa 24 Stunden bleibende Extinktion. Da die Anfangsextinktion E_0 nicht genau gemessen werden kann, wird die Geschwindigkeitskonstante K für jede Einzelmessung nach der Formel

$$K = \frac{1}{\Delta t} ln \frac{E_, - E_\infty}{E_{,,} - E_\infty}$$

berechnet oder auch graphisch ermittelt.

Mit derselben Methode wurden mehrere Versuche mit verschiedenen Pufferlösungen gemacht, die in Tabelle 3 kurz zusammengestellt sind. Der Acetatgehalt ist in allen Versuchen konstant. Die H-Jonenkonzentration ist durch den Gehalt an Essigsäure C_{HAc} variiert worden. Es zeigt sich, dass das

Tabelle 2. Hydrolysgeschwindigkeit von Trianisylmethylperchlorat (T_p) in 50 % Azeton mit Acetatpuffer.

$C_{KAc} = 0{,}294$; $C_{HAc} = 0{,}0237$; $C_{T_p} = 0{,}00136$. (k mit ^{10}log ber.)
K (log nat.) im Mittel rechnerisch 0,0115, graphisch 0,0114.

Zeit Min	E	$2+\log(E-E_\infty)$	$k \cdot 10^4$	Zeit Min	E	$2+\log(E-E_\infty)$	$k \cdot 10^4$
4	0,630	1,788		50	0,377	1,558	51
5	0,620	1,781		60	0,337	1,507	49
6	0,613	1,776	52	70	0,303	1,458	50
7,5	0,603	1,769		80	0,272	1,408	50
9	0,594	1,762		90	0,244	1,358	46
10,5	0,585	1,755	50	100	0,221	1,312	47
12	0,574	1,747		110	0,200	1,265	50
15	0,557	1,733	49	120	0,180	1,215	50
18	0,538	1,718	53	140	0,146	1,114	52
24	0,501	1,686	52	160	0,118	1,009	48
30	0,468	1,655	47	180	0,098	0,914	
40	0,421	1,608	50	∞	0,016	—	

Tabelle 3. Hydrolysegeschwindigkeit von Trianisylmethylperchlorat in 50 % Azeton mit Acetatpuffer.
E_0-E_∞ wahrsch. im Mittel 0,64. ε_0 (Mol. Extinktionskoeff.) 157 bei 4000 Å.
C_{KAc} = 0,294 C.

Nr	K	C_{HAc}	K/C_{HAc}
1 a	0,215	0,470	0,458
1 b	0,220	0,470	0,469
2 a	0,108	0,237	0,456
2 b	0,112	0,237	0,472
3 a	0,0557	0,118	0,471
4 a	0,0228	0,0474	0,481
4 b	0,0220	0,0474	0,464
5 a	0,0114	0,0237	0,481
6 a	0,0057	0,0118	0,482
6 b	0,0057	0,0118	0,482
		im Mittel	0,473

Verhältnis K/C_{HAc} konstant ist, d. h. die Geschwindigkeit ist (in dem untersuchten Gebiet) der H-Jonenkonz. genau proportional, wie Tabelle 1 auch belehrte.

Sämtliche Versuche verlaufen wie der Versuch in Tabelle 2 streng nach einem pseudomonomolekularen Schema. Da dieser Reaktionsverlauf in der verdünnten Lösung a priori anzunehmen ist, kann man umgekehrt aus den gut übereinstimmenden Reaktionskonstanten schliessen, dass für die Lösung das Beersche Gesetz gilt. Hantzsch [1] hat früher diese Gültigkeit auf Grund von sehr primitiven Versuchen behauptet.

Vorläufige Versuche in absolutem Alkohol über die Umsetzung zwischen Trianisylmethylperchlorat und Na-acetat zeigen, dass die Reaktion hier ebenfalls durch HAc katalysiert wird. Das Endprodukt ist jedoch hier unzweifelhaft der Carbinoläther.

HYDROLYSE IN ALKALISCHER LÖSUNG

Diese Versuche können nur ein enges Konzentrationsgebiet umfassen. Ein einwandfreies Puffergemisch haben wir nicht gefunden. Das Lösungsmittel ist wie früher wässriges Azeton.

Die vorläufigen, subjektiven Resultate sind in Fig. 1 Kurve C—D eingetragen und die lichtelektrischen Messungen in Tabelle 4 wiedergegeben. Sämtliche Lösungen sind mit CO_2-freiem NaOH und ausgekochtem Azeton bereitet

Tabelle 4. Alkalische Hydrolyse von Trianisylmethylperchlorat in 50 %igem Azeton. $C_{KAc} = 0{,}294$ *C.*

Nr	C_{NaOH}	K	$K/[OH^-]$
1	0,0758	0,208	2,75
2	0,0379	0,104	2,75
3	0,0190	0,0495	2,61
4	0,00992	0,0210	2,12
5	0,00496	0,00547	1,1
		Wahrsch. Wert	2,7

und enthalten alle 0,294 C Kaliumacetat, um die Jonenstärke einigermassen konstant zu halten. Die Gegenwart von Acetatjonen dürfte keine prinzipielle Änderung im Reaktionsmechanismus verursachen, da die vorläufigen Versuche ohne Acetat ungefähr dasselbe Bild zeigen.

Die Reaktion $[R_3C]^+ + OH' = R_3C \cdot OH$ ist bimolekular. Wenn $[OH^-]$ genügend gross ist, verläuft die Reaktion pseudomonomolekular. In Versuch 1 und 2 ist $K/[OH^-]$ konstant. Obwohl sämtliche Versuche graphisch monomolekular verlaufen, ist das Verhältnis $K/[OH^-]$ für kleineres $[OH^-]$ nicht mehr konstant. Dieses Verhalten ist vielleicht auf mangelnde Pufferkapazität zurückzuführen.

GESCHWINDIGKEIT IN FORMIATPUFFER

Die saure Hydrolyse des Perchlorats lässt sich nur in Puffermischungen genau verfolgen. Es ist dabei an sekundäre Reaktionen zu denken. 1. Das Endprodukt kann (in dem Acetatpuffer) Trianisylcarbinolacetat sein. Präparativ konnte die Sache nicht geprüft werden, da das Produkt nicht krystallisierbar war. Die Bildung des Acetylderivats in der wasserhaltigen Lösung ist aber unwahrscheinlich, da bekanntlich das Tritolacetat sehr leicht verseift wird. Das Perchlorat löst sich auch in Gegenwart von Natriumacetat ohne Farbänderung in Eisessig oder Acetanhydrid auf.

2. Das Trianisylmethylperchlorat ist als ein wahres Salz aufzufassen. Die hohe elektrische Leitfähigkeit in indifferenten Lösungsmitteln spricht u. a. stark dafür. Bei Zusatz von Acetat kann man sich die Bildung eines wenig dissoziierten Komplexsalzes denken etwa wie Silberperchlorat mit Natriumacetat komplexes Silberacetat bildet [8]. Wenn eine solche Verbindung entsteht und viel langsamer mit Wasser reagiert als das Carboniumjon, so ist die gemessene Reaktion die Dissoziationsgeschwindigkeit des Acetatkomplexes. Das scheint aber nicht der Fall zu sein und zwar aus folgenden Gründen.

a. Die kinetischen Vorversuche (Tabelle 1) zeigen, dass die Geschwindigkeit nur von dem Verhältnis HAc/NaAc bestimmt war, nicht aber von der Totalkonzentration (vgl. unten Salzeffekt).

b. Wir machen vergleichende Versuche in einem zweiten Puffer mit einem Anjon, von dem wir annehmen können, dass es eine geringere Tendens hat, komplexe Verbindungen zu bilden. Das gilt für das Formiatjon. Quantitative Komplexuntersuchungen mit Formiaten liegen leider noch nicht vor, halbquantitative orientierende Messungen weisen aber in diese Richtung. Wir haben deshalb einige Hydrolysenversuche mit Trianisylmethylperchlorat in Ameisensäure-Formiatpuffer ausgeführt. Lösungsmittel wie früher 50 %iges Azeton. Es galt dabei einen Acetat- und einen Formiatpuffer mit demselben pH und von derselben Jonenstärke zu bereiten. Als Acetatpuffer wurde die Lösung 2 (Tabelle 3) gewählt. Der Formiatpuffer wurde durch potentiometrische Titration gegen die Acetatlösung als Standard ausprobiert. Es wurde zu diesem Zwecke folgende Kette gebaut:

Au	Acetatpuffer 2 Chinhydron	Ges. KCl	0,294 C K-Formiat Chinhydron	Au

Zu dem rechten Halbelement wurde aus einer Mikrobürette eine azetonhaltige Ameisensäurelösung, die 0,294 C K-Formiat enthielt, zugesetzt, bis die EMK der Kette gleich 0 geworden war. Die Messungen zeigten, dass in dem azetonhaltigen Medium ein Formiatpuffer HFo = 0,0132 C, KFo = 0,294 dieselbe H-Jonenaktivität wie die erwähnte Acetatlösung besitzt.

Die kinetischen Versuche mit jenem Puffer ergaben eine Geschwindigkeitskonstante (log.nat) $K = 0{,}125$. Das ist innerhalb der Fehlergrenzen fast derselbe Wert wie mit dem entsprechenden Acetatpuffer. Es liegt also von kinetischem Standpunkt keine Andeutung vor, dass das Trianisylderivat in den zwei Pufferlösungen in verschiedenen Zuständen resp. Konzentrationen vorliegt. Wir haben durch diese Versuche keine Veranlassung, die Bildung eines Acetatkomplexes anzunehmen, dessen Zerfall geschwindigkeitsbestimmend wäre. Die untersuchte Reaktion ist nach diesen Versuchen eine Umsetzung zwischen dem Carboniumjon und Wasser.

SPEKTRALANALYTISCHE BEOBACHTUNGEN

Hantzsch hat aus seinen Lichtabsorptionsmessungen geschlossen, dass die Triphenylmethylverbindungen in zwei (und nur zwei) scharf gesonderten Gruppen existieren, die diskontinuierlich ineinander übergehen. Von dem Standpunkt der Mesomerie sind aber für ein solches Gleichgewicht z. B.

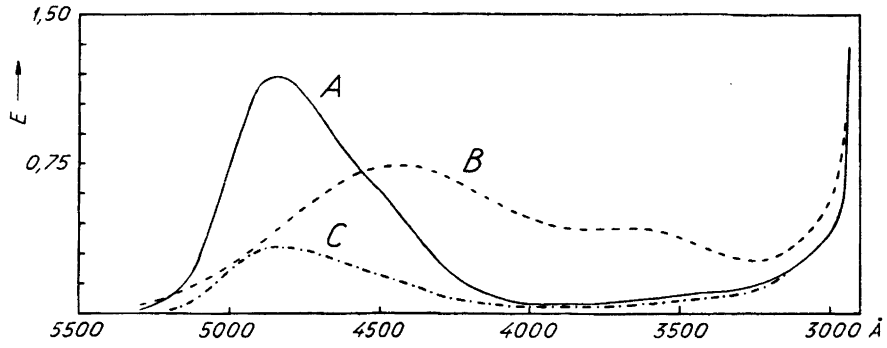

Fig. 2. Lichtabsorption in absolutem Alkohol. A Trianisylmethylperchlorat, B nach Zusatz von 0,1 C NaAc, C nach Zusatz von 0,1 C NaSCN.

$(C_6H_5)_3C \cdot Cl \rightleftharpoons [(C_6H_5)_3C]^+ Cl'$, alle Zwischenstufen denkbar, da es sich nur um Verschiebungen von Elektronbahnen handelt.

Fig. 2 gibt einige Absorptionsspektra in absolutem Alkohol wieder. Sämtliche Lösungen sind in bezug auf Trianisylmethylperchlorat 5 mC, Schichtdicke 1 cm. Diese Perchloratlösungskurven sind nicht gut reproducierbar, da sie stark von Wasserspuren abhängig sind. Alle Kurven von reinem Perchlorat sind aber gleichförmig, und haben ein Maximum bei 4850 Å, wie z. B. Kurve 2 A. Macht man die Lösung 0,1 C auf (wasserfreiem) Natriumacetat, bekommt man ein ganz anderes Kurvenbild, 2 B, mit einem Hauptmaximum bei 4400 Å. Diese Resultate widersprechen also den kinetischen. Es scheint, als ob bei der Einwirkung von Acetat auf das Perchlorat eine neue Verbindung entstanden ist, das sich in optischer Beziehung scharf von dem Carboniumsalz und von dem Trianisylcarbinol (das im sichtbaren Spektrum keine Absorption besitzt) unterscheidet. Bei etwa 3000 Å laufen alle Kurven zusammen. Im Ultraviolett sind sie wieder verschieden. Der Unterschied ist aber weniger charakteristisch und wird deshalb nicht näher besprochen. Setzt man dem Perchlorat 0,1 C *Natriumrhodanid* statt Acetat hinzu, bekommt man die Kurve 2 C. Das Tritylrhodanid ist farblos und ein Nichtleiter, also eine homöopolare Verbindung. Das Trianisylmethylrhodanid wird als ein gelber Körper beschrieben [6]. Da die Kurve 2 C mit 2 A sehr übereinstimmt, scheint das Anisylrhodanid merkwürdigerweise wie das Perchlorat ein Carboniumsalz zu sein, sofern die Lösung nicht ein Gleichgewicht zwischen Carboniumjon und undissoziiertes (farbloses) Rhodanid enthält.

MECHANISMUS DER TRIANISYLMETHYLSALZHYDROLYSE

Obwohl die optischen Versuche eine »Komplexbildung» zwischen dem Trityljon und Acetat angedeutet haben, dürfte die untersuchte von H-Jonen katalysierte Hydrolyse eine Reaktion zwischen Carboniumjon und Wasser sein, da sie auch ohne Gegenwart von Acetat von OH-Jonen in mässiger Konzentration gehemmt wird. Die Hydrolysenkinetik kann ausserdem sehr gut in Anilin-Anilinhydrochlorid-Puffer gemacht werden. Unter dieser Annahme können über den Mechanismus folgende Bemerkungen gemacht werden.

Eine Lösung des Perchlorats enthält, wie Hantzsch [1] einmal angenommen hat, ein Gleichgewicht zwischen Carboniumsalz und Ester:

$$[R_3C]^+ ClO_4^- \rightleftharpoons R_3C \cdot O \cdot ClO_3$$

Über Perchlorsäureester ist nicht viel bekannt. Nehmen wir aber an, das ein solcher Ester sich wie die Carbonsäureester verhält, haben wir eine annehmbare Erklärung für die beobachtete Kettenkurve. Diese Betrachtungsweise scheint aber wenig zufriedenstellend, da es plausibler scheint, dass das Jon wesentlich schneller als der Ester reagiert. Die Annahme einer Esterverseifung ist mit dem gefundenen grossen Neutralsalzeffekt nicht gut verträglich.

Wir möchten lieber annehmen, dass ein Stosskomplex zwischen Triarylmethyljon und Proton entsteht, der als sehr unbeständig schnell mit Wasser reagiert, da die Bindungsenergie $R_3C \rightarrow OH$ gross ist (vgl. Hückel [9])

$$[R_3CH]^{++} + H_2O \rightarrow R_3C \cdot OH + H^+$$

Die Existenz dieser Zwischenstufe entspricht der Bildung von »sauren» Triarylmethylsalzen, die mehrmals in festem Zustande isoliert worden sind.

Der grosse und positive Salzeffekt spricht zugunsten einer Reaktion zwischen zwei gleichgeladeten Jonen; die Bildung des Stosskomplexes würde demnach ordnungsbestimmend sein.

NEUTRALSALZWIRKUNG

Die Verseifung in Acetatpuffern zeigten, dass die Geschwindigkeit, solange C_{Salz} konstant war, genau proportional der $C_{Säure}$ verlief. Bei einer Verdoppelung der Pufferlösungskonzentration wurde ein Geschwindigkeitszuwachs von 10 % beobachtet. Ändert man aber die Jonenstärke des Puffers durch Zusatz von Neutralsalzen, so tritt ein bedeutend grösserer Effekt auf. So haben Natriumchlorid, -perchlorat usw. einen viel grösseren Salzeffekt als das Acetat. Die Versuche sind in Tabelle 5 zu finden. Sie beziehen sich wie früher auf eine 50%-ige Azetonlösung.

Tabelle 5. Neutralsalzwirkung bei der Hydrolyse in 50 % Azeton mit Acetatpuffer. $C_{HAc} = 0{,}0592$, $C_{NaAc} = 0{,}147$ *C.*

Nr	Neutralsalz	C_M	Jonenstärke	C_{ClO_4}	K
1	—	—	0,147	0	0,051
2	$NaClO_4$	0,147	0,294	0,147	0,0741
3	$LiClO_4$	0,147	0,294	0,147	0,0782
4	$Ba(ClO_4)_2$	0,049	0,294	0,098	0,0823
5	»	0,0735	0,368	0,147	0,100
6	NaCl	0,147	0,294	—	0,0704
7	NaSCN	0,147	0,294	—	0,0791

Der Neutralsalzeffekt dürfte sowohl eine Anjonen- wie eine Katjonenwirkung einschliessen. Bei dem Perchlorat steigt die Wirkung deutlich in der Reihe Na < Li < Ba. Mit dem Ba-perchlorat sind die Versuche auf zwei verschiedene Weisen ausgeführt. In einem Versuch (Nr 4) ist die Jonenstärke dieselbe wie in den übrigen (Nr 2, 3, 5, 6). Im Versuch 5 ist die Perchloratjonenkonz. dieselbe wie in 2 und 3. Die genannte Reihenfolge von Na, Li und Ba gilt für beide Fälle. Vergleicht man die drei untersuchten Natriumsalze untereinander, so finden wir die Anjonenreihe $Cl < ClO_4 < SCN$.

Um diese Salzwirkung möglicherweise etwas näher beleuchten zu können, sollen unten einige andere, in nichtwässriger Lösung gemachte Beobachtungen mitgeteilt werden.

Das Triphenylcarbinol (Tritol) ist in Eisessig farblos, erst im Ultraviolett (bei etwa 2600 Å) tritt ein Absorptionsband auf (Fig. 3 A). Versetzt man die verdünnte Tritollösung ($c = 3{,}3$ mC) mit Litiumperchlorat ($c = 1{,}0$ C), wird sie stark gelb gefärbt. Das Absorptionsspektrum ist in Fig. 3 B gezeichnet. Die Höhe der Kurve ist schwierig zu reproduzieren, da sie für Wasserspuren sehr empfindlich ist. (Die Farbstärke kann als ein Reagens auf die Güte des Eisessigs benutzt werden.) Die Lage der Maxima bleibt immer dieselbe (Figur B_1 und B_2). Das ultraviolette Absorptionsband des Tritols wird durch das Perchlorat einwenig höher, sonst nicht merkbar verändert. Setzt man zu dieser gelben Tritol-$NaClO_4$-Lösung $LiNO_3$ oder LiCl ($c = 0{,}2$ C), wird sie sofort fast entfärbt. Kurve 3 C ist die Absorption der chloridhaltigen Lösung. Die Kurve für $LiNO_3$ liegt noch niedriger.

Das Perchlorat hat also auf einer Tritolessiglösung dieselbe Wirkung wie ein Zusatz einer starken Säure, es wirkt, als ob die Essigsäure viel stärker geworden ist. Man kann auch die Annahme machen, dass das Tritol in der Perchloratlösung stärker basisch reagiert. Welche Hypothese vorzuziehen ist,

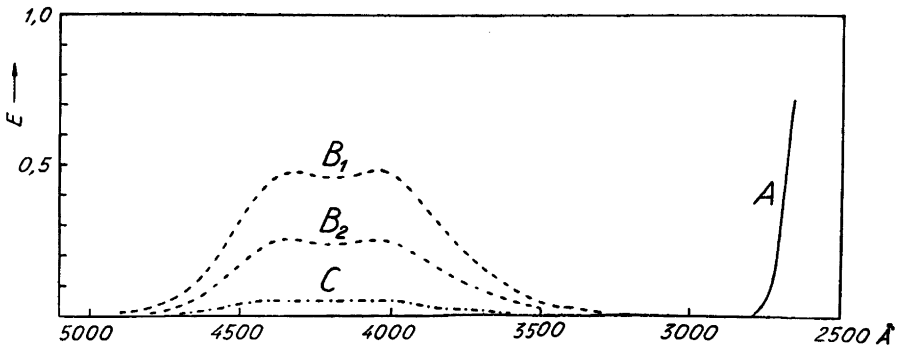

Fig. 3. A Lichtabsorption von Tritol in Eisessig. B_1—B_2 in Gegenwart 1 C $LiClO_4$, C nach Zusatz von 0,2 C LiCl.

mag dahingestellt bleiben. Das grösste Interesse knüpft sich an die Beobachtung, dass Chloride und Nitrate dem Perchlorateffekt entgegenwirken.

Die Perchlorat- und Nitrat-Wirkung haben wir für einige andere säurebasenempfindliche Systeme qualitativ untersucht. Als Beispiel sei angeführt:

1. *Dianisalazetonperchlorat* in reinem oder alkoholhaltigem Azeton wird durch $LiNO_3$ entfärbt.

2. *Dianisalazeton* in Eisessig. Die gelbe Lösung wird durch $NaClO_4$ orange und durch $NaNO_3$ oder $LiNO_3$ wieder gelb.

3. *4,4'-Dimethoxyazobenzol*, $CH_3O \cdot C_6H_4 \cdot N = N \cdot C_6H_4 \cdot OCH_3$, (und das entsprechende Äthylderivat): Die gelbe Eisessiglösung wird durch $NaClO_4$ ($LiClO_4$) orange und nach Zusatz von $NaNO_3$ ($LiNO_3$) wieder gelb. Wird die alkoholische Lösung der Azoverbindung durch eine Spur konz. Schwefelsäure orange gefärbt, so schlägt die Farbe mit $LiNO_3$ um. Setzt man nun $LiClO_4$ in fester Form zu, so wird die Lösung wieder etwas rötlich.

4. *Acetyl-p-aminoazobenzol*, $C_6H_5 \cdot N = N \cdot C_6H_4 \cdot NH \cdot CO \cdot CH_3$, ist in Eisessig gelb. $NaClO_4$ ist ohne Effekt. Färbt man aber die Eisessiglösung mit einer Spur konz. Schwefelsäure orange (partielle Salzbildung), so ruft ein Zusatz von $NaClO_4$ eine Verstärkung der Farbe hervor. Bei Zusatz von $LiNO_3$ wird sie wieder gelb.

Es sind auch Fälle gefunden, wo Perchlorate und Nitrate in derselben Richtung (»als Säuren») wirken.

5. *Rosolsäure* (Corallin) ist in Eisessig orangerot, $NaClO_4$ und $LiNO_3$ machen die Farbe gelb.

6. *Benzal-p-aminoazobenzol*, $C_6H_5 \cdot N = N \cdot C_6H_4 \cdot N = CH \cdot C_6H_5$, ist in Eisessig orangegelb, $LiClO_4$, $LiNO_3$ und auch NaSCN färben die Lösung tief orangerot. Aminoazobenzol verhält sich analog.

Die antagonistische Wirkung von Perchlorat und Nitrat könnte dadurch erklärt werden, dass die Salpetersäure in der Eisessiglösung viel schwächer als Überchlorsäure ist. Die Nitratwirkung wäre mit der von Natriumacetat zu vergleichen. Diese Erklärungsweise dürfte aber gemäss Beispiel 5 und 6 nicht stichhaltig sein. In Wasserlösung dürfte Salpetersäure mit $HClO_4$ und HCl gleichwertig sein [10]. Eine antagonistische Anjonenwirkung bei Dinitrophenolat in Wasserlösung ist von Kortüm [11] beobachtet worden.

ZUSAMMENFASSUNG

1. Die Hydrolyse von *p*-Trianisylmethylperchlorat wird sowohl von H- wie OH-Jonen gefördert. Sie durchläuft also eine »katalytische Kettenkurve», etwa wie die Verseifung von Carbonsäureestern.

2. Die Hydrolyse, in azetonhaltigem Acetatpuffer untersucht, zeigte sich von der Jonenstärke des Puffers sehr wenig abhängig, wurde aber bei Zusatz von Neutralsalzen stark beschleunigt. Es konnte sowohl ein Anjonen- als ein Katjoneneffekt nachgewiesen werden.

3. Es wurde für einige säurebasenempfindliche Stoffe in nichtwässrigen Lösungen eine mitunter antagonistische Wirkung von Perchlorat und Nitrat beobachtet.

4. Der Mechanismus der Anisylperchlorathydrolyse wird besprochen.

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Synthese des α-(3,4-Dimethoxyphenyl)-glycerins

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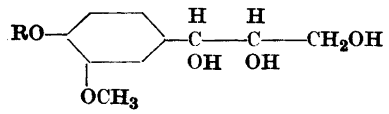
Unsere gegenwärtigen Kenntnisse von der Chemie des Lignins bauen sich auf die gesicherte Tatsache auf, dass das Ligninmolekül aus Guajacylpropaneinheiten — das Lignin der Laubhölzer auch aus Syringylpropaneinheiten — besteht, die in der Seitenkette Hydroxylgruppen tragen und in verschiedener Weise, sowohl durch Verätherung wie durch C—C-Kondensation, miteinander verknüpft sind. Die Anzahl und Stellung der freien Hydroxyle in den C_3-Seitenketten sowie die Art der Verknüpfung zwischen den einzelnen Bausteinen sind sicher nicht einheitlich und im einzelnen noch nicht mit Sicherheit bekannt.

Durch analytische Untersuchungen von Freudenberg [1,2,3] sowie durch die Isolierung von Methoxyessigsäure beim Permanganatabbau methylierter Ligninpräparate (Richtzenhain [4]) ist die Anwesenheit von primären Carbinolgruppen erwiesen. Sekundäre Carbinole in α-Stellung zum aromatischen Ring sind ebenfalls analytisch nachgewiesen worden [2]; sie, bzw. ihre Alkyläther, spielen wahrscheinlich eine hervortretende Rolle beim Übergang des nativen Lignins in Ligninsulfosäure [5–7]. Desgleichen ist die Anwesenheit sekundärer, nicht in α-Stellung stehender Carbinole sowie das Vorkommen tertiärer Carbinole im Lignin nachgewiesen [2].

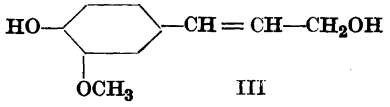
Die analytische Zusammensetzung isolierter Ligninpräparate [8,2,9,10] deutet darauf hin, dass wenigstens ein Teil der Phenylpropaneinheiten Sauerstoffatome, d. h. Hydroxyl- oder Äthergruppen, an allen drei C-Atomen der Seitenkette trägt. Solchen Einheiten liegt demnach ein Guajacylglycerin (I) zugrunde,



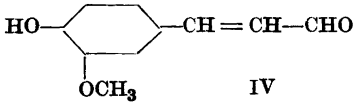
I. R = H II. R = CH_3

in welchem ein Teil der drei aliphatischen Hydroxyle veräthert ist. Das Vorkommen des Guajacylglyceringerüstes im Lignin ist auch wiederholt diskutiert worden [11,12].

Für die Biosynthese des Lignins hat Erdtman [13] die enzymatische Dehydrierung phenolischer C_6—C_3-Körper vom Coniferyltypus mit darauffolgender spontaner Polymerisation der primär gebildeten Radikale bzw. Chinonmethide in Betracht gezogen. Freudenberg [14] hat in der weiteren Verfolgung von Arbeiten von Freudenberg und Richtzenhain [15] gezeigt, dass bei der enzymatischen Oxydation von Coniferylalkohol (III) amorphe, ligninähnliche Produkte entstehen. Von diesem Gesichtspunkt aus betrachtet, kann das Guajacylglycerin (I) formell als einfaches Stabilisierungsprodukt eines (hypothetischen) primären Dehydrierungsproduktes des Coniferylalkohols aufgefasst werden. Es steht auf der gleichen »Oxydationsstufe» wie der Coniferylaldehyd (IV), der — unter Erhaltung seiner ungesättigten Seitenkette — in kleinen Mengen als Endgruppe in das Ligninmolekül eingebaut ist [16].



III



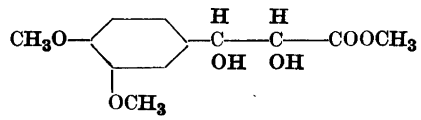
IV

Aus den angeführten Gründen erschien es von Interesse, das Guajacylglycerin bzw. die von ihm abzuleitenden Alkyl- und Aryläther auf ihre Reaktionsfähigkeit hin zu untersuchen und sie mit der des Lignins, z. B. hinsichtlich der Alkoholyse, der Sulfitierung, sowie der Vanillin- und Acetaldehydbildung, zu vergleichen.

Bisher sind jedoch derartige Substanzen nicht bekannt gewesen. Flickinger [17] hat vergeblich versucht, durch Anlagerung von zwei Hydroxylgruppen (mittels Permanganat) oder von zwei Acetoxygruppen (mittels Bleitetraacetat) an die Doppelbindung des Coniferins zu Guajacylglycerinderivaten zu gelangen. Vor kurzem hat Lindgren [18] über Versuche zur Gewinnung des β-Guajacyläthers von II berichtet; auch seine Versuche hatten jedoch nicht zu dem gewünschten Ergebnis geführt.

Es ist uns nun gelungen, das α-(3,4-Dimethoxyphenyl)-glycerin (II), im folgenden kurz als »Veratrylglycerin» bezeichnet, darzustellen. In der vorliegenden Mitteilung beschreiben wir die Synthese dieser Substanz; in folgenden Arbeiten werden wir über einige mit der Ligninchemie in Beziehung stehende Reaktionen derselben berichten. Auch die Darstellung des Guajacylglycerins (I) sowie die Synthese von in der Seitenkette verätherten Guajacyl- und Veratrylglycerinen sind in Angriff genommen.

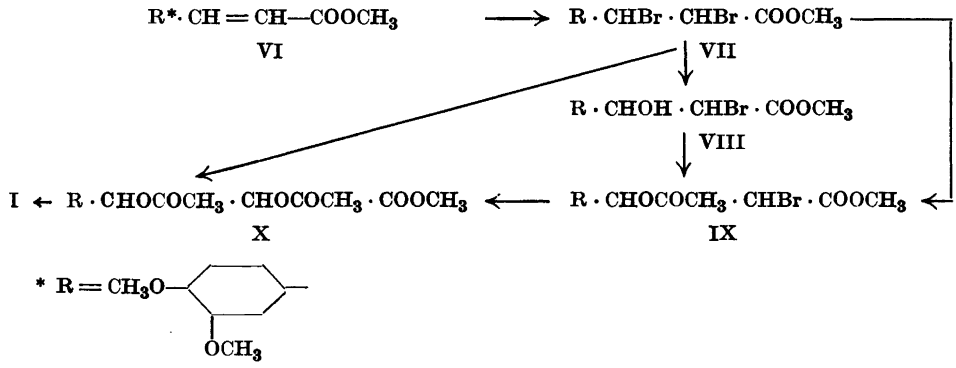
Um zum Veratrylglycerin zu gelangen, beabsichtigten wir, den β-(3,4-Dimethoxyphenyl)-glycerinsäureester V



V

herzustellen und dessen Estergruppe mittels Lithiumaluminiumhydrid zum primären Carbinol zu reduzieren. Versuche, den Ester V aus Methylferulasäuremethylester (VI) durch Permanganatoxydation oder durch Umsetzung mit Bleitetraacetat zu gewinnen, schlugen fehl. Im ersteren Fall blieb die Oxydation nicht bei V stehen, sondern lieferte Veratrumaldehyd und Veratrumsäure, im letzteren Fall trat keine Reaktion ein.

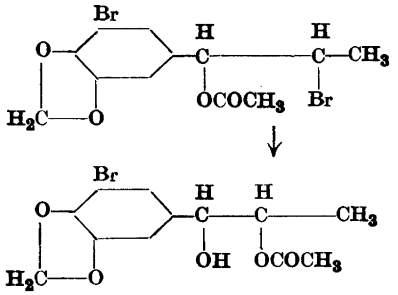
Das Diacetat von V (Formel X) konnte dann auf folgendem Wege gewonnen werden:



Methylferulasäuremethylester (VI) wurde durch Bromieren in Chloroformlösung in das Dibromid VII übergeführt. In VII ist das Bromatom, das sich an dem in α-Stellung zum aromatischen Kern stehenden C-Atom befindet, durch grosse Reaktionsfähigkeit ausgezeichnet. Wie vor allem Wallach [19], Hell [20], Hoering [21, 22] und Werner [23] gezeigt haben, stehen die Halogenide solcher p-Alkoxyaryl-carbinole hinsichtlich ihrer Reaktionsfähigkeit den Halogeniden der p-Oxyaryl-carbinole, wie sie um die Jahrhundertwende von Zincke und v. Auwers eingehend untersucht worden sind, nur um weniges nach. So liess sich auch das Dibromid VII durch kurzes Erwärmen mit einer Mischung von Aceton und Wasser fast quantitativ in das Carbinol VIII überführen. Durch Umsetzen von VII mit Kaliumacetat in Eisessiglösung entstand schon bei Zimmertemperatur das Monoacetat IX; daneben wurden allerdings noch ölige, uneinheitliche Produkte gebildet, und die Ausbeute an reinem

Monoacetat IX betrug nur 25—30 % der berechneten. In etwa der gleichen Ausbeute wurde das Monoacetat IX durch Acetylieren des Carbinols VIII mittels Essigsäureanhydrid und Kaliumacetat bei 100° erhalten. Durch Behandeln mit Essigsäureanhydrid-Pyridin bei Zimmertemperatur wurde das Carbinol VIII fast quantitativ in das Monoacetat IX übergeführt.

Der Austausch des zweiten, fester gebundenen Bromatoms gegen Acetoxyl bereitete zunächst einige Schwierigkeiten. Die direkte Umsetzung des Dibromids VII mit 2 Mol. Kaliumacetat in Eisessig bei 80—100° führte zu einem nicht kristallisierenden öligen Gemisch, und bei der analogen Umsetzung des Monoacetats IX mit 1 Mol. Kaliumacetat wurden neben viel öligem Produkt nur sehr geringe Mengen des gewünschten Diacetats X in kristallisierter Form erhalten. Dass diese Umsetzungen nicht glatt verliefen, konnte auf dem Eintreten verschiedener Nebenreaktionen beruhen. Hoering [22] hatte z. B. die eigentümliche Beobachtung gemacht, dass beim Erwärmen des vom Isosafrol abgeleiteten Monoacetats XI mit Natriumacetat in Eisessig nicht das eigentlich zu erwartende Diacetat, sondern — unter Verseifung der α-ständigen Acetoxygruppe — das Oxy-acetat XII entstand:



XI

XII

Es war denkbar, dass der mit XII analoge Körper auch in unserem Falle auftrat und vielleicht zum Teil durch Abspaltung von Wasser weiterreagierte. In der Absicht, die Anhäufung eines solchen Oxy-acetats und dessen weitere Reaktionen zu verhindern, vollzogen wir die Umsetzung des Dibromids VII bzw. des Monoacetats IX mit Kaliumacetat-Eisessig in Gegenwart von Essigsäureanhydrid. Auf diese Weise wurde das Diacetat X (Schmp. 102—103°) in Ausbeuten von 12 bzw. 25 % der Theorie erhalten. Es erwies sich schliesslich am vorteilhaftesten, das Carbinol VIII mit überschüssigem Essigsäureanhydrid und 2—3 Mol. Kaliumacetat zu acetylieren und das gebildete Monoacetat IX — ohne es zu isolieren — durch Zusatz von Eisessig und weiteres Erwärmen in das Diacetat X überzuführen. Dieses wurde dabei in einer Ausbeute von 30 %, ber. auf das Carbinol VIII, erhalten.

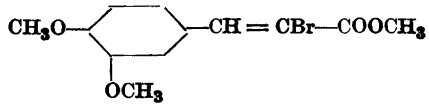
Der Veratryl-diacetyl-glycerinsäureester X wurde sodann mit 3 Mol. Lithiumaluminiumhydrid in Äther behandelt. Erwartungsgemäss wurde dabei nicht nur die endständige Estergruppe zur primären Alkoholgruppe reduziert, sondern es wurden auch die Acetylgruppen reduktiv abgespalten. Aus dem Reaktionsgemisch wurde das Veratrylglycerin (II) in einer Ausbeute von 70 %, ber. auf das Diacetat X, isoliert. Es bildet farblose, in Wasser und Alkohol leicht, in Benzol, Chloroform und Äther schwerer lösliche Kristalle vom Schmp. 109—110°. Von den zwei möglichen Racematen ist offenbar im wesentlichen nur das eine gebildet worden.

Die Konstitution von II ergibt sich aus der Elementaranalyse sowie aus folgenden Befunden:

Die wässerige Lösung der Substanz reagierte neutral. Sie verbrauchte bei pH 7 mit grosser Geschwindigkeit 2 Mol. Perjodat pro Mol. Substanz, wobei die zu erwartenden Spaltprodukte, nämlich Veratrumaldehyd, Ameisensäure und Formaldehyd, gebildet wurden. Die beiden Aldehyde wurden in Form ihrer 2,4-Dinitrophenylhydrazone isoliert. Im Destillat eines Perjodatversuches wurde die für 1 Mol. Ameisensäure pro Mol. Veratrylglycerin berechnete Menge Permanganat verbraucht.

Im Anschluss an die voranstehend beschriebene Synthese des Veratrylglycerins seien noch einige im Zusammenhang damit ausgeführte Versuche mitgeteilt, deren Ergebnisse von einigem Interesse erscheinen.

Die oben (S. 244) erwähnte Acetylierung des Carbinols VIII mittels Essigsäureanhydrid und Kaliumacetat führte nur bei vorsichtiger Ausführung zum Monoacetat IX; wurde das Reaktionsgemisch zum Sieden erhitzt, so erhielt man statt dessen in guter Ausbeute das Bromferulasäurederivat XIII.



XIII

Im Gegensatz zum Methylferulasäuremethylester VI addiert das Bromderivat XIII nicht Brom. Die Richtigkeit der Formel XIII ergibt sich aber — ausser aus der Elementaranalyse — auch aus der Ultraviolettabsorption, welche der des Methylferulasäuremethylesters VI gleicht, sich aber von der des Oxykörpers VIII und der des Veratrylglycerins II deutlich unterscheidet (Fig. 1).

Wie oben erwähnt, lassen sich beide Bromatome in VII — wenn auch mit abgestufter Reaktionsgeschwindigkeit — gegen Acetoxyl austauschen. Das in α-Stellung zum aromatischen Kern gebundene Bromatom setzt sich bereits bei gewöhnlicher Temperatur mit Kaliumacetat-Essigsäure um, das in β-Stellung

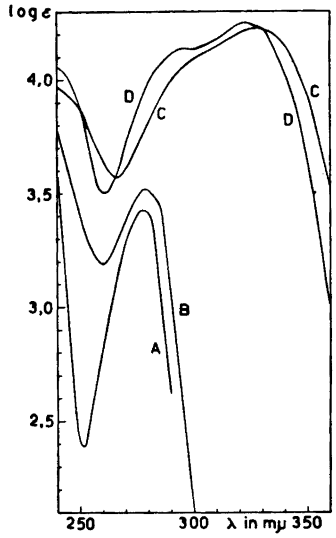
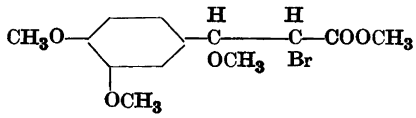
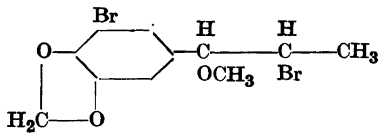


Fig. 1. Ultraviolettabsorption (in 96 %-igem Äthylalkohol).
A. Veratrylglycerin (II).
B. α-Brom-β-oxy-dihydroferulasäure-methyläther-methylester (VIII).
C. α-Brom-ferulasäure-methyläther-methylester (XIII).
D. Ferulasäure-methyläther-methylester (VI).

gebundene Bromatom, im Monoacetat IX, erst in der Wärme, z. B. mit Kalium- oder Silberacetat in Eisessig während 1—2 Stunden auf dem Wasserbad. Überraschenderweise zeigte sich dagegen, dass das β-Bromatom im Carbinol VIII selbst bei 6-stündigem Erwärmen mit Kaliumacetat in Eisessig auf 100° nicht reagierte. In Analogie hierzu erwies sich auch, dass das β-Bromatom im Methyläther XIV,



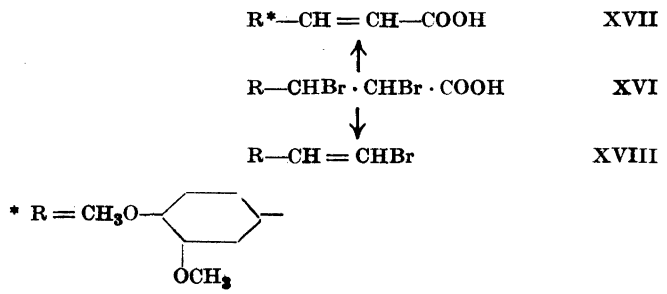
XIV



XV

der durch Behandeln des Dibromids VII mit Methanol glatt gebildet wird, äusserst stabil gebunden ist. Bei mehrstündigem Erhitzen mit Silberacetat in siedendem Eisessig trat keine Reaktion ein. Selbst nach 33-stündigem Erhitzen mit Silberbenzoat in siedendem *o*-Xylol (Kp. 145°) wurde die Substanz XIV unverändert zurückerhalten. Eine ähnliche Stabilisierung des β-Bromatoms durch eine Äthergruppe in α-Stellung findet man in einem von Hoering [22] beschriebenen Versuch, in welchem die Substanz XV sich bei der Umsetzung mit Natriumacetat in Eisessig oder in Äthanol bei 120° als resistent erwies.

Versuche, vom Dibromid der freien Methylferulasäure (XVI) ausgehend, zur Veratrylglycerinsäure zu gelangen, schlugen fehl, da die Carbonsäure XVI sich schon in den ersten Reaktionsstufen ganz anders verhielt als der Ester VII. Beim Behandeln mit Aceton-Wasser wurde — vermutlich durch spontane Abspaltung von HOBr aus primär entstandenem Carbinol — Methylferulasäure (XVII) gebildet. Eine derartige Reaktion ist bereits früher [24] bei ähnlichen Verbindungen beobachtet worden.



Beim Versuch, durch Umsetzen des Dibromids XVI mit Kaliumacetat in Eisessig das in Nachbarstellung zum Kern gebundene Bromatom gegen Acetoxyl auszutauschen, entstand schon bei gewöhnlicher Temperatur unter Abspaltung von HBr und CO_2 das Bromvinylveratrol XVIII. Zincke und Leisse [25] haben die gleiche Reaktion beim Dibromid der 3,5-Dibrom-4-oxy-zimtsäure gefunden.

Die Umsetzung des Dibromids XVI mit Methanol verlief dagegen normal; sie führte — unter gleichzeitiger Veresterung der Carboxylgruppe — zum Körper XIV.

EXPERIMENTELLER TEIL

Ferulasäure-methyläther-methylester (VI)

In eine Aufschlämmung von Ferulasäuremethyläther [26] in der 5-fachen Gewichtsmenge abs. Methanol wurde HCl-Gas ohne Kühlung eingeleitet, bis die Säure in Lösung gegangen war. Man liess über Nacht bei Zimmertemperatur stehen und kühlte auf 0° ab, wobei der Ester ausfiel. Aus Methanol umkristallisiert, derbe Prismen vom Schmp. 68—69°. Ausbeute 87 %. Für den auf anderem Wege hergestellten Ester geben Tiemann und Will [27] den Schmp. 64° an.

α,β-Dibrom-dihydroferulasäure-methyläther-methylester (VII)

Zu einer Aufschlämmung von 0,5 Mol Methylferulasäuremethylester (VI) in 500 ml Chloroform wurde 1 Mol Brom langsam zugegeben, wobei die Temperatur unter 15°

gehalten wurde. Der Ester ging in Lösung. Das nach Abdampfen des Chloroforms i. V. zurückbleibende Öl erstarrte kristallinisch. Aus Chloroform-Hexan umkristallisiert, Prismen vom Schmp. 100—101°. Ausbeute 83 %.

$C_{12}H_{14}Br_2O_4$	Ber.	C 37,73	H 3,69	Br 41,83	OCH_3	24,37
	Gef.	» 37,46	» 3,84	» 41,65	»	24,51

α-Brom-β-oxy-dihydroferulasäure-methyläther-methylester (VIII)

Eine Lösung von 10 g Dibromid VII in 50 ml Aceton wurde mit 50 ml Wasser versetzt und 10 Min. unter Rückfluss gekocht. [Während der Reaktion tritt Bromaceton als Nebenprodukt auf. Das hierfür erforderliche Brom dürfte zum Teil durch Zerfall des gebildeten HBr entstehen; möglicherweise zerfällt auch etwas Oxykörper VIII durch Abspaltung von HOBr (vgl. hierzu Hoering [24]), die ihrerseits HBr zu Br_2 oxydieren könnte]. Beim Abkühlen fiel der Oxykörper VIII aus. Aus 50 %-igem Aceton umkristallisiert, derbe Prismen vom Schmp. 136—137°. Ausbeute 92 %.

$C_{12}H_{15}O_5Br$	Ber.	C 45,14	H 4,74	Br 25,02	OCH_3	29,17
	Gef.	» 45,33	» 4,68	» 25,16	»	29,29

α-Brom-β-acetoxy-dihydroferulasäure-methyläther-methylester (IX)

1. In eine Aufschlämmung von 7,64 g Dibromid VII (0,02 Mol) in 50 ml Eisessig wurden 2,0 g wasserfreies Kaliumacetat (0,02 Mol) portionsweise zugegeben. Die Substanz ging dabei in Lösung, während KBr allmählich ausfiel. Nach mehrstündigem Stehen wurde vom KBr abgesaugt, das Filtrat i. V. eingeengt und mit Wasser verdünnt. Das ausfallende Öl erstarrte teilweise. Nach Umkristallisieren aus Methanol Stäbchen vom Schmp. 64—66°. Ausbeute 28 %.

$C_{14}H_{17}O_6Br$	Ber.	C 46,56	H 4,74	Br 22,13	OCH_3	25,78
	Gef.	» 46,36	» 4,78	» 22,69	»	25,72

2. 12,8 g Oxykörper VIII wurden mit 125 ml Essigsäureanhydrid und 8,0 g wasserfreiem Kaliumacetat 1 Stunde auf dem siedenden Wasserbad erwärmt. Nach Abkühlen und Zerlegen mit Eis wurde das abgeschiedene Öl in wenig 80 %igem Äthylalkohol aufgenommen. Nach mehrtägigem Stehen im Kühlschrank waren ca. 25 % der berechneten Menge an Monoacetat auskristallisiert.

3. 3,19 g Oxykörper VIII wurden in 30 ml Pyridin mittels 6 ml Essigsäureanhydrid acetyliert. Nach Zerlegen mit Eiswasser wurde ein allmählich erstarrendes Öl erhalten. Ausbeute fast quantitativ.

α,β-Diacetyl-β-(3,4-dimethoxyphenyl)-glycerinsäure-methylester (X)

1. Eine Lösung von 7,64 g Dibromid VII (0,02 Mol) in Eisessig und 12 ml Essigsäureanhydrid wurde mit 4,0 g wasserfreiem Kaliumacetat (0,04 Mol) 6 Stunden auf dem siedenden Wasserbad erhitzt. Von ausgefallenem Kaliumbromid wurde abgesaugt, die

Lösung i. V. eingeengt, mit Wasser versetzt, und das ausgefallene Öl in Chloroform aufgenommen. Die Chloroformlösung wurde mit Na_2SO_4 getrocknet und i. V. eingedampft. Der ölige Rückstand erstarrte teilweise beim Verreiben mit Hexan. Die vom restlichen Öl abgesaugten Kristalle wurden aus abs. Äthanol umkristallisiert. Täfelchen vom Schmp. 102—103°. Ausbeute 12 %.

$C_{16}H_{20}O_8$	Ber.	C	56,45	H	5,92	OCH_3	27,35
	Gef.	»	56,27	»	5,89	»	27,50

2. Eine Lösung von 18 g Monoacetat IX (0,05 Mol) in einem Gemisch von 160 ml Eisessig und 140 ml Essigsäureanhydrid wurde mit 7,5 g wasserfreiem Kaliumacetat (0,075 Mol) 2 Stunden auf dem siedenden Wasserbad erhitzt. Nach dem Abkühlen wurde das Essigsäureanhydrid mit Eis zerlegt. Nach Einengen i. V. und Zugabe von Wasser fiel ein zähes Öl aus, das in wenig 80 %-igem Alkohol gelöst wurde. Nach mehrtägigem Stehen im Kühlschrank waren ca. 25 % der berechneten Menge an Diacetat X auskristallisiert.

3. 7,2 g Monoacetat IX (0,02 Mol) in 100 ml Eisessig wurden mit 3,5 g Silberacetat (0,021 Mol) unter Turbinieren während 5 Stunden bei 80° gehalten. Vom gebildeten AgBr (3,75 g = 0,02 Mol) wurde abgesaugt, i. V. eingeengt, mit Wasser versetzt und das ausgefallene Öl in mit wenig Wasser verdünntem Methanol gelöst. Nach Animpfen kristallisierten 1,38 g Diacetat X (Ausbeute 20 %).

4. 6,4 g Oxykörper VIII (0,02 Mol) wurden in 50 ml Essigsäureanhydrid gelöst und nach Zusatz von 5,0 g Kaliumacetat (0,05 Mol) 1 Stunde auf dem Wasserbad erwärmt. Hierauf wurden 50 ml Eisessig zugesetzt, wobei das Kaliumacetat in Lösung ging. Das Erwärmen wurde nun 1 ½—2 Stunden lang fortgesetzt, wobei KBr ausfiel. Nach Erkalten wurde mit Eis zerlegt und i. V. stark eingeengt. Der viskose Rückstand wurde wiederholt mit Wasser durchgeknetet und schliesslich aus 80 %-igem Äthanol kristallisiert. Ausbeute 30 %.

α-(3,4-Dimethoxyphenyl)-glycerin (II)

Eine Aufschlämmung von 6,5 g (0,17 Mol) $LiAlH_4$ in 600 ml abs. Äther wurde unter Rückfluss gekocht (Calciumchlorid- und Natronkalkrohr!). Zwischen Kolben und Rückflusskühler war ein Extraktionsrohr eingeschaltet, in dem sich ein grobporiger Glasfiltertiegel mit 17 g Diacetat X (0,05 Mol) befand. Durch den rückfliessenden Äther wurde die Substanz im Laufe von 4—6 Stunden gelöst und so mit dem $LiAlH_4$ zur Reaktion gebracht. Nach Abschluss der Reduktion wurde auf 0° gekühlt und die gebildete LiAl-Verbindung unter stetigem Durchleiten von Stickstoff durch tropfenweisen Zusatz von 200 ml Wasser bei 0° zerlegt.

Die Ätherschicht wurde mit Na_2SO_4 getrocknet und zur Trockene eingedampft. Der teilweise kristallisierte Rückstand wurde in Chloroform gelöst. Die wässerige Suspension wurde zentrifugiert und die Fällung einige Male an der Zentrifuge mit Wasser gewaschen. Die vereinigten wässerigen Lösungen wurden mit Essigsäure auf pH 7 neutralisiert und i. V. eingedampft. Der Rückstand wurde mit Chloroform erwärmt und die erhaltene Lösung klarfiltriert. Die erwähnte, hauptsächlich aus anorganischem Material bestehende Fällung enthielt noch ziemlich viel Veratrylglycerin. Sie wurde deshalb dreimal mit Alkohol aufgekocht und heiss abgesaugt. Die vereinigten alkoholischen Extrakte wur-

den i. V. eingedampft und der Rückstand in Chloroform gelöst. Die so erhaltenen drei Chloroformlösungen wurden vereinigt, auf kleineres Volumen eingeengt und mit Äther versetzt. Das Veratrylglycerin (II) fiel in bereits ziemlich reinem Zustande aus. Aus Chloroform oder Chloroform-Äther Blättchen vom Schmp. 109—110°. Ausbeute 70 %.

$C_{11}H_{16}O_5$	Ber.	C	57,89	H	7,07	OCH_3	27,19
	Gef.	»	57,94	»	7,12	»	27,02

Oxydation mit $NaBiO_3$. Beim Zusatz von Natriumwismutat zu einer Eisessiglösung von Veratrylglycerin ging das Wismutat unter Entfärbung in Lösung. Die Reaktion ist nach W. Rigby [28] charakteristisch für Glykole. Pro Mol Veratrylglycerin wurden 2 Mol, d. i. die berechnete Menge, $NaBiO_3$ verbraucht.

Oxydation mit Perjodsäure.

a) *Bestimmung des Perjodatverbrauchs.* Eine Lösung von 22,8 mg Veratrylglycerin (0,1 Millimol) in 3 ml Wasser wurde mit 3 ml einer 0,09 M $NaJO_4$-Lösung (0,27 Millimol) versetzt. Nach 15 Min. bei etwa 20° wurden 20 ml Pufferlösung von pH 7 und überschüssiges KJ zugesetzt. Nach einigen Minuten wurde das ausgeschiedene Jod mit Thiosulfatlösung titriert. Es wurden 1,35 ml 0,1050 N $Na_2S_2O_3$-Lösung verbraucht. In einer Blindprobe ohne Veratrylglycerin betrug der Verbrauch an $Na_2S_2O_3$-Lösung 5,15 ml. Im Oxydationsversuch waren somit 0,2 Millimol $NaJO_4$ verbraucht worden (ber. 0,2 Millimol).

Der gleiche Perjodatverbrauch ergab sich nach 30 Min. langem Stehen der Versuchslösung.

b) *Identifizierung von Veratrumaldehyd und Formaldehyd.* Eine wässerige Lösung von 114 mg Veratrylglycerin (0,5 Millimol) wurde mit 228 mg Perjodsäure, $HJO_4 \cdot 2H_2O$, (1 Millimol) versetzt. Nach zweistündigem Stehen wurde ein Überschuss einer Lösung von 0,3 % 2,4-Dinitrophenylhydrazin in 2 N HCl zugegeben. Die orangerote Fällung wurde nach 4 Stunden abgesaugt und mit 80%-igem Äthanol aufgekocht. Der unlösliche Rückstand bestand aus Veratrumaldehyd-2,4-dinitrophenylhydrazon (Schmp. 258—260°, Mischschmp. keine Depression); Ausbeute 68,3 mg, d. i. 83 % der berechneten Menge. Das alkoholische Filtrat gab beim Stehen eine goldgelbe Fällung vom Schmp. 162—164°, die mit Formaldehyd-2,4-dinitrophenylhydrazon (Schmp. 166—167°) keine Depression gab. Mit dem 2,4-Dinitrophenylhydrazon des Acetaldehyds (Schmp. 166—168°) lieferte die Substanz eine deutliche Schmelzpunktserniedrigung. Die Ausbeute an Formaldehyd-2,4-dinitrophenylhydrazon war 12,1 mg, d. i. 81 % der ber. Menge.

c) *Bestimmung der gebildeten Ameisensäure.* 45,6 mg Veratrylglycerin (0,20 Millimol) in 5 ml Wasser wurden mit 10 ml einer 0,09 M $NaJO_4$-Lösung 5 Stunden bei etwa 20° oxydiert. Unverbrauchtes $NaJO_4$ und gebildetes $NaJO_3$ wurden durch Zugabe von 2 ml 20 %-iger Bleinitratlösung gefällt. Die gebildeten Aldehyde wurden durch Zusatz von 150 mg Dimedon (1 Millimol) in 20 ml heissem Wasser und 16-stündiges Erwärmen auf dem Wasserbad gebunden. Die abgekühlte Lösung wurde filtriert, die Fällung quantitativ ausgewaschen und die erhaltene Lösung unter wiederholtem Zusatz von Wasser i. V. destilliert. Um Überspritzen zu vermeiden, wurde eine mit Glasperlen beschickte Kolonne verwendet. Das in Sodalösung aufgefangene Destillat wurde mit $KMnO_4$ titriert.

Verbraucht: 7,0 ml 0,064 N $KMnO_4$, entspr. 0,21 Millimol HCOOH (ber. 0,20 Millimol).

α-Brom-ferulasäure-methyläther-methylester (XIII)

12,8 g Oxykörper VIII (0,04 Mol) wurden mit 125 ml Essigsäureanhydrid und 8 g Kaliumacetat 1 Stunde unter Rückfluss gekocht. Beim Zerlegen mit Wasser wurde Substanz XIII in kristallisierter Form erhalten. Aus Äthanol umkristallisiert, Nadeln vom Schmp. 82–83°. Ausbeute 75 %.

$C_{12}H_{13}O_4Br$	Ber.	C	47,86	H	4,35	Br	26,55	OCH_3 30,91
	Gef.	»	47,80	»	4,46	»	26,50	» 30,94

α-Brom-β-methoxy-dihydroferulasäure-methyläther-methylester (XIV)

1. Dibromid VII wurde in überschüssigem Methanol bei Zimmertemperatur gelöst. Die Lösung wurde i. V. eingeengt und die nahezu quantitativ ausfallende Substanz aus Methanol umkristallisiert. Tafeln und Prismen vom Schmp. 89–90°.

$C_{13}H_{17}O_5Br$	Ber.	C	46,86	H	5,15	OCH_3	37,26
	Gef.	»	46,67	»	5,23	»	37,10

2. In eine Lösung von α,β-Dibrom-dihydroferulasäure-methyläther (XVI)[29] in Methanol wurde unter Kühlung trockener Chlorwasserstoff eingeleitet. Nach mehrstündigem Stehen wurde i. V. eingeengt, wobei Substanz XIV ausfiel. Ausbeute 90 %.

3,4-Dimethoxy-β-brom-styrol (XVIII)

3,68 g α,β-Dibrom-dihydroferulasäure-methyläther (XVI) (0,01 Mol) wurden in Eisessiglösung mit 1,0 g wasserfreiem Kaliumacetat (0,01 Mol) umgesetzt. Nach 4-stündigem Stehen wurde von ausgefallenem KBr abgesaugt, i. V. eingeengt und mit Wasser verdünnt. Das erhaltene Öl erstarrte. Aus Hexan umkristallisiert, Nadeln vom Schmp. 63–65°. Ausbeute 80 %.

$C_{10}H_{11}O_2Br$	Ber.	C	49,40	H	4,56	Br	32,87	OCH_3 25,54
	Gef.	»	49,16	»	4,57	»	32,59	» 25,47

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The Chlorine Oxidation of Glycosides

I. Oxidation of Methyl β-Glucoside

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The use of chlorine as a bleaching agent is of great technical importance. In the cellulose industry it is employed to remove non-cellulosic material from wood pulp and to destroy colouring matter which is retained in the fibres. This process, however, combined with subsequent alkali treatment of the pulp, involves considerable losses of cellulose [1]; the precise manner in which the cellulose is thus degraded is at present unknown, and an investigation of the reactions concerned is therefore of obvious interest.

Existing knowledge of the effect of halogens on carbohydrates is chiefly confined to the monosaccharides [2]. The reaction between free sugars and halogens has been extensively studied under various conditions, and the conversion of glycosides into uronides by the action of hypobromite has been observed. The action of free chlorine or bromine on simple glycosides has, however, apparently not been studied (reactions occurring in the aglycone are disregarded), and little is known of their action on polysaccharides in general.

In considering the problem of the degradation of cellulose by chlorine, it is an open question whether the attack of the halogen occurs at especially weak points in the cellulose molecule such as have been suggested to exist, or merely at statistically distributed centres throughout a whole chain of equivalent glucose units. However it seemed that in any case the most satisfactory approach to the problem would be to study first the action of chlorine water on some simple glycosides, as model substances from which crystalline reaction products might be expected. The first of these investigations has been carried out with methyl β-glucoside.

An aqueous solution of methyl β-glucoside was subjected to a slow continuous stream of chlorine gas, passing at a rate sufficient to keep the solution

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saturated. At regular intervals the optical rotation of the solution was determined, and the values obtained clearly showed that a reaction was taking place. The initially negative rotation steadily increased, approximately following a first order reaction, and reached a maximum after about 14 days; after this the rotation slowly decreased. During the reaction hydrochloric acid was formed, and also organic acids as was indicated by the fact that the optical rotation of the solution changed considerably on neutralisation. The amounts of these acids present were periodically determined; the total concentration of acids, estimated by neutralisation, was always greater than the concentration of hydrochloric acid, determined by Mohr titration. Portions of the solution were also treated at intervals with periodic acid; the consumption of reagent, initially 1.9 moles per mole of methyl β-glucoside, steadily increased and reached a maximum value of 3.7 at about the same time as maximum optical rotation was observed, after which it decreased. This high periodic acid consumption pointed to the presence of such possible oxidation products as gluconic and glucuronic acids, both of which would theoretically consume 4 moles of the reagent. Gluconic acid was actually isolated from the reaction mixture (after 14 days' chlorination) as the calcium salt and was characterised as the phenylhydrazide; from the yield of these derivatives it was concluded that at least 50 % of the methyl glucoside had been converted to gluconic acid during the oxidation. The identity of the product was confirmed by oxidation of the calcium salt by Ruff's method to give D-arabinose. However as both the optical rotation and the periodic acid consumption were found to pass through maximum values, it was clear that gluconic acid could not be the only product of the reaction, and it was decided to attempt analysis of the mixture by paper partition chromatography.

The chromatographic experiments were carried out with a butanol-acetic acid-water mixture as solvent. A solution of methyl glucoside, which had been chlorinated until it just showed the maximum values referred to above, was treated to remove hydrochloric acid. It then gave a chromatogram which, on development with ammoniacal silver nitrate, exhibited five distinct spots. Two of these were identical with the spots given by a solution of D-gluconic acid chromatographed under the same conditions. Alternative development of these chromatograms with methyl orange caused only the upper spot in each case to appear as a red colouration, the lower spot being absent; the former thus corresponds to gluconic acid itself and the latter to a lactone. The equilibrium between the acid and the lactone in solution is established sufficiently slowly to render chromatographic separation possible, but nevertheless when silver nitrate was used as developing reagent there was always a faint colour between the two spots. Two other spots on the chromatogram of the reaction

mixture were found to be given also by 5-ketogluconic acid * under the same conditions, one again doubtless corresponding to the free acid and the other to a lactone. These spots, from both the unknown and the authentic sources, could be alternatively developed by resorcinol in alcoholic hydrogen chloride, a specific reagent for ketoses.

Similar experiments were carried out with a solution of methyl β-glucoside which had been chlorinated for a period of 40 days. The chromatograms obtained showed that this also contained both gluconic acid and 5-ketogluconic acid but suggested that the proportion of keto-acid present was now rather higher than in the solution oxidised for 14 days only. It therefore appeared probable that the primary product of the oxidation of methyl β-glucoside is gluconic acid and that the latter undergoes further oxidation to give the keto-acid. This conclusion was supported by the results of an additional experiment in which gluconic acid itself was oxidised with chlorine for 14 days under the same conditions as the methyl glucoside. The resulting solution gave a chromatogram which when developed with ammoniacal silver nitrate was almost identical with the chromatogram of oxidised methyl glucoside, showing all five spots found on the latter including the two corresponding to 5-keto-gluconic acid and its lactone. It is of interest to note that Hart and Everett [3] have shown that in the prolonged oxidation of glucose by bromine water, the gluconic acid first formed can be further oxidised to 5-ketogluconic acid. The formation of the keto-acid was finally confirmed by its isolation as a barium salt from the solution of methyl glucoside oxidised for 40 days, and subsequent conversion to the brucine salt which had physical properties in agreement with those recorded in the literature [3].

The compound corresponding to the fifth spot which appeared on the chromatograms of both oxidised methyl glucoside and oxidised gluconic acid has not been identified, but it is certainly only a minor product of the reaction.

The formation of gluconic acid by the action of chlorine upon methyl β-glucoside is an unexpected result. The reaction cannot proceed by way of initial hydrolysis of the glycoside group and subsequent oxidation since it has been shown that at room temperature and in the presence of hydrochloric acid of the maximum concentration produced during the oxidation (i. e., about 2N), methyl β-glucoside is quite stable. The actual mechanism of the oxidation is at present obscure, but it is possible that it proceeds by way of a chloro-derivative of the basic structure shown in the inset, which is formed slowly but undergoes rapid hydrolysis to give ultimately gluconic acid. In this way

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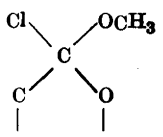

the formation of the latter would approximate to a first order reaction as is found in practice. Some analogous substances to this hypothetical intermediate are described in the literature, for example bromoformaldehyde dimethyl acetal, $BrCH(OCH_3)_2$[4].

The action of chlorine on methyl α-glucoside has also been investigated under similar conditions to those employed with the β-isomer. The reaction was very much slower than with the latter, and after 32 days 66 % of the glucoside could be recovered unchanged. Chromatography of the solution, however, indicated the presence of small amounts of gluconic acid. This difference in reactivity is not unexpected, since it has been shown that while α- and β-glucose both yield gluconic acid by the action of bromine water, the reaction with the β-isomer is 35 times faster than with the α-sugar [5].

On the basis of these results it is possible to formulate a tentative explanation of the degradation of cellulose by chlorine, but further studies which are now in progress on the oxidation of other glycosides, *e. g.* cellobiosides, should render more definite conclusions possible.

EXPERIMENTAL

Action of chlorine on methyl β-glucoside

A slow stream of chlorine gas was passed through a 0.5 M solution of methyl β-glucoside at room temperature with the exclusion of direct light. At appropriate time intervals small portions of the solution were withdrawn, the excess of chlorine removed by aeration for 10—15 minutes, and the following determinations carried out:

a. Determination of optical rotation (2 dm tube).

b. Estimation of total acid present, by titration of the solution (1 ml) with 0.1 *N* sodium hydroxide solution, with gentle warming and using phenolphthalein as indicator, until a permanent pink colour resulted. (Before the final end-point was reached in each titration, vanishing end-points were obtained.)

c. Estimation of hydrochloric acid present, by neutralisation of the solution (1 ml) with sodium bicarbonate and then titration with 0.1 *N* silver nitrate solution by Mohr's method.

d. Determination of periodic acid value. The solution (1 ml) was treated with an excess of a 0.1 *M* solution of periodic acid for 24 hours. After neutralisation with sodium bicarbonate the excess of periodic acid was determined by the arsenite-iodine method. The results of these determinations are shown in Table 1, and in addition the amount of acids present other than hydrochloric acid, calculated by difference from values (b) and (c).

At longer time intervals larger portions of the solution were removed for qualitative examination. After the chlorination had proceeded for 14 days, one such portion was withdrawn and the excess of chlorine removed by aeration. The solution (A) was then found to have a moderately strong reducing power towards Fehling's solution, but tests

for the presence of carbonyl compounds with the usual reagents, *e. g.* 2,4-dinitrophenylhydrazine, gave negative or inconclusive results.

Preparation of calcium salts

The solution (A) (10 ml) was neutralised with silver carbonate, filtered and then saturated with hydrogen sulphide, excess of which was removed by aeration after filtering off silver sulphide. The resulting solution (a small portion of which was reserved for chromatographic examination, see below) was neutralised with calcium carbonate whilst warmed on the water bath, and then filtered; the filtrate was concentrated under reduced pressure to a volume of 5 ml, and the residue poured with stirring into alcohol (100 ml). The calcium salts (0.87 g) thus precipitated were amorphous and attempts to purify them by crystallisation were unsuccessful. The product showed $[\alpha]_{20}^{D}$ + 7.5° in water, c = 1; the value recorded for calcium gluconate is + 8.5°. It was found to contain no methoxyl group, which demonstrated that methyl glucuronide was not present in the reaction mixture.

Table 1. Chlorination of methyl β-glucoside.

Reaction time, days	α_D	Conc. of HCl, equiv./l	Conc. of other acids, equiv./l	HIO_4 consumption, mol./mol.
0	− 6.26°	0	0	1.91
1	− 3.96	0.38	0.12	2.50
2	− 2.55	0.58	0.20	2.96
4	− 0.84	0.83	0.26	3.43
6	+ 0.33	1.04	0.37	3.62
10	+ 1.37	1.35	0.43	3.68
14	+ 1.57	1.62	0.53	—
19	+ 1.50	1.86	0.58	3.58
25	+ 1.43	2.25	0.72	3.34

Isolation of gluconic acid as the phenylhydrazide

Another portion (10 ml) of the solution (A) was freed from hydrochloric acid as described above and the final filtrate concentrated to a volume of about 10 ml. Glacial acetic acid (1.5 ml) and phenylhydrazine (1 ml) were added, the mixture heated on the water bath for 1 hour and then filtered while still hot to remove amorphous material. The solution was allowed to stand overnight at 0° and the yellow crystals which had separated were then collected and washed with ether. The product (0.66 g) was dissolved in boiling alcohol (350 ml), filtered while hot, and the filtrate kept at 0° for 3 hours. Colourless crystals (0.39 g, 27 % of the theoretical yield) were then obtained, which after two further recrystallisations from alcohol had m. p. 196° * (decomp.), undepressed on

* All melting points uncorrected.

admixture with authentic gluconic acid phenylhydrazide, and $[\alpha]_D^{20}$ + 13° in water, c = 1 (the value given in the literature is $[\alpha]_D$ + 12° in water, c = 2).

$C_{12}H_{18}O_6N_2$ (286.3)	Calc.	N 9.79
	Found	» 9.89

Preparation of D-arabinose from the calcium salts

The calcium salts, prepared as described above, (2 g) were oxidised with hydrogen peroxide in the presence of ferric sulphate according to the method of Hockett and Hudson [6]. This gave D-arabinose (0.1 g) which after recrystallisation from absolute methanol had m. p. 152—154°, undepressed on admixture with authentic material. The low yield of arabinose obtained was probably due to the small scale on which the reaction was carried out; under the same conditions authentic calcium gluconate (2 g) gave D-arabinose (0.2 g).

Isolation of 5-ketogluconic acid as the brucine salt

After the chlorination of methyl β-glucoside had proceeded for 40 days, a portion (50 ml) of the solution was withdrawn and the hydrochloric acid present removed as described above. It was then treated with an excess of barium carbonate at 40—45° for 2 hours, filtered, and the filtrate concentrated under reduced pressure to a volume of 5 ml. Alcohol (40 ml) was added, and after a few hours the precipitated barium salts were collected, washed with alcohol and ether and dried. Yield, 1.70 g. The barium salts (1.0 g) were dissolved in water (2—3 ml) and the barium removed quantitatively by addition of N sulphuric acid. After filtration, alcohol (12 ml) was added and then a 10 % alcoholic brucine solution (38 ml). After allowing the mixture to stand at 0° overnight, the crystalline brucine salt was collected, washed with alcohol and ether. Yield, 0.8 g. Recrystallisation from water gave brucine 5-ketogluconate as needles, m. p. 172—173°, $[\alpha]_D^{20}$ —26° ± 2° (micro-determination). Hart and Everett [3] give m.p. 174—175°, $[\alpha]_D$ —24°.

Action of hydrochloric acid on methyl β-glucoside

A 0.5 N solution of methyl β-glucoside in 2 N hydrochloric acid was kept at room temperature and the optical rotation periodically determined. After 23 days the α_D value had only changed from —6.36° to —6.24° (2 dm tube). The solution was then freed from hydrochloric acid in the usual way, and after evaporation to dryness under reduced pressure and recrystallisation of the residue from alcohol a 95 % recovery was obtained of methyl β-glucoside, identical with the starting material.

Action of chlorine on D-gluconic acid

A 5 % aqueous solution of D-gluconic acid (prepared from the δ-lactone) was treated with chlorine gas under the same conditions as methyl β-glucoside. After the reaction had proceeded for 14 days, the hydrochloric acid present was removed as in previous experiments, and the solution obtained subjected to chromatographic examination (see below).

Action of chlorine on methyl α-glucoside

This experiment was carried out exactly as described earlier for the β-glucoside, using the same concentration of substance. After the passage of chlorine had been continued for 32 days, the optical rotation of the solution had only changed slightly, from the initial α^D value of + 30.82° to + 29.26° (2 dm tube). The periodic acid consumption showed no change within the limits of experimental error. The solution was then freed from hydrochloric acid, and a portion examined chromatographically (see below). The remainder was evaporated to dryness under reduced pressure and the residue recrystallised from alcohol to give a 66 % recovery of unchanged methyl α-glucoside.

Chromatographic examination of the reaction mixtures

The chromatographic experiments were carried out on Whatman No. 1 paper, using as solvent a mixture of butanol (40 %), acetic acid (10 %) and water (50 %). The solutions from the oxidation reactions, after removal of hydrochloric acid as described, were adjusted to concentrations of 4—5 % with respect to the carbohydrate taken; the solutions of reference compounds used were all approximately 2 %. One drop of the appropriate solution was used for each chromatogram. After application of the drops, the papers were normally allowed to remain in the vapour of the aqueous phase for one hour before the apparatus was charged with the other phase, and the chromatograms were then run for an average time of 16—18 hours. The papers were dried at 100°, then developed. The following developing reagents were employed: ammoniacal silver nitrate (5 %), resorcinol (3 %) in alcoholic hydrogen chloride (5 %), and methyl orange (ordinary indicator solution). In the case of the first two reagents, the papers were heated at 110° after spraying; in the case of methyl orange the papers were dried for a prolonged period (1 hour) before spraying to remove residual acetic acid. The silver nitrate solution proved very successful as a developing reagent for gluconic acid in spite of the absence of reducing groups in the latter.

Table 2. Chromatographic experiments.

Substance chromatographed	Reagent	I	II	III	IV	V
Chlorinated methyl β-glucoside	$AgNO_3$	+	+	+	+	+
	Resorcinol	—	—	+	+	—
	Methyl orange	—	+	+	—	—
Chlorinated gluconic acid	$AgNO_3$	+	+	+	+	+
Chlorinated methyl α-glucoside	$AgNO_3$	—	(+)	—	—	(+)
Gluconic acid (authentic)	$AgNO_3$	—	+	—	—	+
5-Ketogluconic acid (authentic)	$AgNO_3$	—	—	+	+	—
	Resorcinol	—	—	+	+	—

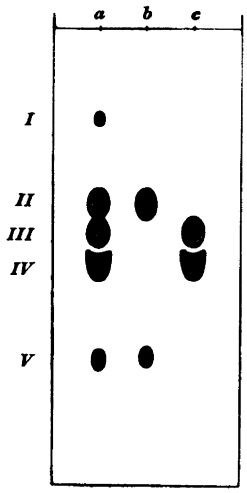

Fig. 1. Typical chromatograms.

a = Chlorinated methyl β-glucoside
b = Authentic gluconic acid
c = » 5-ketogluconic acid

The results of the chromatographic experiments are summarised in Table 2. In addition to the reference compounds mentioned in the table, glucuronic acid, saccharic acid, glucose and arabinose were also chromatographed simultaneously with the methyl β-glucoside mixture, but no indications of the presence of these substances in the latter were obtained. (The chromatograms were developed with ammoniacal silver nitrate in the case of the two acids, and with aniline hydrogen phthalate reagent in the case of the aldoses.)

Explanation of table. The Roman numerals refer to the five spots which appear on the chromatogram of oxidised methyl β-glucoside, as shown in the accompanying diagram (Fig. 1). Presence or absence of these spots on the other chromatograms is indicated by a positive or negative sign respectively; the two positive signs in brackets demote weak colourations.

SUMMARY

The action of chlorine water on methyl β-glucoside has been studied and it has been shown that the chief product is D-gluconic acid, which is slowly oxidised further to 5-ketogluconic acid.

Methyl α-glucoside also seems to undergo a similar reaction but at a very much slower rate.

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On the Accuracy of Moving-Bomb Calorimetry

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In bomb calorimetry it is usually possible to obtain a very high reproducibility within a series of determinations made with the same apparatus and in accordance with the same method. A comparison between values of heats of combustion as measured by different methods (apparatus) often reveals differences between the values obtained. We have determined heats of combustion of compounds containing halogens or sulfur using two different calorimeters both with moving bombs, one designed for the combustion of halogen compounds and the other for sulfur-containing substances. These two apparatuses have now been compared, using the same substances both for calibration (benzoic acid) and measuring experiments (paraffin oil). In this way we have been able to estimate the influence from such systematic errors, which are of different order of magnitude in the two apparatuses and which appear, when burning substances containing carbon, hydrogen and oxygen. Specific systematic errors arrising from the presence of halogen or sulfur are not possible to discover by a direct comparison and will not be treated in this communication.

There is also a particular reason for a comparison of the two methods. In accordance with Washburn's suggestion [1], the Permanent Committee on Thermochemistry of the International Union of Chemistry recommended the use of standardized experimental conditions [2]. When burning halogen- or sulfur-containing substances, one essential factor has to be changed, *viz.* the amount of liquid introduced into the bomb. The Committee recommends 3 ml of water per litre of bomb volume; for halogen compounds we have been forced to use between 70—110 ml and for sulfur compounds 35 ml per litre of bomb volume. Therefore, in the treatment of the Washburn corrections the heat of solution of carbon dioxide becomes of paramount importance. This correction has to be calculated indirectly. This way of calculation together with the difference in magnitude of the CO_2-correction for the different methods

rendered it especially desirable to compare corrected values of the heat of combustion obtained by the two methods on the same sample of a suitable substance.

APPARATUS AND METHOD

The accurate determination of heats of combustion of compounds containing halogen or sulfur requires a change in construction of the usual type of bomb calorimeter. The main reason for this change is that the final state, after the combustion is completed, has to be uniform, *i. e.* thermodynamically defined, otherwise it is not possible to compute the heat of combustion under standard conditions.

Chlorine-containing compounds give about 20—30 % free chlorine and 70—80 % hydrogen chloride as combustion products. By the method of Berthelot a solution of arsenic trioxide is introduced into the bomb and the chlorine will be reduced to HCl, so that the final state will be made up of a diluted hydrochloric acid containing hydrated arsenic trioxide and pentoxide.

However, the reduction could not be quantitative until Smith introduced the quartz-wool method [3]. Here, the surface between the liquid and gaseous phase is greatly increased and the reduction is rapid and complete. In stationary combustion bombs the concentration of the HCl will be different in different parts of the bomb.

Substances containing bromine give about 96 % free bromine and 4 % HBr. Owing to the great quantity of free bromine present, the quartz-wool method is not suitable for the complete reduction of the bromine in this case, and bromo-compounds have hitherto not been burned with modern precision in stationary-bomb systems.

Organo-sulfur substances give sulfuric acid of different concentration in different parts of the bomb. For calorimeters with stationary bombs this causes an appreciable error due to the great heat of dilution of sulfuric acid.

Calorimeters with rotating bomb systems will overcome the difficulty resulting from ill-defined final states. Several such calorimeters have been constructed (Popov and Shirokich [4], Smith and Sunner [5], Sunner [6], Smith and Bjellerup [7], Sunner [8]). Common to all of them is the fact that the bomb liquid will wash the inside of the bomb more or less thoroughly and that they must work with rather much liquid in the bomb.

A uniform final state is reached and the heat evolved, $-\Delta E_B$, when the reaction between the following initial and final states takes place, may be determined with a reproducibility of about 0.01 %:

A. Actual bomb process:

I. *Initial state* (at the initial temperature 20.00 °C):

1. The substance in liquid or solid state under a pressure of 30 atm of oxygen.

2. Bomb liquid, which is water (organo-sulfur substances) or a solution of arsenic trioxide in water (for halogen compounds) under a pressure of 30 atm of oxygen. The gas-phase is saturated with water vapor.

3. Oxygen, compressed to 30 atm. The bomb liquid is saturated with oxygen.

II. *Final state* (at the final temperature, 21.00 or 21.35° C):

1. The gas-phase contains the excess of oxygen, part of the carbon dioxide and water vapor.

2. The liquid phase is composed of a diluted sulfuric or halogen acid with dissolved carbon dioxide and oxygen in equilibrium with the gases at their respective partial pressures in the gas phase. In experiments with halogen-containing substances the bomb liquid will also contain a mixture of arsenious and arsenic acids. However, the thermochemically most valuable reaction takes place between the following states (the heat of combustion with the substances in their standard states, $-\Delta Hc^{\circ}_{293.16.}$).

B. Idealized process (occuring at a constant temperature of 20.00 °C and at a constant pressure of 1 atm of both reactants and reaction products):

$$C_aH_bO_cX_d \text{ (sol or liq)} + (a + b/4 - c/2)\ O_2 \text{ (gas)} \rightarrow$$
$$\rightarrow aCO_2 \text{ (gas)} + b/2\ H_2O \text{ (liq)} + d/2\ X_2 \text{ (liq or gas)};$$
$$C_aH_bO_cS_d \text{ (sol or liq)} + (a + b/4 - c/2)\ O_2 \text{ (gas)} \rightarrow$$
$$\rightarrow aCO_2 \text{ (gas)} + b/2\ H_2O \text{ (liq)} + dS \text{ (rhomb.)}$$

In order to transform process A into process B the following corrections must be made:

1. The Washburn corrections. Washburn calculated the different corrections which have to be applied for the transformation of the actual bomb process into the idealized process for substances containing carbon, hydrogen and oxygen [2].

For compounds containing halogen or sulfur in addition, it is possible to recalculate the Washburn corrections to a final state consisting of pure carbon dioxide at 1 atm pressure and a diluted acid (halogen or sulfuric acid) of the actual concentration under 1 atm pressure.

2. Corrections for the heat of formation of the diluted acid from free halogen or sulfur, water and oxygen according to the equations:

$(n+1)\ H_2O\ (liq) + X_2\ (gas\ or\ liq) \rightarrow 2HX,\ nH_2O\ (liq) + 1/2\ O_2\ (gas)$
$(n+1)\ H_2O\ (liq) + S\ (rhomb) + 3/2\ O_2\ (gas) \rightarrow H_2SO_4,\ nH_2O\ (liq)$

These corrections are calculated either from combustion experiments (sulfur, Sunner [8]) or from data in the literature (halogens). A detailed calculation of the corrections applied for sulfur compounds is given elsewhere (Sunner [8]).

When burning substances containing halogen or sulfur, about 10 to 30 times as much bomb liquid has been used, as recommended by the Permanent Committee on Thermochemistry. (The amount of substance and the oxygen pressure may be chosen according to the recommendations.) The recalculation of the Washburn corrections to the actual conditions valid for compounds containing halogen or sulfur, shows that the heat of solution of carbon dioxide is by far the most important correction term. With 10 ml of bomb liquid in a bomb of 280 ml volume, this correction lies between 7 and 5 cal and with 30 ml of bomb liquid the correction lies between 19 and 14 cal depending on the amount of carbon dioxide formed during the combustion.

Therefore, it was found conveniant to treat this correction separately. The solubility of carbon dioxide in liquids of proper compositions was determined in a range of conditions actually found in the combustion experiments and at different temperatures. The molar heat of solution of carbon dioxide was calculated from the solubility data at different temperatures. The weight of substance burned gave the number of moles of carbon dioxide formed, and from this value the pressure was deduced. The amount of carbon dioxide dissolved was calculated and thus the heat of solution was found.

The accuracy of the calculation of the heat of solution is difficult to estimate. However, the fact that, when burning bromo-compounds we use 30 ml of bomb liquid and, when burning sulfur compounds, we use only 10 ml of liquid in the bomb made it possible to check the usefulness of the correction method.

The magnitude of the correction depends on the amount of carbon in the substances burned and the amount of liquid in the bomb. The carbon content ranges between 50 milliatoms of carbon (aromatic compounds) down to about 30 milliatoms (pure alifatic substances). The heat equivalents of our calorimeters have been determined by burning benzoic acid which has a high carbon content. The correction for dissolved carbon dioxide has thus a high value. We have then burned the same paraffin oil corresponding to about 30 milliatoms of carbon. Here the correction is about 75 % of the correction in the case of benzoic acid. If the calculations of the corrections for the heat of

Table 1.

Investigator	L. B. (L. Bjellerup)	S. S. (S. Sunner)
Apparatus	A modification of the apparatus described by Smith and Bjellerup [7] *)	Described by Sunner [8]
Temperature of the environment	Constant 21.3 ± 0.001 °C	Constant 21.6 ± 0.001 °C
Bomb	Two-parted, Pt-lined 275 ml volume	Two-parted, Pt-lined 282 ml volume
Bomb liquid	30 ml 0.062-molar As_2O_3	10 ml pure water
Temperature reading system	See Sunner [8]	ditto
Temperature increase	20.00 – 21.00 °C	20.00 – 21.35 °C
Length of main period	21 min.	10 min.
Heat equivalent	4 700 cal/°C	3 900 cal/°C
Correction for the formation of HNO_3	1 – 2 cal	7 – 9 cal
Heat exchange correction in % of the total heat evolved	1.1	0.8
The isothermal combustion given at	20.00 °C	20.00 °C
Heat of solution of carbon dioxide per mole of CO_2 in N normal acid (HBr or H_2SO_4)	4 600 cal 0 to 0.15 N HBr 0.25 to 0.10 N As_2O_3 0 to 0.15 N As_2O_5	4 550 cal 0 to 1.2 N H_2SO_4
Solubility of carbon dioxide in mole per ml of bomb liquid and per atm pressure	3.72×10^{-5} 0 to 0.15 N HBr 0.25 to 0.10 N As_2O_3 0 to 0.15 N As_2O_5 at 21.0_0 °C	$(3.75 - 0.35n + 0.07n^2) \times 10^{-5}$ $0 \leq n \leq 1.2$ at 21.3_5 °C

* A detailed description will be published later.

solution of carbon dioxide were significantly erroneous, the heats of combustion of the paraffin oil obtained with the two methods should be different.

The desired accuracy of the heats of combustion, 1 part in 10 000, will be diminished if the systematic errors in the heat of solution of carbon dioxide exceed about 10 %. A random error of 1 % gives rise to a discrepancy in the heat of combustion of paraffin oil of about 3 parts in 10 000.

It has, of course, not been possible to check the two methods against each other by burning compounds containing bromine or sulfur. However, the solubility of carbon dioxide in diluted sulfuric acid or diluted hydrobromic acid in arsenious-arsenic acids is about the same as in pure water and arsenious acid respectively. It therefore seems probable, that errors introduced with bromine or sulfur are negligible regarding the over-all accuracy of the correction in question.

The two methods are compared in Table 1.

In the halogen-apparatus the inner lid is made of ebony: this explains why the main period is so long as 21 minutes. The quantity of nitric acid formed is several times greater in the sulfur-apparatus. The presence of a certain amount of nitrogen is necessary when burning sulfur-containing substances otherwise the oxidation to sulfuric acid will not be complete. The gas phase therefore contains 2.6 % nitrogen in this case, which explains the rather high HNO_3-correction.

Both calorimeters were placed in the same room with constant temperature, 21.3 °C. They were operated independently, one by Bjellerup (for halogen) and the second by Sunner (for sulfur).

EXPERIMENTAL

Table 2 gives data on the combustion of benzoic acid and paraffin oil. The determinations in Table 3 and 4 (L. B.) were done between August 1949 and September 1950 and the determinations in Table 5 and 6 were completed in February 1949 (from Sunner[8]; for paraffin oil the values had to be recalculated owing to the use of an erroneous value of the density of the oil).

The amounts of benzoic acid differ, due to different net bomb volumes, but are in accordance with the recommendation given by the Permanent Committee on Thermochemistry. The results in Tables 4 and 6 are in very good agreement, better than 1 part in 10 000. The standard deviation of the mean is calculated to 0.7 cal (L. B.) and to 0.4 cal (S. S.) and the difference between the paraffin oil values is thus negligible.

Table 2.

Investigator	L. B.	S. S.
Benzoic acid	N. B. S. lot 39 f	The same sample
Amount of benzoic acid	734 mg	822 mg
Amount of carbon in the benzoic acid	42 milliatoms	47 milliatoms
Correction for the heat of solution of carbon dioxide	19.2 cal	6.9 cal
Washburn corrections * (Wc)	2.5 cal	3.3 cal
Paraffin oil	b. p. $\geq$ 150 °C at 0.3 mm Hg	The same sample
Amount of paraffin oil	425 mg	474 mg
Amount of carbon in the paraffin oil	30 milliatoms	33 milliatoms
Correction for the heat of solution of carbon dioxide	14.0 cal	5.1 cal
Washburn corrections * (Wc)	0.5 cal	0.8 cal

* Exclusive the correction for dissolved carbon dioxide.

Table 3. Benzoic acid. (L. B.)

$S_F = 0.4$ cal/I. U.*

Determination Nr.	Mass of sample mg	ΔT (corr) I. U.	Corr. ** cal	Wc cal	CO_2 cal	S_B^0 cal/I. U.
135	734.58	0.99413	20.5	2.5	19.2	4707.7
137	733.19	0.99202	19.9	2.5	19.2	4708.3
136	734.04	0.99289	19.0	2.5	19.2	4708.7
140	734.50	0.99384	20.8	2.5	19.2	4708.9
139	735.08	0.99438	20.5	2.5	19.2	4709.7
138	733.40	0.99144	17.6	2.5	19.2	4710.0

Mean: 4 708.9 cal/I. U.

Table 4. Paraffin oil. (L. B.)

Determination Nr.	Mass of sample mg	$S_B + S_F$ cal/I. U.	ΔT (corr) I. U.	Corr. ** cal	Wc cal	CO_2 cal	$-\Delta Ec^0_{293.16}$ cal/g
132	424.65	4709.7	0.99644	18.5	0.5	13.9	10 973.7
219	424.74	4709.6	0.99711	20.6	0.5	13.9	10 973.8
217	424.28	4707.5	0.99652	20.4	0.5	13.9	10 974.6
133	425.16	4709.7	0.99824	20.2	0.5	14.0	10 976.3
134	424.91	4709.7	0.99709	17.3	0.5	13.9	10 977.1

Mean: 10 975.1 cal/g

* I. U. = interval unit = temp.diff. between the 1.97 and 2.97 scale divisions on the Beckmann thermometer.

** Heat of combustion of cotton thread and heat of formation of HNO_3.

Table 5. Benzoic acid. (S. S.)
S_F = 0.6 cal/I. U. *

Determination Nr.	Mass of sample mg	ΔT (corr.) I. U.	Corr. ** cal	Wc cal	CO_2 cal	S^0_B cal/I.U.
10	821.16	0.99493	19.7	3.3	6.9	5 246.2
7	822.53	0.99526	18.6	3.3	6.9	5 246.8
9	822.63	0.99530	18.3	3.3	6.9	5 246.9
5	823.28	0.99632	19.6	3.3	6.9	5 247.0
8	822.58	0.99543	19.5	3.3	6.9	5 247.1

Mean: 5 246.8 cal/I. U.

Table 6. Paraffin oil. (S. S.)
$S_B + S_F$ = 5 247.6 cal/I. U. *

Determination Nr.	Mass of sample	ΔT (corr.) I. U.	Corr. ** cal	Wc cal	CO_2 cal	$-\Delta Ec^0_{293.16}$ cal/g
5	473.56	0.99536	19.8	0.8	5.1	10 975.3
3	473.50	0.99553	21.3	0.8	5.1	10 975.4
4	473.63	0.99573	20.9	0.8	5.1	10 975.6
2	474.48	0.99750	20.6	0.8	5.1	10 976.2
6	473.86	0.99560	17.0	0.8	5.1	10 977.0

Mean: 10 975.9 cal per g mass ***.

* I. U. = interval unit = temp.diff. between the 0.75 and 2.10 scale divisions.

** Heat of combustion of cotton thread, heat of formation of HNO_3 and HNO_2.

*** A redetermination of the heat of combustion of the same paraffin oil with this calorimeter gave a mean of 10 975.6 cal per g mass (Mr B. Lundin).

SUMMARY

When using moving-bomb calorimeters for the determination of heats of combustion for substances containing halogen or sulfur, in addition to carbon, hydrogen and oxygen, two kinds of systematic errors will appear: one of a specific character depending on the presence of halogen or sulfur, and another of a general character which will cause erroneous results for all substances burned. The practical importance of the last type of errors in methods currently used in this laboratory has been investigated by comparing results from two different, independently operated moving-bomb calorimeters. The heat-equivalents were determined with N.B.S. benzoic acid, and paraffin oil was used for comparison. The mean values of the heat of combustion of the paraffin oil were found to be 10 975.1 and 10 975.9 cal per g mass. The difference is less than the sum of the standard deviations of each series, 1.1 cal. It seems therefore justified to assume that general systematic errors do not influence the accuracy of our methods.

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Physikalisch-chemische Messungen an Poly-Methylphenylenen

(Vorläufige Mitteilung)

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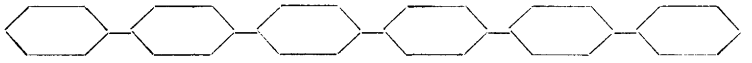
Polyphenylene sind Substanzen die aus verknüpften Benzolringen bestehen Solche Moleküle können die allgemeine Form wie z. B. das



p-Sexiphenyl

haben [1].

Die Benzolringe sind lediglich um die vorhandenen Einfachbindungen also bei *p*-Polyphenylenen in der Längsachse des ganzen Moleküls drehbar.

Gemäss der heute allgemein geltenden Meinung (Vorländer, Kuhn [2,3]) sind diese *p*-Phenylketten stäbchenförmige Moleküle, deren Benzolringe nicht in einem Winkel zueinander stehen, sondern geradlinig angeordnet sind.

Es gelang bisher auf Grund verschiedener Reaktionen solche Produkte, die etwa 7—8 Benzolkerne in *p*-Stellung [4] enthalten und andere bei denen bis 16 Ringe in *m*-Stellung [4] verknüpft sind, zu isolieren und gut zu definieren. Goldfinger [5] findet bei der Einwirkung von metallischem Natrium auf *p*-Dichlorbenzol Gemische von Polyphenylenen die nach kryoskopischen Messungen im Mittel etwa bis 34 Ringe enthalten und vorwiegend in *p*-Stellung verknüpft sein sollen.

Alle derartigen Produkte konnten aber infolge ihrer äusserst geringen Löslichkeit bisher nur sehr wenig untersucht werden.

Durch Einführung seitenständiger Methylgruppen gelang es nun Kern und Gehm [6,7] lösliche Polyphenylene herzustellen. Sie kondensierten 4,4′-Dijod-3,3′-dimethyl-diphenyl nach der Ullmann'schen Reaktion mit Kupfer.

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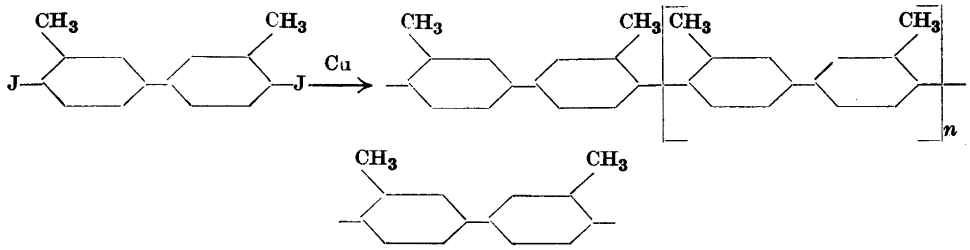


Auf Grund dieser Reaktionsgleichung sollten diese neuen Substanzen die eingangs angegebene lineare Konstitution haben. Es handelt sich um Moleküle mit in *p*-Stellung verknüpften Benzolkernen, die löslich sind, weil jeder Benzolring eine Methylgruppe trägt. Für diese neuen Substanzen wurde der Name Poly-Methylphenylene vorgeschlagen.

Solche von Kern und Gehm in Mainz hergestellten Poly-Methylphenylene wurden am hiesigen Institut mit den für makromolekulare Substanzen hier üblichen Methoden untersucht. Aus dem aus dem Reaktionsgemisch ursprünglich mit Anisol (Kp 155° C) extrahierten Produkt wurde durch Suspendieren in Benzol (40° C) der darin lösliche Anteil (88 %) gewonnen. Dieser wurde durch Ausfällen mit Methanol aus benzolischer Lösung in sieben gröbere Fraktionen (F_1—F_7) zerlegt.

Orientierende Diffusions- und Sedimentationsmessungen in verschiedenen Lösungsmitteln (Benzol, Toluol u. Brombenzol) zeigten, dass die höchstmolekulare Fraktion F_1 Verbindungen enthält, die geeignet sind, mit Hilfe der Ultrazentrifuge näher studiert zu werden.

Diese erwähnte Fraktion F_1 wurde daher, um sie weiter und schärfer zu unterteilen, in Benzol von 40° C gelöst (1 g/300 ml). Bei dieser Temperatur wurde unter lebhaftem Umrühren langsam Methanol (50 ml) bis zur ersten auftretenden Trübung zugegeben. Dabei hatte sich die Temperatur auf etwa 35° C erniedrigt. Eine abermalige Erwärmung auf 40° C liess die Lösung wieder klar werden. Beim langsamen Abkühlen auf Zimmertemperatur schied sich ein Teil des Polymeren klebrig aus und konnte durch Zentrifugieren von der überstehenden klaren, braunen Lösung leicht getrennt werden. Abermaliges Auflösen in Benzol und Eingiessen in etwa die 7—8 fache Menge Methanol liess den Niederschlag, Fraktion S_1, flockig und gut zentrifugierbar ausfallen. Weitere sechs Fraktionen (S_2—S_7) wurden auf diese Art gewonnen und umgefällt. Der Methanolzusatz betrug jeweils 12—20 ml. Die letzte Fraktion S_8 wurde durch Eindampfen und Eingiessen der konzentrierten Lösung in die 10-fache Menge Methanol erhalten.

Alle Fraktionen sind jodhaltig. Die höhermolekularen liefern braune benzolische Lösungen, während die niedrigsten Fraktionen sich mit gelber Farbe lösen.

Kondensiert man das Ausgangsmaterial nach der Wurtz-Fittigschen Synthese mit Natrium in Toluol (Kern und Gehm), so erhält man Produkte, die sich wesentlich von den mit Kupferpulver hergestellten Substanzen unterscheiden. Der Jodgehalt des benzollöslichen Gemisches ist höher als der des nach Ullmann hergestellten unfraktionierten Präparates.

Diese mit Natrium hergestellten Verbindungen wurden aus Benzol mit Methanol grob in fünf Fraktionen (W-F 1 bis W-F 5) unterteilt, von denen nur drei näher untersucht wurden. Die Fraktionen unterscheiden sich hinsichtlich ihrer Schmelzbereiche von einander und schmelzen allgemein tiefer als die mit Kupfer kondensierten Produkte (Tabelle 5. u. 6.).

I. PHYSIKALISCH-CHEMISCHE MESSUNGEN AN MIT KUPFER KONDENSIERTEN POLY-METHYLPHENYLENEN

A. Diffusions- und Sedimentationsmessungen

a. *Diffusionsmessungen*

Die Diffusionsmessungen wurden in einer Claesson-Zelle [8] unter Anwendung von Lamm's Skalenmethode [9] vorgenommen. Als Lösungsmittel wurde Benzol oder Toluol verwendet, die Temperatur auf 20 ± 0,05° C gehalten. Die Diffusionskonstanten D_A und

Tabelle 1. Diffusions- und Viskositätsmessungen an einzelnen Fraktionen der S- und der F-Reihe.

Fraktion	In Benzol D_m 10^7	In Benzol D_A cm²sec⁻¹	In Toluol D_m 10^7	In Toluol D_A cm²sec⁻¹	g/100 ml	$[\eta]$	Schmilzt zwischen °C
Unfraktioniert	36,8	24,0		26,8 *		0,100	150–180
S1			3,52	2,74		0,396	
F1	5,82	5,06		6,5 *	0,54	~ 0,27	280–330
	5,90	5,90			0,27		
S3			8,38	7,02	0,25	0,264	
				6,88	0,25		
F2	18,9	18,1	20,5	20,4		0,135	240–270
				20,1 *			
F3	28,9	28,3		32,1 *	0,55		200–235
	29,0	29,1			0,275		
F5	38,4	40,2		44,6 *		0,053	170–190
F7	80,8	74,0		82,2 *			schmierig
Ausgangs-material	93,9	97,0		110 *			

* Auf Toluol berechnet.

Tabelle 2. Physikalisch-chemische Messungen an den Fractionen S1—S8.

Fraktion	mg	D_m 10^7 cm^2	D_A sec^{-1}	s°_{20} in S	M	$[\eta]$	f/f_0
S1	71	3,52	2,74	8,25	270 000	0,396	2,97
S2	152	5,34	5,11	4,40	77 200	0,316	2,42
S3	161	8,38	7,02	(2,87)	(36 700)	0,264	2,26
S4	135	9,41	8,99	2,35	23 400		2,04
S5	108	11,0	10,4	1,73	(15 800)	0,192	2,06
S6	63		11,1	1,55	12 600		2,04
S7	75	12,5	12,7	1,36	9 300		1,94
S8	137	18,5	17,6	1,10	5 600	0,139	1,68

D_m, nach der Flächen- und Momentmethode berechnet, sind in Tabelle 1. angegeben. Man erkennt, dass das ursprüngliche Reaktionsprodukt sehr inhomogen war, speziell wenn man beachtet, dass das Ausgangsmaterial, 4,4'-Dijod-3,3'-dimethyl-diphenyl, mit einem Molekulargewicht von 434 durch ein D_A von 110 gekennzeichnet ist.

An Fraktion S 3 wird gezeigt, dass die Reproduzierbarkeit der Diffusionsmessungen recht gut ist, besonders wenn man bedenkt, dass die Substanz noch inhomogen ist, was die Bestimmung unsicher macht. Die Konzentrationsabhängigkeit der Diffusionskonstanten ist nicht gross genug, um hier erkannt zu werden (F 1 und F 3).

b. *Sedimentationsmessungen*

Die Sedimentationsmessungen wurden in der Ultrazentrifuge nach Svedberg ausgeführt [10]. Die Werte der Sedimentationskonstanten s° sind, auf 20° C bezogen, angegeben (Tabelle 2. Spalte 5.) *.

Arbeitet man mit Toluollösungen so erhält man, wie aus dieser Tabelle hervorgeht, Sedimentationskonstanten von über 8,25 bis 1,10 für Fraktionen deren D_A zwischen $2{,}74 \times 10^{-7}$ und $17{,}6 \times 10^{-7}$ liegt.

Bei Messungen an Fraktion F_3 mit $D_A = 32 \times 10^{-7}$ liess sich kein Sedimentationsdiagramm erhalten.

Die Konzentrationsabhängigkeit von s_{20} ist, wie die Kurven (Abb. 1.) zeigen, nur bei den höhermolekularen Fraktionen beträchtlich. s°_{20} ist auf die übliche Weise durch Extrapolieren dieser Kurven ermittelt. (Bei Fraktion S_1 liegt s°_{20} sicherlich höher als der durch die lineare Extrapolation gefundene Wert 8,25. Leider liess er sich wegen des geringen Dichteunterschiedes zwischen Lösung und Lösungsmittel und der damit verbundenen Störungen durch Konvektionen nicht genauer festlegen.)

c. *Partielles spezifisches Volumen*

Das partielle spezifische Volumen wurde in Toluol bei 25 ± 0,05° C mit einem Ostwald Pyknometer bestimmt. Zur Berechnung diente die von Lewis und Randall angegebene Methode [10] (Gleichung 109). Die Messungen wurden an zwei verschiedenen Fraktionen F_2 und F_6 vorgenommen. Die spezifischen Volumina beider gelösten Substanzen sind innerhalb der Messgenauigkeit bei gegebener Konzentration gleich. Das partielle

* Alle Sedimentationskonstanten sind in Svedberg-Einheiten angegeben, $1\ S = 10^{-13}$ c.g.s.

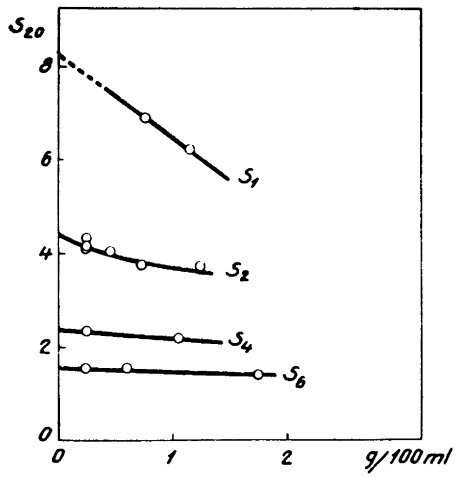


Abb. 1. Die Sedimentationskonstanten in Abhängigkeit von der Konzentration.

spezifische Volumen ist somit praktisch unabhängig vom Molekulargewicht. Sein Wert beträgt 0,841 $\pm$ 0,006 $cm^3\ g^{-1}$.

d. *Molekulargewichte*

Die Molekulargewichte wurden mit Hilfe der Svedbergschen Formel

$$M = RTs^\circ/D\ (1-\overline{V}\ \varrho)$$

ermittelt. Zur Berechnung für die Fraktionen S_3, S_5, S_7 und S_8 wurde aus Zeitmangel die bei C = 0,25 g/100 ml gefundenen Werte der Sedimentationskonstanten verwendet. Die konzentrationsabhängigkeit von s_{20} ist bei diesen Fraktionen, wie die Abb. 1. zeigt, nicht sehr wesentlich. Abb. 2. gibt den Zusammenhang von s°_{20} mit dem Molekulargewicht an.

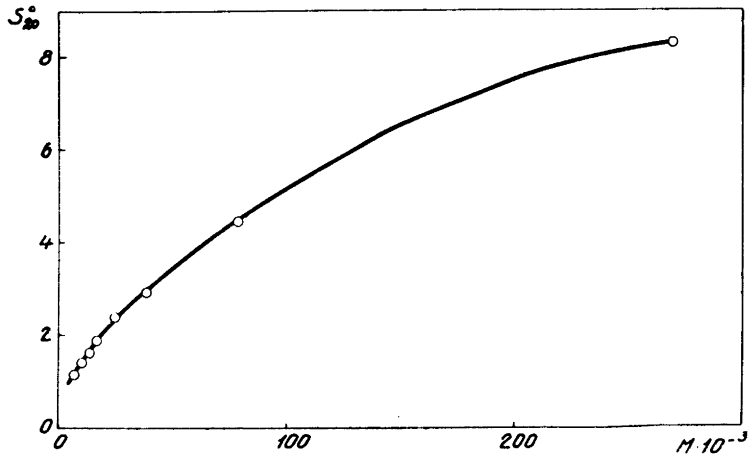


Abb. 2. s°_{20} in Abhängigkeit vom Molekulargewicht.

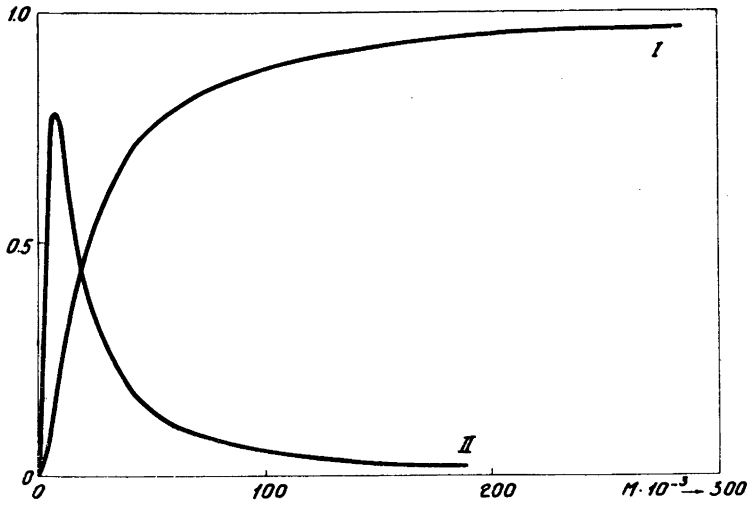


Abb. 3. Fraktionierung der Fraktion F_1.
I. Integraleverteilungsfunktion;
II. Massenverteilungsfunktion.

Die Integrale- und die Massenverteilungsfunktion, die sich mit Hilfe dieser Molekulargewichte aus der oben geschilderten Fraktionierung von F_1 ergeben, sind in Abb. 3. dargestellt. Man erkennt wie uneinheitlich diese Spitzenfraktion war.

B. Osmotische Messungen

Die osmotischen Messungen wurden mittels eines einfachen Osmometers nach Schulz [11] durchgeführt. Als Lösungsmittel diente Toluol. Die Membran bestand aus Zellulose und wurde mit Hilfe von Aceton entwässert. Die Versuchstemperatur betrug 20 ± 0,05° C.

Die Konzentrationsbestimmung der Lösung erfolgte nach beendeter Messung durch Eindunsten einer gewogenen Menge Lösung und nachfolgender Vakuumtrocknung des Rückstandes bis zur Gewichtskonstanz. Bei der Berechnung wurde das oben angegebene partielle spez. Volumen angewandt.

Aus der Abb. 4. erkennt man die Abhängigkeit des osmotischen Druckes von der Konzentration. Aus diesen Kurven ergeben sich die μ-Werte [12] der Tabelle 3.; man erkennt daraus, dass Toluol kein gutes Lösungsmittel ist.

Ein Vergleich der osmotisch gefundenen Molekulargewichte mit den ultrazentrifugalen Messungen ist in Tabelle 3. gegeben. Die Werte wurden mit den extrapolierten Grössen π/C und s_{20} für $C \to 0$ berechnet. Die Übereinstimmung beider Messungen ist gut, besonders wenn man bedenkt, dass die Fraktionen noch recht polymolekular sind.

C. Viskositätsmessungen

Mit einem Ostwaldviskosimeter wurden in Toluol bei 20 ± 0,05° C die Viskositäten bestimmt. (Auslaufzeit für Toluol 228,1 sec.) Die Messungen wurden in einem Konzentrationsbereich von 1 g/100 ml bis 0,075 g/100 ml vorgenommen und die Eigenviskosität $[\eta]$ auf die übliche Weise durch Extrapolieren festgelegt.

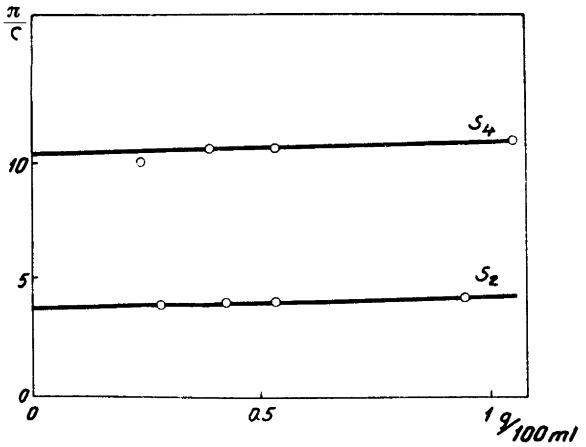


Abb. 4. π/C in Abhängigkeit von der Konzentration in Toluol bei 20 ± 0,05° C.

Tabelle 3. Vergleich der nach den verschiedenen Methoden gefundenen Molekulargewichte.

Fraktion	$M_{osm.}$	$M_{sed.}$	μ
S2	64 000	77 200	0,47
S4	23 200	23 400	0,47

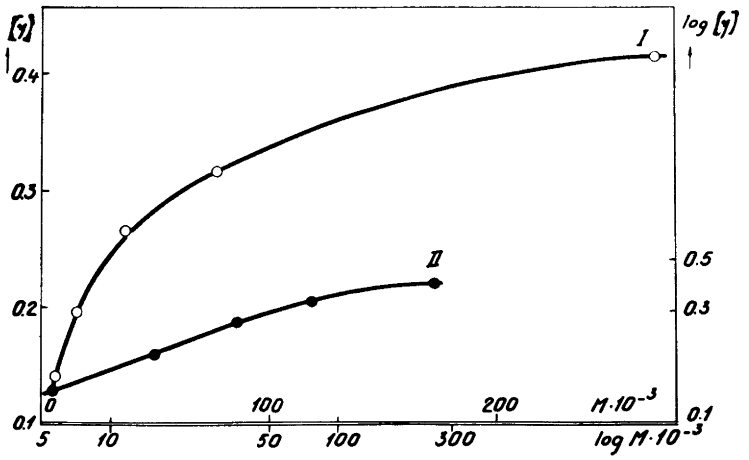


Abb. 5. Die Eigenviskosität $[\eta]$ in Abhängigkeit vom Molekulargewicht.
I. in linearer und
II. in log/log Darstellung.

Abb. 5. zeigt den Zusammenhang zwischen $[\eta]$ und dem Molekulargewicht (U Z-Messungen) in linearer und log/log Darstellung.

D. Absorptionsmessung in U. V. Licht

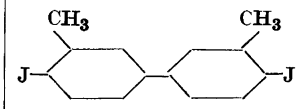
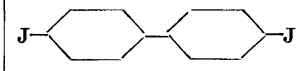
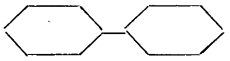
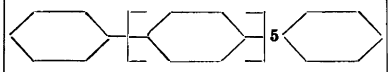
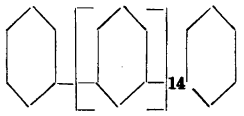
Goldfinger [5] findet für seine in der Einleitung erwähnten Produkte eine starke Absorption bei 3 000 Å.

Misst man die U. V.-Absorption an verschiedenen Fraktionen von Poly-Methylphenylenen (Beckman Photometer Modell D U) in Chloroform, so erhält man Kurven, die über den gemessenen Bereich von 2 400 bis 4 000 Å je ein Maximum bei verschiedener Wellenlänge zeigen.

Die Tabelle 4. gibt die gefundenen und einige der Literatur entnommenen Werte wieder. Es zeigt sich bei den mit Kupfer kondensierten Produkten ein deutlicher Unterschied mit der Kettenlänge. Der Einfluss der Methylgruppen auf die Absorption an Diphenylderivaten ist nicht sehr erheblich.

Eine Diskussion dieser Erscheinungen soll, bis weitere Messungen vorliegen, aufgeschoben werden.

Tabelle 4. Absorptionsmaxima der U. V. Spektren von Poly-Methylphenylenen und Polyphenylenen in Chloroform.

Substanz	D_A 10^7 cm^2 sec^{-1}	Molekul. gew.	Abs.Maxima Å	Autor
F4	~ 35	~ 3 000	2 740	
S1	2,7	270 000	2 790	
	110		2 720	
			2 725	
			2 520	
			2 515	(13)
Polyph. n. Goldfinger		2 700	3 000 *	(5)
		458	3 175	(13)
		1 218	2 550	(13)

* Lösungsmittel unbekannt.

II. PHYSIKALISCH-CHEMISCHE MESSUNGEN AN MIT NATRIUM KONDENSIERTEN POLY-METHYLPHENYLENEN

Die Untersuchungen an den mit Hilfe von Natrium kondensierten Produkten wurden analog der oben erwähnten Messungen ausgeführt. Die D_A Konstanten sind in Tabelle 5. Spalte 5 angegeben. Die Änderung von D_A mit der Konzentration ist auch hier nicht gross genug, um erkannt zu werden (W-F 1). Eine Konzentrationsabhängigkeit von s_{20} (zwischen 0,5 und 2,6 g/100 ml) liess sich nicht beobachten, (Tabelle 5. Spalte 7 und 8). Zur Berechnung des Molekulargewichtes (Tabelle 6. (W-F 1)) mit Hilfe der Diffusions- und Sedimentationskonstanten diente das oben angegebene partielle spezifische Volumen.

Vergleicht man die nach beiden Methoden erhaltenen Produkte (Tabelle 6.), so erkennt man, dass das mit Natrium kondensierte Produkt W-F 1 bei gleichem D_A durch ein grösseres s_{20}, d. h. grösseres Molekulargewicht und eine wesentlich verringerte Viskosität gekennzeichnet ist. Der niedrige $[\eta]$-Wert wird vermutlich auf Verzweigung der Moleküle zurückzuführen sein; auch der tiefere Schmelzbereich der ganzen W-F-Reihe kann so erklärt werden.

Die Absorptionsmaxima dieser Produkte im ultravioletten Licht liegen für W-F 1 bei 2 680 Å und für W-F 5 bei 2 670 Å; d. h. auch hier erhält man andere Werte, als sie für mit Kupfer kondensierten Substanzen gefunden wurden.

III. DISKUSSION

Aus den vorliegenden Messungen geht hervor, dass die untersuchten Substanzen Molekulargewichte zwischen 1 000 und 300 000 haben.

Die Frage nach der Gestalt der Moleküle in Lösungen muss vorerst dahingehend beantwortet werden, dass sie wohl nicht stäbchenförmige, starre

Tabelle 5. Physikalisch-chemische Messungen an mit Natrium kondensierten Poly-Methylphenylenen.

Fraktion	Schmilzt zwischen °C	$[\eta]$	Diffusionsmessungen in Toluol			Sedimentationsmessungen in Toluol	
			D_m	D_A	g/100ml	s_{20}	g/100ml
W-F-Gemisch	145—160	0,05	21,8	20,8	0,28		
W-F 1	180—230	0,05	13,6	11,9	0,294	2,18	0,59
			13,6	12,1	0,147	2,21	1,60
						2,22	2,62
W-F 3	150—185						
W-F 5	135—155			31,5	0,292		

Tabelle 6. Vergleich der mit Kupfer und der mit Natrium kondensierten Poly-Methylphenylene.

Substanz	Kond. Mittel	D_A	s°_{20}	M	$[\eta]$	Schmilzt zwischen °C
S5	Kupfer	10,4	1,83	15 800	0,192	
S7	Kupfer	12,7	1,36	9 300		
S8	Kupfer	17.6	1,10	5 600	0,139	250—280
W-F 1	Natrium	12,1	2,20	16 400	~ 0,05	180—230

Gebilde sind, wie die Reaktionsgleichung erwarten liesse und es das Ziel der organisch präparativen Arbeit war. Wendet man die Beziehungen

$$D_A \cdot M = K_1 \log M - K_2$$

und

$$s^\circ = K_3 \log M - K_4$$

wie sie Riseman und Kirkwood[14] für stäbchenförmige Moleküle ableiten, an, so erhält man, wie die Abb. 6. zeigt, keinen linearen Zusammenhang zwischen den gefundenen Grössen, sondern gekrümmte Kurven.

Bessere Übereinstimmung der Messungen mit der Theorie findet man für die W. Kuhn und H. Kuhn'sche Beziehung[15]

$$s^\circ = K_1 + K_2\sqrt{M}$$

und

$$1/D_A = K_3 + K_4\sqrt{M}$$

für »undurchspülte Knäuel» (Abb. 7.).

Die Poly-Methylphenylene sind allerdings keine normalen »Knäuel», denn die Eigenviskositäten steigen anders an, als bisher für geknäuelte Moleküle gefunden wurde (Abb. 5.). Die K' Werte, die sich aus der Konzentrationsabhängigkeit der Viskosität gemäss

$$\eta_{sp/c} = [\eta] + K' [\eta]^2 c \quad \text{(Huggins}^{16}\text{)}$$

errechnen lassen, liegen zwischen 0,89 und 1,3, was auf grosse Starrheit der Molekeln hindeutet. Aus den osmotischen Messungen könnte man auf Grund des μ-Wertes (Tabelle 3.) annehmen, dass Assoziation eingetreten ist. Auch

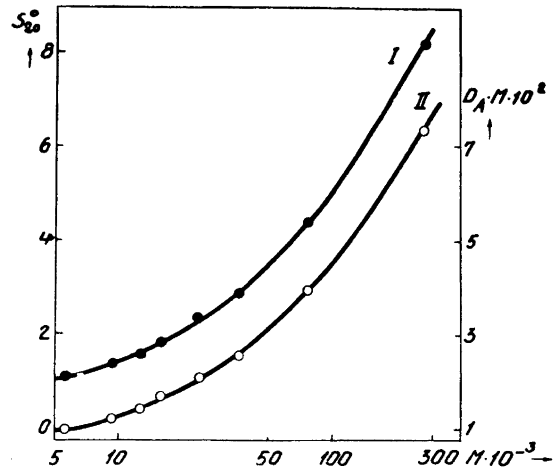


Abb. 6. I. s°_{20} in Abhängigkeit vom log M;
II. $D_A \cdot M$ in Abhängigkeit vom log M.

die ziemlich niedrigen f/f_0 Werte (Tabelle 2.) können auf Assoziation zurückgeführt werden. Diese assoziierten Teilchen müssten aber sehr stabil sein, denn zwischen 20° C und 60° C war die Temperaturabhängigkeit der Viskositätszahl nicht deutlich zu beobachten (Kern und Gehm [7]).

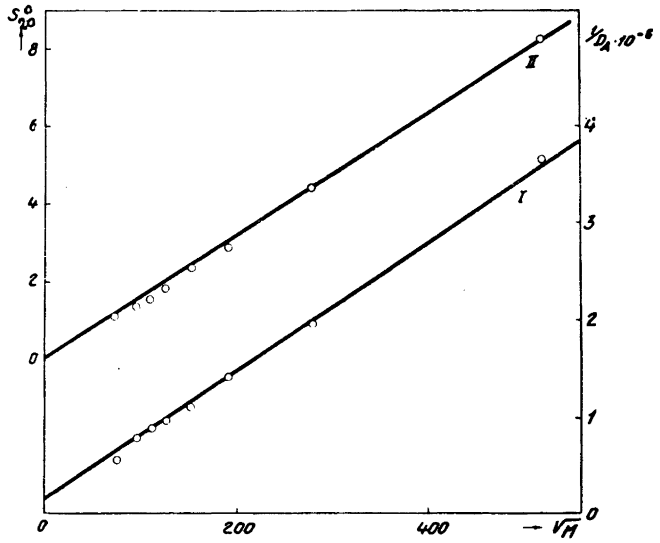


Abb. 7. I. Die Abhängigkeit von $1/D_A$ von $\sqrt{M}$.
II. Die Abhängigkeit von s°_{20} von $\sqrt{M}$.

Es ist also schwierig, eine definitive Aussage über die Gestalt dieser Moleküle oder Assoziate zu machen. Es ist auf Grund der Untersuchungen nicht unwahrscheinlich, dass die Makromoleküle der Poly-Methylphenylene, wie sie aus der angewandten Reaktion stammen, ziemlich starr aber nicht linear sind. Es könnte sich vielleicht um kompakte, verhakte Gebilde handeln. Eine endgültige Antwort auf diese Frage lässt sich erst dann geben, wenn besser fraktionierte und auch nach anderen Methoden hergestellte Poly-Methylphenylene eingehend untersucht sind, ein Problem, das zur Zeit weiter bearbeitet wird.

ZUSAMMENFASSUNG

Diffusions- und Sedimentationsmessungen an fraktionierten, benzollöslichen Poly-Methylphenylenen, die nach der Ullmannschen Reaktion durch Kondensation von 4,4′-Dijod-3,3′-dimethyl-diphenyl mit Kupferpulver hergestellt wurden, ergeben, dass das Reaktionsgemisch Substanzen vom Molekulargewicht etwa 1 000 bis 300 000 enthält. Die aus diesen Untersuchungen resultierenden Molekulargewichte wurden durch osmotische Messungen bestätigt.

Die Beziehung zwischen dem Molekulargewicht und den Sedimentations- sowie Diffusionskonstanten, sowie die Ergebnisse der Viskositätsmessungen sind nicht leicht zu erklären und lassen vermuten, dass die untersuchten Makromoleküle nicht lineare stabförmige Gebilde sind, sondern vielleicht recht starre Knäuel.

Poly-Methylphenylene, die nach der Würtz-Fittigschen Synthese mit Natrium hergestellt sind, unterscheiden sich wesentlich von den nach Ullmann kondensierten Produkten.

Der Verfasser möchte nicht versäumen, dem Vorsteher des Institutes, Herrn Professor Stig Claesson, für die ihm gebotene Gelegenheit, diese Arbeit unter seiner Anleitung hier ausführen zu können, vielmals zu danken. Tekn. lic. Kirsti Granath danke ich besonders für ihre wertvolle Unterstützung und Einführung in die Messtechnik der hier angewandten Methoden. Herrn Assistent Evald Hellman und Fräulein Anna-Lisa Norling verdanke ich viele wertvolle Ratschläge und die Ausführung der zahlreichen Berechnungen. Das Schwedische Institut, Stockholm, gewährte mir ein Stipendium, das meinen Aufenthalt in Uppsala ermöglichte; auch hierfür meinen aufrichtigen Dank.

Herrn Professor Dr. W. Kern, Universität Mainz, danke ich für sein reges Interesse an diesen Untersuchungen.

Diese Arbeit wurde im Rahmen meiner noch nicht eingereichten Dissertation ausgeführt.

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The Activation of Alkaline Phosphatase from Different Organs by Phosphorylated Vitamin D_2

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In an earlier paper[1] a method was described of phosphorylating Vitamin D_2. The phosphorylated compound is soluble in water in concentrations of up to 5×10^{-4} *M*. The increase in solubility after phosphorylation makes it possible to investigate the action of Vitamin D on an *in vitro* system, which is not possible with substances which are totally insoluble in water. Hitherto it has been possible to study the effect of α-tocopherol [2-4] and certain oestrogenic hormones [5] on some *in vitro* systems with the aid of the phosphorylated and in water soluble compounds.

In the following experiments it is shown that phosphorylated Vitamin D_2 (D_2P) activates alkaline phosphatase from the kidneys, intestines, and bones. This effect of Vitamin D seems to be of interest because of the vitamin's specifically antirachitic mode of action.

METHODS

Crystalline Vitamin D_2 (obtained from AB Ferrosan, Malmö) was phosphorylated in accordance with a previously described method[1]. The phosphorylated compound is considerably more sensitive to light than the non-phosphorylated and upon irradiation products are formed with properties differing from those of the original substance. The sensitivity is greatest if the phosphorylated vitamin is kept in a water solution. Absorption in the ultraviolet region, which for D_2P reaches a maximum at 2620–2650 Å, is shifted toward the shorter wave-lengths at the same time as the coefficient of extinction is reduced. The ability to activate alkaline phosphatase disappears promptly after irradiation.

Alkaline kidney phosphatase of a high degree of purity was prepared from the kidneys of calves in accordance with a modification of Albers' and Albers' [6] method developed by van Thoai, Roche, and Sartori [7]. The enzyme contained 10.7 % nitrogen.

Intestinal alkaline phosphatase of a relatively high degree of purity was prepared from the intestines of calves according to Schmidt and Thannhauser [8].

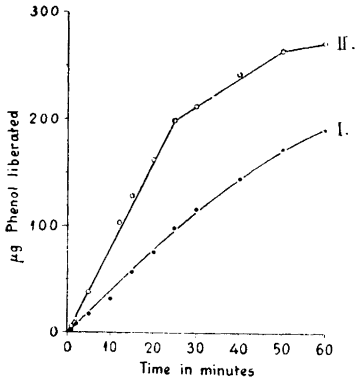

Fig. 1. This figure demonstrates the activation of alkaline kidney phosphatase with phosphorylated Vitamin D_2. Curve I shows the amount of liberated phenol in an incubation without vitamin. Curve II shows the amount of liberated phenol in an incubation with vitamin. Total volume 12.5 ml. Each of the incubation solutions contained 0.25 mg enzyme. D_2P concentration 4.8 × 10^{-5} M. Substrate concentration 6.4 × 10^{-3} M. pH 10.0.

Bone phosphatase was prepared from the bones of rabbits according to Martland and Robison [9]. Since this enzyme was remarkably impure in comparison with phosphatase from the kidneys and intestines, large quantities were required in order to split the substrate. Further purification was nevertheless considered unnecessary, since a noticeable activation of the enzyme was obtained under the influence of D_2P.

Enzyme activity was determined according to Buch and Buch [10]. Phenylphosphate was used as substrate. The incubations were carried out in a carbonate-tetraborate buffer. At pH 10.0 the concentrations of the buffer substances were 1.8 × 10^{-2} M for sodium carbonate and 2.0 × 10^{-3} M for sodium tetraborate. The temperature in all experiments was 37° C.

RESULTS

Fig. 1 shows the effect of the phosphorylated Vitamin D_2 on the activity of alkaline kidney phosphatase. In incubation without addition of the vitamin, substrate splitting proceeds throughout the experiment as an ordinary enzymatic reaction. After 60 minutes there is no distinct evidence of a decline in the speed of the reaction. The addition of D_2P changes the course of the reaction. In comparison with the control experiment, there is a considerable increase in the initial enzymatic activity. After 20 minutes twice as much substrate has been split as in the experiment without the addition of the vitamin. Thereafter there begins a period in which the velocity of the reaction is the same as in the control experiment. Finally the curve rather rapidly comes to an asymptote, meaning that a state of equilibrium is reached considerably more quickly in the experiment with the added vitamin than in the control experiment. Presumably the decline in the activity of the phosphatase in the course of the experiment is due to the fact that the total capacity of the enzyme is exhausted more rapidly after the addition of D_2P than without D_2P. Maximal activation of alkaline kidney phosphatase was not obtained in

Table 1. Activation of intestinal alkaline phosphatase under the influence of D_2P.

Time	μg Phenol liberated		Percent activation
	Without D_2P	With D_2P	
5 min.	16	35	119
10 min.	66	85	29
20 min.	86	84	0

Total volume 5.0 ml. The incubation solution contained c. 1 mg enzyme. Substrate concentration 1.5×10^{-3} *M*. In the experiment in which D_2P was added its concentration was 4.3×10^{-5} *M*. pH 10.0.

this experiment, since this occurs at pH about 9.7 and the experiment was carried out at pH 10.0.

Table 1 demonstrates that intestinal alkaline phosphatase is also activated by D_2P. As in the experiment with kidney phosphatase, the activation is greatest during the first minutes and declines thereafter.

The effect of D_2P on bone phosphatase is shown in Table 2. In both incubations the velocity rate of the enzymatic reaction was relatively low because the enzyme preparation was insufficiently pure. Hence there was no tendency in either of the experiments to diminution of the speed of reaction during the 60 minutes the experiment lasted. Also in this experiment the activation is more pronounced during the first period of incubation. The activation of alkaline bone phosphatase obtained after addition of D_2P is much higher than for kidney phosphatase.

Table 2. Activation of bone phosphatase under the influence of D_2P.

Time	μg Phenol liberated		Percent activation
	Without D_2P	With D_2P	
15 min.	13	60	360
20 min.	14	68	390
30 min.	42	89	110
45 min.	68	120	76
60 min.	85	135	59

The incubations were carried out in the same way as in the preceding experiment. 5 mg impure enzyme to each incubation. Concentration of $D_2P \times 10^{-5}$ *M*. Substrate concentration 6×10^{-3} *M*.

Table 3. The effect of varying amounts of D_2P on alkaline kidney phosphatase.

Concentration D_2P	Percent activation	
	Incubated 5 min.	Incubated 10 min.
$1.17 \cdot 10^{-4}$ *M*	54	40
$5.75 \cdot 10^{-5}$ *M*	54	42
$1.17 \cdot 10^{-5}$ *M*	21	40
$5.75 \cdot 10^{-6}$ *M*	0	0
$1.17 \cdot 10^{-6}$ *M*	0	0

Total volume 3.7 ml. The incubation solution contained 80 μg enzyme. Substrate concentration 4×10^{-3} *M*. pH 8.5.

Table 3 shows the D_2P concentration required to produce an activation of alkaline kidney phosphatase. The relatively low value for the increase in activity after addition of the phosphorylated vitamin is due to the fact that the incubations were carried out at pH 8.5. The concentration 5.75×10^{-5} *M* seems to give maximal activation; higher concentrations produce no increase in the activity of the enzyme. At a lower D_2P concentration, for example 1.17×10^{-5} *M*, the vitamin has given after 10 minutes' incubation the same increase in the amount of hydrolyzed substrate as at higher vitamin concentrations. The low value for the percentual activation after five minutes' incubation would, however, seem to indicate that the concentration was not sufficiently high to produce the initial increase in activity found at higher concentrations. Upon further lowering of the vitamin concentration, D_2P completely loses its ability to activate the phosphatase.

Table 4 demonstrates an experiment with bone phosphatase where enzyme concentration was low. In this case the initial activation is less than in

Table 4. The effect of D_2P on alkaline bone phosphatase at low enzyme concentration.

Time	μg Phenol liberated		Percent activation
	Without D_2P	With D_2P	
15 min.	10	13	30
20 min.	13	24	85
30 min.	19	43	126

The amount of enzyme 2 mg in each incubation. Besides enzyme concentration, the incubations were carried out in the same way as in the preceding experiment with bone phosphatase.

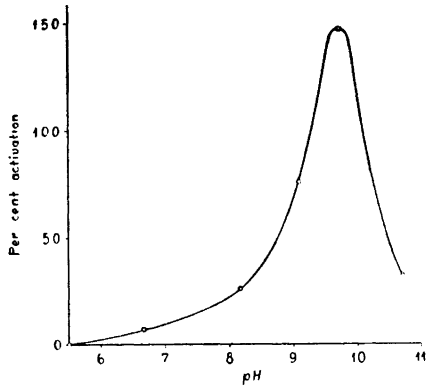

Fig. 2. This figure shows the increase in activity of alkaline kidney phosphatase obtained with D_2P at different pH's. Total volume 3.3 ml. 0.2 mg enzyme in each experiment. Substrate concentration 4 × 10^{-3} M. D_2P concentration 5.17 × 10^{-5} M. Incubation time 10 min.

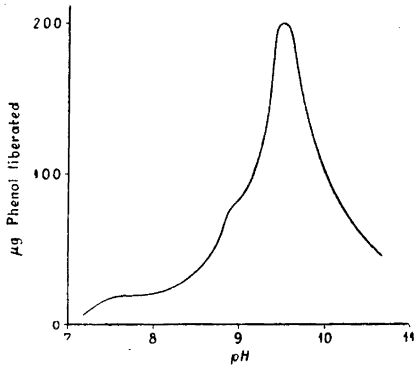

Fig. 3. Activity of alkaline kidney phosphatase at different pH's. Incubation period 10 min.

experiments with higher enzyme concentrations. The result is the same as when the D_2P concentration is about $1.17.10^{-5}$ *M*.

Fig. 2 shows that the extent of the increase in the activity of the alkaline kidney phosphatase produced by D_2P is manifestly dependent on the hydrogen ion concentration. After 10 minutes' incubation the activation is greatest at pH 9.7, thus the pH, at which the enzyme itself, according to Fig. 3 reaches its optimal activity. At this optimum D_2P brings about an increase of c. 150% in the activity of the enzyme. On either side of this pH optimum the activation brought about by D_2P declines with remarkable rapidity. The curve for the increase in activity produced by the vitamin at different hydrogen ion concentrations follows in every respect the curve obtained for the activity of the pure alkaline kidney phosphatase at different pH's. This indicates that the increase in activity, to which D_2P gives rise is specific.

DISCUSSION

If normal ossification is to take place, the animal must be able to absorb and retain a sufficient amount of phosphate, and this must then be utilized by the bone-forming tissue. In order that as much phosphate as possible may be available for ossification, resorption from the intestines must be high, and renal

excretion as slight as possible. The loss of phosphate in the urine is probably regulated by an increase or reduction in the reabsorption in the kidney tubules. The greater part of the phosphate which a growing animal retains is used to build up the skeletal system; hence the amount retained must depend primarily on the power of the osteogenic tissue to absorb phosphate and that of the intestinal and tubular epithelium to resorb it. The degree of phosphate resorption from the intestines also depends on the form in which the phosphate occurs. The phosphate compounds must be somewhat soluble in the intestines, and it must be possible to split organic phosphorus compounds.

The absorption of phosphate in a living cell is an active process rather than a simple diffusion [11]. This presupposes a number of enzymatic reactions in which the phosphatases are most probably involved. Robison [12] has advanced the theory that bone phosphatase is necessary for ossification. If in *in vitro* experiments with growing bones the phosphatase is inhibited, no precipitation of the bone salts takes place in the metaphysis [13]. Rickets can be induced experimentally by providing rats with beryllium chloride at the same time that their supply of phosphate is diminished [14-16]. *In vitro* experiments by Sobel and associates [16] have shown that in beryllium rickets there is a local effect on the bone which hinders calcification. Thus, the etiology of beryllium rickets must involve more than a diminished phosphate resorption from the intestines due to the formation of insoluble beryllium phosphate. Cloetens [17] has shown that a 10^{-3} M solution of beryllium chloride inhibits bone phosphatase. The inhibiting effect of soluble beryllium salts on alkaline kidney phosphatase has been investigated by Klemperer and associates [18] and on intestinal, bone, and kidney phosphatase by Grier and associates [19]. This inhibition, which is independent of the phosphorus containing substrate, is greatest within the area of the hydrogen ion concentration, where the enzymatic activity is optimal. Therefore, it seems possible that beryllium salts can produce experimental rickets because of their ability to inhibit alkaline phosphatase in the intestines, kidneys, and bones.

Robison [20] has found that the phosphatase content is higher in the bones of rachitic animals than in those of normal animals. In rickets the alkaline phosphatase in the serum often increases before any other symptoms appear [21]. Upon administration of Vitamin D the serum phosphatase value gradually returns to normal at the same time as other pathological changes disappear [22]. Morris and associates [23] have assumed that the bones require abnormally large amounts of phosphatase enzyme when there is a Vitamin D deficiency. The increase in the serum phosphatase content should be secondary and conditioned by enzyme from the skeletal system. Vitamin D is necessary for normal ossification; besides regulating the absorption and loss of phosphate, the

vitamin also has a direct effect on the skeleton itself. It seems possible that the effect of Vitamin D on the phosphate metabolism may be due to an effect on alkaline phosphatase. The changes, which are obtained through administration of Vitamin D, are reactions which can be conditioned by changes in the activity of the alkaline phosphatase. Cohn and Greenberg [24] have assumed that Vitamin D facilitates the transition from organic to inorganic phosphate and can in this way exert a direct influence on ossification. Vitamin D undoubtedly influences the resorption of phosphate from the intestines. To a certain extent rats can utilize the phosphate in phytin. Their ability to do this declines considerably if they contract rickets but is rapidly restored after administration of Vitamin D [25]. Harrison and Harrison [26] have been able to show that the administration of Vitamin D to rachitic dogs increases the reabsorption of phosphate in the kidney tubules.

If the activation of alkaline phosphatase from the kidneys, intestines, and bones which is obtained *in vitro* after the addition of D_2P also occurs *in vivo*, it is possible that this effect explains the changes in phosphate metabolism which are observed after the administration of Vitamin D to a rachitic animal. The possibilities for phosphate resorption are improved, since the phosphate esters which the intestinal epithelium cannot resorb are more effectively split. There may also be a direct effect on the resorption mechanism in the intestines. The utilization of endogenous phosphate becomes more effective inasmuch as less is lost in the urine. The possibilities for absorption of phosphate by growing bones are improved.

SUMMARY

1. *In vitro*, phosphorylated Vitamin D_2, which to a certain extent is soluble in water, activates alkaline phosphatase from the kidneys, intestines, and bones. The activation is highest for bone phosphatase. At pH 10.0, where the effect of D_2P is not optimal, the increase in activity is c. 400 % after 20 minutes incubation time.

2. Optimal activation of alkaline kidney phosphatase is obtained within the hydrogen ion concentration area in which the enzyme has its pH optimum.

3. The significance of this activation of alkaline phosphatase is discussed with reference to the vitamin's ability to cure rickets.

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The Renewal of Phosphate in Acid-Soluble Nucleotides in the Liver and the Brain

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Presumably the rate at which phosphate in different nucleotides * is renewed *in vivo* depends on the function of the compounds. In quantitative determination of the acid-soluble phosphorus compounds in a tissue, fractionation based on differences in the solubilities of barium, mercury or silver salts [1–3] of these compounds is usually employed. Generally this method is not sufficiently accurate if the specific activity of several compounds in the same extract is to be determined exactly, inasmuch as it is impossible to obtain the different compounds in pure enough form. This is particularly true if the concentrations of the substances to be investigated are low. The experimental errors become greatest in determination of the specific activity of a compound with a low turnover rate. The admixture of only very small amounts of a compound with a rapid turnover results in completely erroneous values. Kalckar [4] has described a method of determining different phosphate esters and their labeling degree with the aid of specific enzymes. Use of this method is restricted, however, by the fact that only certain phosphorus compounds are suitable and that as a rule purified enzymes are required.

By means of paper chromatography it is possible to separate certain phoshorus compounds such as nucleotides in a nucleic acid hydrolysate [5, 6], dinucleotides [7], and some carbohydrate esters [8]. With this method Hummel and Lindberg [7] have been able to show that phosphate bound to FAD has a high turnover rate in a respiring liver homogenate.

* In this report the following abbreviations are used:
ATP for adenosine triphosphoric acid;
ADP for adenosine diphosphoric acid;
AMP for adenosine-5-phosphate;
DPN for diphosphopyridine nucleotide;
FAD for flavin adenine dinucleotide;
NMN for nicotinamide mononucleotide.

In the following experiments the specific activity of phosphate was determined in the acid-soluble nucleotides of the brain and the liver following chromatographic purification.

METHODS

Material. — The following compounds were used as test substances: adenine, Hoffman—La Roche, Basel; adenosine, Lemke Co., N. Y; AMP, synthetic *; adenosine-3-phosphate, a Schwartz product; ATP prepared from rabbit musculature [9] and from the Sigma Chemical Co., St. Louis; ADP, Sigma Chemical Co.; DPN prepared according to Williamson and Green [10]; hexokinase prepared according to Kunitz and McDonald [11]; potato adenyl pyrophosphatase (apyrase) prepared according to Kalckar [12].

*Choice of solvent system***. — The most suitable eluating substance for separating the adenine compounds occurring in a biological extract has been found to be the following mixture: 66 % isobutyric acid (purified before use by fractionated distillation), 33 % water, and 1 % concentrated aqueous ammonia, (v/v). The pH value of the solvent system is 3.8. During the c. 8 hours for which the chromatogram should run at a temperature of 20° C, no hydrolysis of ATP or ADP takes place. Several buffered systems with pH values between five and seven were tested, but they were unsuitable, as separation of the desired compounds could not be obtained.

Chromatographic method. — For the most part the procedure described by Consden, Gordon, and Martin [13] was followed: *i. e.*, chromatography in closed glass vessels in accordance with the downward-running principle. The temperature was kept as constant as possible. Munktell's No. OB filter paper was used. Three times a volume of 8 μl was applied to the paper with a micropipette, and after each application the spot was dried under an infrared lamp. In this manner up to 200 μg of dissolved substance can be applied to the same chromatogram. When the front of the solution was 5 cm from the lower edge of the paper, chromatography was concluded.

Demonstration of different components. — After the papers were removed from the chromatographic chamber, they were dried in warm air at a temperature of 70° C, rinsed in ether to remove the isobutyric acid, and dried again. The purines were localized by absorption of the light from a Mineralight fluorescent lamp with high intensity at 2400—2600 Å. They were also localized with uranyl acetate [14]. The distribution of radioactive phosphate on the chromatogram was determined according to Lindberg and Hummel [15]. Radioactivity measurements were carried out on a Scaler 64 Electronic Counter (A/S Brüel and Kjaer, Copenhagen, Denmark) which gave 3×10^8 impulses/min. per millicurie P^{32}.

Extraction of different components. — Spots with purine, radioactivity, or both were cut out and thereafter extracted twice in 5 ml of water, each time for four hours. This extraction was by no means quantitative, but was used because the admixture of foreign substances was avoided. The isobutyric acid remaining in the water solution was removed by shaking the solution with an equal volume of ether three times. The ether was removed

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* Kindly furnished by Dr. M. Baddiley.

** During the preparation of the manuscript the same method has been published by Magasanik, B., Vischer, E., Doninger, R., Elson, D., and Chargaff, E. *J. Biol., Chem.* **186** (1950) 37, for separation and estimation of ribonucleotides in a nucleic acid hydrolysate.

from the water phase by aeration, after which the solution was evaporated to the volume desired. As a rule the spots from five to ten chromatograms were extracted simultaneously.

Preparation of solution suitable for chromatographic separation of phosphate-labeled nucleotides in the liver and brain. — White rats, weighing c. 100 g, were injected with 0.3 millicuries of radioactive phosphate as Na_2HPO_4 containing a negligable amount of phosphate. In liver experiments the injections were carried out intraperitoneally and in brain experiments subarachnoidally [16]. After the desired period the animals were drowned in solid carbon dioxide chilled acetone. Prepared brain or liver [17] was pulverized in a mortar chilled with solid carbon dioxide. The pulverized tissue was extracted with 12 % (w/v) trichloroacetic acid (TCA). The ice cold TCA extracts were neutralized to pH 7.0 with sodium hydroxide, whereafter they were precipitated with Hg^{++} at pH 4.0. Complete precipitation was obtained after the sample had been stored in the refrigerator for 12 hours. The precipitate was washed with 1 % (w/v) mercury acetate, pH 4.0, suspended in 2 % (w/v) TCA, and decomposed with hydrogen sulphide. The mercury sulphide was filtered off and the hydrogen sulphide removed by aeration. As the solution. obtained still contained impurities it was reprecipitated with Hg^{++} at pH 4.0. The precipitate was treated as before. The final solution obtained contained nucleotides almost exclusively. There was no FAD as this compound was quantitatively adsorbed by the mercury sulphide. The bulk of the TCA in the solution was removed by shaking it three times with ether. The ether was removed from the water phase by aeration The solution obtained was directly applied to chromatograms.

Splitting of labeled DPN into two mononucleotides. — Four brains from rats, which were given subarachnoidal injections of radioactive phosphate [16] two hours before being sacrificed, were extracted with 12 % (w/v) TCA. The extracts were precipitated with mercury acetate at pH 4.0 and the precipitate decomposed with hydrogen sulphide following suspension in 2 % (w/v) TCA. The mercury sulphide was filtered off, and the hydrogen sulphide removed by aeration. The solution obtained contained most of the DPN originally present in the brains. As carrier, 15 mg unlabeled DPN was added to the solution, whereafter it was precipitated with lead acetate at pH 4.5. DPN was then purified by repeated shaking up in *p*-cresol [18]. The purified DPN was obtained in a water solution without other phosphorus compounds. Cresol was removed by four shakings of the solution with ether. The solution was incubated with washed granules from rabbit kidneys, whereupon DPN according to Kornberg and Lindberg [19] was split into AMP and NMN. After fixation in 10 % (w/v) TCA, the NMN was purified [19].

Determinations. — The total phosphate was determined according to Martin and Doty [20] following combustion in sulphuric acid with hydrogen peroxide. The specific activity (*i.e.*, the number of impulses per μg P) was determined in accordance with a method previously [21] described. The adenylic acid was determined in accordance with Kalckar's method [22] and the DPN and NMN fluorimetrically with quinine sulphate as standard [23].

RESULTS

The chromatogram with pure substances and their R_f *values.* — Table 1 shows the R_f values of the different adenine compounds in the solvent system employed. Separation was good and the different compounds appeared in well delimited spots.

Table 1. R_f values of different adenine compounds. — A solution containing the compounds listed in the table was applied to a strip of filter paper and chromatographed in a mixture of water, ammonia, and isobutyric acid. (cf. Methods).

Compound	R_f Values
ATP	0.12
ADP	0.25
AMP	0.41
Adenosine-3-Phosphate	0.51
Adenosine	0.80
Adenine	0.90

Apparently ATP was not hydrolyzed when the chromatogram ran; a fresh ATP preparation gave only one spot. ATP which had been stored as the solid Ba-salt at room temperature for two months gave two or three different components. In addition to ATP there was always one spot with the same R_f value as ADP and usually another with the R_f value of AMP. Earlier investigations have also indicated that ATP is split if it is stored or irradiated. Bailey [24] has found that on storage inorganic pyrophosphate splits off from ATP, and according to Carter [25] adenine is formed following ultraviolet irradiation.

Separation of nucleotides from the liver. — Fig. 1 shows the separation of acid-soluble compounds precipitable with Hg at pH 4.0 occurring in the liver. The spots marked on the figure indicate the area with absorption of ultraviolet light. From the amount of activity registered on the same figure in different parts of the chromatogram it appears that all the components giving absorption of ultraviolet light are bound to labeling phosphate.

On the chromatogram there are seven different peaks of activity given by the following compounds.

The compound giving the first peak has not been identified since the compound was not obtained in sufficiently pure form because of its low R_f value. Presumably it is also hydrolyzed during chromatography. The substance has high absorption in ultraviolet light, maximum at 2600 Å, and is precipitable as Ba-salt at pH 8.0. Probably one or more phosphate groups of this compound are renewed at a rate of the same order of magnitude as the acid-labile phosphate of ATP. The compound may be identical with an unknown compound which, according to Bailey [24], exists in all ATP preparations.

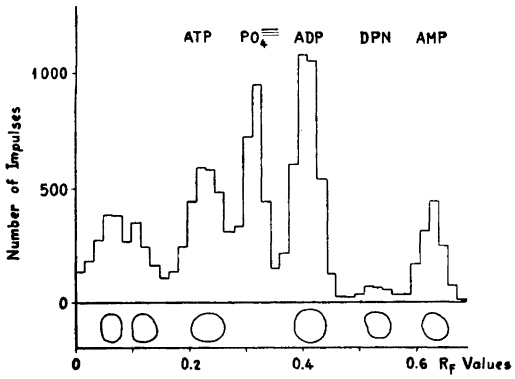

Fig. 1. The migration patterns of phosphorus compounds, insoluble at pH 4.0 as Hg-salts, in a TCA extract of rat liver, after chromatography with the water-ammonia-isobutyric acid mixture described in the text. The spots indicate the areas with absorption of ultraviolet light. The P^{32} values are expressed as impulses per minute. The number of impulses was determined for each ½ cm of the strip of filter paper. The radioactive phosphate was injected 60 minutes before the animal was sacrificed.

It was also impossible to identify definitely the compound which gave the second activity peak. The substance was labeled at a relatively low level. The greater part of the radioactivity found in this spot came from the extended first spot. The compound absorbed ultraviolet light and gave a positive ninhydrin reaction. The Ba-salt was soluble at pH 8.0. The compound might be an aminophosphate, possibly ethanolamine phosphate, which has been isolated from the intestines [26].

ATP gave the third activity peak. If the solution was incubated with hexokinase before chromatography this spot disappeared.

Orthophosphate gave the next peak. This was the only phosphorus compound in the sample which failed to absorb ultraviolet light. Radioactive orthophosphate added to an unlabeled sample before chromatography appeared at this place.

The following activity peak was given by ADP. This peak increased in height following incubation of the sample with hexokinase.

The two last activity peaks were given by DPN and AMP. To separate these two compounds it is necessary that the sample solution is completely free of inorganic salts and that the isobutyric acid used is freshly distilled.

No compounds with higher R_f values containing radioactivity or with absorption of ultraviolet light were observed. Thus, there was no demonstrable amount of adenosine-3-phosphate.

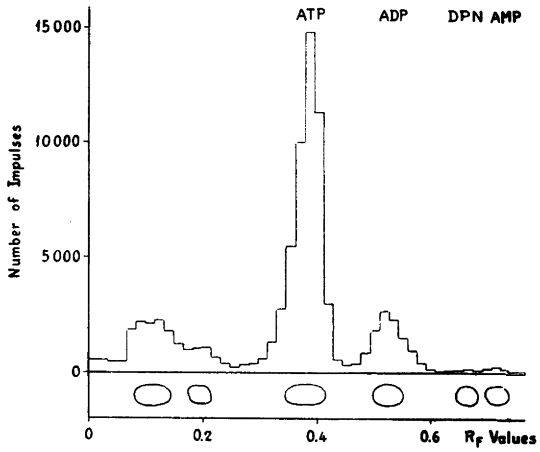

Fig. 2. The migration patterns of phosphate compounds, insoluble at pH 4.0 as Hg-salts, in a TCA extract of rat brain, after chromatography in the same solvent system as in the experiment with liver. The spots indicate the areas with absorption of ultraviolet light. The values are determined and expressed as in Fig. 1. The radioactive phosphate was injected 45 minutes before the animals were sacrificed.

Following incubation with apyrase both the ATP and the ADP peaks disappeared, the former before the latter, being in agreement with the findings of Kalckar [27] that the terminal phosphate group in ATP is earlier split off than the second. Adenosine appeared in agreement with previous investigations [24, 28], according to which apyrase preparations always contain non-specific phosphatase.

Separation of nucleotides from the brain. — Fig. 2 shows a chromatogram of a solution prepared from the brain in the same way as that from the liver. Orthophosphate failed to appear on this chromatogram, which is in accordance with Kerr's observation [29] that orthophosphate in a TCA extract from the brain is not precipitated as mercury salt at pH 4.0. Furthermore there are the same compounds as in the Hg-insoluble fraction of a liver extract. The proportionate amounts of radioactivity in ATP and ADP are wholly different, however. In the liver a larger amount is bound to ADP than to ATP. This is in agreement with other investigations; in the liver the quantity of ADP is considerably greater than the quantity of ATP [30, 31], while in the brain the greater part of the adenylic polyphosphate is ATP [29].

The specific activity of phosphate in different acid-soluble nucleotides — Tables 2 and 3 demonstrate the relative specific activities of the phosphate in AMP and DPN in relation to the specific activity of acid-labile ADP phosphate in

Table 2. The specific activity of the phosphate in adenine compounds from the livers of rats. — Substances purified by means of paper chromatography were extracted, after which the specific activity of the phosphate was determined. See Methods.

Time in minutes after injection of P^{32}	60		90 [c]
Compound	Specific activity	Relative specific activity [b]	Relative specific activity [b]
ADP	270		
AMP	130	32	38
DPN	61	15	17
Labile P of ADP [a]	410		

a) Calculated from the values for total ADP-P and AMP-P.
b) Specific activity of the labile P of ADP = 100.
c) Mean of two determinations.

the liver and brain, assuming that the acid-stable phosphate in ADP has the same specific activity as the phosphate in AMP. The specific activity of AMP's phosphate calculated in this way is the same as reported earlier [32] for the acid-stable phosphate in ATP in relation to the acid-labile. Thus AMP's phosphate is renewed at the same rate as the acid-stable phosphate in ATP. The tables also show that the mean value of the specific activity of DPN's two phosphates is half that of AMP's both in the liver and the brain.

Table 3. The specific activity of the phosphate in adenine compounds from the brains of rats. — Purification and determination as in the liver experiments.

Compound	Specific activity [b]	Relative specific activity [c]
ADP	2 400	
AMP	810	20
DPN	400	10
Labile P of ADP [a]	4 000	

a) Calculated as in the liver experiments.
b) 45 minutes after injection of P^{32}.
c) Specific activity of the labile P of ADP = 100.

Table 4. Labeling degree of the phosphate in DPN bound to adenosine and nicotinamide ribose. — Following the addition of unlabeled DPN as carrier, DPN with radioactive phosphate was purified from a TCA extract of four rat brains. The purified DPN was split into two mononucleotides with washed granules from rabbit kidneys. The NMN formed was partially purified. The animals were sacrificed two hours after the subarachnoidal injection of radioactive phosphate. The values are expressed in micromoles.

	NMN [a]	AMP [a]	Number of impulses per micromol P	Number of impulses per micromol AMP-P
Before purification of NMN	21.6	21.6 [b]	71	141
After partial purification of NMN	18.3	8.4	43	138

a) Free or as DPN.
b) Not determined.

Table 4 shows that the activity in DPN is found exclusively in the phosphate group which is bound to adenosine. Thus the phosphate bound to nicotinamide ribose has not been renewed. The phosphate in DPN which is bound to adenosine, therefore has both in the liver and the brain the same specific activity as the phosphate of AMP and the acid-stable phosphate of ATP.

DISCUSSION

The adenosine-bound phosphate of DPN is renewed *in vivo* although at so slow a rate that this phosphate group cannot directly enter into the phosphate-transferring system.

During the formation of two mononucleotides DPN can be split in two ways.

$$\text{DPN} + \text{pyrophosphate} \rightleftharpoons \text{NMN} + \text{ATP} \quad (1)$$

$$\text{DPN} \rightharpoonup \text{NMN} + \text{AMP} \quad (2)$$

The first of these reactions, described by Kornberg [33], is reversible and is catalyzed by an enzyme found in liver and yeast. The other, described by Kornberg and Lindberg [19], is an irreversible pyrophosphorylysis and is catalyzed by an enzyme purified from potatoes [34].

According to Schrecker and Kornberg [35] FAD can be split according to the following reaction.

$$\text{FAD} + \text{pyrophosphate} \rightleftharpoons \text{flavin mononucleotide} + \text{ATP} \quad (3)$$

Hummel and Lindberg [7] have reported, that a component, containing FAD and having a very high specific activity, can be isolated chromatographically from a liver homogenate which has been incubated under aerobic conditions and in the presence of radioactive orthophosphate. AMP and the flavin mononucleotide were not labeled at all, thus the FAD molecule cannot have obtained its high specific activity according to Reaction (3).

Investigations in this laboratory * have shown, that the highly labeled phosphate bound to FAD is considerably more acid-labile than the phosphate in the FAD molecule itself. Hence the flavin adenine dinucleotide isolated is probably a larger molecule than FAD; possibly an intermediary in Reaction (3). The high specific activity indicates that such a compound is renewed so rapidly that it can function as phosphate transferer in oxidative phosphorylation. Transferal of phosphate taking place in this way must be associated with a very rapid splitting and resynthesis of FAD.

The turned over phosphate in DPN has the same specific activity as the acid-stable phosphate in ATP, for which reason the splitting and resynthesis of DPN must take place more rapidly than the turnover of the phosphate of AMP. The speed of these reactions may be of the same order of magnitude as the renewal of one or more phosphate group in the FAD compound [7]. Since any transfer of phosphate through FAD in accordance with Reaction (3) must result in reversible splitting of the molecule, the rapid splitting and rebuilding of DPN may also be caused by a transferal of phosphate. Cross and associates [36] formulated the hypothesis that coenzyme pyrophosphate is formed primarily in oxidative phosphorylation. From the same laboratory there has been reported conjugation of DPN [37] and of an extremely labile phosphate fraction [38] to the oxidative enzyme complex in washed granules.

SUMMARY

1. A chromatographic method of purifying acid-soluble adenine nucleotides has been developed.

2. The phosphate of AMP is renewed in the liver and brain at the same rate as the acid-stable phosphate in ADP and ATP. In DPN the phosphate bound to adenosine is renewed at the same rate as the phosphate in AMP; the other phosphate is not labeled during short experiments. Thus DPN must be split and resynthesized at a rapid rate. The possibility that DPN transfers phosphate through its splitting is discussed.

* Ernster, L., Lindberg, O., and Zetterström, R. Unpublished experiment.

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Studies in the Tropolone Series

IV. Stipitatic, Puberulic and Puberulonic acids

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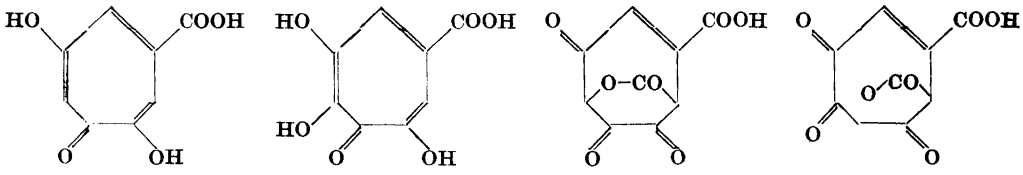
In a previous communication [1] ultra-violet absorption spectra were reported for α-, β- and γ-thujaplicins in neutral and alkaline solutions, and some general physico-chemical properties of the tropolone ring system were discussed in the light of this and other data. As briefly mentioned in a recent note [2], the investigation has now been extended to the natural compounds stipitatic, puberulic and puberulonic acids.

These acids were first isolated from mould cultures by Raistrick *et al.*[3,4], and as a result of their studies these workers concluded that stipitatic acid might "belong to a class of compounds not previously encountered among mould metabolic products, except possibly for puberulic acid" [3]. Stipitatic and puberulic acids have recently been shown by Todd and co-workers to have the structures (A)[5,6] and (B)[5,7] respectively (or tautomerides thereof) in conformity with the earlier suggestions of Dewar [8,9].



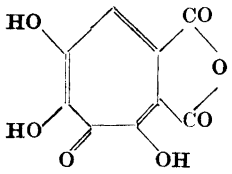
(A) (B) (C) (D)

Birkinshaw and Raistrick [4] ascribed the formula $C_8H_4O_6$ to the yellow puberulonic acid, which they believed to be intimately related to puberulic acid. However, they did not succeed in transforming one compound into the other. Todd and co-workers showed [5,10,7] that puberulonic acid, on heating

to 100° in aqueous solution, loses one molecular equivalent of CO_2 and is converted into puberulic acid. The formula was accordingly amended to $C_9H_4O_7$.

Todd *et al.*[5,7] proposed structure (C) or an isomeric γ-lactone structure (D) for puberulonic acid. These formulae were based on certain chemical properties of the substance, *e. g.* its easy conversion into puberulic acid, its reported resistance towards acetylating agents [4], and the formation of a condensation product with *o*-phenylenediamine [5,10].

The light absorption properties of puberulonic acid, however, favour another structure (E) for the substance, or a tautomeric form, still containing the tropolone ring. This structure was originally proposed [2] on the basis of the ultra-violet investigation to be reported in this paper, and it has now been confirmed by infra-red spectrographic studies [11].



(E)*

A preliminary comparison of the U. V. absorption in organic solvents of stipitatic, puberulic and puberulonic acids and the thujaplicins showed a remarkable similarity between them all. This, together with Dewar's previous suggestion of tropolone structures for stipitatic [8] and puberulic [9] acids, suggested to the present author that puberulonic acid also might be a tropolone derivative (*cf.* Raistrick, Bakerian lecture, May 1949 [12]).

Ultra-violet absorption curves for aqueous solutions of stipitatic [6], puberulic [10] and puberulonic [10] acids have been published by Todd *et al.* and similar results (Fig. 1) were obtained in these laboratories except for puberulic acid **, for which a sharper short-wave maximum was found. The general similarity in light absorption is obvious from these curves, but it is evident that water of undefined pH is not a suitable solvent for a detailed investigation of the absorption properties of easily ionizable substances.

The structures (C) and (D) contain α-diketone groupings, which will cause a yellow colour. Low absorption maxima in the visible region are exhibited by α-diketones in general (*e. g.* diacetyl in hexane: log $\varepsilon = 1.3$ at ca 430 mμ [13]; camphorquinone in *cyclo*hexane: log $\varepsilon = 1.6$ at 478 mμ [14]). Puberulonic acid, however, shows a much more intense long-wave absorption (Figs. 1 and 8), which cannot be explained by the simple —CO—CO— chromophore of (C). Likewise in (D), the unsaturated system —CO—CO—CH = C(COOH)— is branched and not necessarily planar and thus constitutes a complex chromophore that could hardly give rise to the intense long-wave absorption of puberulonic acid.

* The symbol (E) will subsequently be used to indicate any of the four tautomerides.

** According to a private communication from Professor Todd (January 18th, 1950), the published extinction curve for puberulic acid in water is erroneous. He does not intend to publish a correction, and accepts the revised data discussed herein.

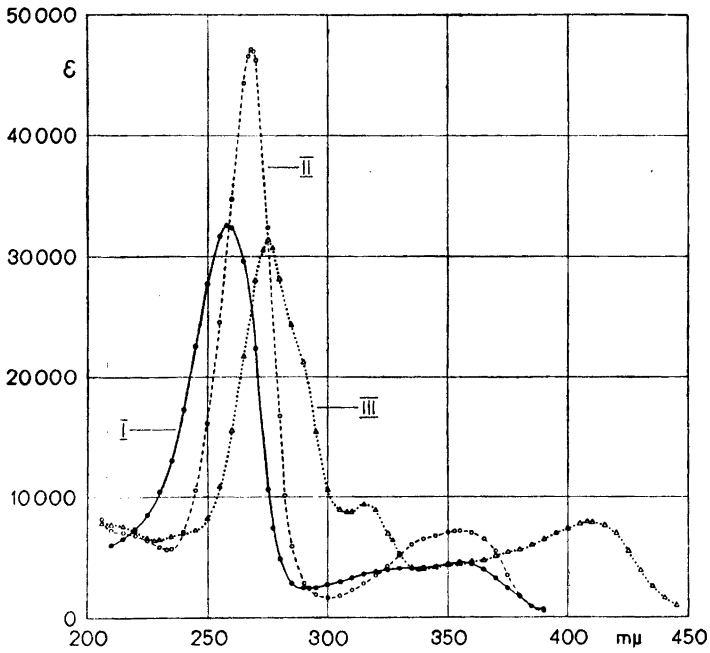


Fig. 1. Stipitatic acid (I), puberulic acid (II), and puberulonic acid (III) in aqueous solution.

Considerations of this kind necessitated the construction of a new formula as a working hypothesis. (E) is the only structure that combines the composition $C_9H_4O_7$ with the tropolone skeleton and can explain the transformation into puberulic acid. Attempts were made to confirm this structure by a study of the hydrolysis and dissociation of puberulonic acid, and particularly the ultra-violet absorption data for the various ions. For comparative purposes and in view of the chemical as well as the spectral [1] relationship between aromatic substances and tropolones, it was also of interest to study the absorption properties of stipitatic and puberulic acids, following step by step the influence of ionization of the various acidic groups by using buffered solutions of adequately varied pH.

EXPERIMENTAL

Prior to investigation, the stipitatic acid specimen * was recrystallized three times from water. This had no significant effect upon the absorption. The puberulic and puberulonic acid samples were used without further purification.

* Pure samples of the three acids were kindly provided by Professor H. Raistrick.

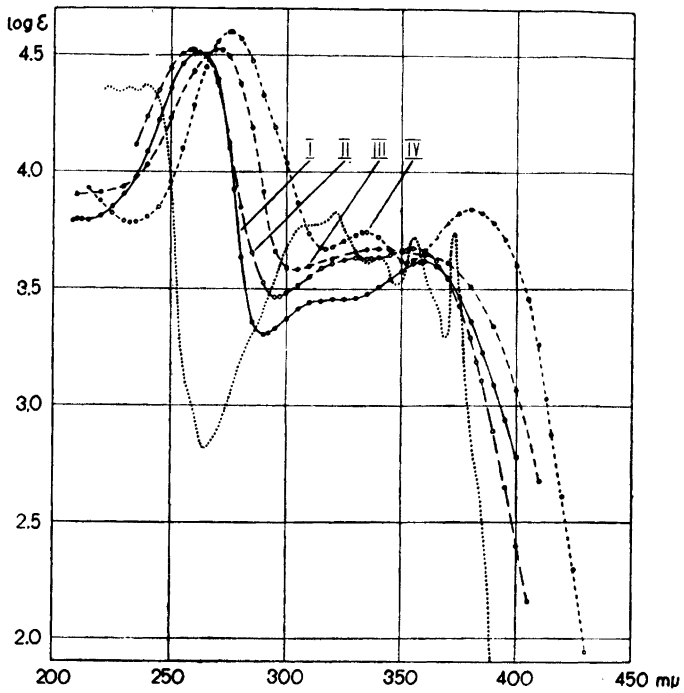


Fig. 2. Stipitatic acid in N-hydrochloric acid (I), at pH 4.8 (II), at pH 7.9 (III), and at pH 12.0 (IV). Dotted line: tropolone in cyclo*hexane* [1].

The following solvents were employed: distilled water, absolute ethanol, purified for spectral purposes [15], dioxan, refluxed with potassium hydroxide and fractionated, phosphate buffer solutions of pH 5.7, 7.7, 7.9, 11.2 and 12.0, phosphate — citric acid buffer solutions of pH 3.5, 4.4 and 4.8, and an ammonia — ammonium chloride buffer solution of pH 8.2.

A Beckman Model DU spectrophotometer with calibrated cells was used for the measurements. The absorption properties of some of the alkaline solutions gradually changed on storing, so that, unless otherwise stated, these solutions were investigated as soon as possible after preparation — at critical wavelengths within a few minutes.

RESULTS AND DISCUSSION

Stipitatic, puberulic and puberulonic acids are all almost insoluble in saturated hydrocarbons. A direct comparison of the undissociated compounds with tropolone and the thujaplicins in such solvents is therefore impossible.

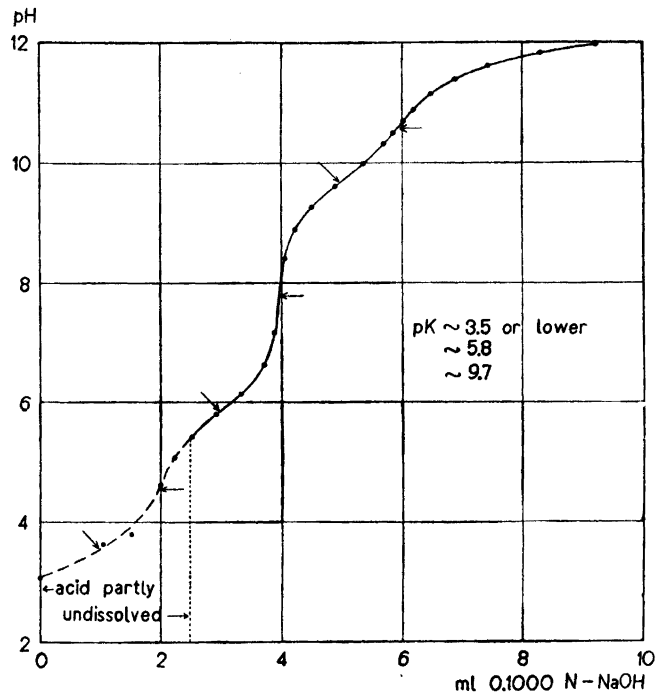


Fig. 3. Potentiometric titration of stipitatic acid.

The extinction curve of unionized stipitatic acid in N-hydrochloric acid solution shows the general features of the curve for tropolone in *cyclo*hexane [1] (Fig. 2), except for vibrational fine structure. However, the absorption intensity at about 300—325 mμ is considerably diminished, and the short-wave absorption band exhibits a combined hyperchromic and bathochromic displacement, obscuring the deep minimum of the tropolone curve. It is seen that the simultaneous introduction of a hydroxyl and a carboxyl group into tropolone — in contrast to benzene [16] — does not cause a bathochromic displacement of the long-wave absorption band.

The effect of dissociation of the various acidic groups of stipitatic acid was studied on the basis of the potentiometric titration curve (Fig. 3), obtained by adding sodium hydroxide solution to a well-stirred aqueous suspension of the acid. In Fig. 3, horizontal arrows indicate equivalence points: the lower ones giving at the same time the pH values for maximum concentrations of the monovalent and bivalent ions. Curves II and III in Fig. 2 are the corresponding extinction curves, whereas IV is the curve for fully ionized stipitatic acid.

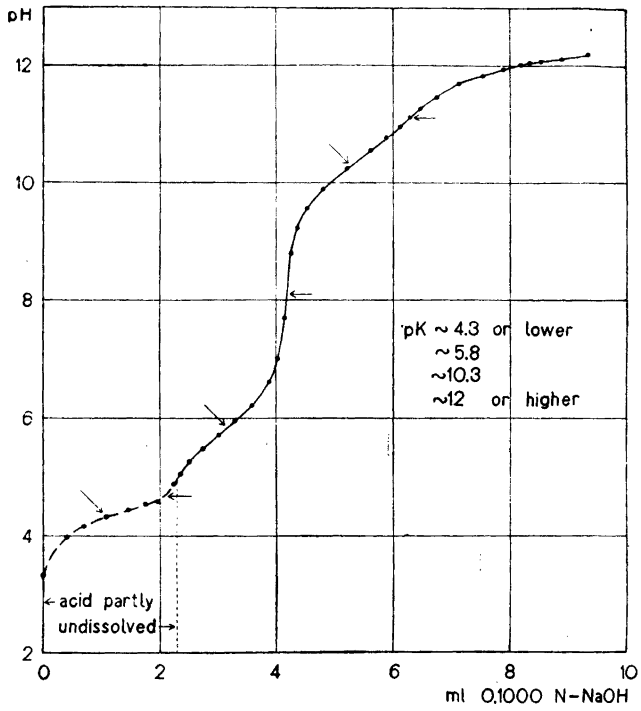


Fig. 4. Potentiometric titration of puberulic acid.

There can be no doubt that the lowest pK value must be assigned to the dissociation of the carboxyl group. The isolated hydroxyl group must be assumed to come next in acidity — pK ca 5.8 — whereas the other hydroxyl group (in *m*-position to the carboxyl or to the isolated hydroxyl group, depending upon the tautomeride preferred) will be more weakly acidic due to the influence of the neighbouring oxygen atom. The pK value of about 9.7 found for this hydroxyl group also conforms with the expectation that dissociation of the "olone" hydroxyl group must take place with greater ease in tropolone itself (pK ca 7 [17–18]) than in a negative ion of stipitatic acid.

Comparison of the curves I and II shows that the dissociation of the carboxyl group causes a small hypsochromic shift, as is usual with carboxylic acids. However, dissociation of the first hydroxyl group (curve III) reverses this effect, displacing the entire curve about 15 mμ towards the red. The last dissociation step (curve IV), involving the "olone" hydroxyl group, leads to a further bathochromic displacement, splitting of the long-wave band into two separate peaks, and a considerable increase in ε, especially in the band close

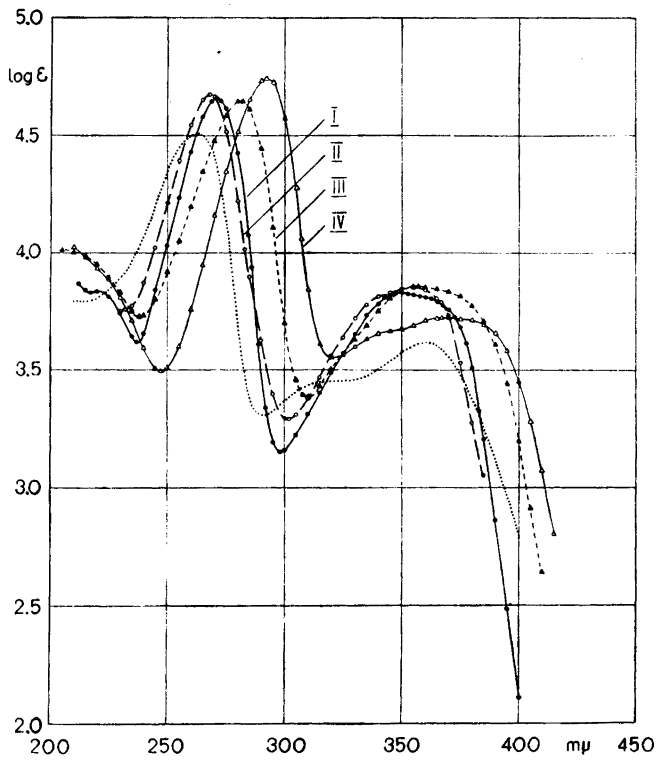


Fig. 5. Puberulic acid in N-hydrochloric acid (I), at pH 4.4 (II), at pH 8.2 (III), and at pH 11.2 (IV). Dotted line: stipitatic acid in N-hydrochloric acid.

to the visible region. The division of the broad long-wave absorption band into two peaks recalls the effect of ionization upon the extinction curves of the thujaplicins [1], and is probably characteristic of the elimination of the proton from the "olone" grouping of the tropolones.

Puberulic acid, being a hydroxy-stipitatic acid, contains four ionizable groups. Its titration curve, obtained in the same way as the one for stipitatic acid, is shown in Fig. 4. Like formula (A) for stipitatic acid, formula (B) for puberulic acid is only one of the two possible tautomerides, both containing the tropolone skeleton, which can be constructed for this acid. Under these circumstances it is not possible to assign with certainty each of the pK values of 5.8 and higher to an individual hydroxyl group. It can only be stated that the dissociation having pK 5.8 refers to a hydroxyl group non-adjacent to the CO-group.

Fig. 5 shows the extinction curve for unionized puberulic acid and the approximate curves for its mono-, di-, and tri-valent anions. Puberulic acid has a

steeper extinction curve than stipitatic acid (Fig. 5), the short-wave maximum is displaced about 10 mμ towards the red, and there is no indication of a maximum at about 315 mμ. Tropolone and the thujaplicins [1] have prominent maxima in this region, and in stipitatic acid the maximum still exists although it is less intense. Its disappearance in the case of puberulic acid may perhaps be due to the influence of the neighbouring hydroxyl group on the „olone" system.

Dissociation of the carboxyl group of puberulic acid causes the well-known slight hypsochromic displacement (Fig. 5, curve II). The effect of dissociation of the first hydroxyl group is also the expected one — a marked red-shift of the entire curve. The next ionization step (curve IV, pH 11.2) produces not only another shift in the same direction but also an intensification of the short-wave maximum and an indication of a division of the long-wave band into two peaks.

It has not been possible to demonstrate the effect of dissociation of the last hydroxyl group of puberulic acid, since in strongly alkaline media as for example N-sodium hydroxide solution the substance is too unstable to allow a determination of the absorption curve by ordinary methods. The extinction curve obtained by a rapid determination at pH 12.0 was, however, very similar to that found at pH 11.2.

Puberulonic acid is not unaffected by alkali. Its solutions have been reported to undergo various colour changes on alkalisation and reacidification [19,10]. Barger and Dorrer [19] attempted to determine the basicity but obtained ambiguous results. Recently, Todd *et al.*[10] have published a potentiometric titration curve for the substance showing that it undergoes some change on storing at any pH between approximately 7 and 10. It was suggested that this change might involve the opening of a lactone ring.

The potentiometric titration of puberulonic acid has been repeated in these laboratories, using about 20 mg of the acid in 20 ml of water. The titration extended over several days altogether and gave a result differing from that mentioned above. In Fig. 6, filled dots indicate the pH readings for the solution at equilibrium; open dots indicate the initial pH values after each addition of alkali. The acid dissolved gradually when the first mole of sodium hydroxide was added, and the pH readings plotted in Fig. 6 were only reached after some minutes of stirring. At pH 3.6—3.7 all the acid was dissolved. During the addition of the second mole of alkali (pH 4—6), the initial pH values were still not stable, indicating that some change in the molecule was gradually taking place. The end values were reached within about ten minutes in the beginning of this interval, in about an hour at the end. At about pH 6 the solution had become almost colourless. Addition of the third mole of alkali

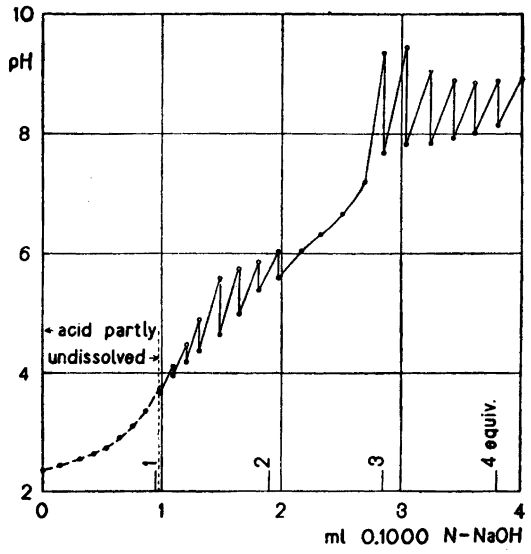
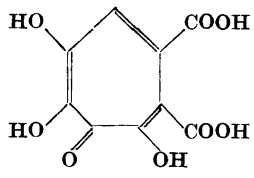


Fig. 6. Potentiometric titration of puberulonic acid.

gave no abnormal effect. The fourth mole caused another reaction, which was, however, much slower than the first one, and since oxygen was not excluded, may have involved an oxidation. The nature of this process was not studied further.

The reaction taking place at pH 4—6 can be explained on the basis of the structure (E) as a gradual hydrolysis of the anhydride to give the corresponding dibasic acid (F), or the tautomeric forms, a process which is complete at pH 5.6. The titration cannot show whether hydrolysis takes place to any considerable extent even below pH 4, because, if it occurred in this region it would be a rapid process and its pH effect would be obscured by that of the dissolution of the substance.



(F)*

In order to get a clear picture of the process of hydrolysis we must consider the equilibrium between the anhydride and the diacid in aqueous solution as well as the ionization of both (E) and (F). At a low pH practically no ions are present but only uncharged molecules (E) and (F) in unknown proportions. As the pH increases, the relative proportions of these molecules remain, of course, unchanged, whereas their total concentrations diminish due to ion formation. After the addition of one mole of alkali most of the material is present in the form of the monovalent ions "E^-" and

* (F) hereafter symbolizes any one of the four tautomerides.

"F^-", the proportion of each depending upon the unknown values of the equilibrium and dissociation constants concerned. The reactions that take place on further addition of alkali must involve the transformation "E^-" → "F^{--}" (via intermediates), since the second ionization constant of (F) must be higher than that of (E). At pH 5.6, the solution at equilibrium will contain predominantly the ion "F^{--}", which is then ionized further on addition of more alkali.

In order to verify this interpretation of the titration data for puberulonic acid, the U. V. absorption curves were determined for solutions of this substance in an inert solvent (dioxan) and in a series of buffered aqueous solutions, the pH values of which were selected with reference to the titration curve. The extinction curves obtained for solutions at equilibrium are shown in Fig. 7.

Curve I in Fig. 7 (dioxan solution) must be that of the anhydride with no appreciable admixture of the dibasic acid. In Fig. 8 it is reproduced, together with the curves for tropolone, stipitatic and puberulic acids in non-ionizing solvents. A comparison shows that the main difference between the curve for puberulonic acid and that for puberulic acid is a marked red-shift of the whole curve. This conforms with the presence of an extra substituent in the nucleus and the conjugation of the anhydride ring (*cf.* below).

The second absorption curve in Fig. 7 (pH 0) is exhibited, not only by solutions obtained by dissolving puberulonic acid in N-hydrochloric acid directly, but also when a freshly prepared solution of pH 14 is acidified to pH 0. The absorption of the anhydride at approximately 295 and 315 mμ is still distinguishable in this strongly acidic aqueous solution. However, two new maxima appear at 277 and 375 mμ. These are located at shorter wavelengths than the corresponding anhydride maxima just mentioned. This is in agreement with the assumption that they are caused by the dicarboxylic acid (F), since other anhydride — diacid pairs, *e. g.* maleic [20] and phthalic [21], show similar absorption differences. A comparison of curve II with the dotted absorption curve of undissociated puberulic acid (Fig. 7) also supports this interpretation.

The distribution of absorption intensity along curve II indicates the remarkable situation that the anhydride and the acid are present in comparable concentrations. Although organic acid anhydrides are in general rapidly and completely transformed into the corresponding acids on treatment with water, some cases of slow hydrolysis are well known, as for example phthalic anhydride. An interesting sequence of increasing stability towards water was described by Rivett and Sidgwick [22]: maleic anhydride is hydrolyzed rapidly, citraconic anhydride more slowly, and pyrocinchonic anhydride in aqueous solution gives an equilibrium with only a small proportion of pyrocinchonic

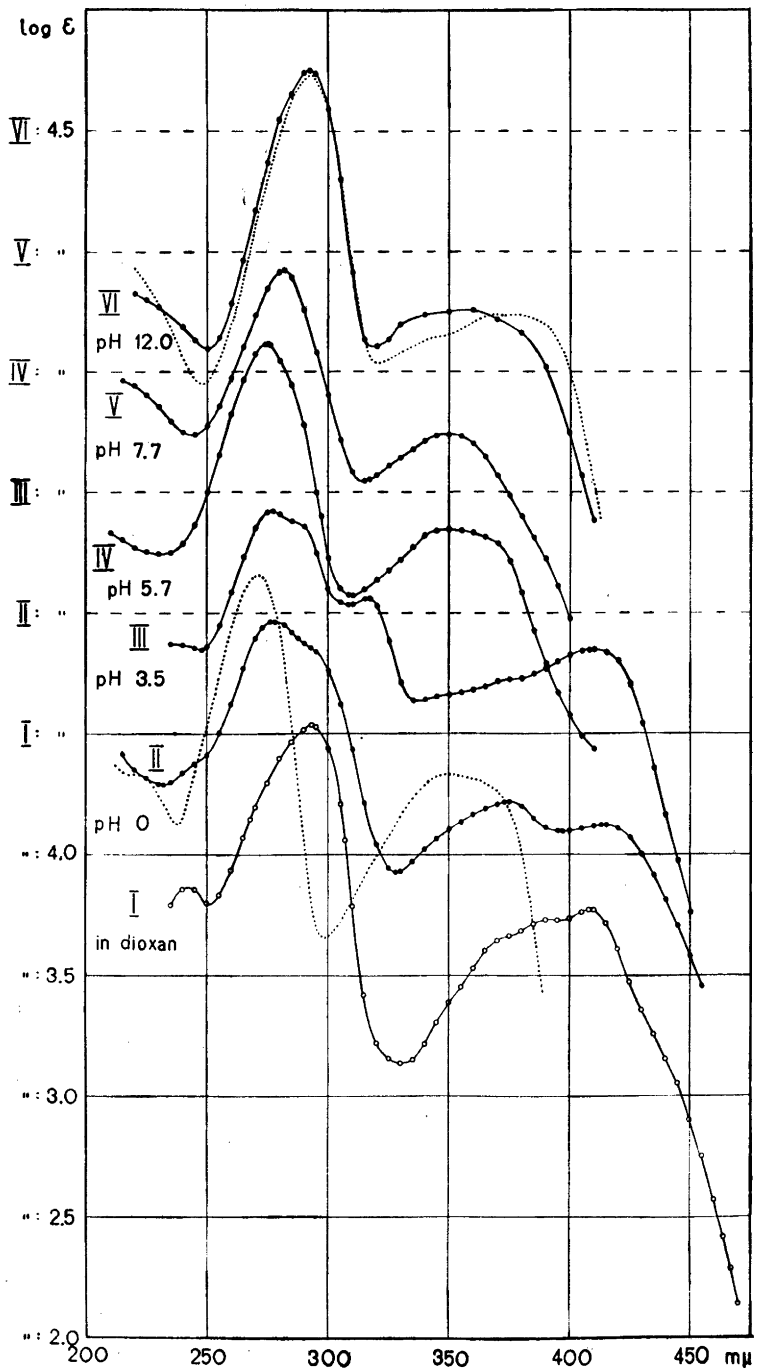


Fig. 7. Puberulonic acid in dioxan (I), in N-hydrochloric acid (II), at pH 3.5 (III), at pH 5.7 (IV), at pH 7.7 (V), and at pH 12.0 (VI). Dotted lines: puberulic acid in N-hydrochloric acid and at pH 12.0 respectively.

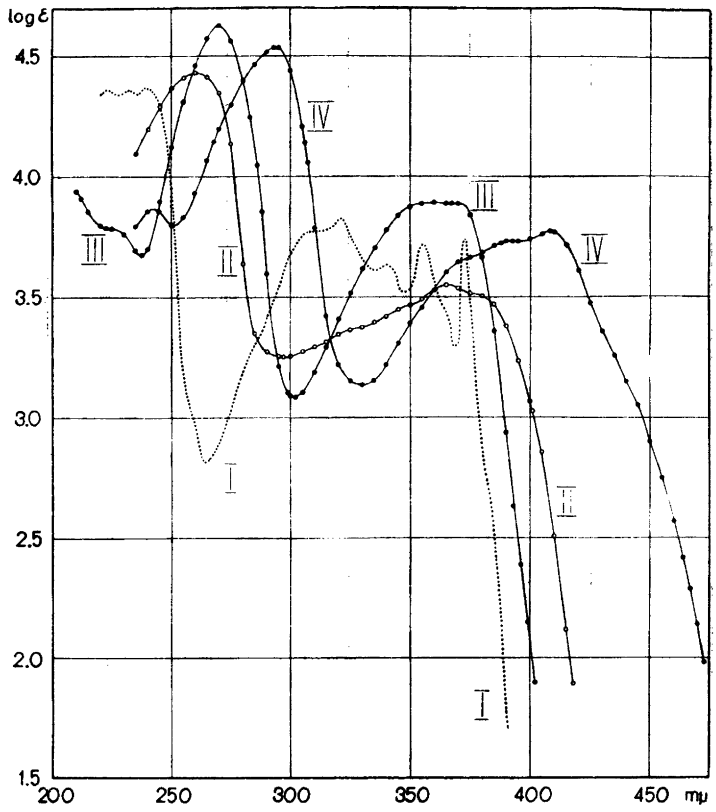


Fig. 8. Tropolone in cyclo*hexane* [1] *(I), stipitatic acid in dioxan (II), puberulic acid in ethanol (III), and puberulonic acid in dioxan (IV).*

acid. Verkade [23] reported that tetramethyl succinic anhydride passes away with the vapour on boiling a solution of tetramethyl succinic acid, and Δ^1-tetrahydrophthalic acid [24] exhibits the same phenomenon. Other examples of anhydrides that are stable in the presence of water are some highly substituted fulgides [25] and cantharidin [26].

In none of these cases have the proportions of acid and anhydride in equilibrium at room temperature been reported to be approximately equal. The proportion of anhydride seems to increase with temperature.

A common feature of the anhydrides mentioned above is substitution of the hydrogen atoms of the anhydride ring by larger groups, which probably increase the stability of this ring by steric or other influences. The seven-membered ring of puberulonic acid may well exhibit the same effect.

The third absorption curve in Fig. 7 is that of the equilibrium mixture at pH 3.5. According to the above interpretation of the titration curve this mixture should contain mainly the ions "E^-" and "F^-" together with some of the uncharged molecules (E) and (F) and the ion "F^{--}". The new and striking feature of curve III is the maximum at 317 mμ, which is not present in any of the other curves, but is found in ordinary aqueous solutions of puberulonic acid (Todd *et al.*[10] and Fig. 1). Very probably it is due to the monovalent anion of the anhydride (E), in spite of the fact that it lies further from the strongest maximum of undissociated puberulonic acid (curve I) than would be expected on the basis of the demonstrated effect of hydroxyl ionization of stipitatic and puberulic acids (Figs. 2 and 5). A support of this interpretation is provided by the fact that the maximum at 317 mμ is more intense immediately after the addition of excess of buffer solution of pH 3.5 to a dioxan solution of the substance. Moreover, the maximum is absent immediately after acidifying an alkaline solution to pH 3.5. The explanation of these phenomena is, of course, that ionization takes place instantaneously, whereas the equilibrium (E) $\rightleftharpoons$ (F) is established only after some time.

The maximum at 277 mμ in curve III together with the flat absorption at about 350 mμ must be due mainly to the ion "F^-". The "shoulder" at 290 mμ is probably caused by some undissociated anhydride present. Ionization of (E) apparently causes some increase of absorption in the visible region.

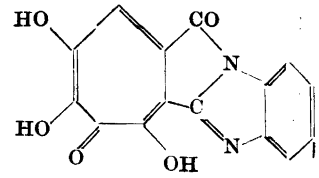
Curve IV (Fig. 7) corresponds to the point on the titration curve where two moles of alkali have been added and the anhydride has been completely transformed into the dibasic acid. The doubly charged anion "F^{--}" should, therefore, predominate in this solution. This conforms very well with the extinction curve, which shows no indication of the presence of the anhydride or its anion. The short-wave maximum has undergone the slight hypsochromic shift expected to occur upon dissociation of the carboxyl groups.

In the solution of pH 7.7 (curve V) the first dissociation of a hydroxyl group in the acid has taken place, resulting in a red-shift of the short-wave maximum. Further dissociation (curve VI) increases this effect and brings the curve very close to that of the trivalent anion of puberulic acid (dotted line).

Thus, it is seen that the ultra-violet absorption data are entirely consistent with the tropolone anhydride structure for puberulonic acid. The lactone formulae, on the other hand, do account for the formation of the acid (F) in alkaline solution, but hardly explain the establishment of an equilibrium in aqueous solution between approximately equal parts of puberulonic acid and (F). An equilibrium involving one of the lactones is improbable from the point of view of energy considerations, because the tropolone ring, being almost aromatic in nature, must possess a high resonance energy. Moreover, on the

basis of formula (C) or (D) a feasible interpretation cannot be found for the maximum at 317 mμ.

Chemical evidence is not necessarily against the anhydride formula. The acetylating difficulties may be overcome by finding suitable conditions, and the condensation product with *o*-phenylenediamine [5, 10] is not necessarily a quinoxaline. The most probable structure is (G) — or a tautomeric form (*cf.* above) — which would be analogous to the products obtained by condensing *o*-phenylenediamine with, for example, cantharidin [27], diphenylmaleic anhydride [28] or hexahydro phthalic anhydride [29].



(G)

SUMMARY

Stipitatic, puberulic and puberulonic acids have been titrated potentiometrically and their U. V. absorption properties have been investigated in organic solvents, and in aqueous buffer solutions of various pH. The influence of the dissociation of the various acidic groups of stipitatic and puberulic acids on the U. V. absorption curves conforms on the whole with expectations based on their structures and previous work on related substances.

It has been shown that the lactone formulae suggested for puberulonic acid by other workers are not compatible with its properties as demonstrated in the present work. Instead, a tropolone anhydride formula (E) is proposed to account for its spectral similarity to stipitatic and puberulic acids, for the anomalies encountered in titration of the substance, and for its U. V. absorption properties at various hydrogen ion concentrations. Spectral evidence discloses the existence of an equilibrium in aqueous solution at room temperature between comparable amounts of puberulonic acid and the corresponding dicarboxylic acid.

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Dependence of the Peptic Hydrolysis of Zein at Different pH on the Protein Concentration

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In examining the so-called plastein formation we have paid attention to the degree and velocity of hydrolysis of zein at different pH while pepsin acts as a hydrolyzing enzyme. This question has gained more importance since the formation of plastein was shown to be an enzymatic synthesis of polypeptides and hence, a reverse to hydrolysis [1-3]. The chemical nature of the insoluble fraction remaining of the hydrolysis of zein has also been a subject of our research.

EXPERIMENTAL

Zein was hydrolyzed at pH about 1, 2, 3, and 4 with cryst. pepsin. Parallel experiments were made at each pH with 2 g of zein in 80 ml, 800 ml, and 1 200 ml of water acidified with HCl. The amount of pepsin was always 5 mg (0.7 mg N) per 1 g zein. In order to avoid zein becoming lumpy and thus disturbing the reaction velocity, the finely ground zein was mixed with quartz sand before putting it in the solution. In this way consistent results were obtained in parallel experiments. The quantity of hydrochloric acid at pH 3 and especially at pH 4 was so small that the pH rose rapidly during the hydrolysis. The acidity of the solutions was followed with repeated determinations of pH by means of a glass electrode and hydrochloric acid was added when necessary. pH could thus be maintained with an accuracy of + 0.4. A slight rise in pH occurred also at pH 1 and pH 2, but no acid was then added.

The hydrolysis of zein was followed by determining the nitrogen brought to the solution.

The chemical nature of the solid substance remaining in the solution at the end of the experiment was examined

1) by determining the total N and the amino N of the substance. The latter was determined as a rule by means of the Cu method of Pope using the coefficient 0.14. The agreement between this and van Slyke method is satisfactory at least in this particular case.

2) by determining the solubility of the precipitate in 60 % alcohol. Zein dissolves in it whereas the polypeptides which are formed in the plastein synthesis are soluble only to a smaller extent.

3) by comparing the x-ray diagrams of zein, plastein, and the precipitate insoluble in the hydrolysis solution. The x-ray diagrams were taken, as described in the previous paper [4], from 1—2 mm thick samples pressed from dry powder.

The dependence of the plastein synthesis on the pH was determined by using a peptic hydrolysate of zein for the starting material. It was prepared by letting cryst. pepsin act for 28 days on a zein suspension acidified with formic acid. pH was at the start 1.78, at the end 2.15. 96.1 % of N was brought to the solution and amino-N was 10.1 % of total N, thus the average size of peptides corresponded to 9.7-peptides.

The clear solution was evaporated to a small volume and by adding alkali (NaOH) or acid (formic acid) a pH series was arranged with pH 1, 2, 3, 4, 5, and 6. Each solution contained 40.0 mg N per 1 ml. The results appear from Table 1.

Table 1. Dependence of plastein precipitation on pH. Experimental time 1 h.

pH	Volume ml	Total N mg	N mg/ml	Cryst. pepsin mg	Precipitate			Loss of amino-N mg	Loss of amino-N, % of initial amino-N
					Dry matter mg	N mg	N % of total N		
1	11	440	40	30	—	—	—	0	0
2	11	440	40	30	42.5	5.44	1.23	1.3	1.4
3	11	440	40	30	317.8	40.67	9.24	6.67	7.2
4	11	440	40	30	637.0	80.9	18.38	14.03	15.1
5	11	440	40	30	389.8	49.9	11.34	6.69	7.3
6	11	440	40	30	126.5	16.10	3.65	4.17	4.5

The peptide size of the hydrolysate was so large that a prolonged hydrolysis would have caused a rise in the amino-N. But within 1 h the synthesis is distinct.

In another experiment where zein was hydrolyzed at pH 1 for 60 days and the average peptide-size of the hydrolysate corresponded to 4.2-peptides the drop in amino nitrogen was 2.2 % at pH 1.5 within 1 h and 4.6 % within 2 and 20 h. The N-concentration of the hydrolysate was in this experiment 53.4 mg per ml.

The dependence of the plastein precipitation on the N-concentration of the hydrolysate is illustrated by the curve in Fig. 1. It does not give a right picture of the corresponding dependence of the plastein synthesis because zein was hydrolyzed in a hydrochloric acid solution at pH 1.2—1.5 and plastein was precipitated from the salt-containing hydrolysate at pH 4. A great amount of small peptides is then precipitated at least in higher concentrations in addition to the synthesized polypeptides [5]. The amount of the plastein precipitate is thus greater than that corresponding the synthesis. We shall deal with this question in another connection at a greater length. The interrupted line respresents the synthesis calculated from the decrease of amino nitrogen. The values have been obtained from experiments made in another connection and not from parallel experiments, hence they are approximate.

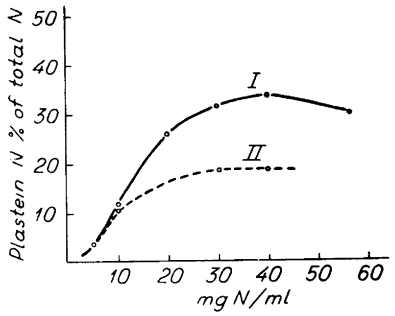

Fig. 1. Dependence of plastein formation at pH 4 on the N-concentration of zein hydrolysate.
I Precipitated N % of total N of the hydrolysate.
II Decrease of amino-N.

RESULTS

The results of the pepsin hydrolysis of zein in different concentrations and at different pH are presented by curves in Figs. 2. It can be seen from them that at pH 1 and pH 2 the zein concentrations of 375 mg N/100 ml, 37.5 mg N/100 ml, and 25 mg N/100 ml have allowed an almost complete (97 %) hydrolysis in the highest concentration and a complete hydrolysis in a 10- and 15-fold dilution. At pH 3 and 4, on the other hand, zein was only partly brought to the solution in the highest concentration. At pH 4 only about 40 % of the zein was brought to the solution during 13 days and the direction of the curve shows that the percentage rises then very slowly with prolonged time. The degree of hydrolysis, again, rises distinctly while the dilution in-

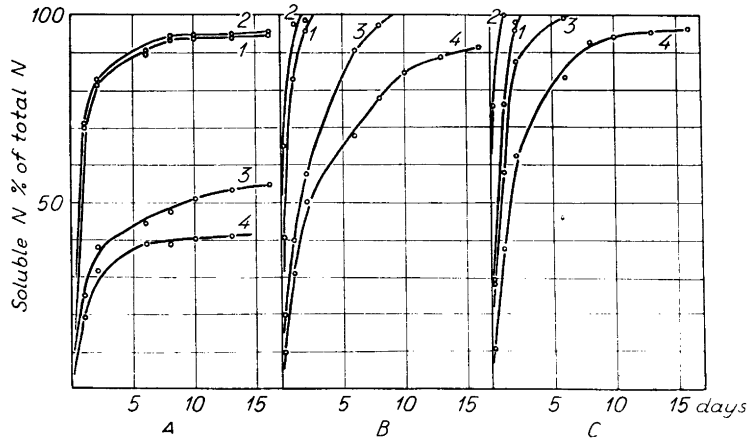

Fig. 2. Hydrolysis of zein at different pH and different concentrations. The numbers indicate the pH of the solution during hydrolysis.
A = Zein concentration 3.75 mg N/ml
B = » » 0.375 » »
C = » » 0.25 » »

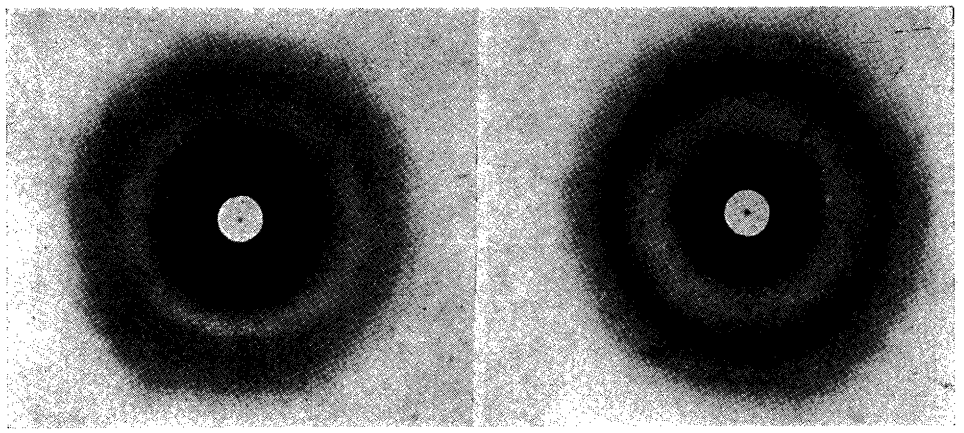

Fig. 3. X-ray diagram of zein (left) and plastein (right) precipitated from the pepsin hydrolysate of zein. The average peptide size of plastein about 50-peptides.

creases. In a 15-fold dilution the zein is almost entirely brought to the solution in 16 days.

These results indicate that a reaction reverse to hydrolysis prevents hydrolysis at pH 3 and especially at pH 4 which is the optimum of the plastein synthesis. At pH 1—2 the synthesis is very weak and experimentally detectable only in much more concentrated solutions than the highest one used in this

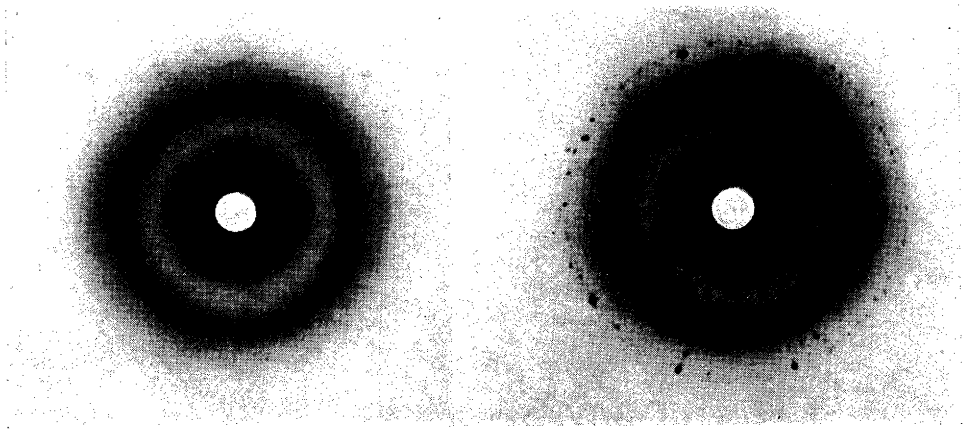

Fig. 4. X-ray diagram of a mixture of zein and plastein (1 : 1) (left) and insoluble residue, which was left over on hydrolysis of zein at pH 4 (right). The spots in the figure are caused by quartz sand particles among the preparation.

work. A complete or nearly complete hydrolysis of zein is then natural. At pH 4, again, the plastein synthesis is experimentally detectable even in such a peptide concentration which is brought about by hydrolysis in the highest zein concentration used in this work. As 40 % of zein nitrogen has been brought to the solution the hydrolysis contains 1.5 mg N per 1 ml. In a zein hydrolysate which contained 1.55 mg N per 1 ml, pepsin produced during a longer time small precipitate whose amino-N was 3.7 % of total N. Plastein synthesis proceeds thus weakly in this peptide concentration.

The insoluble substance which remains over of the hydrolysis at pH 4 dissolves only partly in 60 % alcohol at room temperature. Since zein dissolves quantitatively in this solvent the insoluble substance cannot be exclusively zein. Amino nitrogen of this substance is also higher (in different preparations 0.6—1.6 % of total N) than in zein (about 0.25 % of total N). It is evident, therefore, that the insoluble fraction contains considerably — in experiments of longer duration perhaps chiefly — polypeptides. These may be either products of synthesis or partly high-molecular products of zein hydrolysis, which have not decomposed further. Our concept of the course of protein hydrolysis and plastein synthesis shows the formation possibilities of the insoluble substance in the pepsin hydrolysis:

$$\text{Protein} \rightarrow \underset{\text{(plastein)}}{\text{Polypeptides}} \rightleftharpoons \text{Low-molecular peptides}$$

The x-ray diagrams of zein, plastein and the insoluble substance in pepsin hydrolysis as well as of the mixture of plastein and zein (1 : 1) are given in Figs. 3 and 4.

The rings are sharper in the diagram of the insoluble substance than in zein, but nevertheless slightly more diffuse than in plastein. The x-ray research thus leads to the same result as the determinations of amino nitrogen and solubility in regard to the chemical nature of the precipitate.

The ratio of equilibria in the pepsin hydrolysis of zein is very complicated. The system contains at first only insoluble zein, then growing amounts of soluble peptides and later increasing amounts of polypeptides, which are precipitated. The precipitate then contains simultaneously zein and polypeptides.

SUMMARY

Comparative experiments on the hydrolysis of zein by the action of pepsin at different pH and in different concentrations of zein have shown that at pH 1 and 2 the nitrogen of zein is almost or entirely brought to the solution in concentrations of 3.75 mg N, 0.375 mg N and 0.25 mg N per 1 ml. On the

other hand, at pH 3 and especially 4 a considerable or the major part of nitrogen is in the insoluble residue.

The amino nitrogen of the insoluble residue is considerably higher than that of zein and the residue dissolves only partly in 60 % alcohol, in which zein dissolves completely. Accordingly, the residue contains considerably polypeptides (plastein). The x-ray diagrams lead to the same result in regard to the nature of the residue as determinations of amino nitrogen and solubility.

The results suggest that the insoluble residue in the pepsin hydrolysis of proteins at pH 3 and most distinctly at pH 4, which is optimum to the plastein synthesis, is due to the formation of insoluble polypeptides.

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Short Communications

Calibration of an Electrometric Method for the Determination of Cholinesterase Activity

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The intention of this investigation was to test the accuracy of Michel's [1] method for the determination of cholinesterase activity by means of continuous registration of the pH change. Michel determines the pH change in a buffered solution caused by the acetic acid from the hydrolysis of acetylcholine. He is of the opinion that the rate of the pH change is a satisfactory measure of the enzyme activity, which is true if the interest is limited to the comparison of different enzyme activities determined in the same buffer solution. However, it seemed more reasonable to use the change in the acetic acid concentration per unit time. This measure makes it possible to compare results obtained from different buffer solutions. Furthermore, we will also describe a method that makes it possible to convert measured pH changes into changes of the acetic acid concentration.

Apparatus. For the determination of pH we used a glass electrode in combination with a saturated calomel electrode. This cell was connected to a potentiometer of the type PHM 22d, Radiometer, Copenhagen. To make it possible to record the continuous change in pH a "Brown recorder" was connected to the potentiometer.

The determination of the change in the acetic acid concentration as a function of pH demanded the use of an "Agla micrometer syringe" driven by a synchronous motor and giving 10 μl per minute. Temperature was kept at 25° C in the solutions by an oil thermostat.

Experiments. The buffer used was prepared according to Michel and had the following composition. 4.1236 g (0.02 moles) sodium barbital, 0.5446 g (0.004 moles) KH_2PO_4 and 44.730 g (0.60 moles) KCl were dissolved in 900 ml of water. 10 ml of 0.1000 *M* HCl were added and the solution made up to 1 000 ml with water. The water used in all solutions was redistilled in pyrex glass ware.

The enzyme preparation used was a hemolysate of human erythrocytes prepared according to Augustinsson [2]. Heparinised blood was centrifuged and the corpules washed twice with 0.9 % NaCl and made up to blood volume with water. 1 ml of this solution was diluted to 20 ml with water. Acetylcholine chloride was used as a substrate of which a 0.11 *M* solution was made immediately before addition.

3 ml of the erythrocyte hemolysate and 3 ml of buffer were mixed. They were then placed in the oil bath for 15 minutes to obtain temperature equilibrium. After that 0.6 ml acetylcholine solution was added and the pH registration started. In experiments with tetraethyl pyrophosphate (TEPP) the inhibitor was added half an hour before the acetylcholine.

The apparatus was calibrated in the following manner. The pH scale was set against three standard buffer solutions (pH 7.98, 7.40 and 7.00). By means of the

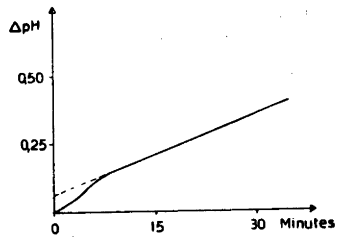

Fig. 1. The variation of pH as recorded by our apparatus in an experiment with uninhibited cholinesterase.

micrometer syringe acetic acid was infused in a solution where the substrate was substituted by water of the same volume. The acetic acid was 0.1379 *M*. The rate of the addition of the acetic acid was of the same magnitude as by the enzymatic decomposition.

Results. The method was tested in an experiment, where the enzymatic activity was determined as a function of various TEPP concentrations. The activities were then determined from the slopes of the lines obtained by the "Brown recorder". The velocity of the recorder paper (0.332 cm/minute) and the aforementioned calibration diagram can be used for the calculation of the "activities" expressed in μ moles of acetic acid per ml per minute.

A deviation from the straight line obtained by the recorder was observed during the first five minutes (see Fig. 1), and this deviation became more permanent, if the

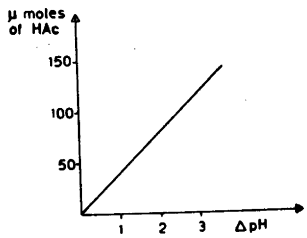

Fig. 2. The calibration curve gives μ moles of acetic acid as a function of pH.

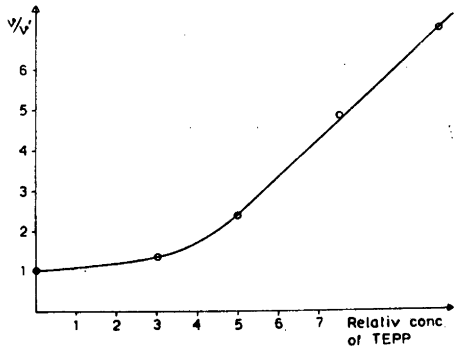

Fig. 3. Inhibition of erythrocyte cholinesterase as a function of the TEPP concentration.
v = reaction rate with uninhibited enzyme.
v' = » » » inhibited enzyme.
1 rel. unit for TEPP conc. = $2.86 \cdot 10^{-9}$ M.

protein content was increased. In experiments with enzyme preparations of low activity, where the concentration of the hemolysate must be increased in order to obtain a measurable slope of the line this deviation may cause erroneous determinations. Under any circumstances it is not advisable to use the points taken during the first five minutes, if point measurements are made according to Michel [1] or Davies and Rutland [3]. A buffer solution which is known not to contain inhibiting substances [4–5] but contains activators as calcium and magnesium ions and which is more sensitive to changes in the hydrogen ion concentration will possibly make this method more useful for enzyme solutions of low activity.

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Crystalline Phosphoserine from Casein Hydrolysate

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In connection with recent investigations of phosphopeptone in this institute [1] we have tried to test by chromatographic methods the homogeneity of these preparations. In the hydrolysate of electrodialyzed phosphopeptone we could identify two hydroxyamino acids, serine in large amounts and threonine in small amounts, and a priori the possibility could not be excluded that both were esterified with phosphoric acid.

The fact that the phosphoric acid in casein is esterified on the alcoholic group of serine is ascribed to Lipmann, who isolated an amorphous silver salt of serine phosphoric acid from casein, following hydrolysis by boiling with 2.5 *N* hydrochloric acid [2]. In his paper [3] Lipmann gives the following analytical figures for the silver salt:

Calc.	C	7.12	H	0.99	N	2.76	P	6.12	Ag 63.8
Found	»	7.56	»	1.24	»	2.79	»	5.65	» 61.2

Phosphothreonine is about as resistant toward acid hydrolysis as phosphoserine [4] and in our view the possibility that small amounts of phosphothreonine might be present in such a preparation could not be excluded. If present it would possibly be proven by means of paper chromatographic analysis.

EXPERIMENTAL AND RESULTS

The preparative method of Lipmann [3] was followed with a few modifications. 50 g samples of casein were hydrolyzed, neutralized, and precipitated as described by Lipmann. The barium precipitate was thoroughly extracted three times with CO_2-free water and the filtrate concentrated *in vacuo* to a small volume. The reaction was adjusted to pH 4.6 by addition of glacial acetic acid, and the phosphorus-containing compounds precipitated by addition of a saturated solution of lead acetate until no more precipitation occurred. The clear supernatant liquid was removed by centrifugation and the precipitate suspended in water and decomposed by means of hydrogen sulphide. The lead sulphide was centrifuged off and the solution concentrated *in vacuo* to a small volume and precipitated with ethanol and ether in the usual manner. The precipitate was electrodialyzed in an apparatus similar to that described by Theorell and Åkesson [5]. When the anode fraction was concentrated first *in vacuo*, and subsequently over silica gel in a desiccator, a crystalline precipitate was obtained. (Fig. 1.) The precipitate was recrystallized from water, dried to constant weight in a desiccator at 25° C over silica gel, and analyzed.

$C_3H_8O_6NP$

Calc.	C	19.46	H	4.32	N	7.57	P	16.76	
Found	»	19.36	»	4.26	»	7.68	»	15.80	

Total nitrogen was determined by the micro-Kjeldahl method using $KMnO_4$ as a catalytic agent. The usual micro-Kjeldahl procedure with hydrogen peroxide as a catalyst gave low values. On heating the substance in desiccator at 105° C for 3 hours, low nitrogen values were obtained by the Dumas-method. Lipmann [3] reported that his silver salt of phosphoserine lost a large amount of nitrogen when it was dried at 100° C. The quotient of Van Slyke nitrogen to total nitrogen was 1.0.

Phosphorus was determined according to Teorell [6]. A combustion time of 4 days was found to be necessary. On heating, the crystals gave a m. p. of 167° (decomposition). The optical rotation a_D^{23} was + 7.2° (water; *l*, 1; c. 4.17). With two dimensional chromatograms a single spot corre-

Fig. 1.

sponding to the position of a synthetic specimen of phosphoserine was observed. Serine was completely set free by hydrolysis with 2 *N* HCl at 120° for 24 hours. When large samples of the hydrolysate (1 mg) were analyzed chromatographically, a very faint alanine spot was observed in addition to the large serine spot. At present it is not possible to decide whether the alanine was present as part of a phosphoserine peptide in the crystalline preparation, or formed from phosphoserine during hydrolysis. The phosphopeptides left in the residue after crystallization of phosphoserine are being investigated with the interest focused primarily on the question of whether or not phosphothreonine is present in the mixture.

The investigation was supported by a grant from the Swedish Medical Research Council. The authors are indebted to Mr. W. Kirsten for the micro carbon and hydrogen analysis, to Mr. S. Eklund for the nitrogen analysis and to Mr. E. Lindberg for the phosphorus analysis.

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On Homospecific Liver Pyrophosphatases

BO NORBERG

Biochemical Department, Karolinska Institutet, Stockholm, Sweden

Recent investigations[1] have demonstrated the existence of four isodynamic pyrophosphatases in rat liver. Swanson [2], however, found only two pyrophosphatases in rat liver. It therefore seems of interest to report some further studies on these enzymes in rat liver and in liver of other species. At the same time a correction regarding the magnesium ion concentration in the previous experiments [1] is given.

The livers of freshly killed animals were used throughout. The assay of pyrophosphatase activity was performed at 37° C as previously described [1]. At pH values below 6 the "third method" was used, *i. e.* the inorganic phosphate liberated was isolated as hydroxyl apatite before estimation. At higher pH values the phosphorus was determined directly on an aliquot of the protein-free filtrate obtained according to the "second method". The pyrophosphate concentration in the enzym digestion mixture was kept at 10^{-3} *M* in all experiments.

For the acid range up to pH 5.8 acetate buffers were used, from pH 6 to 7.6 cacodylate buffers (Plumel [3]) succesfully replaced maleic acid and collidine buffers. Over pH 7.6 ammonium chloride-ammonia buffers were used.

The activating reagent was prepared from a stock solution of molar $MgSO_4$ by dilution with 0.05 *M* buffer. When a new stock solution was prepared (autumn 1950) the enzyme values were lower than before. A check-up study showed that the Mg ion concentration giving maximum activation was about 0.01 *M* instead of 0.2 *M*. As the earlier studies were clearly made with optimal activation con-

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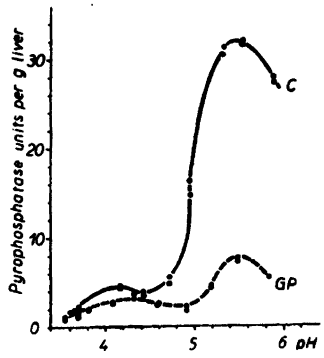

Fig. 1. Acid pyrophosphatases in liver. Dots and fully drawn line: cat liver. Circles and dotted line: guinea pig.

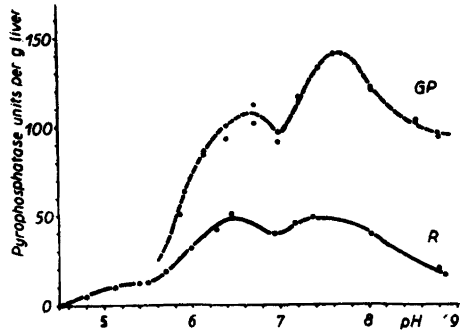

Fig. 2. Pyrophosphatases in liver. Dots and fully drawn line: rabbit liver. Circles and dotted line: guinea pig.

centration the old stock solution which was prepared by a technician, and which I had not controlled, apparently was only one twentieth of the intended concentration. With the proper correction my earlier results should be comparable with other investigations, however.

The pH-activity curves prepared under the conditions described show two peaks in the acid region at about 4.2 and 5.5, as is evident from Fig. 1. With hamster and rabbit liver there is a plateau at pH 5 to 5.5. The curve then rises steeply to the maximum of the neutral pyrophosphatase. In several experiments with rat and guinea pig liver the pH 5 enzyme is indicated by a hump in the curve only. The pH 5 pyrophosphatase may therefore easily be overlooked.

With collidine (method 3) and with cacodylate as buffers the neutral pyrophosphatase has its maximum at pH 6.6 to 7.0. On the alkaline side the optimum appears between pH 7.6 and 8 (Fig. 2). These two pyrophosphatases are some ten times more active than the acid homospecific enzymes with activities from 50 to more than 300 units per g liver (1 unit = 1 micromol of phosphorus liberated per minute in the test system).

Distinction of the homospecific pyrophosphatases by other properties than the pH optima is possible, *e. g.* through selective inactivation [4] or inhibition [1]. The pyrophosphatase pH 4 is inhibited by 0.01 *M* Mg^{++} from 10 to 54 % in guinea pig liver. The pH 5 pyrophosphatase, on the other

Table 1. Pyrophosphatase activities before and after acetone precipitation. Expt. 143 aqueous homogenate pH 6.88. Expt. 193 homogenate in 0.2 % ammonia and 0.003 M $MgCl_2$. Expt. 223 homogenate in 0.2 % ammonia pH 9.2.

	I. Activity at pH 5.2			II. Activity at pH 6.8			Activity ratio I/II		
Expt. no.	143	193	223	143	193	223	143	193	223
Original homogenate	22.0	18.6	20.2	100	99	67.7	0.22	0.188	0.298
40—75 % acetone ppt	8.41	4.45	0.26	23.9	65.6	3.75	0.35	0.068	0.07

hand, is activated considerably, the increase in activity amounting to about 200 %. As the neutral pyrophosphatase is also strongly activated by magnesium it seems essential to demonstrate the mutual independence of the pyrophosphatase 5 and the neutral one. This was tried on rat liver homogenates by fractional precipitation with acetone at 0° C. Comparison between the original activities and the activities of the proteins precipitated by 40 to 75 % acetone demonstrates the requested independence as appears from Table 1. The activity ratio may be changed in opposite directions by slight changes in pH etc. This means that the two enzymatic activities are differently affected, *i. e.* a selective partial inactivation occurs.

This is further evidence for the existence of an acid pyrophosphatase with optimum at pH 5 – 5.5 in addition to the three earlier known isodynamic enzymes. The enzyme seems to be present not only in rat liver but also in the liver of guinea pig, hamster, rabbit and cat.

This investigation has been supported by funds from the State Medical Research Council. I am indepted to P. O. Bethge for technical assistance.

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New Salts of Benzylpenicillin with Organic Bases

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It is known that benzylpenicillin forms crystalline well defined salts with several organic bases, and salts with secondary [1] and tertiary [1–4] as well as primary [1,5–7] amines have been described.

In an attempt to find bases, which might be useful for the isolation of penicillin, a further series of organic amines have been investigated. The penicillin salts were all prepared in essentially the same way; namely, by the addition of a slight excess of the base, dissolved in acetone to a solution of pure free benzylpenicillin in amyl acetate-acetone. Some of the salts separated in crystalline form, others as oils, as shown in the following table.

Base	Reaction product with benzylpenicillin
N-Monomethylethylenediamine [8]	oil
N-Monoethylethylenediamine [8]	»
2-Methylimidazoline [9]	crystals
2-Ethylimidazoline [9]	oil
2-Propylimidazoline [9]	crystals
2-Amylimidazoline [9]	oil
2-Benzylimidazoline	»
2,4(5)-Dimethylimidazoline	»
2-Ethyl-4(5)-methylimidazoline	crystals
2-Propyl-4(5)-methylimidazoline	»

The preparation and properties of the dialkylated imidazolines referred to will be described in a forthcoming communication in this Journal.

Only one example of the preparation of the benzylpenicillin salts will be given in detail.

The 2-propylimidazoline salt of benzylpenicillin. 5.0 g of sodium benzylpenicillin was dissolved in 20 ml of water at 0 – 5° C, 40 ml of cold amyl acetate was added, and then, with vigorous shaking, 4.5 ml of 60 % phosphoric acid. The aqueous layer was separated and extracted with an additional 10-ml portion of cold amyl acetate. The combined

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2-Benzylimidazoline	»
2,4(5)-Dimethylimidazoline	»
2-Ethyl-4(5)-methylimidazoline	crystals
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cold amyl acetate solutions were dried with sodium sulphate, filtered, and diluted with 50 ml of dry acetone. To this solution was added, with stirring, during a period of about 15 minutes, a 10 % solution of 2-propylimidazoline in dry acetone, in amount sufficient to produce a marked green colouration of bromthymol blue indicator. During the addition, the 2-propylimidazoline salt of benzylpenicillin began to crystallize. The mixture was set aside in the refrigerator overnight, then the salt was collected, washed with small amounts of acetone, and dried *in vacuo* at 20°. Yield: 6.2 g of colourless, small needle-like crystals, $[\alpha]_D^{20}$ + 231° (c = 1 in water); m. p. 114° (decomp.); pH of aqueous solution, 5.25; biological assay 1 290 U/mg.

The compound is hygroscopic and very soluble in water and ethanol, soluble in chloroform.

$C_{16}H_{18}O_4N_2S \cdot C_6H_{12}N_2$ (446.3)

Calc.	C	59.15	H	6.77	N 12.55
Found	»	59.60	»	6.86	» 12.41

The 2-methylimidazoline salt of benzylpenicillin. 5.0 g of sodium benzylpenicillin yielded 5.8 g of colourless, rod-like crystals in rosettes, $[\alpha]_D^{20}$ + 242° (c = 1 in water); m. p. 80°; pH of aqueous solution, 4.98; biological assay 1 350 U/mg.

The salt is more hygroscopic than the 2-propylimidazoline salt and it has not been possible to obtain in a completely pure state, as is evident from the analytical figures. The compound is very soluble in water, ethanol and chloroform.

$C_{16}H_{18}O_4N_2S \cdot C_4H_8N_2$ (418.3)

Calc.	C	57.38	H	6.26	N 13.39
Found	»	56.64	»	6.66	» 12.66

The 2-ethyl-4(5)-methylimidazoline salt of benzylpenicillin. 5.0 g of sodium benzylpenicillin yielded 5.3 g of colourless, prismatic crystals, $[\alpha]_D^{20}$ + 232° (c = 1 in water); m. p. 141° (decomp.); pH of aqueous solution, 5.75; biological assay 1 270 U/mg.

The salt relatively non-hygroscopic and very soluble in water and ethanol, soluble in chloroform.

$C_{16}H_{18}O_4N_2S \cdot C_6H_{12}N_2$ (446.3)

Calc.	C	59.15	H	6.77	N 12.55
Found	»	59.06	»	6.95	» 12.49

The 2-propyl-4(5)-methylimidazoline salt of benzylpenicillin. 5.0 g of sodium benzylpenicillin yielded 6.3 g of colourless, rod-like crystals, $[\alpha]_D^{20}$ + 224° (c = 1 in water); m. p. 132° (decomp.); pH of aqueous solution, 6.15; biological assay 1 240 U/mg.

The salt is relatively non-hygroscopic and very soluble in water and ethanol, soluble in chloroform.

$C_{16}H_{18}O_4N_2S \cdot C_7H_{14}N_2$ (460.3)

Calc.	C	59.96	H	7.01	N 12.17
Found	»	60.24	»	7.12	» 11.94

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The Mechanism of Alcohol Dehydrogenase Action

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The preparation of horse liver alcohol dehydrogenase (ADH) in a pure and crystalline state [1] has enabled us to carry out an extensive investigation on the reaction velocities and equilibria in the system:

$$DPN + C_2H_5OH \overset{ADH}{\rightleftharpoons} DPNH + H^+ + CH_3CHO$$

It was observed that the absorption band at 340 mμ in the free DPNH moved down to 325 mμ when DPNH was bound to ADH [2]. This phenomenon made a much more detailed study of the enzyme mechanism possible than in any DPN-enzyme system before.

Spectrophotometric measurements revealed that two molecules of DPNH were bound to one molecule of ADH (M = 73 000) at pH 7—9. The bonds are loosened at higher pH, so that around one molecule of DPNH is bound per mole of ADH at pH 10.

The molar concentration of ADH is so high in the liver that it approaches the DPN concentration. Our studies on the reaction equilibria with varied pH and [ADH] showed that the ratio between the dissociation constants of the DPN · ADH and DPNH · ADH complexes varied from 200, at pH 7, to a value slightly above 1 at pH 10. The redox potentials of the ADH-coferment complex were calculated from the equilibrium values to $E_0' = -0.196$ V at pH 6.4; -0.208 V at pH 7; -0.244 V at pH 8; -0.302 V at pH 9; and -0.351 V at pH 10. These potentials are much higher than for the free DPN—DPNH, and approach the potential level of the ethanol-acetaldehyde. This effect greatly facilitates the oxidation of ethanol in the ADH-system.

The slopes $\frac{d E_0'}{d pH}$ for the ADH-coferment complex indicate that an acid group had a pK' of 10 in the reduced, and pK' 7.8 in the oxidized complex.

This acid group was identified as sulfhydryl, linked to the reduced pyridine ring of DPNH, probably its ring nitrogen atom.

G. Wald in a recent, personal communication to us pointed out that the ADH activity was inhibited by *p*-chloromercurobenzoic acid. The inhibition was reversed by glutathione. We found that the absorption maximum of ADH-bound DPNH immediately shifts from 325 to 340 mμ on the addition of 0.003 *M* *p*-chloromercurobenzoic acid. Our conclusion is that this shift is caused by a bond between ADH—SH and pyridine being broken. This bond is necessary for the enzyme activity.

To our knowledge this is the first observation of SH-groups in apoenzymes interacting with coferments.

Determinations of the association and dissociation velocity constants of ADH · DPNH were carried out by the aid of the band shift by one of us (H. T.) and B. Chance in Philadelphia. Equations were derived for the kinetics of the enzyme systems. All experimental observations hitherto seem to fit with this theory.

A detailed report of this work will be published in this journal.

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New Books

W. Theilheimer, *Synthetische Methoden der Organischen Chemie, Repertorium 3.* S. Karger Ltd. Basel (New York), 1949. 412 pages. Price Swiss fr. 40.—.

This is the third of a series of volumes, the first two of which appeared in 1945 and 1948 respectively, the object of which is to record selected new procedures or valuable improvements on existing methods for the preparation of organic compounds. The earlier volumes covered the literature for the periods 1942—44 and 1945—46 and the present volume 1946—47.

The material is arranged according to the method of formation of an individual bond, the different types of reaction being denoted by a system of symbols devised by Weygand. Thus methods of establishing H—O, H—N, H—S, H—C, O—N, O—S O—C, N—N, N—C, Hal—C, S—C, and C—C linkages by reaction involving addition, elimination, ring opening, ring closure, or rearrangement are reviewed. In the examples chosen, the starting materials, reaction conditions and yields are indicated very briefly, but the original paper, to which the reference is given, must be consulted for more information.

Thus, for example, the formation of an indene derivative by establishment of a C—C-link by a rearrangement involving ring closure under the influence of boron fluoride: In eine Lsg. von 3,4-Di-(p-oxyphenyl)-hexadien-2,4 (formula given) in Chlf. unter Kühlung mit Eiswasser 12 Min. bis zur bis zur Sättigung BF_3 eingeleitet u. noch 18 Min. bei Zimmertemp. stehengelassen → 1-Methyl-2-(p-oxyphenyl)-3-äthyl-6-oxyinden (formula given). Roh-A, 92 %. (W. B. s. E. Adler u. B. Hägglund, Ark. Kemi, 19 A, Nr. 23 (1945).)

These publications are, of course, of great value to research chemists engaged on synthetic problems as they provide a readily accessible method of seeking out analogous reactions in the nowadays formidably voluminous chemical literature, and a general perusal of the series gives considerable insight into modern advances on the synthetic side of organic chemical science. The future popularity of "Theilheimer" is dependant on the completeness of its coverage, and the critical sifting of the modern literature, and in the three volumes which have appeared to date, the author has shown that he has been able to master this difficult task most capably. The index is admirably complete, and is at the same time a cumulative index for the three volumes.

H. Erdtman

F. Feigl. *Chemistry of Specific, Selective and Sensitive Reactions.* Translated from the German manuscript by R. E. Oesper. Academic Press Inc., New York, 1949. xiv + 740 pp. $13.50.

This new book by the founder of the spot test method of qualitative analysis is the theoretical counterpart of the author's laboratory manual *Qualitative Analysis by Spot Tests* (Elsevier, 1946). The book presents "an attempt to summarize our knowledge of the scientific background of the specificity, selectivity and sensitivity of analytical procedures". Specific reactions are such which, under defined experi-

mental conditions, indicate one specified component; selective reactions are those which are characteristic not for one, but for a limited number of substances.

The material is divided into twelve chapters, of which the most valuable ones appear to be those on "The masking and demasking of reactions", "Enhancement of reactivity of compounds and reaction systems", and "The effect of certain atomic groupings on the specific and selective activity of compounds in inorganic analysis" (mostly organic reagents). These three chapters alone contain a wealth of useful material, which should be of interest to inorganic chemists in teaching as well as research. They do, however, make up only about half of the book, and the remaining chapters are not of the same high quality. Especially the large (180 pp.) chapter on "Surface effects in analytical chemistry", although containing much interesting experimental material, is too wordy, and lacks in clarity.

The precursor of the present treatise is Feigl's *Specific and Special Reactions* (Elsevier, 1940. Ca. 200 pp., $3.50). It is the subject matter of that book which, partly revised and enlarged, constitutes the best chapters of the present one. The reviewer would almost have preferred to miss the new chapters and rather pay a more reasonable price.

In spite of these objections, the book is highly recommended for reference use. It represents a unique source of information for everyone interested in analytical chemistry.

Olav Foss

Analytical Chemistry and Chemical Analysis 1948. Proceedings of the International Congress on Analytical Chemistry, Utrecht, June, 1948. Reprinted from *Anal. chim. Acta* 2 (1948) 417–854. Elsevier Publishing Company, Inc., New York — Amsterdam — London — Brussels, 1949.

The Congress was attended by about 40 British, 16 Belgian, 1 Czechoslovak, 7 Danish, 17 French, 4 Swedish, 5 Swiss and some 250 Dutch chemists and physicists. There were five Sections dealing with: 1. General methods and standardisation (11 papers); 2. Electrical methods (11); 3. Emission spectrography; 4. Optical measurements and physical methods of separation (13); 5. Microbiological methods and detection of traces (10). All papers except those of Section 3 are reproduced in the book.

It is not possible to discuss the separate papers here; however, the Opening Adress by the President of the Congress, C. J. Van Nieuwenburg, deserves mentioning. In the Adress, "Analytical chemistry and chemical analysis, now and in the future", he discriminates between chemical analysis (routine work) and analytical chemistry (analytical research). Regretting that this science has a low prestige in several European countries, he says that 'Even now a great many universities think that they can do with analytical training simply as a preparatory course, as a simple "technique", which they grudgingly admit has to be mastered before starting "really scientific" work, but which does not deserve a full academic chair. As often as not the situation in industrial laboratories is even worse. They cannot get a sufficient number of analytical specialists from the universities'. One of the purposes of the Congress was therefore to strengthen the reputation of the analytical science by showing, as stated in the Preface, 'the great changes analytical chemistry has undergone in recent years. Because of the demand for greater speed and accuracy and the need for handling small quantities of material, the classical methods based on gravimetric and volumetric procedures are rapidly being replaced or supplemented by physicochemical and physical techniques. The status of analytical chemistry is being raised to a much higher level, and analyti-

cal chemistry is becoming a science for the specialist.

Modern analysis, with its interests in optical, electrical and microbiological methods, makes very high demands in the training of its practitioners, and it was stressed by speakers from several countries that the training of analytical chemists is a long way behind the requirements of modern industry'.

Olav Foss

Elastomers and Plastomers. Edited by R. Houwink. Elsevier Publishing Company, Inc. I. *General Theory.* 495 pp. II. *Manufacture, Properties and Applications,* 515 pp. (24 fl.).

The two volumes are to be followed by a third on testing and analysis and containing a tabulation of properties.

They will be of interest to all who are interested in that broad science of polymers, which has become so extremely useful to mankind. It is written by a number of the best authorities in the field and the exposition is clear and rather easy to follow.

The first chapter in Vol. I deals curiously enough with a survey of the economic aspects, but for the more or less theoretically minded reader it is useful at once to be reminded of the economic consequences of his fellow-scientists efforts.

The rest of Vol. I contains a mass of valuable material.

The different chapters are well balanced in length according to their importance for the subject. Thus the chapter on the chemical kinetics of "polyreactions" comprises about 100 pages, and the chapter on mechanical proporties and on "physics and structure" about 60 pages each. For scientists who are interested in kinetics the above mentioned chapter is of special interest. One finds here presented in a handy form an account of the main features of the different mechanisms, which have proved valid for the understanding and the technical development of the different types of polymerization-reactions. It is a pleasure to read this well written account of an application of the theory of chain reactions to peaceful ends. This and all the other chapters are provided with extensive lists of references.

A few of the chapters in this volume seem more technological than theoretical, but again it is useful for the theorist to see how the chemical and physical proporties of the substances determine the construction of apparatus for the treatment: moulding, hardening a. s. o.

In Vol. II the different plastomers are treated successively, each type in its own chapter. The one (chapter 2) on synthetic resin ion exchangers will be wellcome to many chemists also outside the circle of "plastic-minded" people.

Of course not only products made from artificial polymers, but also products made from natural high polymers are treated. As examples products from natural proteins, natural rubber and natural resin may be mentioned.

Maybe the ordinary chemist will be mostly interested in the productions of the purely synthetic products and their mother substances, *e. g.* formaldehyde and phenol, which are adequately described. One cannot expect to find factory secrets disclosed in a book like this, but with this proviso also this volume seems to meet the needs of the chemist and also the specialist in this branch of chemistry.

The printing and the paper are as they should be in a book as this of high standard.

J. A. Christiansen

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The Reaction Between Carbon Disulphide and Azide Ions

A Kinetic Investigation

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Sommer [1] was the first to observe that carbon disulphide and azide ions interact with the formation of azido-dithiocarbonate ions, $SCSN_3^-$. A kinetic study of this reaction was of interest because Hofman-Bang and Szybalski [2] found that the rate determining reaction step of the carbon disulphide catalyzed iodine-azide reaction was a second order reaction between carbon disulphide molecules and azide ions. Further it was shown [3] that this iodine-azide reaction was a chain reaction with a chain length of about 17 at room temperature, and that the chain length was almost independent of temperature. From these results it could be inferred that the energy of activation of the reaction between azide ions and carbon disulphide could not differ greatly from that of the iodine-azide reaction, and that the ratio between the rate constants of the two reactions would be identical with the chain length of the iodine-azide reaction. The experiments confirmed these expectations.

ANALYTICAL METHOD

Introductory experiments showed that the formation of azido-dithiocarbonate ions was accompanied by the formation of a very small percentage of partly colloidal sulphur, and therefore spectrophotometric measurements were left out of consideration. The formation of these small amounts of sulphur was presumably due to slow decomposition of azido-dithiocarbonate ions. Currier and Browne [4] found that a solution of sodium azido-dithiocarbonate was slowly decomposed according to the scheme:

$$SCSN_3^- \rightarrow N_2 + S + SCN^-$$

Browne and Smith [5] investigated possible methods for the determination of azido-dithiocarbonate ions when no interfering substances were present. The best method was found to be a Volhard titration. The procedure was as follows:

The sample of azido-dithiocarbonate was dissolved in about 125 ml of water, a few drops of conc. nitric acid were added, and thereafter 0.05 *N* silver nitrate solution was added to an excess of about 10 ml. The mixture was allowed to settle for two hours. The precipitated silver azido-dithiocarbonate was removed by filtration and the filtrate and wash water were treated with 2 ml of a 10 % solution of ferric alum which contained one fourth of its volume of nitric acid. The excess of silver was then determined by titration with a standard solution of ammonium thiocyanate to the appearance of a permanent red coloration. The error of this determination was 0.1—0.2 %.

This Volhard titration was modified so that the amount of azido-dithiocarbonate could be estimated when an excess of sodium azide and carbon disulphide was present. In water the solubility of silver azido-dithiocarbonate, $AgSCSN_3$, was found [5] to be 2.2×10^{-4} mole per liter at 25° C. According to Browne *et al.*[6] azido-dithiocarbonic acid, in contradistinction to hydrazoic acid, is a fairly strong acid. So, although the solubility in water of silver azide [7] is only 5.1×10^{-5} mole per liter at 25° C, it was to be expected that

Table 1. Effect of concentration of nitric acid on the Volhard titration of azido-dithiocarbonate ions. 10 ml solution containing 1.025 millimole sodium azido-dithiocarbonate was diluted with 125 ml water or 1, 2 or 4 M nitric acid. Silver nitrate (0.05074 M) was added to an excess of 5—10 ml. After two hours the mixture was filtered, and the filtrate and wash water, after addition of ferric alum, were titrated with standard ammonium thiocyanate solution (0.05460 M).

Expt. no.	Conc. of nitric acid	Silver nitrate added, ml	Ammonium thiocyanate added, ml	Azido-dithiocarbonate found	
				millimole	%
1	0	27.29	6.60	1.023	100
2	0	27.67	6.88	1.027	
3	1 *M*	28.04	7.47	1.014	98.6
4	1 »	28.29	7.82	1.007	
5	2 »	28.43	7.82	1.014	99.0
6	2 »	28.37	7.75	1.015	
7	4 »	28.40	7.86	1.009	98.6
8	4 »	28.45	7.86	1.013	

Table 2. Effect of sodium azide and carbon disulphide on the Volhard titration of azido-dithiocarbonate ions in 4 M nitric acid. 10 ml solution containing varying amounts of sodium azido-dithiocarbonate was diluted with 125 ml 4 M nitric acid in which approx. 0.1 g carbon disulphide and 0.2 g sodium azide had been dissolved. Silver nitrate solution (0.05074 M) was added to an excess of 5—10 ml. After two hours the mixture was filtered and the precipitate washed with 50 ml 4 M nitric acid. The filtrate and wash solution, after addition of ferric alum, were titrated with standard ammonium thiocyanate solution (0.05460 M).

Expt. no.	Azido-dithiocarbonate added, millimole	Silver nitrate added, ml	Ammonium thiocyanate added, ml	Azido-dithiocarbonate found	
				millimole	%
1	1.025	27.13	6.46	1.023	99.7
2	1.025	27.65	6.99	1.020	
3	0.5125	17.20	6.69	0.5074	99.3
4	0.5125	18.14	7.51	0.5104	
5	0.2563	14.70	9.05	0.2518	98.4
6	0.2563	15.52	9.80	0.2524	
7	0.1025	10.06	7.47	0.1025	101.0
8	0.1025	10.80	8.12	0.1046	

silver azido-dithiocarbonate could be precipitated in strong nitric acid without co-precipitation of silver azide.

Firstly, experiments were carried out to determine if silver azido-dithiocarbonate was precipitated quantitatively when rather much nitric acid was present. Expt. nos. 1 and 2 in Table 1 represent the analysis of a standard solution of sodium azido-dithiocarbonate. 10.00 ml of this solution was diluted with 125 ml water and the estimation was carried out exactly according to the procedure of Browne and Smith [5]. In expt. nos. 3 and 4, 125 ml 1 *M* nitric acid was added instead of 125 ml water, but the remaining procedure was unchanged. Even when using 4 *M* nitric acid the error was less than 2 %.

In the experiments in Table 2 different, but known amounts of sodium azido-dithiocarbonate were estimated by Volhard titration in 4 *M* nitric acid and in the presence of ca. 0.1 g carbon disulphide and 0.2 g sodium azide. In acid solution carbon disulphide would not be expected to react with silver ions, and no further formation of azido-dithiocarbonate ions would be expected to take place, because azide ions only, and not hydrazoic azid, will react with carbon disulphide. If considerably more than 0.2 g sodium azide was present,

a redbrown colour, due to a complex formed between ferric ions and azide ions, would appear when ferric alum was added. According to Ricca [8] the dominating complex ion seems to be FeN_3^{++}, so it is quite understandable that the redbrown colour did not appear in 4 *M* nitric acid when the added amount of sodium azide was not too large, because the ionization constant of hydrazoic acid is about 2×10^{-5} at 25° C. The redbrown ferric azide colour would have prevented the titration with thiocyanate.

The results (see Table 2) showed that the estimation could be carried out within an error of 1—2 %.

EXPERIMENTAL

The analytical method developed above was used for the determination of the degree of conversion in an aqueous solution of carbon disulphide and an excess of sodium azide. A kinetic experiment was carried out as follows:

Two 500 ml measuring flasks containing sodium azide and carbon disulphide solution respectively, were placed in the thermostat water for the sake of temperature adjustment. The sodium azide solution was made by weighing dried sodium azide and dissolving in the measuring flask. The sodium azide used was analyzed by oxidizing hydrazoic acid to free nitrogen by means of ceric salt [9] in excess. The purity was found to be 99.8 %. The carbon disulphide solution of about the concentration required, was made by dilution of a saturated aqueous solution.

The sodium azide solution was now poured into a 1000 ml bottle with an outlet at the bottom. The outlet was provided with a piece of rubber tubing and a Mohr tubing compressor. The experiment was started by pouring the carbon disulphide solution down into the sodium azide solution. When half the carbon disulphide solution had run out of the measuring flask, a stopwatch was started. Thereafter the bottle was stoppered and shaken for ten seconds, and the mixture was distributed as quickly as possible — through the rubber tubing — among ten 60 ml bottles which were stoppered immediately so that no air at all was present between mixture and stopper, and then placed in the thermostat. The first and the last bottles thus filled were left at room temperature until next day, and then used for the determination of the initial concentration of carbon disulphide.

After a certain time of reaction (t) one of the bottles in the thermostat was opened and 10 to 50 ml of the mixture was taken out with a pipette and run into a suitable amount of nitric acid so that the total volume was 135 ml and the molar concentration of nitric acid 4, whereby further formation of azido-dithiocarbonate ions was prevented. Then the determination of azido-dithiocarbonate was carried out exactly as described above. The most convenient method for the determination of the initial concentration of carbon disulphide was to allow the two samples mentioned above to react until complete conversion of carbon disulphide into azido-dithiocarbonate ions — with a following determination of these. In all cases 24 hours at 20° C were enough to secure complete conversion (more than 99.9 %).

As was to be expected, the reaction turned out to be a second order reaction with respect to azide ions and carbon disulphide molecules. In

Table 3. The bimolecular reaction between azide ions and carbon disulphide molecules at 20° C. a and b are the initial molar concentrations of sodium azide and carbon disulphide respectively; t is the time of reaction in minutes; x is the molar concentration of azido-dithiocarbonate ions at the time t; k is the second order rate constant calculated according to (1).

Expt. no.	1		2		3	
a	0.03079		0.03077		0.06154	
b	0.004127		0.007923		0.01290	
t	$10^3 . x$	*k*	$10^3 . x$	*k*	$10^3 . x$	*k*
15 min			0.83	0.243	2.48	0.236
30 »			1.63	0.256	4.60	0.249
60 »	1.47	0.244	2.81	0.250	7.27	0.241
90 »					8.96	0.235
120 »	2.34	0.237	4.43	0.242	10.64	0.266
150 »					11.24	0.253
180 »	2.91	0.234	5.50	0.240	11.77	0.254
240 »	3.33	0.240	6.22	0.239		
300 »	3.56	0.232	6.69	0.235		
360 »			6.96	0.225		
Average		0.237		0.241		0.248

Table 3 are given the details of three experiments with different initial concentrations. The corresponding rate constant k (for each time of reaction t) was calculated from the integrated second order rate expression:

$$k = \frac{2.303}{t\,(a—b)} \log \frac{b\,(a—x)}{a\,(b—x)}$$

where a is the initial concentration of sodium azide, b is the initial concentration of carbon disulphide, and x is the concentration of azido-dithiocarbonate ions at the time t.

ENERGY OF ACTIVATION

The energy of activation of the formation of azido-dithiocarbonate ions was determined by experiments analogous to those described above. The rate of reaction was determined by experiments analogous to those described above. The rate of reaction was determined in the temperature range 8° to 25° C. In

Table 4. Energy of activation of the reaction between azide ions and carbon disulphide. a and b are the initial molar concentrations of sodium azide and carbon disulphide respectively; t is the time of reaction in minutes; x is the molar concentration of azido-dithiocarbonate ions at the time t; k is the second order rate constant calculated according to (1). $k_{exp.}$ is the simple average of the k values in one experiment. $k_{calc.}$ was calculated from the equation $\log k = H - \frac{A}{T}$, *which is a straight line fitted to the experimental $k_{exp.}$ and T (abs. temperature) values.*

Expt. no.	1		2		3		4		5	
a	0.06154		0.06153		0.06154		0.06154		0.06154	
b	0.01042		0.01378		0.01548		0.01290		0.01246	
Temp. °C	7.68		10.00		15.00		20.00		25.00	
t	$10^3 . x$	*k*	$10^3 . x$	*k*	$10^3 . x$	*k*	$10^3 . x$	*k*	$10^3 . x$	*k*
15 min.							2.48	0.236	4.04	0.440
30 »							4.60	0.249	6.70	0.446
45 »									8.65	0.468
60 »			2.99	0.0679	5.74	0.1322	7.27	0.241	9.71	0.455
75 »									10.43	0.443
90 »					7.78	0.1359	8.96	0.235		
120 »	3.28	0.0527	5.38	0.0704	9.20	0.1340	10.64	0.266		
150 »							11.24	0.253		
180 »	4.37	0.0511	6.97	0.0680	11.40	0.1362	11.77	0.254		
210 »										
240 »	5.30	0.0506	8.45	0.0700	12.59	0.1311				
360 »	6.49	0.0469	10.22	0.0682						
480 »	7.70	0.0493	11.44	0.0684						
$k_{exp.}$		0.0501		0.0683		0.134		0.248		0.450
$k_{calc.}$		0.0502		0.0684		0.131		0.246		0.452

Table 4 is given the average rate constant ($k_{exp.}$) for each temperature. Using the method of least squares, the numerical values of H and A were calculated according to the equation:

$$\log k = H - \frac{A}{T}$$

where k is the rate constant and T is the absolute temperature, giving:

$$\log k = 15.147 - \frac{4618}{T}$$

By inserting the experimental values for T, values of k ($k_{calc.}$) were calculated. These are also given in Table 4. The energy of activation was:

$$A \times 4.571 = 4618 \times 4.571 = 21100 \text{ cal/mole}$$

The frequency exponent H, which had the value 15.15 using minutes as the time unit, was 15.15—log 60 = 13.37 with seconds as the unit.

At 20° C the rate constant of the carbon disulphide catalyzed iodine-azide reaction [2] was found to be 4.45, while that of the formation of azido-dithiocarbonate ions (see Table 3) was 0.248. The ratio between the two constants is 18.0; the chain length of the iodine-azide reaction was found [3] to be about 17 at room temperature.

The energy of activation of the iodine-azide reaction was found [2] to be 19950 cal, *i. e.* only 1150 cal less than that of the reaction between azide ions and carbon disulphide. These 1150 cal represent the negative energy of activation of the chain length of the iodine-azide reaction.

SUMMARY

The reaction between azide ions and carbon disulphide with formation of azido-dithiocarbonate ions was investigated kinetically. It is a second order reaction with respect to azide ions and carbon disulphide molecules. The energy of activation was found to be 21100 cal/mole. The values of the rate constant confirmed that this reaction is closely related to the carbon disulphide catalyzed iodine-azide reaction.

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Action of Strong Acids on Acetylated Glycosides

VIII *. A New Synthesis of Melibiose

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Melibiose, or 6-glucose α-galactopyranoside, has been prepared by Helferich and Bredereck [1] by the action of galactose bromide tetraacetate upon 1,2,3,4-glucose tetraacetate with quinoline as condensing agent. From these same carbohydrate derivatives, but using silver carbonate as condensing agent, Helferich and Rauch [2] obtained another disaccharide, different from melibiose. As β-galactosides are the usual products when the Koenigs-Knorr synthesis is applied to galactose bromide tetraacetate, these results indicated that melibiose had an α-glycosidic structure. Helferich and Bredereck [1] also condensed galactose bromide tetraacetate with phenol in the presence of quinoline, and were able to isolate phenyl α-galactoside from the reaction mixture.

Melibiose is structurally related to *iso*-maltose, 6-glucose α-glucopyranoside. The latter disaccharide has been prepared from the corresponding β-glucoside, gentiobiose, by transglycosidation (Part V [3]). As the transglycosidation reaction has been successfully performed in the galactose series (Part VI [4]), it seemed possible that melibiose might be prepared from the 6-glucose β-galactoside mentioned above. This synthesis has now been carried out, by the same procedure as was employed for the synthesis of *iso*-maltose. The β-glycosidic disaccharide, in the form of the octaacetate, was treated with titanium tetrachloride in chloroform. In the reaction product, a mixture of disaccharide chloride heptaacetates, the chlorine was replaced by acetoxyl with the aid of mercuric acetate in acetic acid. From the resulting mixture a small amount of almost pure β-melibiose octaacetate could be isolated by successive recrystallizations. Helferich and Bredereck had considerable difficulty in obtaining the

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melibiose octaacetate in a pure state. This was probably due to the fact that β-melibiose octaacetate and β-6-glucose β-galactoside octaacetate have rather similar solubilities and crystallization tendencies and therefore are not easily separated. In the present case there is a further complication, due to the strongly acidic nature of the catalyst, titanium tetrachloride.

It is known that in the presence of aluminium chloride, also a strong acid, the octaacetates of cellobiose and lactose are partly isomerized into other disaccharides [5], two of which, celtrobiose and neolactose, have been isolated. In these cases the glycosidically linked glucose unit has been transformed into the altrose configuration. Now titanium tetrachloride, although not so strong an acid as aluminium chloride (at least it is not as active when used as catalyst in the Friedel-Crafts synthesis [6]), might also be able to catalyze this isomerization; there are moreover no reasons why the effect should be restricted to lactose and cellobiose.

It is in fact reasonable to assume that the acetates of all reducing saccharides, at least those in which the reducing part of the molecule is a glucose unit, may undergo this type of isomerization in the presence of aluminium chloride or titanium tetrachloride. This would be a most undesirable reaction in the synthesis of α-glycosidic saccharides, and it may well be responsible for the low yields obtained in the synthesis of *iso*-maltose and melibiose.

EXPERIMENTAL

β-Melibiose octaacetate

To a solution of β-6-glucose β-galactoside octaacetate (2.3 g) in anhydrous chloroform (70 ml), titanium tetrachloride (3 g) was added. A yellow precipitate was formed, which did not dissolve when the mixture was refluxed for four hours on a glycerol bath, kept at 70°. After cooling, the mixture was poured into ice water (400 ml), whereupon the precipitate dissolved and the chloroform phase became almost colorless. The latter was separated, washed with water, dried over calcium chloride and concentrated to a sirup under reduced pressure. This sirup, together with mercuric acetate (2 g), was dissolved in acetic acid (20 ml) and kept at room temperature. After four hours the solution was poured into ice water (250 ml) and extracted with chloroform (2 × 30 ml). The chloroform solution was washed with sodium carbonate and water, dried over calcium chloride and concentrated under reduced pressure. The residual sirup was dissolved in methanol (5 ml) and the solution kept at 0°, when crystals slowly separated. Three crops of somewhat sticky crystals (total yield 0.95 g), melting between 140—150° and showing $[\alpha]_D^{20}$ + 50° (chloroform, c = 2), were collected. The first crop (0.25 g) was subjected to successive recrystallizations from methanol, and after eight such operations, the product had m. p. 171—172° (uncorr.) and $[\alpha]_D^{20}$ + 100°. One further recrystallization did not change these values. An authentic sample of β-melibiose octaacetate melted at 173—174° and showed $[\alpha]_D^{20}$ + 102°; an admixture with the synthetic product melted at 172—173°. As only a

small amount (25 mg) of the synthetic material remained after the final recrystallization, further purification was not attempted. The specific rotation and melting point values, however, showed that it was substantially pure β-melibiose octaacetate.

SUMMARY

β-Melibiose octaacetate has been prepared from β-6-glucose β-galactoside octaacetate by the transglycosidation method.

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Activation of Aerobic Oxidation in Kidney Mitochondria by Phosphorylated Vitamin D_2

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Despite the great number of investigations concerning the mode of action of vitamin D the problem still remains obscure. Besides influencing the development of the skeleton the vitamin is known to affect the metabolic patterns of other tissues. It seems probable that the different effects of vitamin D primarily depend on the part played by the vitamin on the enzymatic processes concerned with cellular function.

In broadest aspect vitamin D stimulates growth [1]. The retardation of growth by D-avitaminosis is probably due to quite a fundamental disturbance in the metabolic processes. Pathological changes in the carbohydrate metabolism have been observed in D-avitaminosis (rickets) [2–5]. Values on the content of hexose phosphates in resting muscle and in muscle after tetanic contraction, given by Räihä and co-workers [6], indicate that the shift from anaerobic to aerobic metabolism, occurring in muscles with high energy output [7], is delayed in rachitic rabbits. The capacity of work is depressed in muscles from rachitic dogs but returns almost immediately to normal after administration of vitamin D [8]. These muscle experiments seem to give evidence that in D-deficiency there is an impairment of the oxidative phase of carbohydrate metabolism in muscles and that vitamin D plays a role in cellular respiration.

The activity of different enzyme systems in the presence of the phosphorylated water-soluble vitamin D_2 [9] is investigated as a means of obtaining information concerning the vitamin's action on the energy transfer system. In an earlier report [10] evidence has been presented that phosphorylated vitamin D_2 (D_2P) activates purified alkaline phosphatase from kidney, intestine and bone.

This communication deals with results obtained from experiments to determine the effect of D_2P on the oxygen uptake of the respiratory enzyme complex in mitochondria which is known to catalyze all the reactions of the Krebs tricarboxylic acid cycle and fatty acid oxidation [11–12].

EXPERIMENTAL

Preparation of the mitochondria (washed granules):

The granules were prepared by a method similar to that developed by Slater (personal communication) for heart muscle mitochondria. The animals (rabbits or rats) were killed by decapitation and the kidneys were immediately removed. All subsequent operations were performed at 2—4° C. In about 100 ml of an ice-cold 0.11 *M* potassium chloride solution, being 0.05 *M* with respect to sodium fluoride, the kidney cortex was cut into small slices with a pair of scissors. After three washings with 100 ml of the potassium chloride solution containing fluoride the tissue was ground to a fine paste with quartz sand in two volumes of a solution of the following composition: one part of 0.15 *M* KCl, one part of 0.2 *M* NaF and two parts of 0.065 *M* phosphate buffer of pH 7.3. The preparation was then centrifuged in an International refrigerated centrifuge for five minutes at about 600 × *g*. The residue was then ground once again with two volumes of the same solution as before and thereafter recentrifuged at the same gravity. The supernatants were combined and admitted to high speed centrifugation at about 20 000 × *g* for 15 minutes. The supernatant was discarded, and the residue fraction washed once by resuspension in a 0.11 *M* KCl solution, being 0.05 *M* with respect to NaF, followed by resedimentation for 15 minutes at 20 000 × *g*. The supernatant was carefully decanted and the washed mitochondria were taken up in a sufficient amount of ice-cold 0.2 *M* glycylglycine buffer of pH 7.3 to yield a suspension suitable for experimental purpose.

Other materials: Vitamin D_2 was phosphorylated by the method described earlier [9]. Since the phosphorylated vitamin, especially when stored in water solution, is extremely labile, the D_2P was tested before use by means of its ability to activate purified alkaline kidney phosphatase. Destroyed D_2P is not capable to give such an effect.

Glucose-6-phosphate of purity 0.90 was obtained as follows: glucose-1-phosphate was prepared according Sumner and Somers [13], the phosphate ester obtained was then incubated with phosphoglucomutase, purified according to Najjar [14]; the glucose-6-phosphate formed was finally purified as the Ba-salt.

Adenosine-5-phosphate was obtained from the Sigma Chemical Company.

Hexokinase was prepared according to Berger, Slein, Colowick and Cori [15].

Components of the incubation mixture: Incubations with the suspension of mitochondria in glycylglycine were made aerobically and in the precense of K^+, Mg^{++}, fluoride, adenosine-5-phosphate and inorganic phosphate. Glutamate was used as oxidizable substrate and glucose served as phosphate acceptor. Hexokinase was added in order to transfer esterified phosphate to glucose. The pH of the mixture was 7.3; temp. 30° C.

ANALYTICAL METHODS

Manometric measurements of oxygen uptake were made at 30° C in Warburg vessels of conventional design with air as the gas phase. The center well contained 0.2 ml 2 *N* KOH. Glucose and D_2P were pipetted into the side-arms. Flasks were equilibrated

for 15 minutes prior to closing of the taps. Glucose and D_2P were tipped in after closing. Readings were mostly made every ten minutes.

Phosphate was determined by the method of Fiske and Subbarow [16] after fixation of the sample in 7 % (wt./vol.) trichloroacetic acid (TCA).

RESULTS

The effect of D_2P in a concentration of 2×10^{-5} *M* on the oxygen uptake of the mitochondria suspension is shown in Fig. 1. In comparison to the control experiment there is a considerable activation *.

The maximum effect of the vitamin is exerted immediately after it is mixed with the enzyme complex; thus it must be rapidly associated to the enzyme system. After 30 minutes of incubation, the oxygen uptake is almost the double in comparison to the experiment without D_2P. After the lapse of about 90 minutes, the uptake per unit of time is the same in both experiments. The velocity of the reaction thus becomes the same in the two incubation mixtures after the initial activation caused by D_2P at the start of the experiment.

The effect of D_2P on oxygen uptake when incubating mitochondria in the presence of limited amounts of substrate is shown in Fig. 2. The relative initial activation given by D_2P is about the same as in the experiments with unlimited amounts of substrate. When the substrate is almost completely oxidized the rate of oxygen uptake declines considerably and the curve of the uptake comes rather rapidly to an asymptote being almost of the same level in both experiments. The asymptote, however, was reached quite a bit earlier in the experiment with D_2P, indicating that the entire oxidation of glutamate to carbon dioxide, water and ammonia was performed at a more rapid rate when D_2P was present. Since the oxygen uptake thus was the same in both experiments, the increased oxygen uptake after addition of D_2P cannot be due to an oxidation of the vitamin. Such conditions may also be denied by the low amount of D_2P present (5×10^{-2} micromoles).

The experiments referred in Figs. 1 and 2 have been performed with mitochondria from healthy rabbits. Preliminary investigations, presented in Table 1, have shown a much greater activation by D_2P in experiments with kidney mitochondria from rachitic rats **.

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* Throughout this paper the term activation has been used to indicate an increase in the rate of oxygen uptake in the entire system.

** Rickets was produced by giving young rats, weighing 40—50 g, a rachitogenic diet [17] for a period of six weeks.

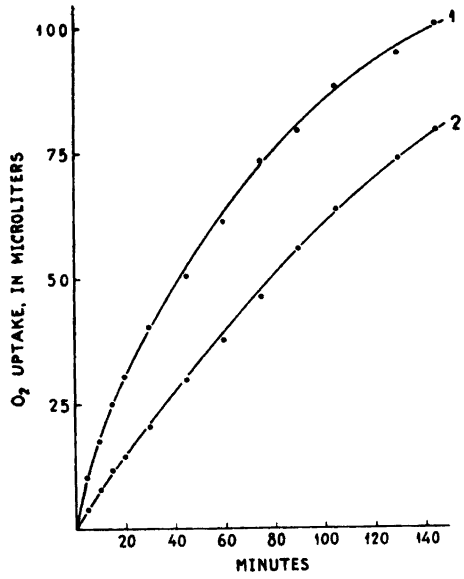

Fig. 1. Stimulation of respiration of rabbit kidney mitochondria by D_2P.
Curve 1 represents an incubation with D_2P.
Curve 2 represents an incubation without activator.

The initial volume in the main compartment of each Warburg vessel was 2.0 ml. The mixture contained 0.3 m of the enzyme suspension (the mitochondria from two kidneys had been suspended in 8 ml 0.2 *M* glycylglycine buffer of pH 7.3), 80 micromoles of KCl, 6 micromoles of $MgCl_2$, 100 micromoles of NaF, 5.0 micromoles of orthophosphate, 0.4 micromoles of adenosine-5-phosphate*, 30 micromoles of sodium glutamate and 0.01 ml hexokinase solution. In the side-arms 30 micromoles of glucose and in the experiment with D_2P 5×10^{-2} micromoles of the activator. The volume in the side-arms 0.5 ml. The taps were closed after an equilibration period of 15 minutes. The contents in the side-arms were then tipped into the main compartment. Thus, the final volume of the incubation mixture was 2.5 ml. The vessels were incubated at temp. 30° C with air as the gas phase.

On aerobic incubation of intact mitochondria in the presence of an oxidizable substrate the oxidation is coupled to formation of phosphate bound energy [18,19,12], the phosphate esterification accompanying the oxidation being roughly proportional to the amount of substrate oxidized [20]. In the presence of a suitable phosphate acceptor and a phosphate transfer system an accumula-

* The very low concentration of adenosine-5-phosphate has been used since it has been shown by Lindberg and Ernster (to be published) that this concentration is optimal and most physiological for oxidative phosphorylation in mitochondria.

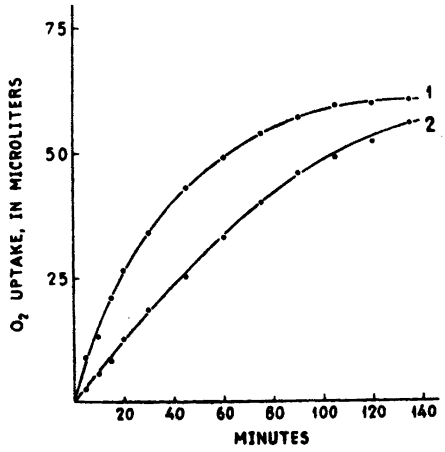

Fig. 2. The activation of oxygen uptake of kidney mitochondria by D_2P with a small amount of oxidizable substrate.
Curve 1 represents an incubation with D_2P.
Curve 2 is from an experiment without activator.

The incubations were performed as in the experiments in Fig. 1 with the exception that each Warburg vessel contained only 0.6 micromoles of sodium glutamate.

Table 1. Stimulation of respiration of kidney mitochondria from rachitic rats by D_2P.

Mitochondria prepared from 8 kidneys were suspended in 4 ml 0.2 *M* glycylglycine buffer of pH 7.3. Each Warburg vessel contained in the main compartment 0.5 ml enzyme suspension, 80 micromoles of KCl, 8 micromoles of $MgCl_2$, 100 micromoles of NaF, 5.0 micromoles of orthophosphate, 0.3 micromoles of adenosine-5-phosphate, 30 micromoles of glutamate, and 0.05 ml hexokinase solution. In the side-arms 30 micromoles of glucose and in the experiment with activator 7×10^{-2} micromoles of D_2P. The experiments were made in the same way as in Fig. 1. Final volume 2.5 ml. Temp. 30° C.

Microlites of O_2 taken up during 15 minutes after equilibration	
With D_2P	Without activator
22 [a]	5
22	5.5
23.5	4.5

[a] Each value represents one determination.

Table 2. The effect of varying concentrations of D_2P on the esterification of phosphate and on the oxygen uptake in aerobic incubations of rabbit kidney mitochondria.

The incubations were made in Warburg vessels. The experimental conditions were the same as in Fig. 1. Manometric readings were made every ten minutes.

Amount of D_2P added. In micromoles	O_2 taken up/10 min. during the first 60 min. In microliters	O_2 taken up/10 min. during the period 60–135 min. In microliters	Amount of phosphate esterified. In micromoles
—	5.9	5.4	3.6
1.10^{-3}	7.1	6.1	3.5
5.10^{-3}	7.7	6.1	3.1
$1.5.10^{-2}$	8.8	5.9	2.2
5.10^{-2}	10.2	5.5	1.0

tion of esterified phosphate takes place. Thus, if the activation of oxygen uptake in mitochondria after addition of D_2P is due to a more rapid utilization of substrate and if no dissociation of electron transport and phosphorylation occurs, the increased activity should increase phosphorylation.

This was, however, not the case, as shown in Fig. 3. The maximal esterification was reached in both experiments after a period of about 25 minutes. After that time the curves showed a tendency to decline. During the first minutes of incubation the phosphate uptake was about the same with and without D_2P. Later on, however, a difference between the two sets of condition was obtained. The net esterification of phosphate was less in the experiment with D_2P than in the other, and this difference seemed to be gradually increasing. The oxygen uptake as controled on a part of each sample in a Warburg vessel was satisfactory in the two sets of conditions throughout the whole experimental period.

Table 2 shows the effect of different concentrations of D_2P on the esterification of phosphate in an experiment continued for a long period. The net uptake decreased considerably with increasing concentration of D_2P. With the highest concentration of D_2P applied (2×10^{-5} *M*) the amount of phosphate esterified was less than one third of that in the experiment without addition of vitamin. The same table also shows the effect on oxygen uptake with varying concentrations of D_2P. Even a concentration as low as 4×10^{-7} *M* gave a significant increase in activity. The activation increased with increasing concentrations of D_2P. In all cases the activation occurred during the first period of incubation, whereas in the later phase there was no significant difference

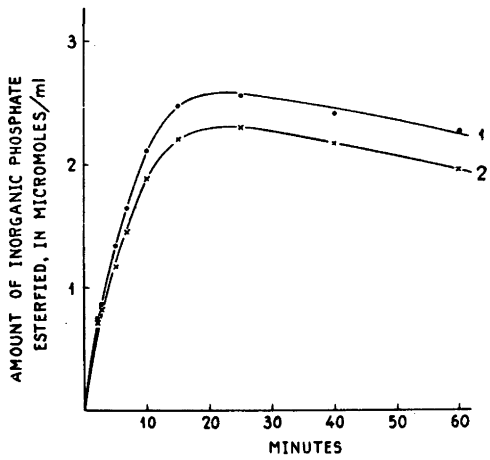

Fig. 3. Net esterification of phosphate in an aerobic incubation of rabbit kidney mitochondria under the influence of D_2P.
Curve 1 represents an experiment without D_2P.
Curve 2 represents an experiment with D_2P in a concentration of 1.2×10^{-5} M.

The incubations were performed under constant stirring in two open Florence flasks of 250 ml, the surface of the incubation mixtures being large enough to permit steady oxygen uptake. Samples of 1 ml were removed and immediately fixed in ice-cold TCA at times given in the figure. The total volume in each flask was 12 ml. Each incubation mixture contained 2.5 ml mitochondria suspension in 0.2 *M* glycylglycine buffer (mitochondria from two kidneys were suspended in 5.5 ml of the buffer solution), 520 micromoles of KCl, 36 micromoles of $MgCl_2$, 600 micromoles of NaF, 40 micromoles of orthophosphate, 1.5 micromoles of adenosine-5-phosphate, 30 micromoles of sodium glutamate, 180 micromoles of glucose, and 0.1 ml of the hexokinase solution. The pH of the mixtures being 7.3. Temp. 30° C.

between the five groups. Thus, the ratio of esterified phosphate to oxygen taken up decreased with increasing amounts of D_2P added.

The decreasing tendency of the amount of esterified phosphate after a long period of incubation (*cf.* Fig. 3) indicates the presence of a non-inhibited phosphatase in the enzyme preparation. Swanson [21] has demonstrated a phosphatase specific for glucose-6-phosphate in mitochondria preparations. Since D_2P has been shown to activate alkaline phosphatase from different organs [10], it may be possible that the diminished net uptake of phosphate is due to an activated break down of phosphoric esters.

Table 3 shows that in the mitochondrial suspension there was a marked initial increase in the phosphatase activity towards glucose-6-phosphate after the addition of D_2P. The activation was of the same "initial" type as that

Table 3. The effect of D_2P on the phosphatase activity in rabbit kidney mitochondria.

The incubations were made in closed test tubes. The final volume in each experiment was 7.0 ml. Samples of 1.0 ml were taken with time intervals given in the table. 0-values were calculated from the amount of orthophosphate present in the mitochondria suspension and in the glucose-6-phosphate solution before mixing. Each mixture contained 1 ml mitochondria suspension in 0.2 *M* glycylglycine buffer of pH 7.3 (the mitochondria from two kidneys were suspended in 6 ml of the buffer solution), 580 micromoles of KCl, 20 micromoles of $MgCl_2$, and 20 micromoles of glucose-6-phosphate. In the experiment with D_2P the vitamin was present in a concentration of 1.3×10^{-5} *M*. Temp. 30° C.

Time min	Amount of orthophosphate formed [a]	
	Without D_2P	With D_2P
5	1.6	4.0
10	1.1	8.8
20	5.9	16.0
40	17.0	17.2

[a] In micrograms/ml incubation mixture.

obtained when incubating purified alkaline kidney phosphatase with phenyl phosphate in the presence of D_2P.

DISCUSSION

The exact nature of the activation of oxygen uptake by D_2P cannot be fully understood by means of the results given in this report.

Many steroid hormones, supposed to influence the metabolic patterns by affecting the function of enzyme systems, such as desoxycorticosterone, testosterone, progesterone and others, inhibit the oxygen uptake of tissue slices [22], of homogenates [23], and of isolated enzyme systems such as succinoxidase [24] and D-amino acid oxidase [25]. The specificity of these actions has been questioned, since physiologically inactive steroids also are capable of inhibiting the same systems. It has been supposed that the steroids in some way, specifically or non-specifically, interact with the proteins [24].

A change in the activity of the electron transport system can, according to Keilin and Hartree [26], be caused *in vitro* by compounds which either react directly and specifically with one or more components in the dehydrogenase-cytochrome system, or by those which, by modifying the colloidal state of the enzyme preparations, may markedly increase or decrease the activity. Decreased rate of oxygen uptake in a system, due to suboptimal concentration

of orthophosphate, can, by addition of denaturated globin or of Ca^{++} or Al^{+++}, be brought to the same level as with optimal phosphate concentration; the effect being due to a change in the colloidal structure [26].

If the activation by D_2P is only due to a change in the colloidal state the activation would remain constant for the whole period of incubation; particularly with regard to the fact that the net uptake of phosphate is depressed and thus the phosphate concentration remains nearer the optimum. The concentration of D_2P necessary to induce activation of oxygen uptake was very low. Since D_2P also was shown to interact with the net uptake of phosphate the effect of D_2P is probably not the result of a change in the colloidal state of the system.

The increased oxygen uptake obtained after the addition of D_2P takes place in the first period of incubation, *i. e.*, when the esterification of inorganic phosphate coupled to the electron transport process is most intense. Further on, when the rate of esterification has declined, there is no longer any activation. In long period experiments (*cf.* Table 2) the amount of esterified phosphate is considerably decreased when D_2P is added; after a short period of incubation, however, the difference between the two sets of condition is very little (*cf.* Fig. 3). *I. e.*, the P : O ratio decreases gradually both with and without D_2P. The decline is, however, much greater in experiments with D_2P. In the *in vitro* system applied, the amount of esterified phosphate represents a resultant between the synthesis of new phosphate bonds coupled to the oxidation and the action of splitting enzymes such as phosphatases and ATPase. The balance between these two processes must be changed after the addition of D_2P, since the phosphatase activity is increased. Therefore, it might be possible that the absolute amount of esterified phosphate in the initial phase of the incubation is greater with D_2P than without.

Phosphorylation can be completely inhibited without any effect on the oxidation [27,28]. Thus esterification of inorganic phosphate is not an essential part of oxidation. It might be assumed, however, that an increased possibility for phosphorylation gives a corresponding possibility for increased oxidation. Earlier the hypothesis has been advanced [29] that certain dinucleotide splitting enzymes are directly engaged in the mechanism of phosphorylation. Phosphate transferring enzymes, necessary for oxidative phosphorylation, might *in vitro* in the absence of a suitable acceptor split esters, thus acting as ordinary phosphatases (*cf.* Meyerhof and Green [30]). It seems reasonable that D_2P through activation of enzymes acting as phosphatases when tested *in vitro* but under physiological conditions, *i. e.*, *in vivo*, having a function other than to split phosphate esters, might increase the possibility for aerobic phosphorylation, involving a stimulation of oxidation.

SUMMARY

Phosphorylated vitamin D_2 (D_2P) added to a suspension of kidney mitochondria, containing glutamate as oxidizable substrate, gives an obvious initial activation of oxygen uptake. The net esterification of phosphate coupled to oxidation is almost the same with or without D_2P in the first phase of incubation. Further on, when the phosphorylation rate is declining the net uptake becomes less in the presence of D_2P depending on an activation of phosphatase. The significance of these findings has been discussed.

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Acetic Acid Metabolism in *Escherichia coli*

I. General Features, and the Metabolic Connection between Acetate and Glutamic Acid, Aspartic Acid, Glycine, Alanine, Valine, Serine and Threonine

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In a series of previous publications [1–4] we have described the cultivation of the yeast, *Torulopsis utilis*, on C^{13}—C^{14}-labelled acetic acid as the sole source of carbon, and how the different carbon atoms of the amino acids isolated could be traced back to the origin from the methyl group or the carboxyl of acetate. Since this work has given a great deal of information concerning the metabolic connection between acetate and amino acids in general we have extended it to a similar investigation of the amino acid metabolism in *Escherichia coli*, in order to compare the general features of amino acid interrelationship in these two representatives of lower fungi and aerobic bacteria.

In principle the present study of the formation of amino acids in *E. coli* follows the experimental methods outlined in the *Torulopsis* work. $C^{13}H_3C^{14}OOH$ was prepared and converted to sodium acetate, and the bacteria were cultivated for 3.5 hours with the acetate as the sole source of carbon. After separation of the bacteria, and hydrolysis of the bacterial protein the amino acids were isolated and subjected to chemical degradation in order to determine the C^{13} and C^{14}-content of different carbon atoms, originating from the labelled substrate. Since these procedures have not been previously described in detail we will in the experimental part of this paper give a full account of the methods used.

EXPERIMENTAL

1. The cultivation of *E. coli* on $C^{13}H_3C^{14}OONa$ as the sole source of carbon

50 mg wet weight of bacteria, obtained from ordinary cultivation on Agar-plates [5,6] were transferred to a 1 litre glass-washbottle with a large porous plate in the bottom, containing the following sterilized medium:

Sodium chloride	5 g
Ammonium phosphate, primary	1 »
Potassium phosphate, secondary	1 »
Magnesium sulphate (7 H_2O)	0.2 »
Acetic acid, conc.	2.2 »
Distilled water	1 000 ml

The pH was adjusted to 6.8 with 1 *N* NaOH. The temperature was maintained at 36°, and a current of sterile air was passed through the solution (approximately 500 ml per minute). After three days the wet weight of the bacteria had increased to 3.4 g. The suspension was centrifuged under sterile conditions and the bacteria transferred into a 7 litre Kluyver-flask, made from stainless steel [7], containing 4 litre of the same sterile medium. A vigorous current of sterile air (4 litre per minute) was passed through the suspension by means of a porous sintred glass disc in the bottom of the flask. The top of the conical flask was closed by a stainless steel cover, with an outlet for the air. The pH was kept at 7 by addition every 12 hours of 1 *N* acetic acid.

After a week at 36° the total wet weight of the bacteria had increased to 24.4 g, and this material was centrifuged and transferred to a 30 litre kluyver flask of stainless steel, containing 25 litre of medium. After ten days at 36° the total wet weight was approximately 180 g and the pH during this period was maintained at 7.0 by continual addition of 1 *N* acetic acid.

This amount of bacteria was used as the inoculum for a cultivation on isotope-labelled acetic acid ($C^{13}H_3C^{14}OOH$), the preparation of which has been previously described [1]. The centrifuged bacteria were transferred into fresh medium (25 litres) and cultivated as above for 22 hours, without any further addition of acetic acid. The amount of the latter, being at the beginning $2.2 \cdot 25 = 55$ g (pH 6.8) decreased during the 22 hours to 17 g (pH approximately 8). At the end of the period, 50 g of labelled acetic acid (6.5 atom per cent excess C^{13} in the methyl group, 33 000 counts per minute C^{14}) in one litre of distilled water, was added.

The wet weight of bacteria at this moment was 250 g. A sample of the medium was taken in order to estimate the actual isotope content of the acetate of the medium. This estimation was carried out by water vapour distillation of the sample (200 ml), neutralisation of the acetic acid distilled over, and subsequent isolation as the silver salt, combusted to CO_2 and isolated as barium carbonate. The isotope determination of the barium carbonate sample from total acetic acid gave 2.29 atom per cent excess C^{13} and 13 100 counts per minute C^{14} (per 15 mg of barium carbonate), corresponding to 4.58 atom per cent excess C^{13} in the methyl group and 26 200 counts per minute C^{14} in the carboxyl group.

Table 1. Isotope content of respiratory CO_2, taken at intervals. C^{13} is given in atom per cent excess. C^{14} is given in counts per minute per 15 mg of barium carbonate. $'C^{13}$ and $'C^{14}$ denote isotope content of respiratory CO_2 in per cent of the corresponding C^{13}- and C^{14} content in $C^{13}H_3C^{14}OONa$, the latter being the sole source of carbon. (4.58 per cent excess C^{13} and 26 200 counts/min/15 mg of $BaCO_3$ = C^{14} in the methyl- and the carboxyl groups, respectively.)

Time intervals in minutes	Isotope content C^{13}	C^{14}	$'C^{13}$	$'C^{14}$	$'C^{13}+'C^{14}$	Ratio: $'C^{13}/'C^{14}$
0— 3	0.01	56	0.22	0.22	0.44	1.0
3— 8	0.025	155	0.55	0.59	1.1	1.07
8— 18	0.05	432	1.1	1.3	2.4	1.18
18— 33	0.16	1 005	3.5	3.8	7.3	1.10
33— 53	0.31	2 105	6.8	8.0	14.8	1.18
53— 83	0.46	3 635	10.0	13.9	23.9	1.39
83—128	0.70	4 985	15.3	19.0	34.3	1.25
128—188	1.05	6 035	22.6	23.0	45.6	1.02
188—218	1.15	6 575	25.1	25.1	50.2	1.00

The cultivation on the labelled acetate medium was continued for an additional 3.5 hours, with continous aeration (13 l/min). At intervals samples of the medium and the respiratory CO_2 (trapped as barium carbonate) were taken in order to examine the overall isotope content (see Table 1). At the end of the cultivation experiment the bacteria were centrifuged off, washed by centrifuging three times with Tyrode-solution, once with water, three times with absolute alcohol and three times with dry ether. The resulting dry powder (28 g) had an overall isotope content (by combustion to $CO_2 \rightarrow$ barium carbonate) of 0.08 atom per cent excess C^{13} and 133 counts/min C^{14} per 15 mg of $BaCO_3$.

2. Separation and isolation of amino acids

The dry bacterial material from the 3.5 hour experiment (28 g) was hydrolyzed during 24 hours with 20 % hydrochloric acid, and the hydrolysate worked up by electrodialysis according to Sperber [8]. In Sperber's modification of the electrodialysis procedure the acidic amino acids are trapped in Amberlite IR4, which is present in large amount in the middle compartment of the electrodialysis cell. At the end of the dialysis procedure the Amberlite was filtered off, washed free of neutral amino acids and eluted three times by stirring with 1.5 litre of 1.5 *N* HCl each time. The combined eluates were filtered, treated with a small amount of Norite and evaporated to 12—15 ml. After the saturation with hydrochloric acid gas the solution was kept 24 hours in a refrigerator. The glutamic acid hydrochloride was filtered off on a pre-cooled sintered glass filter and washed twice with one ml of icecold concentrated HCl each time, followed by 15 ml of dry acetone. The crude hydrochloride was recrystallized by dissolving in 3—4 ml of distilled water, the solution being saturated with HCl-gas, and kept over night at 0°. The crystals were filtered off and washed with acetone, and finally dried in vacuum over P_2O_5. Yield 2.3 g. According to a test with paper chromatography no other amino acids were present.

The combined mother-liquors were repeatedly evaporated to dryness in vacuum and redissolved in water, in order to remove excess HCl. The solution was then diluted with water to 100 ml and boiled with excess copper carbonate 15 minutes, filtered while hot and allowed to cool. The residual copper carbonate was treated three times with 20 ml of boiling water, and the filtrates combined with the first filtrate. After three days in a refrigerator the copper salt of aspartic acid, had separated out and was filtered off, washed with 100 ml of cold water, dissolved in 800 ml of boiling water, filtered and kept at 0° three days. The crystals were filtered off, suspended in 40 ml water near the boiling point, treated with H_2S for two hours, and filtered, after the addition of a small amount of Norite. The clear solution was evaporated in vacuum to 10 ml, precipitated with an equal volume of alcohol, the crystals filtered off and washed with alcohol and ether. The aspartic acid, thus obtained, was dried over P_2O_5 in vacuum. The yield was 1.2 g. A paper chromatogram showed the preparation to be free from amino acid contaminants.

The neutral fraction, after tyrosine had been removed by crystallisation [2], was treated with an ion-exchange resin (Dowex 50) and eluted with dilute HCl, according to the directions given by Stein and Moore [9]. The resin (250—500 mesh) was washed repeatedly for one week with 4 *N* HCl, then transferred in a 4 *N* HCl-suspension to a glass column 82 cm long and 8 cm wide, provided with a glass filter in the bottom. The resin was allowed to sediment up to 64 cm from the filter plate and was washed continuously with 1.5 *N* HCl, until the fluid, collected from the column had the same normality. Then an evaporated, tyrosine-free solution of the neutral amino acids (35 ml in 1.5 *N* HCl) was sucked into the uppermost layer of the resin; after which 1.5 *N* HCl was continuously added at a rate adjusted to keep 10 cm of solution above the upper resin layer.

The continuous flow from the column was 2—3 ml per hours per square centimeter of surface area, which corresponded to 100 ml/hour. An automatic fraction collector, time-switch controlled, changed fractions every hour. From each of these 100 ml samples 0.3 ml was withdrawn, evaporated on a watch-glass on the water bath, dissolved in one drop of water and subjected to paper chromatography. In this way we could observe the order in which the amino acids appeared in the eluate, their purity and relative amount. The results of the elution procedure is shown in Table 2.

The fraction containing pure glycine (46—48) were combined, evaporated to dryness, dissolved in 20 ml of water, treated with excess silver carbonate, filtered, treated with H_2S, a small amount of Norite added, filtered and evaporated in vacuum to 2—3 ml. This volume was treated with the same volume of 95 % alcohol, kept at 0° over night, filtered, washed twice with small amounts of 60 % alcohol, then with absolute alcohol. The crystals were dried in vacuum over P_2O_5. The same isolation procedure was used for alanine (fractions 53—59), valine (fractions 69—83), isoleucine (fractions 107—116) and the leucine-isoleucine mixture (fractions 117—121). The isolation of proline differed in that fractions 87—100 were, after removal of HCl with silver carbonate, evaporated to dryness and extracted with absolute alcohol, filtered and the alcoholic solution slowly evaporated at 60° to 3 ml. Precipitation with 8 ml of dry ether brought about crystallisation of the proline. The solution, after 24 hours at 0°, was filtered and repeatedly washed with dry ether.

The phenylalanine fraction (130—135), which was eluted with 4 *N* HCl, were evaporated to dryness and repeatedly evaporated with water in order to remove the main part of HCl before the silver carbonate treatment. After this the fractions were treated like the glycine, alanine and valine preparations.

Table 2. Separation of neutral amino acids on a Dowex-50 column. The figures give the volume of 1.5 N HCl penetrating through the column, the number of fractions collected and the amino acids isolated during the procedure.

ml of 1.5 *N* HCl	Fraction numbers	Amino acids isolated
0— 2 650	1— 23	—
— 3 015	24— 26	unknown
— 3 510	27— 30	—
— 4 360	31— 37	serine + threonine
— 4 900	38— 41	2 unknown
— 5 430	42— 45	—
— 5 780	46— 48	glycine
— 6 210	49— 52	glycine + alanine
— 6 935	52— 59	alanine
— 8 060	60— 68	—
— 9 785	69— 83	valine
—10 000	84— 86	—
—11 740	87—100	proline
—12 640	101—106	—
—13 975	107—116	iso-leucine
—14 635	117—121	iso-leucine + leucine
—15 585	122—129	leucine + phenyl-alanine
—16 325	130—135	phenyl-alanine

The leucine-iso-leucine mixture (117—121) was separated into its components by a repeated run in 1.5 *N* HCl on the same column as a separate procedure.

The separation of the mixture of serine and threonine in the fractions (31—37) was done according to Ehrensvärd, Reio, Saluste and Stjernholm [3] by treating the mixture at 120—130° with hydroiodic acid and red phosphorous in a bomb tube during 5 hours. The threonine remained intact, whereas the serine was almost quantitatively converted to alanine. The separation of threonine and alanine was done on a Dowex column as described for the main neutral fraction. Yield 380 mg of pure threonine and 300 mg of alanine *ex* serine.

In the different paper chromatography tests of the fractions from the columns we followed in principle the original method given by Consden, Gordon and Martin [10]. A description of the muoifications used in connection with this work will be published by one of us (S).

3. Degradation of amino acids

In the course of the degradation of the different amino acids two procedures were used throughout, namely total combustion to CO_2,[3], the isotope content of which represents the mean value of that of *all* carbon atoms, and ninhydrine treatment according to Van Slyke *et al*[11], whereby the α-carboxyl is selectively split off as CO_2 (except in the case of aspartic acid, where *both* carboxyls are liberated as CO_2). For the isotope determination in other carbon atoms of the different amino acids special methods have been

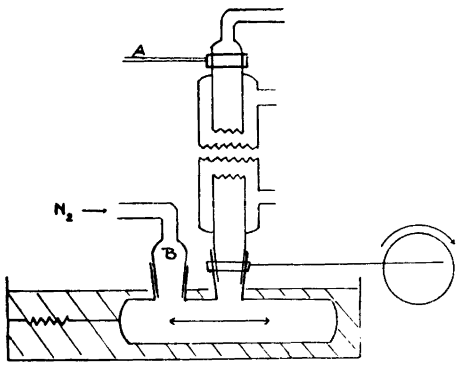

Fig. 1. The apparatus is clamped in A and shaken at 5 periods/sec. Sodiumazid is added through B at intervals. The liberated CO_2 passes through the condensor into a trap at —35° and then into baryta solution.

worked out for each single case all of them implying the conversion of one or several carbon atoms to CO_2, and the subsequent isotope determination of the latter.

The CO_2 from each degradation procedure was trapped in saturated baryta solution as $BaCO_3$, washed and dried at 120°. At first the C^{14}-content was determined on 15 mg samples of uniform layers of $BaCO_3$ on 1 cm^2 discs under standard conditions, by means of a Tracergraph Geiger-counter, with automatic sample changer. The C^{14}-values are given in *counts per minute per 15 mg of* $BaCO_3$. The C^{13} determination was carried out on the same samples, by mixing the 15 mg of $BaCO_3$ with 50 mg of dry KH_2PO_4 in the bottom part of a pyrex tube, 15 cm long, 5 mm in diameter, closed in one end, the other fused to a ground glass joint. A 5 mm thick layer of asbestos wool covers the sample, and above the asbestos 30 mm of P_2O_5, covered with another asbestos layer, serves as water absorbent. The tubes could be attached directly to a mass-spectrometer, and by gently heating of the carbonate-phosphate mixture a certain amount of CO_2 could be liberated for determining of the mass-ratio 44/45. As a suitable standard of $BaCO_3$ of present state biological origin [12] we used a $BaCO_3$-preparation made by combustion of ethyl alcohol, made by fermentation. The C^{13}-values are given in *atom per cent excess.*

Glutamic acid was degraded by:

1. total combustion
2. ninhydrine treatment
3. Schmidt degradation to α-γ-diamino-butyric acid.

The latter procedure implies a modification of the method of Adamson [13], carried out in semi-micro scale. In a rapidely shaken flask of the type presented in Fig. 1. 800 mg of glutamic acid (made from the hydrochloride by treatment with aniline in 30 % alcoholic solution), 3.5 ml of conc. H_2SO_4 and 5 ml of pure chloroform were treated with 600 mg of sodium azide, added in portions during 3 hours. The temperature of the water bath was kept at 48° and the CO_2 liberated from the γ-carboxyl of glutamic acid was swept out by a slow stream of CO_2-free nitrogen. A trap at —50° condensed the evaporated chloroform and the CO_2 was absorbed in saturated baryta solution. In order to ascertain whether the barium carbonate sample thus obtained really represented the γ-carboxyl, the α-γ-diamino butyric acid in the reaction mixture was isolated by dilution of the latter with water, neutralization and chromatographic separation on a Dowex-50 column, by elution at first with 1.5 *N* HCl, then with 4 *N* HCl. The fractions collected were

Table 3. Isotope content of different carbon atoms of aspartic acid and glutamic acid, isolated from E s c h e r i c h i a c o l i, grown on $C^{13}H_3C^{14}OONa$ as the sole source of carbon. C^{13} in atom per cent excess, C^{14} in counts per minute per 15 mg of barium carbonate.

Acetate (substrate)		Carbohyl group	C^{13}	C^{14}
		Carboxyl group	—	26 200
		Methyl group	4.58	—
A.	Aspartic acid,	total combustion to CO_2	0.14 ± .01	246 ± 10
B.	» »	$\alpha+\beta$-carboxyls 1 : 1, by ninhydrin	0.09 ± .01	447 ± 20
C.	» »	$\alpha+\beta$-carboxyls 4 : 1, by chloramine T	0.09 ± .01	451 ± 20
D.	» »	α-carboxyl, calculated from B, C	0.09 ± .015	453 ± 30
E.	» »	β » » » » »	0.09 ± .015	441 ± 30
F.	» »	$\alpha+\beta$-carbon atoms, calc. from A, B	0.19 ± .02	39 ± 39
G.	» »	β-carbon atom, from iodoform, combusted to CO_2	0.155 ± .01	4 ± 4
H.	» »	ditto, corrected with factor 1.5, according to [4]	0.23 ± .03	6 ± 6
A.	Glutamic acid,	total combustion to CO_2	0.15 ± .01	297 ± 15
B.	α-γ-diamino-butyric acid di-hydrochloride, comb.		0.165 ± .01	133 ± 7
C.	Glutamic acid,	α-carboxyl, by ninhydrin treatment	0.10 ± .01	572 ± 25
D.	» »	γ-carboxyl, calc. from A and B		953 ± 45
E.	» »	γ-carboxyl, directly as CO_2 from Schmidt reaction	0.005	911 ± 45
F.	» »	α-β-γ-carbon atoms, calc. from A, C, and F (mean value)	0.22 ± .02	2 ± 2

checked with paper chromatography. About 70 % of glutamic acid was recovered in the first 40 fractions; the later contained 270 mg of the diamino acid dihydrochloride, m. p. 195° (recorded 195—196°), obtained in pure state. By calculation the isotope content of the CO_2 from the combusted samples of α-γ-diamino butyric acid dihydrochloride and glutamic acid will give the value of the γ-carboxyl of glutamic acid. The result is shown in Table 3.

Aspartic acid was degraded by:

1. total combustion
2. ninhydrine treatment yielding CO_2 from both carboxyls in a ratio of 1 : 1 [3].
3. treatment with Chloramine T, liberating CO_2 from the α- and the β-carboxyls in a ratio of 4 : 1 [3].
4. treatment with hypochlorite and conversion of the liberated acetaldehyde to iodoform, representing the β-carbon atom.

The latter procedure is a modification of the method of Langheld [14]. 200 mg of aspartic acid was dissolved in 10 ml water and the solution cooled down to 0°. 10 ml of an ice-cold sodium hypochlorite solution in a molar ratio of 1 : 1, was added and the mixture allowed to stand for 10 minutes. The solution was added during 2 minutes through a separatory funnel to a boiling solution of 600 mg of KH_2PO_4 in 50 ml of water, the acetaldehyde distilled over and condensed as water solution in an ice-cooled receiver of a U-tube type. The dimensions of the latter were: length of each vertical part 500 mm. inner diameter of the tube 7 mm. At the bend of the bottom part a constriction, 1 mm in diameter, was made and in the upper parts of the vertical sections two bulbs, each of 25 ml volume, were attached in order to prevent backsuction.

Table 4. Isotope content of different carbon atoms of alanine, serine, glycine and valine, isolated from E s c h e r i c h i a c o l i, grown on $C^{13}H_3C^{14}OONa$ as the sole source of carbon. Before the degradation procedure serine has been transformed to alanine. C^{13} in atom per cent excess, C^{14} in counts per min. per 15 mg of $BaCO_3$.

Acetate (substrate)		C^{13}	C^{14}
	Carboxyl group	—	26 200
	Methyl group	4.58	—
Alanine, total combustion to CO_2		0.125 ± .005	132 ± 7
»	carboxyl, by ninhydrin treatment, CO_2	0.09 ± .005	351 ± 15
»	α- and β-carbon atoms, calculated mean value for each atom	0.14 ± .03	22 ± 22
»	ditto, calculated from acetaldehyde-thio-semicarbazone, comb. to CO_2	0.12 ± .01	8 ± 8
»	β-carbon atom, from iodoform	0.11 ± .02	12 ± 12
Serine (as alanine), total combustion to CO_2		0.13 ± .01	128 ± 6
» » »	carboxyl, by ninhydrine	0.08 ± .005	308 ± 15
» » »	α- and β-carbon atoms, calculated mean value	0.155 ± .03	38 ± 30
» » »	ditto, calculated from acetaldehyde-thio-semicarbazone, as CO_2	0.13 ± .01	6 ± 6
» » »	β-carbon atom, from iodoform	0.15 ± .02	11 ± 11
Glycine, total combustion to CO_2		0.12 ± .01	178 ± 15
»	carboxyl, by ninhydrin treatment	0.08 ± .005	321 ± 20
»	methylene group, calculated from above	0.16 ± .015	35 ± 25
Valine, total combustion to CO_2		0.085 ± .01	80 ± 8
»	carboxyl, by ninhydrine treatment	0.065 ± .005	258 ± 15
»	side chain carbon atoms, calc. mean value	0.09 ± .02	35 ± 20
»	ditto, calc. from iso-butyraldehyde-dinitro-phenyl-hydrazone, comb. to CO_2	0.12 ± .01	7 ± 7

Table 5. Isotope content of different carbon atoms of threonine, isolated from Escherichia coli, grown on $C^{13}H_3C^{14}OONa$ as the sole source of carbon. C^{13} in atom per cent excess, C^{14} in counts per min. per 15 mg of $BaCO_3$.

Acetate (substrate)		C^{13}	C^{14}
	Carboxyl group	—	26 200
	Methyl group	4.58	—
A.	Threonine, total combustion to CO_2	0.145 ± .005	251 ± 15
B.	» carboxyl by ninhydrine treatment	0.09 ± .005	441 ± 20
C.	» α-β-γ-carbon atoms, calculated mean value	0.16 ± .01	188 ± 20
D.	» β-γ-carbon atoms, calculated from acetaldehyde-thio-semicarbazone, combusted to CO_2	0.175 ± .015	226 ± 15
E.	» α-carbon atom, calc. from C, D	0.14 ± .03	(110 ± 45)
F.	» γ-carbon atom, from iodoform	0.06 ± .005	279 ± 15
G.	» ditto, corrected with factor 1,5, according to [4]	0.09 ± .01	418 ± 40
H.	» β-carbon atom, calc. from D, G	0.25 ± .03	32 ± 32

A total of approximately 15 ml of distillate were collected and diluted to exact 25 ml. The aldehyde content was determined on a 1 ml-sample according to Peters and Van Slyke [15]. Part of the solution, corresponding to 20 mg of acetaldehyde was treated with slightly less than theoretical amount of thio-semi-carbazide in 3 ml of water and the solution was kept in a stoppered flask at 35° for 12 hours. The solution was then continuously extracted with ether for four hours, and the ether extract evaporated to dryness. Usually the dry product had the right melting point (146°) for acetaldehyde-thio-semicarbazone. In some cases it was recrystallized from 50 % (aldehyde-free) methanol.

The rest of the solution was subjected to hypo-iodite treatment in order to isolate the methyl group of acetaldehyde as iodoform. These degradation procedures were checked [3] with synthetic $C^{13}-C^{14}$-labelled aspartic acid and acetaldehyde, $HOOC^{14}C^{13}H_2CH(NH_2)COOH$ and $HOC^{14}C^{13}H_3$.

The isotope values of the CO_2 from the combustion of the acetaldehyde-thio-semicarbazone, multiplied with 3/2 represents the mean value of both carbon atoms of acetaldehyde, and thus the two middle atoms of aspartic acid. The CO_2 from the combusted iodoform represents the β-carbon atom. (In view of a certain contamination of the 50 mg samples of iodoform a correction factor of 1.5 has been used for the isotope values of the carbonate from iodoform [3].)

The result of the degradation of aspartic acid is shown in Table 3.

Alanine (and thus also alanine *ex serine*) was degraded by total combustion, ninhydrin treatment and isolation of the acetaldehyde as thio-semi-carbazone, as described under aspartic acid and in a previous paper [3]. The iodoform from part of the aldehyde was combusted to CO_2. The result is shown in Table 4.

Valine was combusted to CO_2 and treated with ninhydrine. The iso-butyraldehyde resulting from this reaction was isolated as 2-4-di-nitro-phenyl hydrazone. The isotope values of the CO_2 from the combustion of the latter, multiplied by 10/4 represents the mean value of the four non-carboxylic carbon atoms of valine. The result, together with the values from the degradation of

Table 6. Survey of the distribution of C^{13} and C^{14} in the carbon structures of arginine, histidine, lysine, leucine, iso-leucine, proline, tyrosine and phenyl-alanine, isolated from Escherichia coli, grown on $C^{13}H_3C^{14}OONa$ as the sole source of carbon. C^{13} in atom per cent excess, C^{14} in counts per min. per 15 mg of $BaCO_3$.

Acetate (substrate)		C^{13}	C^{14}
	Carboxyl group	—	26 200
	Methyl group	4.58	—
Arginine, total combustion to CO_2		0.095 ± .005	191 ± 10
» carboxyl, by ninhydrine treatment		0.10 ± .005	429 ± 20
» guanido-carbon		0.02 ± .01	100
» carbon atoms 2,3,4,5, mean value		0.11 ± .015	132
Histidine, total combustion to CO_2		0.075 ± .005	47 ± 5
» carboxyl, by ninhydrine treatment		0.09 ± .005	8 ± 8
» carbon atoms 2,3 and ring, mean value		0.07 ± .01	55 ± 8
Lysine, total combustion to CO_2		0.105 ± .005	115 ± 8
» carboxyl, by ninhydrine treatment		0.065 ± .005	250 ± 10
» carbon atoms 2,3,4,5,6, mean value		0.11	65 ± 5
Leucine, total combustion to CO_2		0.055 ± .005	123 ± 12
» carboxyl, by ninhydrine treatment		0.010 ± .005	538 ± 20
» carbon atoms 2,3,4,5,5′, mean value		0.065	<40 ± 10
Iso-Leucine, total combustion to CO_2		0.05 ± .005	78 ± 7
» carboxyl by ninhydrine treatment		0.035 ± .005	228 ± 15
» carbon atoms 2,3,4,4′,5		0.055	48 ± 10
Proline, total combustion to CO_2		0.115 ± .005	296 ± 10
» carboxyl, by ninhydrine treatment		0.095 ± .005	415 ± 25
» ring carbon atoms, mean value		0.12 ± .01	266 ± 35
Tyrosine, total combustion to CO_2		0.06 ± .005	84 ± 4
» carboxyl, by ninhydrine treatment		0.07 ± .005	269 ± 12
» carbon atoms 2,3 and ring, mean value		0.06 ± .01	61
Phenyl-alanine, total combustion to CO_2		0.055 ± .005	83 ± 4
» » carboxyl, by ninhydrine treatment		0.02 ± .005	507 ± 20
» » carbon atoms 2,3, and ring, mean value		0.06 ± .01	30

Glycine, is also shown in Table 4.

Threonine was degraded by total combustion, ninhydrine treatment, and periodate oxidation according to Shinn and Nicolet [16] and Ehrensvärd, Reio, Saluste and Stjernholm [3]. From the liberated acetaldehyde, representing the β- and γ-carbon atoms, the iodoform obtained by hypoiodite treatment represents the γ-carbon atom. The result is shown in Table 5.

The isotope value of the CO_2 originating from total combustion and ninhydrine treatment of *proline*, *leucine*, *isoleucine*, *tyrosine*, *phenylalanine*, *arginine*, *histidine* and *lysine* are recorded in Table 6. An account of further degradation of these amino acids will be given in the next paper of this series.

RESULTS AND DISCUSSION

Acetate metabolism and the interrelationship with glutamic acid, aspartic acid and alanine

The C^{13}—C^{14}-content of the respiratory CO_2, taken at intervals during the cultivation, together with the C^{13}—C^{14}-content of the different carbon atoms of glutamic acid, aspartic acid, alanine and serine (Tables 1, 3 and 4) give the general features of the aerobic metabolism pattern in *E. coli*. The work of Lichstein and Cohen [17] has provided evidence that glutamic acid, aspartic acid and alanine in *E. coli* are, at least to some extent, equilibrated with the corresponding keto-acids through trans-amination reactions. Thus the assumption may be justified that the individual labelling patterns of the three amino acids will be found in the corresponding keto-acids: α-ketoglutaric acid, oxal-acetic acid and pyruvic acid. This reasoning is furthermore supported by the fact that the three amino acids mentioned are assumed to have originated from the keto-acids by a few-step sequence, involving reductive amination and transamination [18–20]. Thus the isotope pattern of glutamic acid, aspartic acid and alanine will in the following discussion be regarded as representative (in qualitative respect) of the corresponding labelling of α-keto-glutaric acid, oxal-acetic acid and pyruvic acid.

As seen from Table 1 the respiratory CO_2 has a $'C^{14}/'C^{13}$-ratio varying from 1.0 during the first period after addition of the labelled acetate to 1.25—1.0 during the last hours of the experiment. The ratio of around 1.0 should appear in the β-carboxyl of oxal-acetate, as a consequence of the β-carboxylation of pyruvate, for which Wood *et al.*[21, 22] have given strong evidence. We find a $'C^{13}/'C^{14}$-ratio of 0.86—0.88 in *both* carboxyls of aspartic acid, suggesting that the oxal-acetate from pyruvate and CO_2 is equilibrated with a symmetrical metabolite. This is in full accordance with the findings of Wood and the earlier work of Nishina, Endo and Nakayama [23], who demostrated the synthesis in *E. coli* of fumarate *via* malate from pyruvic acid and radioactive CO_2.

Since the C^{13} and C^{14} values of the carboxyl and the α- and β-carbon atoms of alanine are in almost complete correspondance with those of the α-carboxyl, the α- and β-carbon atoms of aspartic acid, the corresponding labellings of pyruvic and oxal-acetic acid will consequently be the same, which agrees with the assumtion of a Wood-Werkman equilibrium in *E. coli*. A direct oxidative condensation of two acetate-molecules to succinate, as proposed by Slade and Werkman [24] and Kalnitzsky, Wood and Werkman [25] should, however, bring about a high C^{14}-content and almost no C^{13} in the carboxyls of succinate. Assuming that oxal-acetate could be formed from this succinate one would expect a very high $'C^{14}/'C^{13}$-ratio in the carboxyls of the latter. Our experiments show that this ratio for oxal-acetate (as aspartate) and pyruvate (as alanine) is of the same order as that of the respiratory CO_2, and consequently, give no support for any direct acetate → succinate transition in *E. coli in vivo*. It must be borne in mind that the experiments of the authors mentioned [24], [25] has been carried out on cell-free enzyme preparations.

With regard to the α-keto-glutaric acid, the isotope labelling of which being indicated by the C^{13}—C^{14}-values of glutamic acid (Table 3), its origin from oxal-acetate and acetate *via* citric acid seems to be supported by the fact that the γ-carboxyl has about the double C^{14}-content, as the α-carboxyl, with almost no C^{13} ($'C^{14}/'C^{13} > 32$). Accepting the view that the first step of citric acid formation involves a condensation between the methyl group of acetate and the keto-group of oxal-acetate it is obvious that the acetate part will show up a more intense isotope content than the oxal-acetate residue, the latter being continuously diluted by non-labelled material from the carbohydrates of the original inoculate, and being labelled from acetate in a series of reactions, involving several steps. Since the work of Potter and Heidelberger [26] have confirmed the hypothesis of Ogston [27] that the apparent symmetrical citric acid does *not* undergo any randomization on its enzymatic conversion to α-ketoglutaric acid, the latter should consequently possess a higher C^{14}-content in its γ-carboxyl than in its α-carboxyl; the former originating directly from the acetate carboxyl, the latter indirectly, being introduced via CO_2.

Summing up, our results are in complete accordance with the assumtion that:

1. Oxal-acetate originates from pyruvate through β-carboxylation.
2. Oxal-acetate could be transformed, via a reversible series of reactions to a symmetrical metabolite [26] (fumarate or succinate).
3. By means of α-condensation with acetate oxal-acetate is the precursor of α-ketoglutarate via citric acid and the other members of the tri-carboxylic acid series. Thus, all steps but one in the scheme of Krebs [28] as modified by

Wood [29,30] for the cyclic decarboxylation of acetate-oxal-acetate are in accordance with the isotope data that we have found for alanine, aspartic acid and glutamic acid. The remaining step, the conversion of α-ketoglutarate to succinic acid, by decarboxylation and dehydrogenation, is, however, supported by the fact that at least as much methyl groups of acetate (indicated by C^{13}) as carboxyl groups (C^{14}) are converted to CO_2, the ratio being, as said, 1 : 1. Since there is no evidence of a direct oxidation of methyl groups of acetate to a carboxyl, the most probable route seems, at present, to be the conversion of α-ketoglutarate to succinic acid, whereby the C^{13}-labelled α-keto-group of α-ketoglutarate will appear as one of the carboxyls of succinate. The work of Ajl and Werkman [31] on *E. coli* indicates a conversion of α-ketoglutarate in this direction. Since succinic acid is a symmetrical molecule, its equilibration with oxal-acetate will label both carboxyls of the latter, with C^{13} and C^{14} in a constant ratio. Consequently the respiratory CO_2 arising from the β-decarboxylation of oxal-acetate and the decarboxylation of oxal-succinate and ketoglutarate, will contain a certain C^{13}-content originating from the methyl groups of acetate. So far none of our results, based on isotope distribution, of the general metabolic pattern of *E. coli* and (by previous work) of *Torulopsis utilis* [1,4] are contradictory to the assumtion of a cyclic decarboxylation mechanism of the Krebs type in these micro-organisms.

The rôle of formate in general metabolism is, in part, connected with the glycine-serine equilibrium [32–34], at least in higher organisms. If these assumptions are also valid for *E. coli*, the isotope labelling of the β-carbon atom of serine should be an indication of the origin of formate. In our experiments, the pure C^{13}-labelling of this carbon atom points to the origin of formate entirely from the methyl group of acetate. This conclusion presupposes that glycine plus formate (or formaldehyde) are the main precursors of serine; if, however, serine arises from another source, the split to glycine and formate will make the β-carbon atom of serine the immediate precursor of formate, likewise C^{13}-labelled. In both cases the formate has its origin from the methyl group of acetate, which hardly fits with the idea of the phosphoroclastic split of pyruvate into acetate and formate. (The latter should in that case originate from the carboxyl of pyruvate, and thus from the carboxyl of acetate [25,35]. Formate from this source would be C^{14}-labelled and some of this C^{14} should consequently be detected in the β-atom of serine, which is not the case in our experiments. It is not excluded, however, that a minor amount of C^{14}-labelled formate is formed from pyruvate, but the main part of formate (or formaldehyde) in *E. coli* seems to be derived from the C^{13}-labelled methyl group of acetate.)

It must be pointed out that the main features of the metabolic utilization and break-down of acetate in *E. coli* presupposes a continuous supply of oxal-

acetate for the combination and cyclic degradation of acetate. The isotope values are in accordance with this view, but no evidence has been produced concerning *the net conversion* of acetate to pyruvate and oxal-acetate. Naturally, a considerable part of the pyruvate and oxal-acetate are produced from the breakdown of the carbohydrate material. This is shown in Table 1, where only 25.1 + 25.1 = 50.2 % of the respiratory CO_2 originates from acetate. (The rest originates from non-labelled material of the inoculate.) But since there has been a considerable growth of the organism, there must exist a pathway, whereby acetate will *produce* pyruvate in excess over what arises by decarboxylation of the recycling oxal-acetate. This route is at present unknown, but we know with certainty that the net result is the formation of a pyruvate, the α- and β-carbon atoms of which originate from the methyl group of acetate.

Main principles of amino acid formation

The C^{13}—C^{14}-labelling of the amino acids recorded in Tables 4, 5 and 6 give some hints of the formation of amino acids in general. The origin of the *dicarboxylic amino acids* and *alanine* has already been discussed, and is most probably connected with the amination and transamination of the corresponding keto-acids. The analogous labelling of the α-carbon atom and the carboxyl of *glycine* and *serine* point to a close connection between these amino acids, probably via a formylation-deformylation equilibrium. Yet the direct pathway to either glycine or serine from acetate is still obscure. The other member of the β-hydroxy-amino acids, *threonine*, has a labelling indicating that the two middle atoms have mainly originated from the methyl group of acetate, whereas the carboxyl and the γ-carbon atom are derived from the acetate carboxyl [36]. The case of the C^{14}-labelled γ-methyl group is interesting, but not wholly unexpected, because the same labelling has been recorded in threonine, isolated from *Torulopsis*, grown on $C^{13}H_3C^{14}OOH$ [4, 36]. It has been proposed [37] that the γ-methyl group has arisen by a γ-β-OH-shift in *homoserine*, the γ-CH_2OH of which must consequently originate from a carboxyl, and thus be C^{14}/C^{13}-labelled [4].

Among the branched-chained amino acids the isotope data for *valine* (Table 4) clearly show that the whole side-chain originates from the methyl group of acetate, and that the γ-β-γ^1-part cannot be an acetone residue, contrary to expectations. The same labelling is recorded for valine from *Torulopsis* [4]. This formation of a carbon atom system with one C-atom connected to three others, all of them originating from the methyl group of acetate, point to the existance of a metabolic mechanism, hitherto unknown. On the other

hand the data for *leucine* and *iso-leucine* show, like in *Torulopsis*, that one of the sidechain carbon atoms must hold a slight C^{14}-content. The localization of this atom will be described in a later publication; most likely the C^{14}-content is not in the α-position. It has been shown that in *Neurospora* the distal carbon atom of the ethyl group in isoleucine originates from the carboxyl of acetate.[38]

The case of the two aromatic amino acids is interesting. The C^{13}—C^{14}-values of total carbon and the carboxyl-group point to at least one C^{14}-labelled atom in the ring, which is in accordance with the corresponding labelling of tyrosine from *Torulopsis* [2]. On the other hand, the carboxyls of *tyrosine* and *phenyl-alanine* possess definitely different labellings. The tyrosine carboxyl has a $'C^{14}/'C^{13}$-ratio = 0.67, indicating the ordinary equilibrium with the respiratory CO_2 (= 1.0), whereas the corresponding quotient for phenyl-alanine is = 4.4. Actually, the C^{14}-content of the latter is considerably higher (507) than in the tyrosine carboxyl (269) and lower in C^{13} (0.02 against 0.07). It seems likely that the non-aromatic part of these two amino acids is formed by different routes, one of them involving a direct coupling-in of an acetate residue (phenyl-alanine), the other involving the attachment of a pyruvate residue to the aromatic system. (It is not at all certain that the two different 3-carbon chains are virtually attached to the same point in the original, preformed aromatic ring system at the formation of phenyl-alanine and tyrosine.) The view, that we have expressed in a previous paper [2], that the benzene-ring of tyrosine could be formed from the closure of an alifatic, branched chain system, could also be consistent with the cyclisation of a derivative of glucose. The ring labelling, that we found in tyrosine from *Torulopsis*; C^{14} in 4, (3.5), could be identical with the expected labelling in glucose (3.4), when $C^{13}H_3C^{14}OOH$ is the substrate [29, 30]. This view has been considered by Bloch and Gilvarg [39] in a study of the formation of the aromatic system of tyrosine and phenyl-alanine in *Saccaromyces*. We intend to make a detailed investigation of this problem in connection with the present work.

Proline contains at least one C^{14}-labelled atom in the pyrrolidine ring, the position of which is uncertain for the present time. Assuming *one* C^{14}-labelled atom, this must have a C^{14}-content of about the double of that of the carboxyl, which would point to glutamic acid as the precursor, possibly *via* ornithine, as assumed by Rittenberg and Shemin [40].

The further degradation of *arginine*, *lysine* and *histidine* will be described later. We will only point out that the carboxyl of histidine, like in *Torulopsis* [3,4], is derived from the *methyl* group of acetate and not from the carboxyl, which is an exception to the rule that, in general, amino acid carboxyls are derived from the acetate carboxyl, *via* CO_2 or directly.

CONCLUSIONS

The over-all picture of acetate metabolism in *E. coli*, as reflected by the different C^{13}—C^{14}-values of the different carbon atoms of various amino acids is in its *qualitative* aspect strikingly in accord with the corresponding picture from *Torulopsis* [4, 5]. Comparing the data from Table 7 with the analogous compilation of data in the work of Ehrensvärd, Reio, Saluste and Stjernholm [4] (recorded in Table 8, *l. c.*) we find, for example, that the $'C^{14}/'C^{13}$-ratio for

Table 7. Survey of the isotope content of 15 amino acid carboxyl groups, compiled from tables 3,4,5 and 6, and a comparison with the isotope content of the respiratory CO_2 during the last hour of the cultivation experiment (from table 2). C^{13} is given in atom per cent excess, C^{14} in counts per minute per 15 mg of $BaCO_3$. $'C^{13}$ and $'C^{14}$ denote isotope content in per cent of the corresponding isotope content of the methyl group and the carboxyl of the $C^{13}H_3C^{14}OONa$, present as substrate in the medium at the start of the experiment.

	Reference	C^{13}	C^{14}	$'C^{13}$	$'C^{14}$	$'C^{14}/'C^{13}$
Acetate, carboxyl	table 1	—	26 200	—	100	1.00
» methyl group	»	4.58	—	100	—	
Respiratory CO_2 83—128 min. after start of exp.	»	0.70	4 985	15.3	19.0	1.25
128—188	»	1.05	6 035	22.6	23.0	1.02
188—218	»	1.15	6 575	25.1	25.1	1.00
Glutamic acid, γ-COOH	table 3	0.005	911	0.11	3.48	> 31.5
» » α-COOH	»	0.10	572	2.18	2.22	1.02
Aspartic » α-COOH	»	0.09	453	1.96	1.73	0.88
» » β-COOH	»	0.09	441	1.96	1.68	0.86
Alanine, COOH	» 4	0.09	351	1.96	1.34	0.68
Serine, COOH	»	0.08	308	1.75	1.18	0.68
Glycine, COOH	»	0.08	321	1.75	1.22	0.70
Threonine, COOH	» 5	0.09	441	1.96	1.68	0.86
Valine, COOH	» 4	0.065	258	1.42	0.99	0.70
Leucine, COOH	» 6	0.01	538	0.22	2.04	9.3
Iso-leucine, COOH	»	0.035	228	0.76	0.87	1.1
Proline, COOH	»	0.095	415	2.07	1.58	0.76
Tyrosine, COOH	»	0.07	269	1.53	1.03	0.67
Phenyl-alanine, COOH	»	0.02	507	0.44	1.94	4.4
Arginine, COOH	»	0.10	429	2.18	1.64	0.51
Lysine, COOH	»	0.065	250	1.42	0.96	0.68
Histidine, COOH	»	0.09	8	1.96	0.02	0.01
Threonine, γ-carbon atom	table 5	0.09	418	1.96	1.60	0.81
Arginine, guanido group	» 6	0.02	90	0.44	0.34	0.77

most amino acid carboxyls correspond to that of the respiratory CO_2 during the last hours of the cultivation. The only difference is that the quotient of the lysine carboxyl is much higher in *Torulopsis* than in *E. coli*, indicating that the biosynthesis of lysine may proceed over different pathways in those two organisms.

In *quantitative* respect the main difference is that $'C^{14}/'C^{13}$-ratio of the respiratory CO_2 (and thus of most amino acid carboxyls) is approximately 1.0 in *E. coli* and about 1.8 in *Torulopsis* indicating a relatively higher rate of the utilization of the acetate carboxyl for synthetic purposes in *E. coli*. In addition, the overall isotope content, as compared with that of the substrate is considerably lower in *E. coli* than in *Torulopsis* which points to a slower rate of protein synthesis, compared to the rate of aerobic decarboxylation in *E. coli;* the actual utilization of acetate for protein synthesis (= growth) in *Torulopsis* being markedly higher.

With regard to the connection of acetate and glutamic acid, aspartic acid and alanine (representing α-keto-glutaric acid, oxal-acetic acid and pyruvic acid) the C^{13}—C^{14}-labellings of those amino acids from *E. coli* has the same pattern as in *Torulopsis*. The identical labellings of the carboxyls and the α- and β-carbon atoms in aspartic acid, the high C^{14}-value of the γ-carboxyl of glutamic acid, being twice of that of the α-carboxyl, and, finally, that the $'C^{14}/'C^{13}$-ratio for the respiratory CO_2 and that of all α-carboxyls of the amino acids mentioned, are of the same order of magnitude, *all this is in agreement with the assumption of an aerobic decarboxylation mechanism with many features in common with the scheme of Krebs and Wood.* In fact, the recorded data for glutamic acid, aspartic acid and alanine are the same that could be expected in higher biological systems, *e. g.* rat liver [30]. The 2 : 1 ratio of the C^{14}-labelling of the γ- and the α-carboxyls of glutamic acid has an analogy in the observation of Guzman Barron [41] on rats, confirmed by our own, unpublished experiments.

Our data from serine point definitely towards an origin of the β-carbon atom from the methyl group of acetate. If formate is the immediate precursor for this carbon atom [32–34] it must in turn have been derived from the same source.

SUMMARY

1. *Escherichia coli* has been cultivated in large scale under aerobic conditions on isotope labelled acetate ($C^{13}H_3C^{14}OONa$) as the sole source of carbon. By hydrolysis of the bacterial proteins, 15 amino acids have been isolated, all of them containing C^{13} and C^{14} in different atoms originating from the labelled substrate.

2. The carboxyls of all amino acids have been isolated as CO_2 and the C^{14} to C^{13} ratio determined and compared to that of the respiratory CO_2. The latter is 1.0, indicating an equal utilization of the methyl groups and the carboxyl of the acetate. A $'C^{14}/'C^{13}$-ratio of the same order of magnitude is shown to appear in all amino acid carboxyls, except in four special cases. The carboxyls of glutamic acid (γ), leucine and phenyl-alanine, seem to be derived from acetate carboxyl more directly than the rest of the amino acid carboxyls. The carboxyl of histidine is derived from the methyl group of acetate.

3. The 3-carbon chains of phenyl-alanine and tyrosine are probably not formed by identical mechanisms.

4. Total degradation and localization of the isotope content has been performed on glutamic acid, aspartic acid, glycine, alanine, valine, serine, threonine. The result of the isotope determination on barium carbonate samples, derived from one or several carbon atoms of the amino acid structures, indicate a metabolic connection between acetate and these amino acids, which is analogous to that found in representatives for lower fungi, *e. g. Torulopsis utilis.* The isotope distribution in the carbon atoms of glutamic acid, aspartic acid and alanine is compatible with the assumption of a cyclic, aerobic decarboxylation mechanism, analogous to that found in higher organisms.

5. There is some indication that formate in *E. coli* is derived mainly from the methyl group of acetate. The β-carbon atom of serine was found to be entirely C^{13}-labelled.

6. Like in *Torulopsis utilis*, the branched side chain of valine was found to be derived entirely from methyl groups of acetate. The γ-methyl group of threonine originates from the carboxyl of acetate.

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Contribution to the Knowledge of the Alkali Tungsten Bronzes

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In the ample literature on alkali tungsten bronzes a great number of different compounds have been reported (*cf.* Gmelin [1] and Mellor [2]). In 1935, however, it was shown by Hägg[3,4] that all sodium tungsten bronzes of cubic symmetry, described by previous authors, are not separate compounds but members of a continuous series of solid solutions corresponding to a phase Na_xWO_3 of variable composition with a very extended homogeneity range. At the theoretical upper limit of x equal to 1 all the tungsten atoms are in the quinquevalent state and the bronze crystallizes with a complete perovskite lattice. With decreasing values of x, statistically distributed, vacant positions occur in the original sodium lattice and a corresponding number of tungsten atoms acquire the valency of six.

As it was thought very likely that the knowledge of other tungsten bronzes was in several respects inadequate an investigation of these compounds was started at this Institute a few years ago. The aim of these studies has been especially to settle the existence and composition of the existing phases and to find their crystal structures. Generally no attempts have been made to find the extension of the homogeneity ranges of the various phases. It has also been thought of interest to correlate the structures of the bronzes with those of certain related compounds recently investigated here, *viz.* oxides of tungsten and molybdenum [5]. On the following pages a survey will be given of results concerning the preparation and composition of the various phases obtained.

Methods of preparation

The general method of preparing tungsten bronzes is by reduction of polytungstates *. This reaction may be carried out in various ways. The original

* The word polytungstate is here used for substances of composition $Me_2O \cdot nWO_3$ ($n > 1$), without regard to whether they are definite compounds or not.

method, introduced by Wöhler[6], involves heating of the polytungstate in a stream of hydrogen. Wright[7] reduced fused polytungstate with tin, zinc or other elements, while Scheibler[8] used electrolytic reduction of the molten material. Brunner[9] heated mixtures of polytungstate and tungsten dioxide *in vacuo*.

In the present investigation syntheses have been carried out utilizing reduction with hydrogen and tungsten dioxide and by electrolysis. The polytungstates were prepared by heating alkali carbonate with tungsten trioxide. Tungsten dioxide was obtained by heating tungsten trioxide mixed in the proper proportion with tungsten powder *in vacuo*. Platinum * or carbon electrodes were used for the electrolytic reductions.

With most of the syntheses impure products were obtained. The purification of the bronzes comprised repeated boiling alternately with concentrated alkali carbonate solution, concentrated hydrochloric acid, and *aqua regia*, and in certain cases a final boiling with a mixture of concentrated nitric acid and hydrofluoric acid.

Identification of the products

All the samples were investigated microscopically and by means of X-ray powder photographs. The latter were recorded in focusing cameras using monochromatized Cu-$K\alpha$ radiation. Exact unit cell dimensions were obtained from photographs taken in Phragmén-Hägg focusing cameras with Cr-K or Cu-K radiation and expressed in true Å units[10].

Methods of analysis

In a recent paper[11] comments and certain improvements of the methods for analysing tungsten bronzes have been reported. The analyses of all the investigated bronzes except the lithium compounds were performed according to those principles. For the latter compounds the applied methods failed to give faithful results, and we have thus refrained from any definite conclusions concerning the compositions of these compounds. Quite recently, however, Straumanis and Hsu[12] have reported that they have overcome the difficulties connected with the analysis of lithium tungsten bronzes, but so far their method has not been tested here.

INVESTICATED COMPOUNDS

The generally adopted idea that the composition of the alkali tungsten bronzes may be represented by the formula Me_xWO_3 has been fully confirmed

* The platinum electrodes were heavily attacked during the electrolyses.

by our chemical analyses and crystal structure investigations. For all bronzes, the structures of which have been hitherto determined, the constant ratio of tungsten atoms to oxygen atoms equal to 1 : 3 is accounted for by the arrangement of the atoms of the lattices. They have all been found to contain a stable framework of WO_6 octahedra mutually connected by having corners in common, every oxygen atom thus belonging to two octahedra.

Lithium tungsten bronzes

The blue lithium tungsten bronzes $Li_2W_4O_{12}$[9] and $Li_2W_5O_{15}$[8, 9, 13–16] have been reported by previous authors. Blue preparations with lower content of lithium have been considered to consist of mixtures of bronzes (*e. g.* $Li_2W_7O_{21}$)[9].

Perovskite phase. A lithium tungsten bronze, briefly reported in previous papers[5,17], has been synthesized by heating mixtures of lithium polytungstate and tungsten dioxide ($\sim Li_2O \cdot 3WO_3 + 0.3WO_2$) at about 850° C *in vacuo* and also by electrolytic reduction of fused lithium polytungstate ($\sim Li_2O \cdot 2WO_3$, current density at the cathode ~ 0.6 A/cm^2). The product obtained by the first method consisted of a blue or bluish violet crystalline powder, while dark blue crystals of irregular shape were formed by the electrolysis. The powder photographs showed the structure to be of perovskite type and thus isomorphous with that of the cubic sodium tungsten bronze (*v. infra*). The length of the cube edge, being about 3.72 Å, was slightly different for various preparations, thus indicating that the substance has the character of a berthollide. The composition was roughly given to be Li_xWO_3 ($x = 0.4$ or 0.3).

These data have been fully confirmed by the investigations independently carried out by Straumanis and Hsu [12]. These authors furthermore state that the limits of the homogeneity range correspond to values of x equal to 0.57 and 0.31. The two compounds $Li_2W_4O_{12}$ and $Li_2W_5O_{15}$, assumed by previous authors, should thus be members of this series of solid solutions.

Tetragonal phase. In the powder photographs of preparations that had not been reduced as far as those mentioned above, lines of another phase appear together with those of the perovskite phase. The lithium content of the new phase is obviously less than that of the latter. The extra lines are consistent with a tetragonal unit cell with the dimensions $a = 5.14$ Å, $c = 3.80$ Å. The structure is isomorphous with that of tetragonal sodium tungsten bronze II (*v. infra*). This tetragonal lithium tungsten bronze may be identical with a tetragonal phase mentioned by Straumanis and Hsu [12].

Sodium tungsten bronzes

Besides preparations of cubic sodium tungsten bronze of different compositions and colours described by various authors there are also reports of a bronze with the composition $Na_2W_5O_{15}$ forming blue, prismatic needles [15,18] and of samples of blue bronzes of still lower sodium content, assumed to be mixtures of $Na_2W_6O_{18}$ and $Na_2W_7O_{21}$ [9].

Perovskite phase. This phase, investigated by Hägg [4], has been briefly described in the introduction to this paper. It has a very broad homogeneity range ($0.32 \lesssim x \lesssim 0.93$) [4] comprising all the cubic sodium tungsten bronzes reported by previous authors. Later on various values of the limits of the sodium content have been given [19–21]. It must be emphasized that the extension of the homogeneity range may show considerable variations connected with the temperature of preparation of the samples.

Tetragonal phase I. This compound was synthesized by electrolytic reduction of fused sodium polytungstate ($Na_2O \cdot 2.5WO_3$) at about 800° C with a current density at the cathode of 0.3A/cm². The composition of the sample, consisting of rather long blue needles, was $Na_{0.38}WO_3$. For a specimen of similar appearance, prepared by Hägg using another polytungstate mixture ($Na_2O \cdot 3WO_3$), analysis gave the formula $Na_{0.28}WO_3$. This phase evidently is identical with the bronze $Na_2W_5O_{15}$ described by previous authors. Powder photographs showed the symmetry of the structure to be tetragonal and gave the unit cell dimensions $a = 12.102$ Å, $c = 3.752$ Å for the former sample and $a = 12.094$ Å, $c = 3.748$ Å for the latter. These dimensions, however, correspond to a substructure of the real lattice, as was obvious by the occurrence in amply exposed single crystal photographs of very weak reflections, indicating the true unit cell to have the axial lengths $a' = a\sqrt{2}$ and $c' = 2c$. The crystal structure was found to be closely related to that of the tetragonal potassium tungsten bronze (*v. infra*). A report of these studies has been previously published [22].

Tetragonal phase II. By electrolyzing sodium polytungstates richer in tungsten trioxide than those mentioned above ($Na_2O \cdot 3.5WO_3$), dark blue, prismatic crystals of a tungsten bronze were obtained, previously not characterized. The colour after grinding was bluish green. The composition of one of the preparations was found to be $Na_{0.10}WO_3$. The structure was tetragonal with the unit cell dimensions $a = 5.25$ Å, $c = 3.90$ Å. A structure determination has been carried out and will be published elsewhere [23]. The structure is closely related to that of the perovskite phase.

Potassium tungsten bronzes

A violet potassium tungsten bronze, forming needle-shaped crystals, has been described by several authors. Opinions have diverged concerning the formula of this compound, the compositions $K_2W_3O_9$ [15] and $K_2W_4O_{12}$ [13,24,25] being reported. Blue preparations of lower potassium content have been considered to consist of mixtures of bronzes ($K_2W_5O_{15}$, $K_2W_6O_{18}$, $K_2W_8O_{24}$) [9], but similar products have also been regarded as containing potassium octotungstate [13].

Tetragonal phase. By reducing potassium polytungstates with hydrogen at about 600° C a bronze was obtained in the form of very small reddish-violet needles. The alkali content of the product was found to be dependent on the composition of the polytungstate used. Thus by reducing $K_2O \cdot 1.2WO_3$ a preparation K_xWO_3 ($x = 0.57$) was obtained while a polytungstate $K_2O \cdot 3.5WO_3$ gave a bronze with x equal to 0.475. This phase thus seems to be identical with the violet bronze reported by previous authors, the formula $K_2W_4O_{12}$ falling within and $K_2W_3O_9$ slightly outside the observed homogeneity range. The dimensions of the tetragonal unit cell were found to be $a = 12.317$ Å, $c = 3.841$ Å for the preparation $K_{0.57}WO_3$ and $a = 12.285$ Å, $c = 3.833$ Å for $K_{0.475}WO_3$. By heating a mixture of polytungstate and tungsten dioxide ($K_2O \cdot 2WO_3 + 0.5WO_2$) *in vacuo* rather large crystals of the tetragonal phase were obtained, contaminated by the dark blue hexagonal phase *(v. infra)*. The crystal structure of the tetragonal bronze has been determined and described in a previous paper [26].

Hexagonal phase. By heating a mixture of potassium polytungstate and tungsten dioxide ($K_2O \cdot 6WO_3 + 3WO_2$) at about 1100° C *in vacuo* the dark blue compound mentioned above was prepared in a pure state in the form of irregularly shaped crystals. The composition of the product was found to be $K_{0.27}WO_3$ in close agreement with the formula $K_2W_8O_{24}$ proposed by Brunner [9]. The analysis definitely excluded the possibility of this substance being an octotungstate(VI) [13], thus confirming the view of Brunner *. The powder photographs were consistent with an hexagonal unit cell with the dimensions $a = 7.40$ Å, $c = 7.56$ Å. The density value of 6.702 given by Brunner would correspond to a cell content of 6(5.9) formula units of $K_{0.27}WO_3$. An attempt to find the crystal structure of this compound will be made in the near future.

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* The results of the analyses were 4.45 % K + 95.74 % WO_3 = 100.19 % in total, while the contents corresponding to an octotungstate, $K_2O \cdot 8WO_3$, are 4.01 % K and 95.17 % WO_3. For the hexagonal rubidium and cesium tungsten bronzes analyses gave the following results (values corresponding to the octotungstate formulae within brackets): 9.10 % Rb + 91.00 % WO_3 = 100.10 % (8.37 % Rb and 90.85 % WO_3) and 15.44 % Cs + 84.66 % WO_3 = 100.10 % (12.44 % Cs and 86.81 % WO_3).

Rubidium and cesium tungsten bronzes

According to Schäfer [25] a mixture of blue-violet rubidium tungsten bronze ($Rb_2W_4O_{12}$) and dark blue rubidium octotungstate can be prepared by heating rubidium polytungstate in a stream of hydrogen. Nothing is said in the literature about cesium tungsten bronzes.

Hexagonal phases. By heating a mixture of rubidium polytungstate and tungsten dioxide ($Rb_2O \cdot 2WO_3 + 0.3WO_2$) at about 1050° C *in vacuo* dark blue crystals of a rubidium tungsten bronze were obtained. The composition of the preparation was found to be $Rb_{0.27}WO_3$. The same product could also be prepared by reducing $Rb_2O \cdot 3WO_3$ with hydrogen at about 600° C. Powder photographs showed this bronze to be isomorphous with the hexagonal potassium tungsten bronze, the unit cell dimensions of the former being $a = 7.39$ Å, $c = 7.54$ Å. It seems probable that this phase is identical with the „octotungstate" described by Schäfer. No substance corresponding to the compound $Rb_2W_4O_{12}$ has been observed in our experiments.

Similarly by heating cesium polytungstate and tungsten dioxide ($Cs_2O \cdot 4WO_3 + 0.5WO_2$) at about 950° C *in vacuo* a dark blue cesium tungsten bronze of composition $Cs_{0.32}WO_3$ was prepared. This phase was found to be isomorphous with the hexagonal potassium and rubidium bronzes. The unit cell has the dimensions $a = 7.42$ Å, $c = 7.63$ Å.

SUMMARY

A number of alkali tungsten bronzes has been synthesized in pure form. The preparations have been investigated analytically and by means of X-ray methods and the results have confirmed the view that these compounds have the general formula Me_xWO_3 ($x<1$). The observed phases have been found to comprise the majority of alkali tungsten bronzes reported by previous authors. The following structural types have been found:

perovskite type, represented by lithium and sodium [4] bronzes,

degenerated perovskite type of tetragonal symmetry, appearing in the lithium and sodium bronze systems at low alkali metal contents,

tetragonal potassium tungsten bronze type (a related sodium compound of considerably lower alkali content shows a superstructure in comparison with this type), and

hexagonal potassium tungsten bronze type with representatives also appearing in the rubidium and cesium bronze systems.

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The Iron-protein Bonds in Cytochrome c

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Theorell and Åkeson suggested [1] that imidazole groups of histidine residues constitute the heme-linked groups in cytochrome c. The experimental background for this theory was in short as follows:

Amino acid analyses revealed that cytochrome c contained three histidine residues per molecule. Its titration curve showed that two equivalents of base were consumed per mole in the region pH 5.5—8.5, where the histidine imidazoles are normally titrated. A study of the heat of ionization disclosed, however, that other groups in cytochrome c than the imidazoles were also titrated in this pH range. In fact, only one imidazole group was free to combine with an acid or base, while the other two were somehow blocked. This was most easily explained by assuming linkages to the iron atom. Spectrophotometrically five different types of ferricytochrome (I—V, I being the most acid) could be distinguished, the pK values for the transitions being 0.42, 2.50, 9.35, and 12.76 respectively. With increasing acidity of the solution the histidine-iron bonds, present in type III, were released at pH 2.50 and 0.42 and substituted by protons. These low pK values for the ionizations of imidazole groups were explained as due to the influence of the iron atom. The dissociations in the alkaline region were attributed to the imino groups of the imidazole rings. Accordingly two pK values were found (9.35 and 9.85), only the former being optically operable. In agreement herewith ferricytochrome consumed two equivalents more per mole than ferrocytochrome did between pH 8.5 and 10.5. The transformation from type IV to type V appeared at higher alkalinity, where one of the histidine — iron bonds was displaced by a hydroxyl group. Ferricytochrome V and cyanide combine in the ratio 1 : with $K_{app.} = 6.16 \times 10^{-4}$ moles per liter *. Types III—V are diamagnetic, while the ionic bonds between the pyrrols and the iron in types I and II render these types paramagnetic.

* Unpublished experiment by K. G. P.

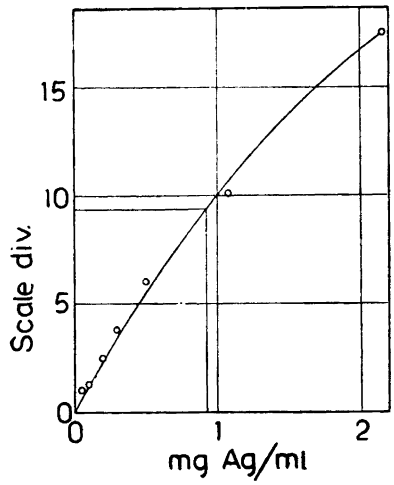

Fig. 1. Determination of silver in the protein moiety of cytochrome c. "Scale div." = difference in galvanometer deflections when the cuvette was filled with 1) water 2) the solution to be examined. Calibration curve made on silver nitrate solutions of given concentrations. The curve is drawn to fit the values. The protein solution, concentrated to 5.0 mg N/ml, gave 9.3 scale divisions, which is equal to 0.93 mg Ag/ml.

Since it is now possible to compare intact cytochrome c with its protein moiety [2], it was of interest to investigate if results from such a comparison were compatible with the above-mentioned theory.

EXPERIMENTS

Cytochrome c of the preparation with 0.424 % iron described in next paper [3] was used for the titration experiments in this paper. The fee prrotein was prepared by treatment of the cytochrome in the usual way [2] with silver sulphate and acetic acid (60°, 90 min.). The protein was then precipitated with ice-cold acid acetone, dissolved in water,reprecipitated, and dialyzed for four days against glass-distilled water. Electrodialysis was tried but found unsuitable, since the protein coagulated irreversibly at the cathode membrane. After the dialysis the clear solution was concentrated by freeze-drying and analyzed.

Electrophoretical examination showed uniform cathodical migration with the velocity 2.55×10^{-5} cm^2 × volt^{-1} × sec.$^{-1}$ (descending boundary, 1 % solution in acetate buffer pH 4.94, ionic strength 0.1).

The stock solution contained 1.998 mg of nitrogen per ml. The cytochrome preparation had been found to possess 145 nitrogen atoms per iron. After the removal of the prosthetic group from the molecule, the nitrogen content thus corresponded to 1.012×10^{-6} moles of the protein moiety per ml.

The silver content of the protein was of a special interest (*cf.* the discussion). Potentiometrical titrations of the ash, dissolved in nitric acid, with chloride gave inconsistent results, but by means of x-ray absorption measurements [4] the value 3.4 silver atoms per 141 nitrogen atoms was found * (Fig. 1). Because of the carbon and the nitrogen of the protein this value is slightly lower than the true value, which is estimated as 3.5—3.6.

A number of titration curves were made on the native cytochrome c and on its protein component. The latter was found to precipitate slightly below pH 5, and the titrations

* The author expresses his thanks to Docent Arne Engström for making the silver determination.

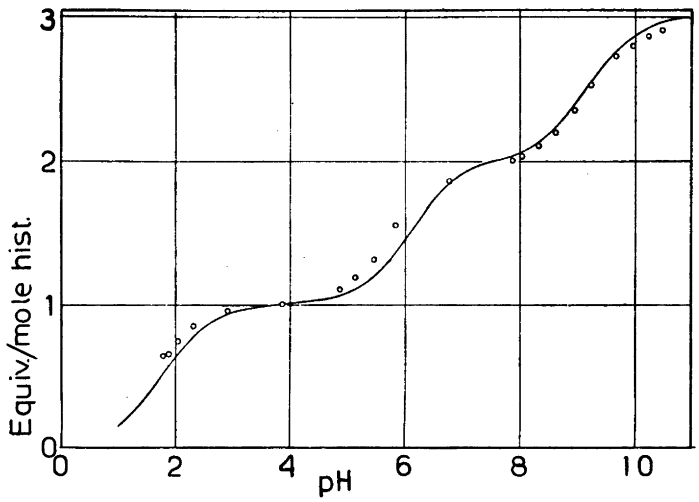

Fig. 2. Titration curve of histidine in glycerol-water 1 : 1 (O). The curve is calculated from pK values for histidine in water (Greenstein, J. P. J. Biol. Chem. 93 (1931) 479), the values being 1.77, 6.10, and 9.18 respectively.

were therefore carried out in water-glycerol. The effect of glycerol on the magnitude of the dissociation constants was studied on histidine (Fig. 2). In spite of the glycerol the protein began to flocculate at about pH 8.5, and consequently no reliable results could be obtained at higher alkalinity than pH 8. Those protein titrations, which were made in pure water between pH 1.7 and 4.5, agreed within this region fairly well with those made in glycerol-water.

One pair of titration curves of cytochrome c and its protein moiety in glycerol-water are given in Fig. 3. The results were reproduced in other experiments. The stock solution of cytochrome c was diluted with water to give a concentration of 1.997 mg of nitrogen per ml (micro-Kjeldahl determination on the diluted solution). The protein solution was taken directly for the titrations. To obtain equivalents of base combining with one mole of the substance to be titrated, the nitrogen values were recalculated to molarities on the basis of 145 and 141 nitrogen atoms per molecule for cytochrome c and its protein moiety respectively. Thus the former contained 9.83×10^{-7} and the latter 10.12×10^{-7} moles per ml. 5.00 ml of the solutions to be titrated (cytochrome, free protein and water in the case of the blank curve) + 5.0 ml glycerol a. g. + 0.05 ml octanol were acidified with 0.28 ml 1.011 *M* HCl. An iron rod, sealed in glass, was put in the vessel for the magnetic stirring. To protect the solution from CO_2 the vessel was closed with a rubber membrane, which also served to keep in position the in- and outlet tubes for the CO_2-free nitrogen stream, the glass electrode, the agar bridge with saturated KCl, and the microburette with sodium hydroxide (2.530 *M*). The tip of the burette and the inlet tube for the nitrogen stream dipped a few mm below the surface of the liquid. The electrode and the potentiometer (Cambridge Instruments) were checked with standard phtalate (pH 3.97) and borate (pH 9.14 at 25° C) buffers before and after each titration. The temperature was kept constant by means of a water thermostat. The results of the titrations are discussed on page 384.

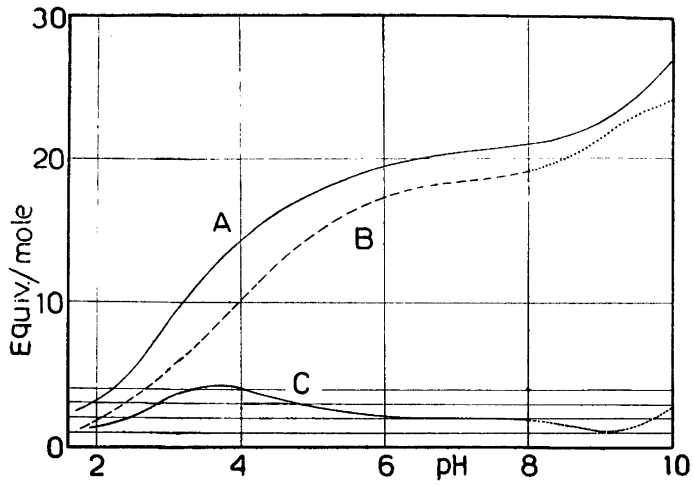

Fig. 3. Titration curves of cytochrome c and its protein moiety in glycerol-water 1 : 1. The positions of the two curves in relation to each other have been given so they should differ by two equivalents at pH 7, where all groups, common to the two substances, are titrated and in addition to them the two ferri porphyrin carboxyl groups in cytochrome c. The absolute positions along the ordinate are arbitrarily chosen. The same applies to the difference curve, and thus only differences in equivalents per mole between two pH values can be obtained. Curve A: Cytochrome c. Curve B: Protein moiety. Curve C: Difference between A and B.

The above-mentioned methods for studies of the heme-linked groups give only indirect information about the nature of these groups. For that reason it was desirable to employ some method, which could give a more direct answer. 1,2,4-fluorodinitrobenzene (FDNB) reacts with the imidazole group of histidine to give DNB-histidine [5]. The amino group will also combine with FDNB, but if this group has been protected by a peptide bond during the exposure to FDNB, histidine, subsequently liberated by acid hydrolysis, contains only two polar groups. Since the amino acid decarboxylases can not attack amino acids with only two polar groups, histidine with DNB bound to the imidazole ring will not be decarboxylated. On the assumption that their linkages to the iron atom would prevent two of the imidazole groups from reacting with FDNB [6], the following experiment was made (Table 1).

Histidine decarboxylase [7] and FDNB [8] were prepared according to given descriptions. The latter substance could not be distilled completely free from the yellow colour, although it was definitely lighter than the commercial preparation. The cytochrome c for this experiment was purified in the same way as the preparation used for the titrations, and had an iron content of 0.426 %. The amount of histidine residues, able to react with histidine decarboxylase, was determined in two hydrolysates of cytochrome with and without pretreatment with FDNB. In the first case 7.44×10^{-6} moles in 5 ml water + 6.25 ml conc. HCl were refluxed on a sandbath for 24 h, cooled, filtered, and evaporated to dryness several times with addition of water. The residue was dissolved in 0.2 *M* acetate buffer pH 4.5 and pH adjusted to 4.5 with 1 *M* NaOH. The final volume was 6.0 ml. Aliquots of this were taken for manometrical determinations of CO_2 evolved due to

Table 1. Determination of free histidine in hydrolysates by means of enzymatic decarboxylation. Temperature + 30° C. Side bulb: 40 mg (dry weight) bacterial cells in 0,50 ml. 0.2 M acetate buffer pH 4.5. Volume in main vessel made up to 3.15 ml with acetate buffer. Histidine solution 8.30 × 10^{-6} moles per ml. Cy hydr. = hydrolysate of untreated cytochrome c. DNB-cy hydr. = Hydrolysate of FDNB-treated cytochrome c.

Material	μl CO_2		
	Found	Calc.	Found %
Blank	<2	—	—
0.75 ml histidine	132	155	85
1.50 » »	274	310	88
		Average recovery 86.5	
		Moles of histidine per mole of cytochrome	
1.00 ml cy hydr.	81	2.63	
2.00 » » »	154	2.50	
1.00 » DNB-cy hydr.	29	0.47	
2.00 » » »	58	0.47	

the action of the enzyme. In the second case 1.95 × 10^{-5} moles of cytochrome c in 5 ml 10 % $NaHCO_3$ were shaken with 1 ml FDNB in 9 ml ethanol for two hours at room temperature [9]. The protein precipitate was washed four times with ethanol and twice with ether. It was then dried and hydrolyzed as above. The final volume of the solution in buffer after the correction of pH was 7.8 ml.

DISCUSSION

The sulphur containing amino acids. Cytochrome c contains six atoms of sulphur per molecule [1]. Two of them derive from methionine, and another two were found in the cystine fraction [10]. The latter two were considered as identical with the thio-ether forming cysteine residues. However, native cytochrome c gives a polarographical wave indicating the formation of a thiol group [11]. The parent group of that thiol can not be methionine [11] or the thio-ether group [12]. Thus it is very likely that cytochrome c contains one cystine in addition to the porphyrin bound cysteine residues. The nitroprusside reaction for free sulphhydryl groups is negative for cytochrome c [1]. In experiments with free amino acids we found that after treatment under the experimental conditions employed for the splitting of the cytochrome, cystine but not methionine gives a positive nitroprusside reaction. Thus the protein moiety of cytochrome c should contain two thiol groups + one disulphide group, or, in

the presence of silver ions, four mercaptide bound silver atoms per 141 nitrogen atoms. Peters and Wakelin [13] found that methionine gave a positive nitroprusside reaction after warming only at slightly alkaline reaction. This means that it might be possible to determine cystine on the basis of the silver content of the protein after digestion at faintly acid reaction and the sum of cystine and methionine after digestion at faintly alkaline reaction. The amount of sulphur found in cystine and methionine should for most proteins be equal to the total sulphur content of the protein. The hydrolysis of the protein with strong acid with the losses of cystine could thus be avoided.

Titration curves. In agreement with the described theory the differences in the titration curves of cytochrome c and its protein moiety can be interpreted in the following way: In intact cytochrome c the group of pK 0.42 is outside the titrable range because the lowest pH at which cytochrome c is stable is 1.7 [14]. From pH 1.7 to pH 4 the intact cytochrome c consumes three equivalents more than the free protein does. These three groups correspond to the two carboxyl groups of the ferri porphyrin + the imidazole group of pK 2.50. In the free protein the ferri porphyrin carboxyl groups are absent, and the imidazole groups are titrated closer to their normal pK value. Hence the free protein should consume two equivalents of base per mole more than the intact cytochrome does between pH 4 and neutrality. The dissociation constant in the free protein at about pH 8 may be attributed to an amino group in the neighbourhood of the porphyrin carboxyl groups as was done in the case of the ferrihemoglobin — globin titrations [15].

A few comments have to be made to this interpretation.

1. Cytochrome c itself is stable within the pH region covered in the titrations [14]. The best evidence for the stability of the protein moiety would of course be to recombine it with the prosthetic group and demonstrate the biological activity of the product. It is, however, very unlikely that a recombination to a substance, possessing the thio-ether bonds, could take place under the conditions usually employed for the recombination of ferri porphyrins with enzyme protein components (aqueous solution of pH about 7). A mixture of the protein moiety and the ferri porphyrin under such conditions gives upon reduction a spectrum, very similar to that of ferrocytochrome c; however, since acid acetone splits off the prosthetic group, no recombination to the structure of cytochrome c can have occurred. For that reason it has not been possible to test the stability of the free protein. The whole discussion has therefore to be based on the assumption that the protein part can stand the treatment without changes in its acid-base combining capacity in addition to those which are caused by the removal of the prosthetic group. The amide nitrogen was unchanged by the silver sulphate — acid acetone treatment.

2. There is evidence in support of the assumption that the porphyrin carboxyl groups in cytochrome c are free to be titrated. Hematoporphyrin can not be prepared from ferri protoporphyrin chloride by means of hydrogen bromide in glacial acetic acid if the porphyrin carboxyl groups are linked to amino acids by peptide bonds [16], but cytochrome c gives readily hematoporphyrin by that treatment [17]. Moreover it seems unlikely that the procedure for the splitting of cytochrome c, employed in this paper, could break a peptide bond. The interpretation of the titration curves presupposed that the ferri porphyrin carboxyl groups were titrated at about pH 3, far below their normal pK value. p$K_{25°}$ for propionic acid in water is 4.87. The di-sodium salt of ferri protoporphyrin hydroxide ("hematin") consumed upon its titration with hydrochloric acid simultaneously two equivalents at about pH 6.5 [18]. No formation of the mono-salt could be seen. In porphyrin c [19] two of the four carboxyl groups were titrated with pK 5.7. In both examples, however, the carboxyl groups were uninfluenced by positive groups in the vicinity. A positively charged—NH_3^+ group in the neighbourhood would increase the tendency of the carboxyl group to split off a proton. In fact, the low pK value for the carboxyl groups indicates that they are influenced, *e. g.* by salt linkages, by positive groups of the protein.

3. If two protein amino groups are sterically close to the ferri porphyrin carboxyl groups, the ionization of pK about 8 should correspond to two equivalents instead of only one. However, one of these groups may of course be strongly basic and not appearing within the examined pH range. It may also be that the flocculation of the protein, beginning just there, could mask one amino group.

4. It should be mentioned that some preparations of cytochrome c consumed more than two equivalents between pH 5.5 and 8.5. The preparation, the titration curve of which is reproduced in Fig. 3, thus consumed 2.9 equivalents. It may be that this specimen was not pure. Since this investigation was finished, cytochrome c with an iron content of 0.47 % has been obtained [20, 21]. The nature of the fraction, removed by these investigators, as well as its relation to the cytochrome molecule, is as yet unknown.

As a whole the results from the titration experiments can be interpreted in agreement with the theory of histidine residues as heme-linked groups, but they do not exclude the possibility that other groups are heme-linked. This possibility will therefore be briefly discussed.

Tsou [22] digested cytochrome c with pepsin at pH 1.5, and isolated a peptide of the average molecular weight 2 500, calculated on the basis of its iron content. This peptide, which was autoxidizable, contained the prosthetic group plus some amino acids. It lacked the absorption band at 280 mμ, deriving

from tyrosine and tryptophane, but it contained histidine according to its paper chromatogram. Thus tyrosine can be excluded as mediator of the protein-iron linkage. Moreover, an ionization due to the hydroxyl group of tyrosine should appear at much higher alkalinity than between pH 4 and neutrality.

The average value of 2.57 histidine residues per mole of cytochrome c was found by the determination with histidine decarboxylase. If this value is corrected for the average recovery of 86.5 %, observed with pure histidine, it will increase to 2.97, in good agreement with the value from amino acid analyses. After the same correction the value for the DNB-cytochrome becomes 0.54. This is considerably lower than the expected value of 2, which was calculated from the assumption that both heme-linked groups were histidine residues. However, cytochrome c is largely denatured by alcohol. Since it was possible that the denaturation could imply a re-arrangement around the iron atom, the following experiment was made. A 2×10^{-5} M solution of cytochrome c in 10 % $NaHCO_3$ was divided into two equal parts. One part was treated with alcohol but without FDNB and dried as described above. It was then re-dissolved to its original volume with water. Both solutions were then reduced with the same quantity of dithionite and aerated. The alcohol treated cytochrome c was rapidly reoxidized, while the other remained reduced. Thus the alcohol treatment itself makes cytochrome c autoxidizable. This means that oxygen somehow can reach the iron, and it might thus also be possible for FDNB to react with at least one of the heme-linked groups. The experiment with FDNB gives therefore only qualitative indication that one of these groups is an imidazole. In the case of hemoglobin [6] the hemin did not protect the histidine residues from reacting with FDNB, and free hemin could be detected in the solution after the exposure to FDNB-alcohol. The better results with cytochrome c can therefore be attributed to the firmer heme-protein bonds.

Thus from experiments, presented earlier [1, 22] and in this paper it can be considered as established that at least one histidine residue is linked to the iron. From Fig. 3 it is obvious that also the other group must have its pK value between pH 4 and neutrality. For this reason a guanidino group can be excluded as heme-linked. Possible groups — in addition to the imidazole group — could be the second carboxyl group of aspartic or glutamic acid. In the free amino acids these groups have the pK values 3.65 and 4.25 respectively in water. In peptides and in solvents of lower dielectricity the values are slightly higher [23]. It is thus possible that one of these acids could constitute the other heme-linked group. It is likely that two different pK values should be found for the groups dissociating between pH 4 and 6.5 if they were one

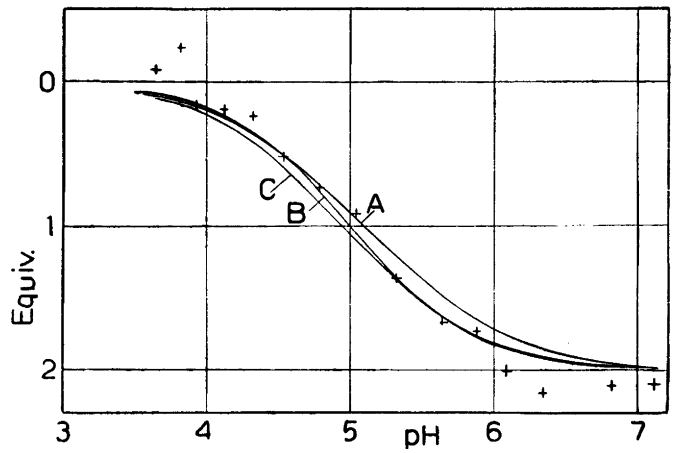

Fig. 4. The difference curve between pH 4 and 7 (excerpt from Fig. 3). + experimentally determined differences. The curves have been drawn on the assumptions that two equivalents are titrated between pH 4 and 7 with the pK values for curve A 4.70 and 5.50, curve B 4.90 and 4.90 and curve C 4.70 and 5.20.

imidazole and one carboxyl group. In Fig. 4 the difference in base-binding capacity between cytochrome c and its protein moiety within this region are plotted against pH. The curves are calculated for the dissociations of two groups of certain pK values. As can be seen the accuracy of the experiments does not permit a definite conclusion about which curve would fit best, though it seems to be the one with two equal pK values.

SUMMARY

1. Cytochrome c and its protein moiety have been titrated in glycerol-water between pH 1.7 and 8. Between pH 1.7 and 4 cytochrome c consumed three equivalents per mole more than its protein moiety. From pH 4 to pH 6 the latter took up two equivalents more, and between pH 8 and 9 another equivalent. The results are compatible with the theory that histidine residues constitute the heme-linked groups, but the possibility that one carboxyl and one imidazole group are linked to the iron can not be excluded from these experiments. When cytochrome c was treated with flurodinitrobenzene, hydrolyzed, and the amount of free histidine determined by means of histidine decarboxylase 0.54 moles of histidine, free from dinitrobenzene, were found per mole of cytochrome c. This result indicates that at least one histidine residue is linked to the iron atom.

2. A discussion of data, available in the literature, reveals that cytochrome probably contains two methionine, two cystine, and two cysteine (thio-ether bound) sulphur atoms. From results, obtained by treatment of the free, sulphur containing amino acids in the same way as was done with cytochrome c, it was to be expected that the protein moiety should contain four silver atoms per mole. The value actually found was 3.5—3.6. It is suggested that this observation could give a new method for the determination of the sulphur containing amino acids.

During a visit to England, made possible by a grant from *Statens Medicinska Forskningsråd*, the author had the opportunity to study the determination of histidine by the decarboxylase method. He wants to express his thanks to dr E. F. Gale and dr J. Tosic at the Unit of Microbiology, Cambridge, for advice and discussions. The author's thanks are also given to prof. H. Theorell for very valuable discussions, and to miss Inger Agerberg for technical assistance.

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The Porphyrin Component of Cytochrome c and its Linkage to the Protein

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Hill and Keilin [1] found that digestion of yeast cytochrome c with a hydrogen bromide-glacial acetic acid mixture gave an ether soluble porphyrin. It was identical with hematoporphyrin, to judge from its spectrum and its loss of water upon heating *in vacuo*. The authors also found that sulphur dioxide in concentrated hydrochloric acid released a porphyrin from cytochrome c which had a spectrum intermediate to the meso- and protoporphyrin types. This porphyrin, "porphyrin c", was soluble in water at all pH values and could not be taken into ether. Zeile [2] and Zeile and Piutti [3] prepared derivatives of protoporphyrin by introducing nitrogen bases in its side chains 2 and 4. Some of these products or their iron complexes behaved in certain respects as cytochrome c or porphyrin c, *e. g.* as regards solubility, spectrum, and reactivity with hydrogen bromide-glacial acetic acid. The dimethyl ester of mesoporphyrin, obtained from cytochrome c hematoporphyrin by means of reduction with hydrogen iodide, gave no melting point depression with mesoporphyrin ester from blood ferri protoporphyrin [4]. Theorell [5, 6], employing an improved technique, prepared porphyrin c with a molecular weight of 890 $\pm$ 10. The ratios total-N : amino-N : S : COOH : Fe (subsequently introduced) were found as 6 : 2 : 2 : 4 : 1. Since there was no nitrogen in excess of what could be accounted for by the amino and pyrrol nitrogen atoms, the bonds between porphyrin and protein could not be mediated by nitrogen.

Porphyrin c gave a sulphide upon melting with solid potassium hydroxide. The nitroprusside reaction for free sulphhydryl groups was negative as well as the polarographical test for a disulphide. It was therefore considered as probable that the sulphur atoms were present in thio-ether bonds. Porphyrin c did not take up any hydrogen in the presence of palladium. The ninhydrine test was positive. It was therefore concluded that porphyrin c was the di-

cysteine adduct of protoporphyrin. The theory was confirmed by the isolation of l-cystine from porphyrin c [7]. The yield of cystine was 40 % of the theoretical for two cysteine adducts. The suspicion that porphyrin c could be an artifact, formed during the acid hydrolysis of cytochrome c [8], could be rejected when it was proved that a twenty-fold lowering of the cytochrome concentration during the hydrolysis did not influence the result [9].

Zeile and Meyer [9] compared two preparations of porphyrin c, obtained in different ways. The "natural" porphyrin c was prepared by hydrolysis of cytochrome c with sulphuric acid, while the "synthetic" porphyrin c was obtained by melting together l-cysteine hydrochloride and di-bromoprotoporphyrin. Both preparations, isolated via their methyl esters, were free from ash and of the elementary composition required by protoporphyrin-di-cysteine. They agreed completely as regards spectra and partition of their esters between ether and acid buffers. Neither preparation could be crystallized. Their optical activities were, however, quite different, $[\alpha]^{17}_{\text{white light}}$ being — 172° for the natural and + 27° for the synthetic porphyrin c in 0.1 % hydrochloric acid. Also the yields of hematoporphyrin from their iron complexes by hydrogen bromide — glacial acetic acid treatment differed, 30 % of the theoretical for the natural and up to 92 % for the synthetic porphyrin c. The hematoporphyrin from the iron complex of natural porphyrin c was optically inactive.

The above mentioned investigators employed methods, which included treatment with a strong acid for the liberation of the porphyrin from the protein. This is to be noticed, since mineral acids are known to catalyze the addition of thiols to alcoholic structures in side chains [10]. There is also an equilibrium between proto- and hematoporphyrin in hot hydrochloric acid [11]. The splitting of cytochrome c with a silver salt [12] involves milder conditions. It seemed therefore to be of interest to re-examine the structure of the cytochrome c porphyrin. The splitting procedure also opened a possibility to determine the positions of the thio-ether bonds in the side chains 2 and 4 of the cytochrome c porphyrin.

EXPERIMENTS

Material

Cytochrome c with an iron content of 0.41 % was prepared from cow hearts [13]. This material was used for most experiments. A part of the sample was further purified by electrophoresis at pH 10.45 and 7.7 [14]. It was dialysed against 0.1 % ammonia until negative sulphate reaction. Finally it was lyophilized, redissolved in a small volume of redistilled water and electrodialyzed. The filtered solution was found to contain 89.2 μg iron and 3.24 mg nitrogen (micro-Kjeldahl) per ml, which corresponds to 145 nitrogen atoms per one iron atom. The dry weight was 21.04 mg per ml, giving an iron content of 0.424 %. The ash content was 0.027 and 0.071 mg after drying and ignition in the gas flame of 0.10 and 0.20 ml respectively.

The light absorption at the wave-length for the top of the α-band of reduced cytochrome c is frequently used for the determination of the concentration of cytochrome c in solutions and to follow spectrophotometrically reactions in which cytochrome c is involved. The value reported in the literature for the molar absorption of the α-band of reduced cytochrome c vary considerably, however [15]. For this reason we considered it to be of interest to re-determine the molar absorption with the Beckman spectrophotometer (type DU). Suitably diluted samples (0.05—0.15 ml to 10.0 ml with $M/15$ phosphate buffer pH 6.8) were reduced in the cuvettes and their absorptions measured with the slit 0.01 mm. On the basis of the iron content the molar absorptions (β) * were found as 6.36×10^7 and 1.76×10^7 $cm^2 \times mole^{-1}$ for the maximum of the α-band and the minimum between the α-and the β-bands respectively. The value 6.36×10^7 has been found by the author for several cow heart cytochrome c preparations. It is advisable to make determinations at every mμ within 548—552 mμ to find the maximum, since the position of the top of the α-band varies a little from one preparation or instrument to another.

The partition of nitrogen between the split products

3.00 ml of the purest preparation, containing 6.94×10^{-4} gramatoms of nitrogen, were digested at 60° with 3.0 ml silver sulphate solution (800 mg salt per 100 ml salt solution) and 0.6 ml glacial acetic acid. After 80 min the mixture was cooled, the protein precipitated with 42 ml acid acetone (1 ml 5 N sulphuric acid to 100 ml acetone) and centrifuged down. The precipitate was redissolved in 3 ml distilled water + 0.4 ml glacial acetic acid and reprecipitated with 30 ml acid acetone. This procedure was repeated once more. The combined acetone solutions were evaporated to about 5 ml and rinsed over into a 10-ml volumetric flask with 3 ml acetic acid and water. The total nitrogen content of the fraction, determined on aliquots of 3 and 6 ml, was found to be 0.367 mg. If complete splitting of the cytochrome c is assumed, this means that the ratio of nitrogen to iron was 5.4 in the ferri porphyrin fraction. No amide-nitrogen was found in this fraction.

The total nitrogen content of the protein fraction was found to be 6.67×10^{-4} gramatoms. The recovery of nitrogen (protein + ferri porphyrin) was thus 6.93×10^{-4} gramatoms.

Elementary composition of the ferri porphyrin from cytochrome c

Cytochrome c with 0.41 % iron was used for these experiments. Of this preparation 1.44 g, corresponding to 75 mg ferri porphyrin (calculated as ferri hematoporphyrin chloride) was treated as above with silver sulphate and

* $\beta = \frac{1}{e} \times \frac{1}{d} \times \ln \frac{I_0}{I}$, where c = concentration in moles per ml solution, d = optical depth of the solution in cm, I_0 and I = intensities of incident and transmitted light respectively.

acetone. Only one acetone extraction was made. The acetone was removed *in vacuo* and the ferri porphyrin flocculated from the acetic acid — water mixture by the addition of sodium acetate. The suspension was left in the cold room overnight to complete the precipitation, then centrifuged down and washed with water, 0.1 M HCl and once more with water. After drying at 60° for three hours and overnight in a desiccator the material weighed 68 mg.

Before any further purification was attempted, the preparation was examined for amino acid residues [16]. 9.1 mg were hydrolyzed for 24 h with 2.2 ml 20 % hydrochloric acid. After the removal of the acid by repeated evaporation to dryness and the addition of a few drops of 4 M sulphuric acid, the filtered hydrolysate was run in a two-dimensional paper chromatogram with the systems phenol-water 4 : 1 and lutidine-collidine-water 1 : 1 : 1 (volume ratios). No amino acids or cysteic acid could be detected with ninhydrine *.

The rest of the material was dissolved in 10 ml methanol, added in small portions with immediate filtration and evaporation to dryness after each addition. The dry material was ground with ether for 30 min and collected on a glass filter. After careful washing with water, the preparation was dried by suction and in a desiccator. The weight was 53.7 mg. To convert any ferri porphyrin hydroxide, which might be present, to the chloride, the preparation was eluted from the filter with 0.01 ml conc. hydrochloric acid in 5 ml acetone. The weight after drying in a current of air and *in vacuo* was 50.5 mg.

The spectrum in methanol is given in Fig. 1.

Analyses:	C	H	N	Fe	Ash
Found (%)	59.8	5.0	7.6	8.1	12.7
Calc. for hematohemin ($C_{34}H_{36}O_6N_4FeCl$)	59.4	5.3	8.1	8.1	11.6

Nitrogen was determined according to Dumas, which method often will give too low values for pyrrol nitrogen. Total conversion of the iron to Fe_2O_3 was assumed for the calculation of the ash weight.

Conversion of the ferri porphyrin to porphyrin

In the same way as described above 46 mg ferri porphyrin were obtained from 1.00 g cytochrome c, the grinding with ether being omitted. We found it, however, to be preferable to substitute the acetate for sodium bicarbonate for

* Dr. S. Paléus gave valuable assistance with the paper chromatogram, which is gratefully acknowledged.

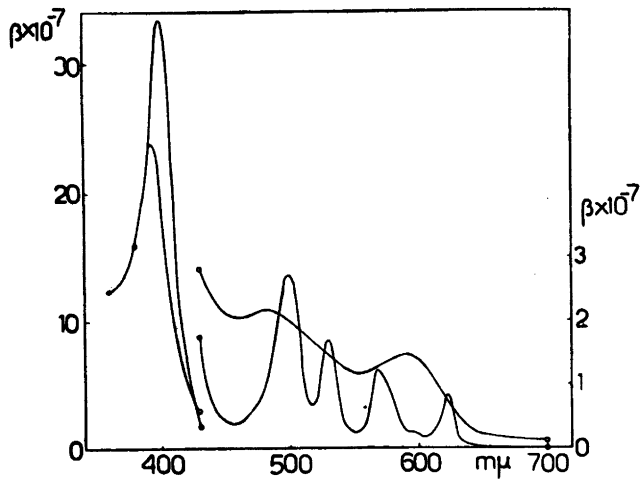
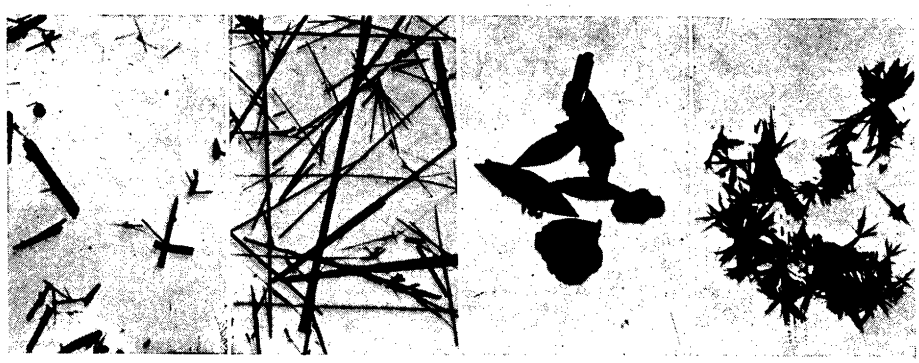


Fig. 1. Hematohemin c in methanol (O—O).

$\beta_{393} = 23.9 \times 10^7\ cm^2 \times mole^{-1}$.
$\beta_{483} = 2.19$ » » »
$\beta_{590} = 1.46$ » » »

Tetramethyl hematoporphyrin c in dioxane (●—●).
$\beta_{400} = 34.9 \times 10^7\ cm^2 \times mole^{-1}$.
$\beta_{499} = 2.88$ » » »
$\beta_{530} = 1.79$ » » »
$\beta_{568} = 1.24$ » » »
$\beta_{623} = 0.83$ » » »
Left ordinate scale for 360—430 mμ, right scale for 430—700 mμ.

the flocculation. Maximal flocculation occurred when 1/5 of the acetic acid had been neutralized (pH 4). The dried ferri porphyrin was divided into four equal parts, which were separately dissolved in 10 ml methanol each. 0.5 g ferrous sulphate was added, and dry hydrogen chloride was vigorously bubbled through the solution for three minutes [17]. After that time no absorption band of the "hemin" type could be detected. The solution was cherryred and showed the acid type spectrum of a porphyrin. The combined solutions were taken into a separatory funnel with chloroform-water-acetate. The chloroform solution was washed three times with 7 % sodium chloride solution, filtered through a chloroform-moistened, double paper and evaporated to dryness. The commonly used washing of the chloroform solution with dilute ammonia was omitted, since it was found to develope a brown discoloration of the porphyrin.



A B C D

Fig. 2. *A = tetramethyl hematoporphyrin c (fraction I), m. p. 144°.*
B = » » » ») m. p. 145°.
C = trimethyl » » (fraction IIB).
D = tetramethyl hematoporphyrin, m. p. 148°.
All specimens were crystallized from chloroform-methanol. Magnification 72 ×.

The material was further purified by chromatography on alumina * grade IV [18]. It was adsorbed on the top of the column from its chloroform ** solution and the chromatogram was developed with chloroform-methanol 200 : 1. Two porphyrin coloured zones appeared, the lower of which (fraction I) descended twice as fast as the upper (fraction II). No other fractions were seen. Obviously the preparation was rather pure, since the brown region at the top of the column, which is generally seen, did not appear.

Fraction I. The total weight was 35 mg. The material crystallized very easily from chloroform-methanol with m.p. 144° (Fig. 2 A), after rechromatography on alumina grade I + II (taken directly from the package) with chloroform as eluant 145° (Fig. 2 B).

Fraction II. No crystals were obtained from the commonly employed solvents, so the material was rechromatographed on alumina grade IV with chloroform-methanol 500 : 1 as eluant. This revealed three fractions (IIA, IIB, and IIC). IIA, brownish and very small, was discarded. IIB and IIC were porphyrin coloured. Both fractions crystallized from chloroform-methanol. IIB appeared at first as very thin equilaterals of m. p. 110°, which

* Savory and Moore Ltd (Aluminium oxide for chromatographic analysis standardized according to Brockmann).

** Chloroform to be used for the chromatograms was washed once with potassium carbonate solution, twice with water, dried and distilled over calcium chloride, and stored in a dark bottle. It was used within two days. Methanol a. g. was employed without pretreatment.

gradually changed in shape (Fig. 2 C). The rhomboedric crystals from fraction IIC, of the same appearence as fraction I, m. p. 144° (Fig. 2 A), melted indefinitely at 202—206°, after drying at 56° for one hour *in vacuo* at 203—206°. Attempts were made to reach a more definite melting point by chromatography on various adsorbents and with various eluants but without success. The spectra of fractions IIB and IIC agreed with the hematoporphyrin dimethyl ester spectrum.

Ferri hematoporphyrin chloride was prepared as described before [19]. For the conversion of it to the corresponding methylated porphyrin it was found, however, that three minutes did not suffice for complete reaction under conditions identical with those employed for the cytochrome ferri porphyrin as regards quantities of reactants *etc.* Not until after fifteen minutes had the sample aquired the pure porphyrin colour. The yield of tetramethyl hematoporphyrin was also low (49 mg from 200 mg ferri hematoporphyrin chloride, = 28 % as compared to 87 % for the cytochrome porphyrin). The difference in reactivity between the two ferri porphyrins was confirmed in several experiments with different preparations. After washing and chromatography as described above, the methylated hematoporphyrin crystallized from chloroform-methanol with m. p. 148° (Fig. 2 D). No change in the shape of the crystals or their melting point was achieved by repeated chromatography.

A ground mixture (1 : 1) of the two substances (m. p. 145° and 148° respectively) melted at 132°.

The visible spectra of the two substances agreed (Fig. 1 and Table 1). Their infrared spectra * are given in Fig. 3.

Analyses:	C	H	CH_3O
Cytochrome porphyrin;			
Fraction I	69.9	7.0	18.6
» IIB	—	—	13.9
» IIC	—	—	13.7
Hematoporphyrin;			
Fraction I	69.8	6.9	18.4
Calculated for			
Hematoporphyrin dimethyl ether dimethyl ester ($C_{38}H_{46}O_6N_4$)	69.7	7.1	19.0
Hematoporphyrin dimethyl ester ($C_{36}H_{42}O_6N_4$)	69.0	6.8	9.9
Hematoporphyrin monomethyl ether dimethyl ester ($C_{37}H_{44}O_6N_4$)	69.3	6.9	14.5

* This result is a part of a more general investigation of the infrared spectra of porphyrins and porphyrin derivatives by H. Theorell and K.-G. Paul (to be published shortly).

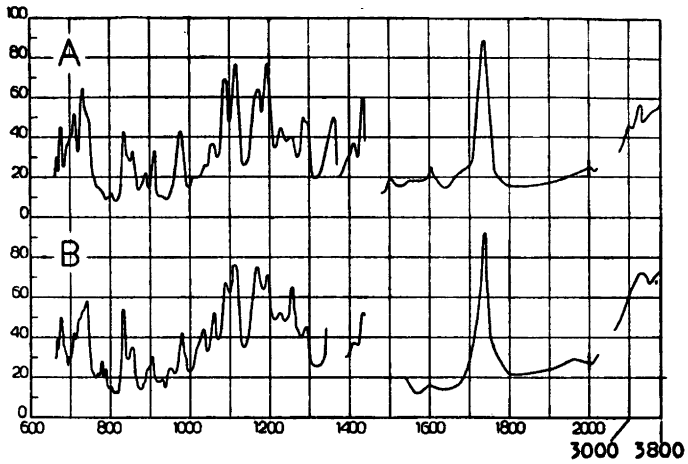


Fig. 3. Infrared spectra of tetramethyl hematoporphyrin c (A) and tetramethyl hematoporphyrin (B). Specimen/paraffine oil = 1/2.5. Thickness of mull in the cuvette 0.025 mm. Abscissa; cm^{-1}. Ordinata; Absorption in per cent.

Optical activity of cytochrome porphyrin and hematoporphyrin

Because of the low intensity of the transmitted light from the relatively concentrated porphyrin solutions which had to be used, a 2 kW carbon-carbon arc was employed as light source. The light was filtered through a 5 cm cuvette with pure water, through Schott Gen. filter nr BG 21 (gray) and in some experiments also through Schott Gen. filter RG 1 (red). A Hilger polarimeter with a 5.00 cm microtube, taking 0.3 ml, was used for the readings.

Determinations on the cytochrome porphyrin (fraction I) were made on two solutions of the same preparation. In the first experiment 2.775 mg substance were dissolved in 1.00 ml purified dioxane [20]. The rotation of this solution was $-0.137° \pm 0.009°$ (S.D.) ($-0.03°$ to $-0.21°$, $n = 30$) with the blank value for pure dioxane in the tube $-0.002° \pm 0.007°$ ($0.08°$ to $-0.07°$, $n = 30$). The rotation due to the substance was thus $-0.135° \pm 0.011°$, which gives the specific rotation $-97.3° \pm 7.9°$. In the second experiment 14.812 mg were dissolved in 1.00 ml glacial acetic acid. Because of the very high light absorption the red Schott filter was not used. The rotation was found as $-0.323° \pm 0.030°$ ($-0.19°$ to $-0.46°$, $n = 9$). When this solution was diluted with one volume of glacial acetic acid (both Schott filtra) the rotation was $-0.184° \pm 0.012°$ ($-0.07°$ to $-0.29°$, $n = 32$). The blank value with glacial acetic acid in the tube was $-0.076° \pm 0.015°$ ($0.02°$ to $-0.14°$, $n = 16$). The actually determined angles were thus after corrections for the

Table 1. Spectra of the tetramethyl compounds of hematoporphyrin and hematoporphyrin c (Beck-Hartridge reversion spectroscope).

Solvent	Tetramethyl hematoporphyrin	Tetramethyl hematoporphyrin c
5 % HCl	593.8	593.1
	573.9	572.3
	550.3	549.7
Aqueous pyridine	623.9	623.7
	568.3	568.3
	535.5	533.4
	500.4	499.7
Chloroform	622.5	622.5
	567.5	567.7
	535.5	533.5
	501.2	499.4

blank $-0.247° \pm 0.034°$ and $-0.108° \pm 0.019°$, giving the specific rotations $-33.3° \pm 4.6°$ and $-29.2° \pm 5.1°$ respectively.

For the determination of the optical activity of the tetramethyl compound of hematoporphyrin 7 mg were dissolved in 1 ml glacial acetic acid. The determined angle was $0.019° \pm 0.020°$ ($-0.10°$ to $0.15°$, $n = 16$) for the solution and $0.026° \pm 0.020°$ ($-0.12°$ to $0.13°$, $n = 16$) for the blank, giving the rotation due to the substance $-0.007° \pm 0.028°$. Thus no significant optical activity could be demonstrated for the synthetic porphyrin. This is in agreement with earlier observations [9].

The positions of the hydroxyl groups of the cytochrome c porphyrin

The loss of water from cytochrome c ferri porphyrin was registered in the following way. 1 ml of a solution of cytochrome ferri porphyrin in methanol, containing about 0.04 mg per ml, was pipetted into each of five Thunberg tubes. Another series was prepared in the same way with hematohemin. The tubes were then dried in a current of air, leaving the substance as a film on the glass walls, evacuated, and immersed in an oil bath of + 140° C. After certain times a pair of tubes were removed, one from each series. They were cooled, and 5.0 ml of a mixture of pyridine and 0.1 *M* NaOH (1 : 3) + some

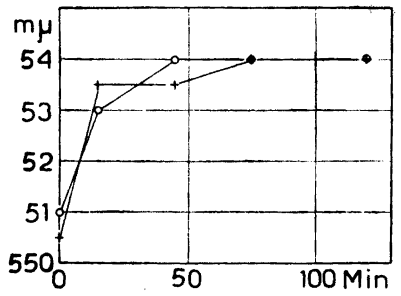


Fig. 4. Shift in wavelength for the top of a mixture of pyridine hemochromes upon heating of the corresponding ferri porphyrins (cf. text).
+ hematohemin c, O hematohemin.

dithionite were added. The spectrum from 545 to 560 mμ of the mixture of the hemochromes was determined. The shift in wave-length of the top of the absorption band, due to the formation of vinyl groups, was plotted against time of heating (Fig. 4).

Tetramethyl hematoporphyrin is converted to protoporphyrin dimethyl ester when heated *in vacuo* at 135—150° C for 5—10 min.[21]. The same was found for the tetramethyl porphyrin from cytochrome c: 9.79 mg of fraction I, previously dried at 56° *in vacuo* over P_2O_5 for one hour, were heated at 140° for 10 min. The loss of weight was 0.94 mg, corresponding to the removal of 1.96 moles of methanol per mole of tetramethyl porphyrin. The residue was chromatographed on alumina grade IV with methanol-chloroform 200 : 1 as eluant. The first fraction that left the column weighed 7.0 mg. It was red, crystallized from chloroform-methanol with m. p. 226°. A freshly prepared sample of protoporphyrin dimethyl ester (m. p. 228°)[17] gave no melting point depression (229°). The spectra of the two substances in chloroform agreed completely. Another three fractions, green, red, and brown respectively, all very small and of about equal strength, appeared on the column but were not isolated. Thus the tetramethyl porphyrin from cytochrome c gives protoporphyrin dimethyl ester upon heating, and behaves in that respect as tetramethyl hematoporphyrin.

The two experiments described above indicate that the two porphyrins in question have their hydroxyl groups bound in the same way. The behaviour of the cytochrome porphyrin upon tritylation should give further evidence for the positions[22]. To 34.0 mg cytochrome ferri porphyrin in 2.2 ml anhydrous pyridine were added 30.3 mg triphenylmethyl chloride (2.2 moles per mole ferri porphyrin) in 1.4 ml anhydrous pyridine. The trityl chloride had previously been recrystallized from acetyl chloride and dried under protection against moisture. The mixture was left in an ice-bath for two hours and then at room temperature overnight. After the addition of 2 ml of water and stand-

ing for another two hours at room temperature the solution was evaporated to dryness on a waterbath (bath temperature not exceeding + 50°, vapour temperature + 27°). The residue was dissolved in acid acetone, the solution filtered, dried, and the residue re-dissolved in methanol. After washing several times with light petroleum and standing overnight under a layer of light petroleum the methanol solution yielded a sediment of darkbrown material with an iron content of 6.47 %. This iron content corresponds to the addition of 0.7 triphenylmethyl groups per ferri porphyrin. The precipitate was insoluble in ammonia. Since the carboxyl groups of ferri protoporphyrin chloride have to be neutralized to 90 % before dissolution in an aqueous medium is possible [23], it is likely that the tritylation has attacked the carboxyl groups. It was easily soluble in pure methanol.

The dimethyl ester of the porphyrin would be the best material for the tritylation studies. However, it has as yet not been possible to develope a suitable procedure for the removal of iron from the cytochrome ferri porphyrin. The yield is very poor (< 10 %) by various methods, and as a rule several fractions of about equal magnitude appear. A few mg, obtained from 40 mg ferri porphyrin by means of the ferrous acetate procedure and subsequent esterification with diazomethane and chromatography, melted at 214°, Hematoporphyrin dimethyl ester (m. p. 213°) gave a melting point depression (198—200°).

DISCUSSION

The cytochrome c ferri porphyrin is easily soluble in alcohols, and under certain conditions it can also be obtained in an aqueous medium (excessive washing of its solution in ether-acetic acid with dilute hydrochloric acid). It might therefore be questioned whether some extra, hydrophilic group(s) might be present. The value 5.4 nitrogen atoms per one iron atom could be an indication thereof. However, this is probably not the case. The ratio of nitrogen to iron was determined in a single experiment, and with the rather large volumes of solvents, which had to be used, it is quite possible that some foreign nitrogen could have entered the solution. It seems also to be difficult to attribute this extra nitrogen to some position in the molecule. Also the "synthetic" hematohemin is more hydrophilic than the proto-, meso-, and deuterohemins. An indication thereof can be seen in the R_f-values of these four substances in paper chromatography with the system methyl ethyl ketone / aqueous buffer of pH 3, which were found as 0.85, 0.95, 0.9, and 0.9 respectively [24]. Accordingly it is not necessary to suppose any hydrophilic groups in addition to the hydroxyl and carboxyl groups.

The solubility in alcohols depends no doubt upon the hydroxyl groups, since the other three substances, mentioned above, are sparingly or not at all soluble in alcohols. This property of hematohemin c * offers a convenient method for the removal of impurities, *e. g.* in isotope experiments [12,25].

Hematohemin c is obviously an isomer of hematohemin. The non-identity of the two substances is evident from 1) their different reactivities during the methanol-hydrogen chloride treatment 2) the melting point depression 3) the optical activity of hematohemin c and 4) their infrared spectra.

The addition of hydrogen bromide to the vinyl groups of ferri protoporphyrin chloride should proceed in such a way that bromine becomes attached to the secondary carbon atoms of the side chains 2 and 4 [26]. This direction of the reaction is favoured by the glacial acetic acid being a strongly polar solvent, and also by the traces of iron which are liberated from the ferri porphyrin. No peroxide is present, and the solubility of oxygen in the hydrogen bromide solution (sp. g. 1.4) is probably low. In his later papers on hematoporphyrin Hans Fischer also gave the structure of hematoporphyrin as possessing two secondary alcoholic groups. The vinyl groups are equally available to the hydrogen bromide from all sides. Random configuration is probable, and the racemic form will thus appear.

When cytochrome c is treated with hydrogen bromide in glacial acetic acid, hematoporphyrin is obtained [1]. This hematoporphyrin is optically inactive [9], in contrast to hematoporphyrin c. If the reasonable assumption is made that the two secondary carbon atoms in question are optically active already in the intact cytochrome c molecule, the silver splitting procedure must imply conditions, which retain this activity and prevent racemization. This protection could be effected by the silver ions. Sulphides are known to form addition compounds with salts of heavy metals, "probably as a result of the donor activity of the sulphur" [27]. In that way the covalent carbon — sulphur bond could become more polar, and thereby exert a directing influence on the hydroxyl groups.

The conversion of an α-hydroxy ethyl group to a vinyl group should proceed much easier than the corresponding reaction with a β-hydroxy ethyl group. Hematohemin has two and hematohemin c at least one α-hydroxy ethyl group, as is evident from its optical activity. Since the dehydration of the two substances proceeds at the same rate (Fig. 4), it is likely that also hematohemin c has two α-hydroxy ethyl groups. The difference between the pyridine

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* To avoid confusion with the iron complex of porphyrin c (= the di-cysteine adduct of protoporphyrin) the name *hematohemin* c is suggested for the ferri porphyrin, which is liberated from cytochrome c by the procedure with silver salt and acid acetone.

proto- and hematohemochromes is 7 mμ, which is exactly the double of the terminal value in our experiment. No experiment has been made to explain the discrepancy. The essential thing is, however, that hematohemin and hematohemin c reacted in the same way. The same conclusion applies of course also to the removal of methanol from tetramethyl hematoporphyrin c by heating.

Helferich [22] has recently reviewed the subject of tritylation. According to this review secondary as well as primary hydroxyl groups can react with trityl chloride in pyridine. However, there is a considerable difference in tritylation velocity between the two kinds of hydroxyl groups, primary alcohols being tritylated much easier. When the reaction is carried out in pyridine with only a slight excess of trityl chloride and at room temperature, the difference is in fact of such an order of magnitude that Helferich concluded: "If an hydroxyl group does not react with trityl chloride in pyridine, it is not a primary hydroxyl group. No instance of an unreactive primary hydroxyl group has yet been reported." With due reservation given to the fact that the experiences on tritylation reactions have been aquired mainly in the field of carbohydrate chemistry, the negative result of the tritylation attempt in this case is interpreted as strongly supporting the idea that the two hydroxyl groups are not bound to the primary carbon atoms of the side chains 2 and 4.

The spectra in visible light of the isomers of coproporphyrin do not differ from each other, and the same applies to the uroporphyrins. The meso- and coproporphyrin spectra are also very similar [28]. It is therefore most unlikely that in the present case an α-β-isomery (or of course the difference between the optically active form and its racemic form) would reflect in the spectra in visible light.

Contrary to what is found for the spectrum in visible light the infrared spectrum can be expected to be influenced by the structural difference between the tetramethyl compounds of hematoporphyrin c and hematoporphyrin [29]. The sharp peak at about 1735 cm^{-1}, found also in the spectra of the free proto-, meso-, deutero-, and hematoporphyrins, is caused by the carbonyl group of the carboxyl group [29]. Accordingly it is not to be expected that the two spectra should differ at this frequency. The structure —C—O—C—, present in ethers and esterified carboxyl groups, gives a band [29] at 1110—1250 cm^{-1}. The bands at 1115, 1170, and 1195—1200 cm^{-1} are found also in the spectra of the dimethyl esters of proto-, meso-, deutero-, and hematoporphyrins, whereas the band at 1090 cm^{-1} is absent in them. It *might* therefore be due to the ether structure at the side chains 2 and 4. Differences between the two curves in Fig. 3 are found in the regions 1 000—1 100 and 1 200—1 300 cm^{-1}. These two spectra will be discussed more detailed together with the spectra of some other porphyrin derivatives elsewhere [30].

Fischer and Müller presented in 1924 a detailed study of tetramethyl hematoporphyrin [21]. When prepared from ferri protoporphyrin chloride by bromination and subsequent substitution of the bromine for methoxyl groups without hydrolysis in water, the substance could appear in four different types A—D with the melting points 185°, 178.5°, 140°, and 110°. The crystals varied, A being needles, B double pyramides, C cubes, and D of miscellaneous forms (bundles of needles, prisms etc). A and D were crystallographically identic. It was possible to convert type A to type D by recrystallization from chloroform-methanol and *vice versa*. Types C and D could be crystallized from the mother liquor of type B crystals. With reference to Willstätter's formula for hematoporphyrin (one hydroxy ethyl and one hydroxy vinyl side chain) Fischer and Müller concluded that the differences between the crystallographical types were due to structural isomery. This is rather startling since their experiments on the removal of methanol by heating *in vacuo* gave a compound, which was identic with ooporphyrin (= protoporphyrin) ester. This result fits well with Küster's hematoporphyrin formula (two hydroxy ethyl groups), which also was referred to. Accordingly it is possible that the different melting points depend upon some other factor, *e. g.* the structure of the crystals.

In the present investigation, where the tetramethoxyl derivative was prepared in a different way and subsequently chromatographed, only one form of the crystals appeared. Its crystallographical examination will be undertaken later on. In any case there is a structural difference between the tetramethyl compounds of hematoporphyrin and hematoporphyrin c.

From the results presented here it is evident that hematohemin c possesses two α-hydroxy ethyl groups. It is most unlikely that the warming in 3 *M* acetic acid and the subsequent treatment with acetone etc. could have introduced any artifacts. Thus Theorell's [6] formula with the cysteine residues attached to the secondary carbon atoms of the side chains 2 and 4 has been conclusively confirmed. It is interesting to note that optical activity, exerted by the asymmetric carbon atoms of amino acids in native proteins, is found also in the case of the asymmetric carbon atoms of the porphyrin. As far as we are aware this is the first case when optical activity has been demonstrated in an isolated, chemically identified porphyrin, free from adducts, which are optically active themselves.

The question about the optical activity of porphyrin c of various origins is not yet clear. A consistent hindrance in earlier investigations was that the available method (hydrogen bromide in glacial acetic acid) for the preparation of hematoporphyrin from either porphyrin c or cytochrome c also possibly converts a certain amount of hematoporphyrin to protoporphyrin, which subsequently via the bromine adduct can give the ordinary hematoporphyrin.

Similar troubles were met with in the acid hydrolysis of cytochrome c, where possibilities for the formation of adducts between porphyrin and cysteine were at hand (α-position with hematoporphyrin, β-position with protoporphyrin) [8,14,31]. The consistent value for the optical activity of porphyrin c, obtained by hydrolysis of cytochrome c with dilute sulphuric acid at various concentrations of the pigment [9], indicates that a resynthesis probably did not occur. However, since in this case the optically active asymmetric carbon atoms of the cysteine residues influenced the rotation, no conclusive evidence as regards α- or β-position of the thio-ether bonds were obtainable. The attempt of Zeile and Meyer [9] to prepare porphyrin c by melting together dibromo protoporphyrin with l-cysteine hydrochloride ought to give a substance with the sulphur bonds in α-positions. The optical activity of this preparation was however, quite different from that found for the preparation from cytochrome c (*cf.* page 390). The experiment can, unfortunately, not be exactly reproduced since no information as regards reaction temperature or time is given. For the complete interpretation of published data concerning porphyrin c, the "synthetic" as well as the different kinds of "natural", it would be necessary to study in detail the equilibrium between hemato- and protoporphyrin in acids of various concentrations and a tvariou stemperatures, and also to elucidate the difference in the mode of action between sulphuric acid and hydrochloric acid on cytochrome c. A more profitable investigation would perhaps be to study the result of the silver digestion on the different types of porphyrin c. Experiments of this kind are being done.

The preparation of an isomer of hematoporphyrin with β-hydroxy ethyl side chains would be of interest in connection with another question. It has been stated [32] that hematohemin does not combine with the apoenzyme of horse radish peroxidase. This statement has been disputed [33] as well as confirmed [34]. When hematohemin c is brought to react with the apoenzyme under suitable conditions a recombination to holoenzyme with the same activity as "synthetic" protohemin horse radish peroxidase takes place [35].

SUMMARY

1. The ferri porphyrin, which is liberated from cytochrome c by treatment with silver salt and precipitation with acid acetone of the protein moiety, is an isomer of hematohemin. The name *hematohemin c* is suggested for this isomer.

2. Hematohemin c is readily converted to the dimethyl ether dimethyl ester of hematoporphyrin c by the procedure designed by Grinstein. There is

a significant difference between hematohemin c and hematohemin in this respect, hematohemin reacting more sluggishly.

3. The non-identity of the two tetramethyl compounds is evident from the above-mentioned difference in reactivity, the melting point depression given by them, and their infrared curves.

4. Hematoporphyrin c is optically active. As far as the author is aware this is the first case, when optical activity has been demonstrated for an isolated, chemically identified, simple porphyrin.

5. Hematohemin and hematohemin c are dehydrated at the same rate upon heating. Tetramethyl hematoporphyrin c is converted to protoporphyrin dimethyl ester by heating *in vacuo*. According to the literature tetramethyl hematoporphyrin gives the same product under the same conditions.

6. The hydroxyl groups of hematohemin c do not seem to react with triphenylmethyl chloride.

7. It is concluded that the side chains 2 and 4 of hematohemin c are α-hydroxy ethyl groups.

8. The two cysteine residues in cytochrome c are linked to the secondary carbon atoms (α-positions) of the side chains 2 and 4.

The author's thanks are due to Prof. H. Theorell for stimulating discussions during the course of this investigation, and to Prof. A. Fredga and Dr. D. Wood for suggestions. He also wants to express his thanks to Prof. B. Holmberg for valuable discussions during this and earlier investigations. Miss Inger Agerberg has given very valuable technical assistance.

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Studies on the Purification and Properties of Aldehyde Oxidase from Horse Liver

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The detection and partial purification of an enzyme in *pig* liver which catalyzed the oxidation of aldehydes but not xanthine has been reported by Gordon, Green and Subrahmanyan [1]. The present report contains studies on a similar enzyme found in *horse* livers. The enzyme was partially purified by fractional precipitations with ammonium sulfate, and potassium phosphate buffer at about pH 7.8 followed by electrophoresis. The procedure resulted in a product which was purified about twentyfold with a recovery of about 20 per cent of the total activity of the starting material.

The most highly purified preparations of the horse liver enzyme possessed an activity on a weight basis that was about equivalent to onethird of the activity obtained by Gordon *et al.*[1] on their best preparation of enzyme obtained from pig liver. However, as judged by the lack of an absorption peak at 405 mμ (see Fig. 1), the horse liver preparation appeared to be quite free of catalase. Catalase was apparently still present in the preparation made by Gordon *et al* .[1] Despite the fact that the purified horse liver enzyme possessed an orange color, it did not possess any pronounced absorption peak from 200 to 500 mμ, except the one at 275 mμ which is attributable to aromatic amino acids (Fig. 1). Absorption bands due to flavineadenine dinucleotide, expected at 380 and 450 mμ [1], were not detected.

Stimulation by ammonium ions

It became apparent quite early in these studies that ammonia or ammonium ions in quite high concentration produced a marked stimulation of the enzyme. This observation arose from experiments designed to test the stability of the

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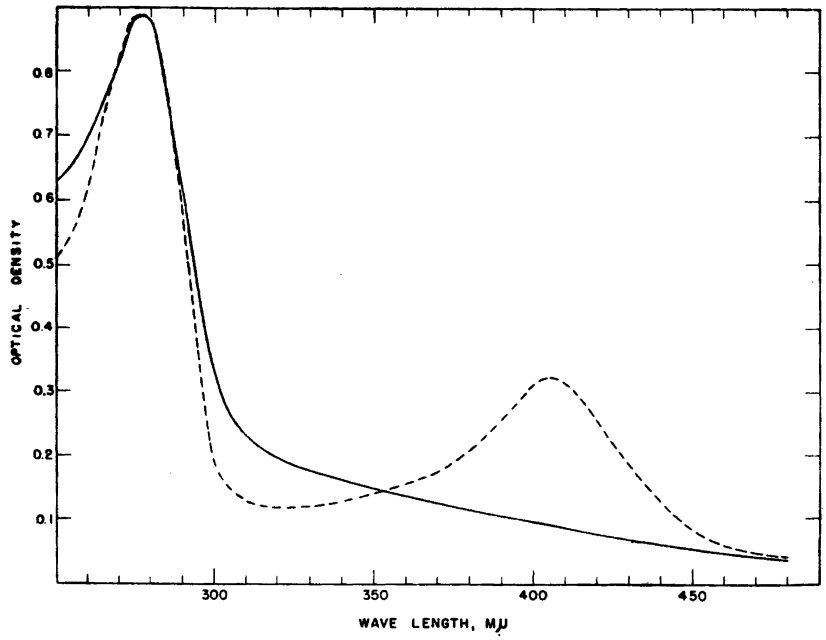


Fig. 1. Absorption spectra of electrophoretically separated fractions from a preparation of horse liver aldehyde oxidase: fraction containing the enzyme activity ———; and fraction containing no enzyme activity — — — —.

enzyme to dialysis. Gordon *et al.*[1] reported the pig liver enzyme to be completely inactivated by dialysis for 24 hours against distilled water at 0°. Acting on the supposition that this inactivation of the pig liver enzyme could have been due to a drop in pH or to removal of salt during the dialysis, a fraction of horse liver enzyme which had been prepared by fractional precipitation with ammoniacal ammonium sulfate[1] was subjected to dialysis at 0° against 0.01 *M* potassium phosphate buffer at pH 7.4. It was noted that the activity of the preparation dropped rapidly during the first 6 hours of dialysis and then remained fairly constant at a level of about 20 per cent of the activity of the original material during dialysis for another 24 hours. However, upon the addition of various amounts of ammoniacal ammonium sulfate to the dialyzed enzyme, the resulting mixtures possessed activities that were equal to or even greater than that of the original undialyzed preparation.

Since the addition of sodium sulfate, sodium chloride or potassium phosphate at the proper pH to dialysed preparations of the enzyme did not bring about an increase in activity, while additions of ammonium sulfate, ammonium chloride and ammonium phosphate at the proper pH did bring about an in-

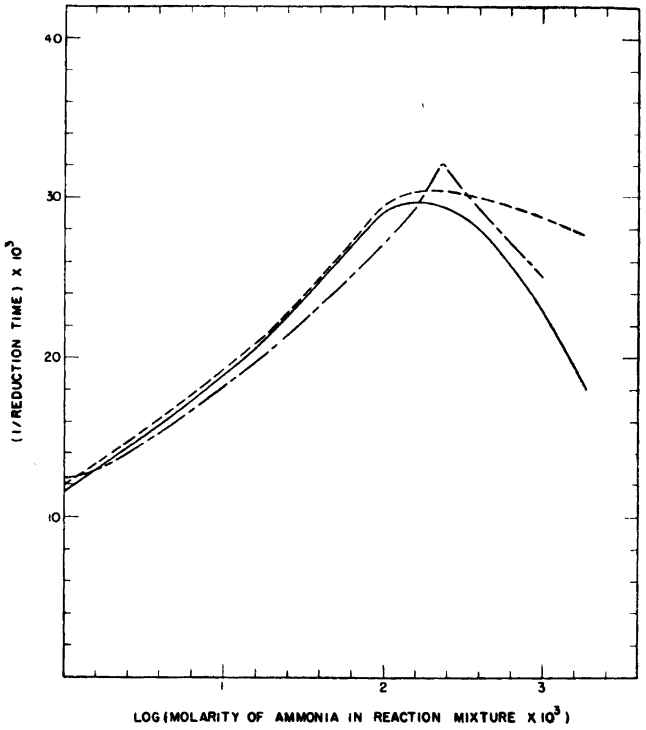


Fig. 2. Effect of ammonia concentration on the activity of horse liver aldehyde oxidase at pH 8.0 with salicylaldehyde as substrate when the ammonia was added as a salt of: chloride ————; sulfate – – – –; and phosphate — — — — — —. The experiment was performed in Thunberg tubes containing 0.1 ml of 0.0113 M methylene blue, 0.1 ml of 0.062 M salicylaldehyde, 0.1 ml of enzyme, 0.9 ml of borate (0.2 M)-phosphate (0.2 M) buffer at pH 8 and 1 ml various concentrations of ammonium salts so as to give the concentrations indicated in the figure.

crease in the activity of dialysed preparations, it was apparent that the stimulation was due to ammonia or ammonium ions. Cognizance was taken of this fact in modifying the methylene blue method of assay of Gordon *et al.*[1] so that an excess amount of ammonia was present in all assays.

Fig. 2 shows the effect of ammonia concentration at pH 8.0 on the activity of a dialysed preparation of horse liver aldehyde oxidase. The results show that the activity of the preparation increased in an almost identical manner regardless of whether the ammonia was added as a salt of chloride, sulfate or phosphate until a concentration of about 0.5 *M* ammonium ion in the reaction mixture was reached. Increasing the concentration of ammonium ions above

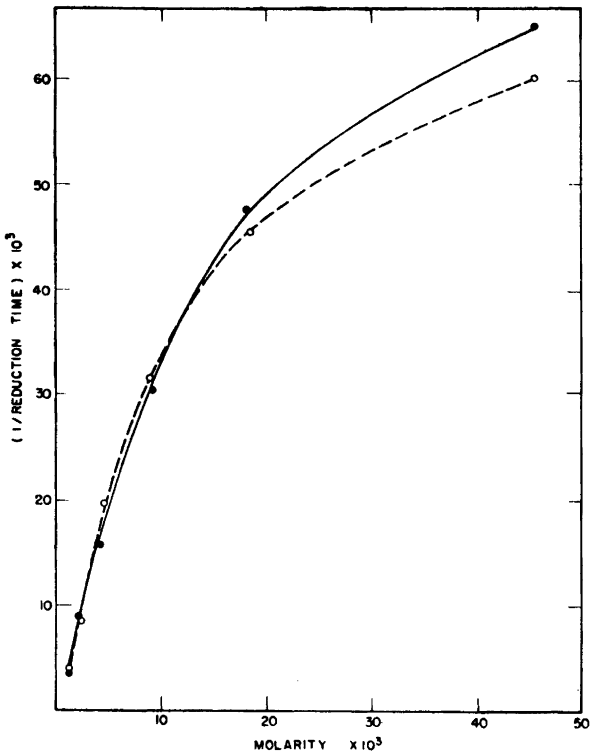


Fig. 3. Effect of substrate concentration on the activity of horse liver aldehyde oxidase: acetaldehyde plus equivalent amounts of ammonia ●——●; and the ammonia derivative of acetaldehyde O— — —O. Experiment performed in Thunberg tubes containing 1 ml of enzyme, 1 ml of 0.2 M borate buffer at pH 8.2, 0.1 ml of 0.0113 M methylene blue and 0.1 ml of solutions of either the ammonia derivative of acetaldehyde or of acetaldehyde plus an equivalent amount of ammonia as $(NH_4)_2HPO_4$.

0.5 *M* in the reaction mixture resulted in an inhibition of activity. This inhibition varied somewhat with the type of salt used.

Because of the effect of ammonia in stimulating the activity of the enzyme, it was thought that the ammonia might take an active part in the reaction, possibly through a mechanism such as that illustrated in the following equation, where M. B. indicates methylene blue:

$$\underset{\text{I}}{CH_3CHO} \xrightarrow{NH_3} \underset{\text{II}}{CH_3C(H)(OH)NH_2} \xrightarrow[\text{M.B.}]{\text{Enzyme}} \underset{\text{III}}{CH_3C(=O)NH_2} + H_2\text{M.B.}$$

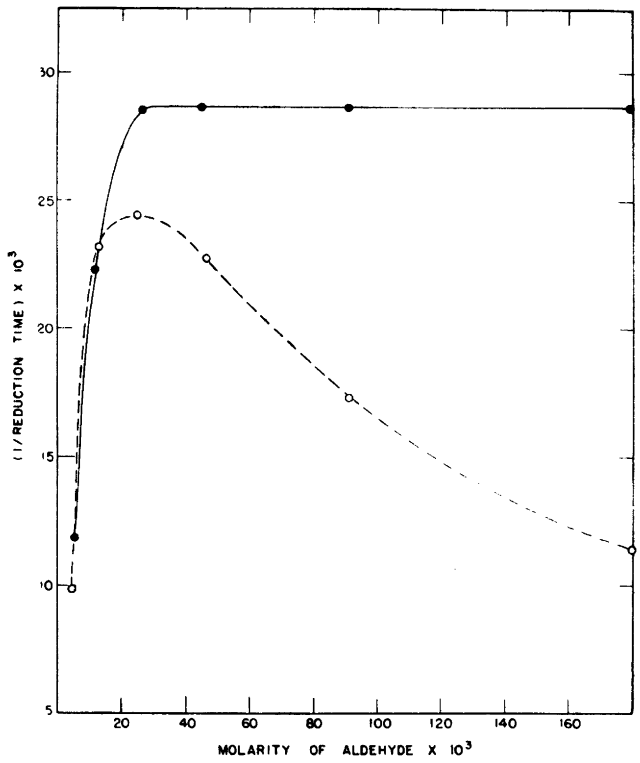


Fig. 4. Effect of substrate concentration on the activity of horse liver aldehyde oxidase in the presence of an excess of ammonium ions: acetaldehyde plus an equivalent amount of ammonia O— — — —O; ammonia derivative of acetaldehyde ●——●. Conditions the same as for Fig. 3 except that 1 ml of 1 M $(NH_4)_2HPO_4$ was used instead of 1 ml of borate buffer.

In order to test this assumption, the ammonia derivative (II) of acetaldehyde was prepared [2] and compared as a substrate at various concentrations with acetaldehyde plus an equivalent amount of ammonia added as diammonium hydrogen phosphate. The results, which are illustrated in Fig. 3, indicated that at least at low substrate concentrations the two substrates were practically equivalent. Essentially the same results were obtained (see Fig. 4) when the ammonia derivative (II) and acetaldehyde were compared as substrates in a medium containing large amounts of ammonium ions. However, at high substrate concentrations, the ammonia derivative (II) was a slightly better substrate than the free aldehyde. Furthermore, the ammonia derivative, unlike the free aldehyde, did not inhibit the reaction when present in an

amount several times that necessary to produce optimum activity. Since the ammonia derivative of acetaldehyde is quite unstable in aqueous solution, one can not conclude that it is being used directly as a substrate. At any rate the derivative (II) does not appear to be greatly superior to the free aldehyde as a substrate.

Since the rather large amounts of ammonia used in these tests precluded detecting the use of ammonia in the reaction by measuring the disappearance of ammonia during the experiment, attempts were made to demonstrate the presence of acetamide (III) in the reaction products. The procedure used by Speck [3] for the determination of glutamine in the presence of ammonia was adapted to the problem. No evidence was obtained for the formation of appreciable amounts of acetamide during the reaction. Thus the results do not support the proposed theory for the stimulatory affect of ammonia on the oxidation of aldehydes by horse liver aldehyde oxidase. They do not, however, eliminate the possibility that ammonia is utilized in some other fashion in the oxidative reaction.

Optimum pH

In Fig. 5 the effect of pH at constant ammonia concentration on the activity of the enzyme is shown when furfural was used as a substrate. Essentially the same results were obtained when acetaldehyde or salicylaldehyde were used as substrates. These results indicate that in the presence of 0.91 *M* ammonium ion in the reaction medium, the optimum pH occurs at 8.1 $\pm$ 0.3. The lower value of pH 7 was reported by Gordon *et al.*[1] for the pig liver enzyme.

Substrates

A number of aldehydes and aldehyde derivatives were tested to see if they would serve as substrates for the action of the partially purified enzyme in methylene blue reduction tests. Acetaldehyde, *formaldehyde*, heptaldehyde, furfural, salicylaldehyde, paraldehyde, benzaldehyde, crotonaldehyde, chloroacetal and pyridoxal possessed some activity as substrates. Quinine [4] and xanthine possessed a very slight but still detectable activity as substrates. Acetone would not serve as a substrate.

The effect of substrate concentration on the activity of the enzyme was investigated for salicylaldehyde, *n*-heptaldehyde, furfural and acetaldehyde. The results of these investigations for acetaldehyde and furfural are shown in Figs. 4 and 6. The results show that there is a rather narrow range of optimum concentration of substrate and that the activity of the enzyme falls off quite rapidly when the optimum concentration is exceeded. The concentration of the aldehyde in the reaction mixture which gave the highest activity is shown in column two of Table 1.

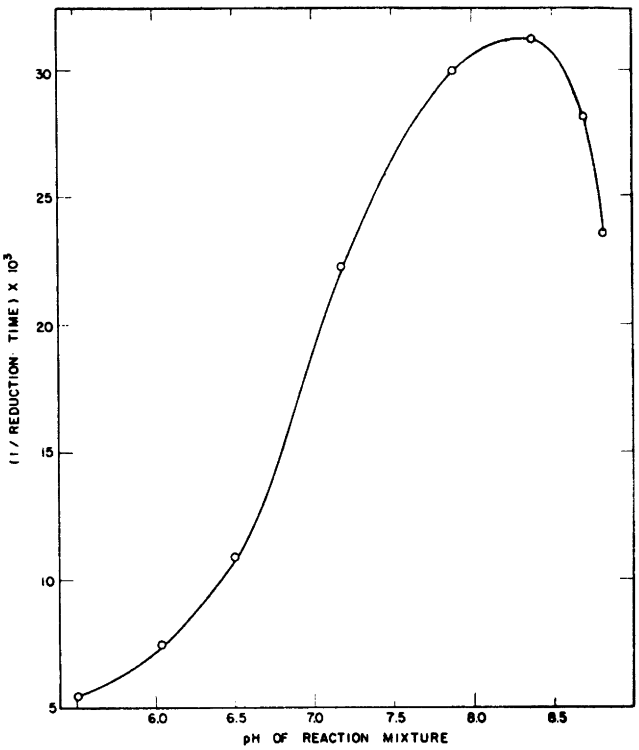


Fig. 5. Effect of pH on the activity of horse liver aldehyde oxidase at an ammonia concentration of 0.91 M in the reaction mixture and with furfural as the substrate. The Thunberg tubes contained 1 ml of enzyme, 0.1 ml of 0.1 M furfural, 0.1 ml of 0.0113 M methylene blue and 1 ml of various ammonium phosphate buffers prepared by mixing various amounts of 1 M $(NH_4)_2HPO_4$ with either 2 M $NH_4H_2PO_4$ or 0.66 M $(NH_4)_3PO_4$. The pH noted is that found on the reaction mixture after reduction of the dye.

It was obvious from these results that estimates of the relative activities of substrates as determined by Gordon *et al.*[1] in which all aldehydes were tested at the same concentration were likely to be misleading. Therefore, acetaldehyde, furfural, salicylaldehyde and *n*-heptaldehyde were compared as substrates at their optimum concentrations against the same enzyme preparation. The results showed that both salicylaldehyde and furfural were much better substrates than acetaldehyde when each was compared at its optimum concentration. *n*-Heptaldehyde on the other hand was a very inferior substrate.

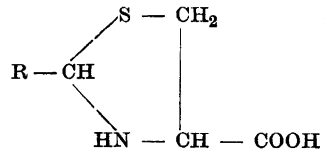
In connection with the studies on the ammonia stimulation effect, it was thought advisable to investigate the use of certain aldehyde derivatives as

Table 1. Relative effectiveness of various aldehydes as substrates for aldehyde oxidase.

Tests were performed in Thunberg tubes in which the main bulb contained 1 ml of enzyme and 1 ml of 1 *M* $(NH_4)_2HPO_4$; and the side bulb contained 0.1 ml of 0.0113 *M* methylene blue and 0.1 ml of a solution of the various aldehydes so as to give, after mixing the contents of the Thunberg tube, the molarity indicated in the table.

Aldehyde	Molarity of aldehyde in test	Relative activity (acetaldehyde = 100 %)
Acetaldehyde	0.046	100 %
Furfural	0.0046	175 »
Salicylaldehyde	0.0028	175 »
n-Heptaldehyde	0.0014	1.8 %

substrates for the enzyme. Of particular interest to the author was the class of compounds called thiazolidines which have recently been reported to occur in nature as an integral part of the penicillin molecule [5]. Several aldehydes were condensed with L-cysteine to give 4-thiazolidine carboxylic acids of the general formula (IV).



IV

The compounds from formaldehyde (IV, R = H), furfural (IV, R = C_4H_3O and benzaldehyde (IV, R = C_6H_5) were prepared according to the directions of Schubert [6]. The compound from acetaldehyde (IV, R = CH_3) was made according to the directions found in the monograph on *The chemistry of penicillin* [7]. All of these 4-thiazolidinecarboxylic acids except the one prepared from formaldehyde served as substrates for the action of the enzyme. However, none of the active thiazolidines were as effective as substrates as the corresponding aldehydes. The thiazolidines needed to be present in much higher concentrations than the free aldehydes in order to give maximum activity. A comparison of furfural and 2-furfuryl-4-thiazolidinecarboxylic acid as substrates is shown in Fig. 6. It is possible that the substrate activity of the thiazolidines is due to a partial reversion of the compounds in solution to aldehydes and cysteine [6,8].

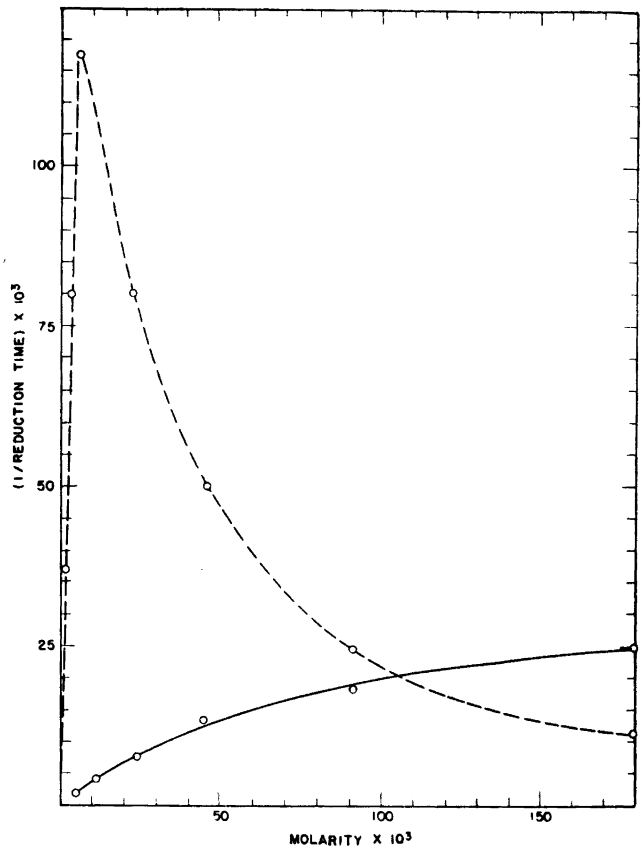


Fig. 6. Effect of substrate concentration on the activity of horse liver aldehyde oxidase: furfural — — — —; and 2-furfuryl-4-thiazolidinecarboxylic acid ————. The Thunberg tubes contained 1 ml of enzyme, 1 ml of 1 M $(NH_4)_2HPO_4$, 0.1 ml of 0.0113 M methylene blue and 0.1 ml of solutions of either furfural or 2-furfuryl-4-thiazolidinecarboxylic acid of various concentrations.

EXPERIMENTAL

Assay method. — In the main bulb of a Thunberg tube were placed the enzyme in a volume of 1.0 ml and 1.0 ml of 1 *M* $(N_4H)_2HPO_4$. In the side bulb of the Thunberg tube was placed 0.1 ml of a solution that was 1 *M* with respect to acetaldehyde and 0.0113 *M* with respect to methylene blue. The tube was evacuated for at least one minute on a water pump, and then placed in a water bath at 37° for 2.0 minutes. The contents of the two bulbs were mixed and the time for complete reduction of the methylene blue was noted. The enzyme contained one unit of activity when the reduction time was sixty seconds under the above conditions. In practice it was found advantageous to use an

amount of enzyme that would cause reduction of the dye in a period of 20 to 40 seconds. This was because a plot of (1/reduction time) against dilution of the enzyme, although linear for crude enzyme, fell off rather rapidly for purified preparations of the enzyme.

Since very crude fractions of the enzyme would bring about reduction of the dye in the absence of substrate, it was necessary in these cases to determine the activity in units in the absence of substrate and subtract this value from the value obtained in the presence of substrate in order to secure a fair approximation of the activity due to the aldehyde oxidase enzyme.

Activity per unit weight. — The activity was referred to the optical density of the solution at 280 mμ. The ratio of the activity in units per cubic centimeter to the optical density at 280 mμ of a 1 centimeter thick solution was used as a measure of purity. For abbreviation this is written as "units/o.d.". Turbid solutions were clarified by centrifugation at high speed in an angle head before making the optical density determinations.

The relationship of the optical density at 280 mμ of a solution to the dry weight of material was determined on a purified fraction of enzyme (0.22 units/o.d.) from which catalase had been removed by electrophoresis. From the results of dry weight and absorption spectra measurements it was found that an optical density reading of 1.88 for a 1 cm thick solution corresponded to 1 mg of material per cubic centimeter of solution. Thus a fair approximation of the activity per unit protein can be obtained by the following equation:

$$\text{units/mg} = (\text{units/o.d.}) \times 1.88$$

In many of the fractionation procedures the removal of catalase was followed by determining the optical density at 405 mμ as well as at 280 mμ. The ratio of absorption at 280/405 was used as a measure of the catalase present. This ratio became progressively larger as the catalase was removed, reaching a value of about 7.4 when a peak at 405 mμ was no longer detected.

Fractionation of horse liver aldehyde oxidase

Starting material. — In the course of this work both fresh and frozen horse livers were used. The frozen horse livers had been stored at — 15° for periods ranging from three to fifteen days. Experience with these two types of material showed the fresh, unfrozen livers to be a much better source of starting material than livers which had been stored for several days in the frozen state. About twice as much enzyme with about twice the specific activity was obtained from fresh livers as was obtained from the frozen livers.

During the early stages of fractionation of this enzyme turbid solutions which could not be readily clarified were encountered. Due to the turbidity of the solutions it was found impossible to fractionate with salt by visual observation of precipitates. Thus it was necessary to add the salt to certain predetermined concentrations and to collect the fractions therefrom. The salt concentrations were determined by measuring the specific gravity of the solutions with floating pycnometers and relating the specific gravity to salt concentration by use of a standard curve. As fairly dilute solutions of protein were used in the fractionations, the contribution of the protein to the density of the solution was quite small. However, in order to make the results strictly reproducible it was found advisable to adjust the protein concentrations of the solutions before fractionating with salt. This was accomplished by diluting the protein solution until the desired optical density reading at 280 mμ was obtained. For most fractionations the solutions were

Table 2. Steps II to V. Fractionation of horse liver aldehyde oxidase.

Step no.	Description	Starting material		Data on fractions obtained		
		Source	Protein conc. *(o.d./ml)*	Salt conc.[d] *moles*	Total activity *(units)*	Specific activity *(units/ o.d.)*
II	2nd fractionation with $(NH_4)_2SO_4$[a]	See Text	108		21 600	0.015
	(a) 1st ppt.[e]			1.56	6 650	0.013
	(b) 2nd ppt.			1.92	10 500	0.023
	(c) M. L.[f]				0	0
III	3rd fractionation with $(NH_4)_2SO_4$[b]	II-b	90		10 500	0.023
	(a) 1st ppt.			1.35	500	
	(b) 2nd ppt.			1.86	9 000	0.031
	(c) M. L.				0	0
IV	1st fractionation with phosphate[c]	III-b	100		9 000	0.031
	(a) 1st ppt.			1.02	1 200	
	(b) 2nd ppt.			1.20	6 250	0.076
	(c) M. L.				810	
V	2nd fractionation with phosphate[c]	IV-b	50		5 800	0.076
	(a) 1st ppt.			0.99	500	
	(b) 2nd ppt.			1.20	4 800	0.160
	(c) M. L.				450	

[a] Solid, technical grade ammonium sulfate was added.
[b] Solid, C. P. grade ammonium sulfate was added.
[c] A 4 *M* phosphate solution (10 % KH_2PO_4 and 90 % K_2HPO_4) was added.
[d] Concentration of the salt in solution in moles per liter, as measured by specific gravity, when the precipitates were formed.
[e] All precipitates were collected by centrifugation.
[f] M. L. indicates the mother liquor from the second precipitate.

diluted so that a 1 cm thick solution would have an optical density at 280 mμ equal to 100, equivalent to 50 to 60 milligrams of protein per cubic centimeter.

Typical fractionation

Preliminary extract. — 11.3 kg of fresh horse liver, which had been freed of fat and arterial tissue, were ground in a meat grinder and extracted with 22.6 kg of tap water at 4° for 16 hours. After removal of the cell debris by centrifugation, the supernatant

liquid measured 23.1 liters and contained 24 000 aldehyde oxidase units with a purity of 0.0118 units/o.d.

Step I. First precipitation with ammonium sulfate. — Technical grade ammonium sulfate in the amount of 35 g per 100 ml of supernatant fluid was added to 23 liters of the supernatant liquid at 4°. After a period of 1.5 hours at 4°, the precipitate was collected by centrifugation. The mother liquor was either discarded or used for the preparation of horse liver alcohol dehydrogenase. The precipitate was dissolved in water to give a solution containing 21 600 units (90 %) with a purity of 0.015 units/o.d.

Steps II to V. — The active precipitate from step I was fractionated twice with ammonium sulfate and then twice with phosphate (10 % potassium dihydrogen phosphate and 90 % dipotassium hydrogen phosphate) as outlined in Table 2.

In the fractionations with ammonium sulfate the pH was purposely not adjusted (*i. e.*, solutions had a pH of about 5.7). Since the enzyme was rather unstable under these conditions, it was necessary to carry out the fractionations with ammonium sulfate as promptly as possible. Although some activity was lost by the fractionation at pH 5.7, the enzyme appeared to be more stable in later stages of purification if it were fractionated at this step without adjusting the pH to 7.5 to 8.5.

When the active material from step V (see Table 2, Fraction V-b) was dissolved in water a nearly clear solution, black-brown in color, was obtained. This solution, when stirred under a strong light, had a sheen resembling that observed during the formation of protein crystals. A similar phenomenon was observed in several preparations at this level of purity. When the solution was centrifuged for 1 hour at 18 000 rpm, a small amount of a bright yellow substance, presumably fat or lipoprotein, accumulated at the surface. This substance, which was apparently the cause for the sheen, did not contain any activity. It could not be completely removed by centrifugation but did gradually disappear on further fractionation with salt.

Material at this or a slightly greater stage of purity was used for most of the electrophoresis experiments.

Steps VI to VIII. — Results of further fractionation with phosphate are described in Tables 3, 4, and 5. In these fractionations the solution was diluted to an optical density of about 70 at 280 mμ and then 4 *M* phosphate was added dropwise with the collection of 4 or 5 fractions containing the activity. Each fraction was analysed for enzyme activity and optical density at 280 and 405 mμ.

The results of the last three fractionations with phosphate are typical of several experiments carried out in this fashion. Material with a specific activity of 0.27 units/o.d. could be obtained quite readily. However, in several instances further fractionation of such material with phosphate gave considerable inactivation of the enzyme. As a result the more purified material although containing less catalase, as judged by the ratio of absorption at 280/405, possessed a lower specific activity than the starting material.

Electrophoresis

Several preparations of a purity of 0.2 units/o.d. were investigated by electrophoresis at pH 7.8 to 8.8. These preparations showed the presence of two main components which could be separated by electrophoresis. The component moving the fastest toward the anode contained the enzyme. The slower moving component possessed an absorption spectrum in agreement with that of catalase. The absorption spectra of fractions separated in this fashion are reproduced in Fig. 1.

Table 3. Step VI. Third fractionation with phosphate.

Fraction	Total units %	Total extinction at 280 mμ %	Units/o.d.	280/405
Starting material	100	100	0.16	5.1
1st ppt.	2	3	0.099	4.9
2nd ppt.	61	42	0.240	5.8
3rd ppt.	26	24	0.177	5.2
M. L.	8	32	0.041	5.0
Total recovery	97	101		

Table 4. Step VII. Fourth fractionation with phosphate.

The second and third precipitates from step VI (Table 3) were combined and refractionated.

Fraction	Total units %	Total extinction at 280 mμ %	Units/o.d.	280/405
Starting material	100	100	0.214	5.5
1st ppt.	15	15	0.212	6.25
2nd ppt.	43	34	0.270	6.25
3rd ppt.	15	12	0.270	5.50
4th ppt.	10	11	0.204	5.25
M. L.	6	28	0.053	4.65
Total recovery	89	100		

Table 5. Step VIII. Fifth fractionation with phosphate.

The second and third precipitates from step VII (Table 4) were combined and refractionated with 4 *M* phosphate.

Fraction	Total units %	Total extinction at 280 mμ %	Units/o.d.	280/405
Starting material	100	100	0.27	5.88
1st ppt.	27	33	0.244	6.95
2nd ppt.	22	25	0.246	7.00
3rd ppt.	9	10	0.252	6.35
4th ppt.	8	10	0.220	5.77
M. L.	7	21	0.094	5.00
Total recovery	73	99		

Experiments were undertaken to determine at what pH these two components separated the most rapidly. The same initial enzyme preparation was subjected to electrophoresis at pH 7.3, 8.3 and 9.5 in buffers possessing essentially the same ionic strength as determined by conductivity measurements. Nearly the same current density was used in all three cases. An automatic compensator was used and set at such a rate as to keep the fastest moving boundary (the enzyme) practically motionless. The rate of separation of the catalase and enzyme boundaries was then determined. At the conclusion of the experiment the identity of the two ractions was checked by enzyme assay and light absorption measurements. From the results of this experiment (Table 6) it is evident that increasing the pH increases the rate of separation of the two boundaries. It also, of course, increases the rate of travel of the two boundaries toward the anode.

Table 6. Separation of enzyme and catalase by electrophoresis.

pH	Type buffer	Applied voltage	Current *ma*	Separation *cm/hour* *
7.3	Phosphate	330	17.5	0.150
8.3	»	330	17.5	0.190
9.5	Borate	300	18.5	0.214

* The separation refers to the images projected on a ground glass scale and not to the actual separation rate in the solution.

Stability

Stability to storage at 4°. — A variety of conflicting results were obtained in stability studies performed on various fractions stored at 4°. As a rule crude preparations (0.04 to 0.10 units/o.d.) at pH 7.5 to 9 could be kept for a period of several weeks at 4° without appreciable loss of activity. Purer preparations (0.1 to 0.2 units/o.d.) were more apt to suffer greater losses of activity during the same period. Inactivation that did eventually occur in these relatively stable solutions was probably due primarily to the development of microorganisms.

However, not all preparations of the enzyme exhibited this relatively great stability to storage. In some cases the activity would decrease as much as 5 to 10 % per day from the day that it was stored. The marked differences in the stability of various preparations could not be definitely traced to any one factor. However, it was noted that nearly all, if not all, of the preparations which had been fractionated with ammonium sulfate without pH adjustment (*i. e.*, at about pH 5.7) in the early stages of the purification exhibited a marked stability to storage.

Stability to lyophilization. — A fraction of enzyme containing 0.04 units/o.d. which had been dialysed against 0.05 *M* phosphate at pH 8.1 was subjected to lyophilization. The material that had been dried in the frozen state retained 66 per cent of the activity of the starting material.

Stability to dialysis. — Considerable variation in the stability of the enzyme to dialysis was noted. The stability to dialysis of various preparations seemed to be related to the stability to storage at 4° of these preparations. Preparations that were stable to storage

could, on the whole, be dialysed for several days in the pH range 7.3 to 9.5 without any large losses of activity. On the other hand preparations which lost from 20 to 50 % of their activity on dialysis also proved to be quite unstable on storage.

Successful dialysis experiments were obtained on all the buffers tried, *i. e.*, phosphate, borate, and ammonia-ammonium sulfate. Thus instability of certain preparations could not be traced to the buffer salt.

Crude preparations tended to be much more stable to dialysis than preparations having a purity of 0.2 units/o.d. or better. However, in several instances preparations of this purity were successfully dialysed.

The activity lost by some preparations on dialysis did not appear to be due to the loss of a prosthetic group or co-factor other than ammonia. Ammonia, of course, was added back to dialysed solutions before assay. For example, one catalase-free preparation of enzyme was dialysed for a week against constant changes of dilute ammonia water at pH 8.8. The enzyme lost all of its activity during this time, but lost none of its total light absorption in the range 500 to 230 mμ. Thus the removal of a prosthetic group, absorbing in the range 230 to 500 mμ, seemed to be eliminated. In another instance, a crude preparation was dialysed in a similar fashion against dilute ammonia water at pH 8.5. During the dialysis, the enzyme lost approximately 50 % of its activity. The dialysate was concentrated to dryness *in vacuo* at 40°. The addition of the residue to the impermeate did not increase the activity of the enzyme. In still another instance, a few cubic centimeters of the original enzyme extract were boiled for five minutes. The insoluble material separated, and the cooled supernatant was added to a preparation which had lost about one-half of its activity on dialysis. The addition of the boiled extract did not increase the activity of the dialysed preparation.

From all of these results it seems safe to conclude that the losses of activity which occur occasionally on dialysis are due to some type of inactivation of the enzyme similar to that occasionally experienced on storage of the enzyme. Certainly there is no evidence for the removal of any co-factor other than ammonia during the dialysis.

SUMMARY

1. A procedure for the partial purification of aldehyde oxidase from horse liver is described.

2. The activity of the enzyme as measured by the methylene blue reduction test is enhanced by the presence of ammonium salts. Studies on the nature of this stimulation by ammonium salts are reported. In the presence of ammonium salts, the enzyme has its optimum activity at pH 8.

3. A number of aldehydes including formaldehyde are effective as substrates for the enzyme. Furfural and salicylaldehyde are about twice as effective as substrates as is acetaldehyde.

4. At high substrate concentration, the ammonia derivative of acetaldehyde is a slightly better substrate than free acetaldehyde. Although L-cysteine derivatives of several aldehydes (thiazolidines) can be used as substrates, they are not as effective as the corresponding free aldehydes.

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Equitransferent Salt Bridge for Elimination of the Diffusion Potential Compared with Saturated Potassium Chloride Salt Bridge

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In a previous paper [1] it has been pointed out that an equitransferent solution of potassium chloride + potassium nitrate will probably be better suited for elimination of the diffusion potential than the saturated potassium chloride solution most frequently used for this purpose. The advantage by using an equitransferent reference solution * is that a convergence of the diffusion potential towards zero is obtained on increasing dilution of the test solution. Otherwise the diffusion potential will include a factor due to the difference of the ion mobilities in the reference solution as it is actually possible to regard the diffusion potential as consisting of two parts, one of which being due to differences between cation and anion mobilities in the test solution, and the other to differences between cation and anion mobilities in the reference solution. The latter part will be equal to zero if the reference solution is equitransferent. If this is not the case, it will increase proportionally with the logarithm of the degree of dilution. It will appear from the present paper that this part of the diffusion potential amounts to approximately 2, 3 and 4 mV for a saturated potassium chloride solution when the concentrations of the test solution are 0.1, 0.01 and 0.001 normal, respectively. This part of the diffusion potential has often been neglected because of wrong assumptions regarding the transference number of potassium chloride. However, this will not always involve that results obtained by the use of saturated potassium chloride on the basis of such assumptions include errors of the order of magnitude indicated, as in

* The word reference solution is used in this paper as a common designation for salt bridge and reference electrode solution.

practice the said part of the diffusion potential will very often occur twice with opposite signs in the experiments so that the error is cancelled out. The error will only be discernible in cases where a test solution is compared with a standard solution the conductance of which materially differs from that of the test solution, or where it is attempted to make extrapolations towards infinite dilution.

Another reason for trying to make the diffusion potentials as small as possible is, however, also that as a rule they are not particularly well defined. Even if it is possible to obtain comparatively reproducible results by observing particular experimental conditions in making the boundary, great diffusion potentials will always be a source of uncertainty as it may be taken for granted that the error which may be involved in an experiment by the diffusion potential must be proportional to the magnitude of same. This should hold in cases where it is tried to calculate the diffusion potential as well as in cases where two diffusion potentials of the same order of magnitude but of opposite directions are anticipated to cancel.

COMPUTED DIFFUSION POTENTIALS

The computations have been made on the basis of Henderson's formula. This formula has been deduced on the assumption of certain ideal conditions of which it can definitely be said that they do not exist in actual practice. The deviations occurring because these ideal conditions are not fulfilled can hardly be expressed by mathematical formulae. Consequently no absolute validity can be attributed to calculated diffusion potentials, they can only be regarded as an orientation about their order of magnitude.

In accordance with Guggenheim and Schindler [2] the ideal values for ion mobilities and activities have been used as the basis for the computations in the present paper. By this procedure the calculations are considerably simplified, and numerous table references are saved as compared with another procedure sometimes used (Bjerrum and Unmack [3]) to the effect of employing the stoichiometric ion concentrations in connection with the ion mobilities at the total ion concentration in question.

According to Henderson's formula the diffusion potential: reference solution/test solution is:

$$E_j = \frac{R\ T}{F\ \log\ e} \cdot \frac{(U - V) - (U_r - V_r)}{(\bar{U} + \bar{V}) - (\bar{U}_r + \bar{V}_r)} \log \frac{(\bar{U}_r + \bar{V}_r)}{(\bar{U} + \bar{V})} \qquad (1)$$

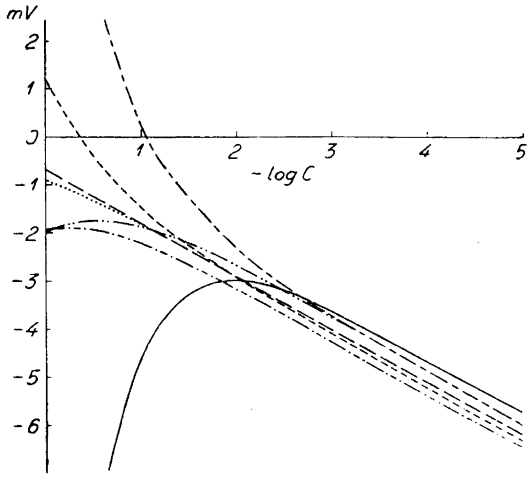

Fig. 1. The diffusionpotential: reference solution/test solution with 4.1 N potassium chloride as reference solution. c = molarity of test solution.

——————— *HCl*
– – – – – – *NaCl*
— — — — *KCl*
— – — – — *NaOH*
..... *KCl + KNO_3*
— ·· — ·· *Na_2SO_4*
— · – · — *CH_3COONa*

where

$$U = \sum_t u_i c_i \qquad V = \sum_t v_j c_j$$

$$\overline{U} = \sum_t u_i z_i c_i \qquad \overline{V} = \sum_t v_j z_j c_j$$

$$U_r = \sum_r u_i c_i \qquad V_r = \sum_r v_j c_j$$

$$\overline{U}_r = \sum_r u_i z_i c_i \qquad \overline{V}_r = \sum_r v_j z_j c_j$$

u_i and v_j being mobilities of cations and anions, respectively, z_i and z_j their valence (taken positive) and c_i and c_j their molarities. Indices t and r refer to the test solution and reference solution, respectively. A positive value of E_j is concomitant with a positive test solution as compared with the reference solution.

If the values (taken from Harned and Owen [4]) for the ion mobilities of 4.1 N saturated potassium chloride and 3.6 N equitransferent solution of KCl + KNO_3, respectively, are inserted, the results obtained at 25° C are:

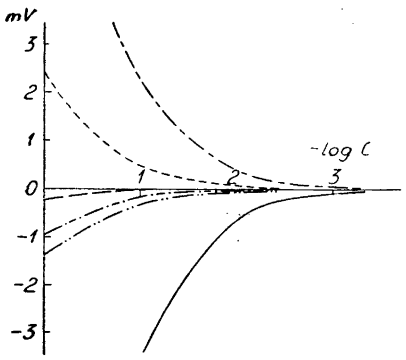

Fig. 2. The diffusion potential: reference solution/test solution with 3.6 N equitransferent solution of $KCl + KNO_3$. c = molarity of test solution.

——— *HCl*
– – – – – – *NaCl*
— — — *KCL*
— – — – *NaOH*
—··—·· Na_2SO_4
— ·–· — CH_3COONa

for 4.1 *N* KCl

$$E_j = \frac{0.05916\ ((U - V) + 11.56)}{(\overline{U} + \overline{V}) - 614.4} \log \frac{614.4}{\overline{U} + \overline{V}}$$

or

$$E_j = \frac{0.05916\ ((U - V) + 11.56)\ (2.7884 - \log\ (\overline{U} + \overline{V}))}{(\overline{U} + \overline{V}) - 614.4} \qquad (2)$$

and for 3.6 *N* equitransferent solution of $KCl + KNO_3$

$$E_j = 0.05916\ \frac{U - V}{(\overline{U} + \overline{V}) - 530.7} \log \frac{530.7}{(\overline{U} + \overline{V})}$$

or

$$E_j = \frac{0.05916\ (U - V)\ (2.7248 - \log\ (\overline{U} + \overline{V}))}{(\overline{U} + \overline{V}) - 530.7} \qquad (3)$$

Equations (2) and (3) are used for calculation of the curves in Figs. 1 and 2 giving a comparison of the diffusion potentials between a number of solutions and the two reference solutions under consideration.

In the calculations the following ion mobilities are used (after Harned and Owen [4]):

H 349.8; Na 50.11; K 73.52; Cl 76.34; OH 197.6; CH_3COO 40.9; NO_3 71.44; $\frac{1}{2}SO_4$ 80.

When the reference solution is 4.1 *N* KCl (Fig. 1), the diffusion potentials for the higher concentrations of the test solution assume values characteristic of the electrolytes concerned. With increasing dilution the curves take a

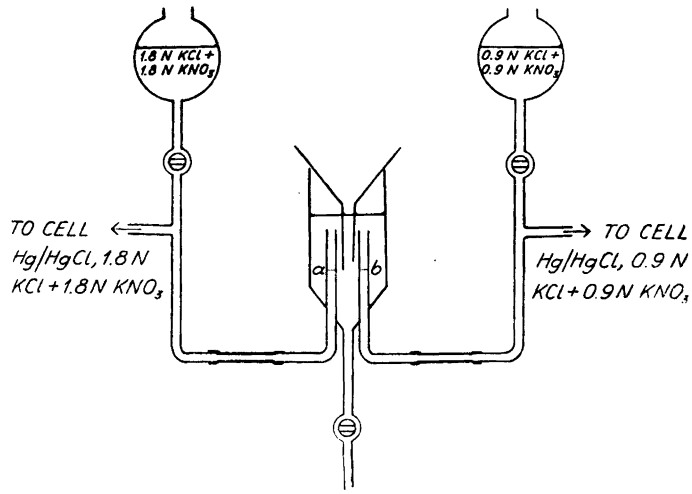

Fig 3. Experimental arrangement. a and b boundaries corresponding to equation (4).

uniform course, the numerical value of the diffusion potential increasing proportionally with the negative logarithm of the concentration.

If on the other hand the reference solution is a 3.6 N equitransferent mixture of KCl + KNO_3 (Fig. 2), the diffusion potential for the higher concentrations will continue to be characteristic of the electrolytes concerned, but with increasing dilution the curves will approach the abscissa asymptotically.

EXPERIMENTS

Measurements have been made in cells of the type:

$$\text{Hg} \mid \text{HgCl, } 1.8\ N\ \text{KCl} + 1.8\ N\ \text{KNO}_3 \mid_{a} \text{test solut.} \mid_{b} 0.9\ N\ \text{KCl} + 0.9\ N\ \text{KNO}_3, \text{HgCl} \mid \text{Hg}$$

The diffusion potentials at a and b are not measurable, but if the difference between the normal potentials of the two calomel electrodes is known, it is possible by means of that difference to determine experimentally the difference between the two diffusion potentials. As the latter difference can also be computed by means of Henderson's formula, this affords a possibility of checking the results obtained by means of the formula.

If the diffusion potentials at a and b are denoted by $E_{3.6}$ and $-E_{1.8}$, $E_{3.6}$ will be given by equation (3) while the corresponding equation for $E_{1.8}$ will be:

$$E_{1.8} = \frac{0.05916\ (U - V)\ (2.4237 - \log\ (\overline{U} + \overline{V}))}{(\overline{U} + \overline{V}) - 265.3} \qquad (5)$$

The experimental arrangement is shown in Fig. 3. The two calomel electrodes were placed in a thermostat at 25° C. The remaining part of the apparatus was placed outside the thermostat. The room temperature was 25° C ± 0.3° C. The measurements were made on a measuring bridge with a mirror galvanometer as zero instrument. Readings were taken immediately after establishment of the boundaries. The measuring apparatus permitted readings with an accuracy of about 0.02 mV; for the weakest solutions it was, however, about 0.05 mV.

Before measurement the vertical tubes intended for the boundaries were washed with the pertinent reference solutions, and the test solution was filled in the vessel. In order to obtain cylindrical symmetry, the boundaries were established, as in previous experiments [1], by compression and subsequent expansion of the rubber tubing shown in the illustration so that the boundaries moved a few cms downwards in the tubes. Then the test solution rendered impure by mixture with the reference solution was removed from the vessel, and replaced by new test solution. Immediately afterwards the reading was taken. The results of the measurements are compared with the computed values of $E_{1.8} - E_{3.6}$ in Table 1.

The difference between the normal potentials of the two electrodes was determined from measurements in equimolar mixtures of potassium chloride + potassium nitrate. At the concentrations 0.001, 0.01, and 0.1 the values 19.91, 19.93, and 19.94, mV respectively, were obtained. The mean value 19.93 was used for determination of the experimental value of $E_{1.8} - E_{3.6}$, the measured potential difference being subtracted from the said figure.

It will appear from the table that there is no complete agreement between the computed and the measured values of $E_{1.8}$—$E_{3.6}$, a fact which may partly find its explanation in that the ideal conditions required for Henderson's formula are not satisfied. But apart from the results obtained with 1 N test solutions, there is a reasonable agreement for the other solutions. The experiments, moreover, confirm, as previously mentioned, that computed values of the diffusion potentials can only be in the nature of an orientation about their order of magnitude.

ON THE USE OF BJERRUM'S EXTRAPOLATION METHOD

The quantity $E_{1.8}$—$E_{3.6}$ dealt with in the preceding section is of particular interest as it may be compared with the so-called Bjerrum extrapolation. The extrapolation method for evaluation of the diffusion potential proposed by Bjerrum [5] in 1905 is based on the assumption that the diffusion potential of an arbitrary dilute solution and a 1.75 N potassium chloride solution will

Table 1. Comparison of computed and measured values of $E_{1.8}-E_{3.6}$. mV.

	Measured potential difference	Computed values			Experimental values of $E_{1.8}-E_{3.6}$
		$E_{1.8}$	$E_{3.6}$	$E_{1.8}-E_{3.6}$	
1 *N* HCl	30.30	− 20.71	− 14.74	− 5.97	− 10.37
0.1 — —	22.80	− 5.77	− 3.63	− 2.14	− 2.87
0.01 — —	20.46	− 1.11	− 0.64	− 0.47	− 0.53
0.001 — —	19.93	− 0.17	− 0.09	− 0.08	0
1 *N* NaOH	12.90	14.76	10.20	4.56	7.03
0.1 — —	18.25	3.74	2.30	1.44	1.68
0.01 — —	19.70	0.67	0.38	0.29	0.23
0.001 — —	19.95	0.10	0.05	0.05	− 0.02
1 *N* NaCl	18.64	3.60	2.39	1.21	1.29
0.1 — —	19.63	0.81	0.49	0.32	0.30
0.01 — —	19.93	0.14	0.08	0.06	0
0.001 — —	19.90	0.019	0.011	0.008	0.03
1 *N* KCl	19.38	0.36	0.24	0.12	0.55
0.1 — —	19.84	0.83	0.050	0.033	0.09
0.01 — —	19.96	0.014	0.0080	0.006	− 0.03
0.001 — —	19.92	0.0020	0.0011	0.0009	0.01
1 *N* CH_3COONa	19.97	− 1.45	− 0.95	− 0.50	− 0.04
0.1 — —	20.01	− 0.31	− 0.018	− 0.13	− 0.08
0.01 — —	19.95	− 0.05	− 0.03	− 0.02	0.02
0.001 — —	19.95	− 0.007	− 0.004	− 0.003	0.02
0.5 *M* Na_2SO_4	21.53	− 1.37	− 0.98	− 0.39	1.60
0.1 — —	20.35	− 0.50	− 0.31	− 0.19	− 0.42
0.01 — —	19.98	− 0.09	− 0.05	− 0.04	− 0.04
0.001 — —	19.95	− 0.014	− 0.007	− 0.007	− 0.02

be double the quantity of the diffusion potential of the same solution and a 3.5 *N* potassium chloride solution. This is used in practice, for example in a hydrogen ion measurement by measurements with 1.75 and 3.5 *N* potassium chloride solutions as salt bridges. The difference between the two measurements is put equal to the diffusion potential and used as a correction for the measurement. The method was proposed on the assumption that a potassium chloride solution is approximately equitransferent, and it is characteristic that the extrapolations found decrease in magnitude with increasing

dilution of the test solution. As it is elucidated by the present findings this does not mean that the diffusion potential decreases with increasing dilution, but has its explanation in that the parts of the diffusion potentials $E_{1.75}$ and $E_{3.5}$ which are due to nonequitransference in the reference solutions are of the same order of magnitude. This again means that the experimental part of the present paper cannot be ascribed any significance for the purpose of an evaluation of the question whether the reference solutions used in the experiments are equitransferent. The assumption that this is approximately the case in an equimolar mixture of KCl + KNO_3 is, so far, exclusively based upon the paper by the present author [1] previously mentioned,

With regard to the applicability of Bjerrum's extrapolation for evaluation of the diffusion potential the above considerations imply that on condition of an equitransferent salt bridge — and only on this condition — the extrapolation will express the order of magnitude of the diffusion potential to the extent originally stated by Bjerrum [3,5] as it appears from the following quotation: "... es ist erlaubt anzunehmen ..., dass man ... aus dem Unterschiede zwischen den mit halbgesättigter und mit gesättigter Chlorkaliumlösung gefundenen Werten auf die Grössenordnung der Fehler schliessen darf, die man begeht, wenn man annimmt, dass eine gesättigte Chlorkaliumlösung das Diffusionspotential eliminiert. Und ich glaube weiter, dass man ... durch die kleine Extrapolation etwas bessere Werte bekommen kann."

As previously mentioned Bjerrum's extrapolation will, however, be of value in many cases even if the salt bridge is not completely equitransferent. This will be the case for example if p_H is measured in a given solution on the basis of a standard solution of the same conductance. Here the extrapolation will not be a function of the absolute magnitude of the diffusion potential, but it will be a measure of the error committed by not making allowance for the diffusion potential.

SUMMARY AND CONCLUSION

The diffusion potentials of some dilute solutions with saturated KCl and equitransferent 3.6 *N* KCl + KNO_3, respectively, as reference solutions are computed. With saturated KCl the diffusion potential increases with extreme dilution of the test solution proportionally with the logarithm of the degree of dilution. With equitransferent reference solution the diffusion potential converges towards zero on dilution of the test solution.

Some computed and experimental values of the quantity: $E_{1.8}$—$E_{3.6}$ for equitransferent reference solution are compared.

Bjerrum's extrapolation method for determination of diffusion potentials affords an orientation about the order of magnitude of the diffusion potential on condition that the reference solution is equitransferent, and only on this condition.

My best thanks are due to Rektor, Professor C. Faurholt for the kind interest he has taken in the present work. Further I wish to thank Professor J. A. Christiansen for discussions of the manuscript.

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Turnover Rates During Formation of Proteins and Polynucleotides in Regenerating Tissues

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The simultaneous appearance of protein and nucleic acid in growing cells was discovered by Miescher [1]. Kossel [2] later contended that a simultaneous formation of basic proteins and polynucleotides is a fundamental reaction in cell nuclei, connected with the processes of cell division, fertilization and inheritance.

More recently several investigators, for example Masing, Fridericia, Brachet, Caspersson *et al.*[3-6], have demonstrated the fact that an increase in the amount of polynucleotides per weight or volume of cell occurs during early stages of growth. However, there is very little information about the appearance of the proteins that are synthesized during growth and formed apparently at about the same time as the polynucleotides.

The authors of the present paper wanted to investigate a possible correlation between the turnover and synthesis of protein and that of polynucleotides during growth. Glycine marked with N^{15} was used as tracer-precursor in these experiments. The rate of protein turnover was measured by the concentration of isotope in the glycine incorporated into the proteins at different stages of regeneration of rat liver. In the same experiment the polynucleotides were degraded and the rate of their turnover measured by the isotope content in the nitrogenous bases.

Hen bone marrow was also used in some experiments in order to study the metabolic changes during growth and differentiation of the blood cells. Re-

sults of these experiments will be mentioned here only in so far as they disclose a certain relationship between the isotope content in amino acids and purine bases.

EXPERIMENTAL

Regeneration of liver

Albino rats of 180 to 200 g were used. The animals were obtained from various sources and constancy of strain could not be controlled. This unfortunate fact may have introduced biological variations. To minimize the effect of this we have pooled the livers from 20—40 rats for the analysis at each regeneration stage. This has also made it possible to include in the analytical plan the small fraction of PNA in the nuclei.

The animals had free access to a mixed diet of milk, bread and oats, both before and after the operation. Their consumption of food was only roughly estimated. The hepatectomized animals began to drink milk about half an hour after the operation but they consumed less food than animals which had not undergone hepatectomy.

Partial hepatectomy was performed in the same way as in earlier work [7]. Isotopic glycine (31 atom per cent excess N^{15}) was injected subcutanously at a level of 100 mg of glycine per 100 g of the body weight at the time of operation. Injections were made every two hours, four times in all, during the last eight hours before the animals were sacrificed. The rats were killed two hours after the last injection of isotopic glycine.

Five groups of animals were used with twenty to forty rats in each depending on the stage of regeneration. After preliminary experiments with smaller groups the following regeneration times (hours of life from operation to death) were chosen: 11, 26, 32, 56 and 170.

The only intentional difference between these groups is the stage of regeneration. Each group received the same amount of isotope during the same period before death.

Table 1. Regeneration at different times after partial hepatectomy.

No. of animals	Regeneration time in hours	Regeneration in per cent and standard deviation
40	11	2 ± 13
30	18	32 ± 19
30	26	50 ± 13
25	24	34 ± 19
25	32	34 ± 14
20	48	102 ± 32
20	56	65 ± 19
20	162	131 ± 71
20	170	142 ± 44

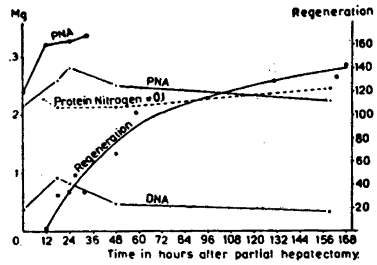


Fig. 1. Mg per 100 mg dry weight of liver. PNA and DNA as phosphorus. Protein as nitrogen. Regeneration as increase in per cent of the weight of the remaining lobe after partia l hepatectomy.

The excised portions of the liver were always weighed. In a separate experiment on 25 rats both the excised and the remaining portions of the liver were weighed; in this experiment the weight of the remaining part was found to be 57.8 % of the weight of the excised part with a standard deviation of 5.7. This figure has been used to calculate from the weight of the excised liver in each case, the weight of the liver remaining after partial hepatectomy at regeneration time zero. From this figure and from the final weight of the liver after regeneration, the amount of regeneration can be calculated in grams of wet weight. In this paper the extent of regeneration is defined as the increase in wet weight of the remaining liver lobe, expressed as per cent increase of its calculated weight at the time of operation.

At each regeneration stage samples of liver tissue were removed for cytological investigations.

In separate experiments the extent of regeneration was determined for regeneration times eight hours less than the regeneration times in the actual isotope experiments (3, 18, 24, 48 and 162 hours). The differences between these figures and those found for regeneration in the isotope experiments were taken as indicative of the extent of regeneration during the last eight hours, *viz.* the period when isotopic glycine was available in the body. In 5 to 10 % of the cases the liver showed extremes of very low or excessive growth. These extremes are included. The variations are too large to allow a determination of the point of maximum growth during the eight hour periods of tracer turnover (Table 1).

The separation of cell nuclei and cytoplasm, the preparations of protein and polynucleotide fractions and the degradation of the latter were carried out by methods described in earlier publications [8]. The purification of the liver cell nuclei was controlled by phase contrast microscopy.

Regeneration of blood cells in the hen was brought about by phenylhydrazine hemolysis. Fifty mg of phenylhydrazine per kg of body weight were injected subcutaneously. After 54—60 hours the frequency of mitosis in the bone marrow had reached its highest value and the relative number of immature cells in the blood stream was at its maximum. At this point intraperitoneal injections of N^{15}-labeled glycine were begun; three injections were made at 6 hours intervals, the total amount injected being 2.0 g per kilogram of body weight. The hen was sacrificed by exsanguination after a further 6 hours period (72 hours from the time of hemolysis). Further particulars of this regeneration and the results on the formation of cellular substances during the blood cell production will be published elsewhere.

Table 2. The isotope content in glycine + serine compared with the sum of that in fourteen of the other amino acids (incl. humin and ammonia). The values are given in atom per cent N^{15} calculated on basis of 100 per cent in administered glycine.

Hen no.	Organ	I Glycine + Serine	II Amino acids	I/II
1	Bone marrow normal hen	19.8	11.6	1.71
3	Bone marrow phenylhydrazine 4 g glycine/kg	43.4	26.4	1.64
1	Liver normal hen	12.9	9.6	1.35
2	Liver phenylhydrazine 2 g glycine/kg	14.2	11.4	1.25
3	Liver	22.2	17.2	1.29

Regenerating liver from rat.

Regeneration time in hours	Glycine + Serine I	Amino acids II	I/II
11	11.13	5.92	1.88
26	16.06	7.17	2.24
32	14.06	10.17	1.38
56	18.22	11.92	1.53
170	14.36	8.54	1.68

Glycine and the other amino acids were separated from hydrolysates of the liver and bone marrow proteins by starch chromatography according to the method of Moore and Stein [9] but on an enlarged scale to permit the isolation of milligram amounts of each amino acid. Details of this separation will form the subject of an other paper [10].

RESULTS

A general picture of the processes of polynucleotide and protein synthesis during different stages of regeneration is given by Fig. 1. The amounts of polynucleotide phosphorus per gram of dry liver tissue were determined by the

Table 3. Correlation of isotope contents of glycine and serine to adenine and guanine in experiments on hens with glycine-N^{15}. The values are given as atom per cent N^{15} calculated on basis of 100 per cent in administered glycine.

Hen no.	Organ	Glycine/ Adenine	Glycine/ Guanine	Serine/Adenine	Serine/Guanine
1	Bone marrow normal hen	$\frac{12.85}{5.17} = 2.49$	$\frac{12.85}{5.01} = 2.56$	$\frac{6.97}{5.17} = 1.35$	$\frac{6.97}{5.01} = 1.39$
2	Bone marrow phenylhydrazine	$\frac{21.24}{6.10} = 3.48$	$\frac{21.24}{7.50} = 2.85$	$\frac{11.59}{6.10} = 1.90$	$\frac{11.59}{7.50} = 1.55$
3	Bone marrow phenylhydrazine, 4 g glycine/kg	$\frac{26.55}{8.56} = 3.10$	$\frac{26.55}{9.68} = 2.74$	$\frac{16.84}{8.56} = 1.97$	$\frac{16.34}{9.38} = 1.74$
1	Liver	$\frac{7.34}{2.66} = 2.76$	$\frac{7.34}{3.65} = 2.01$	$\frac{5.52}{2.66} = 2.07$	$\frac{5.52}{3.65} = 1.51$
2	Liver	$\frac{7.61}{2.11} = 3.61$	$\frac{7.61}{3.08} = 2.47$	$\frac{6.54}{2.11} = 3.10$	$\frac{6.54}{3.08} = 2.12$
3	Liver	$\frac{12.38}{5.04} = 2.44$	$\frac{12.28}{5.48} = 2.24$	$\frac{9.89}{5.04} = 1.96$	$\frac{9.89}{5.48} = 1.80$

method of Hammarsten. The levels of both PNA and DNA show a maximum during early stages of regeneration. When the amount of trichloroacetic-acid (TCA)-insoluble nitrogen was determined no definite changes was observed.

Distribution of isotope in the proteins. The isotope content of the amino acids isolated from the proteins was determined. Table 2 shows a survey of the distribution of N^{15} from administered glycine in the other amino acids. The N^{15} content of isolated glycine is about ten times that of any one of the other amino acids into which N^{15} has presumably been introduced mainly by transamination reactions. The one exception is serine which is known to be synthesized directly from glycine.

When the total amounts of isotopic nitrogen in the amino acids were calculated from the isotope contents and the approximate amounts of amino

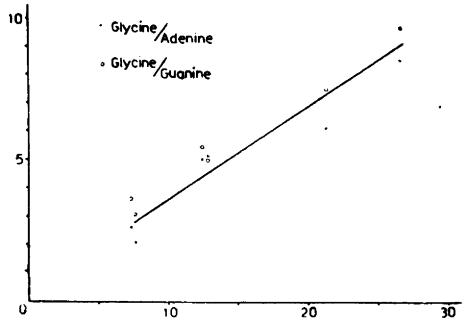


Fig. 2.
Ordinate: N^{15} *content in adenine and guanine.*
Abscissa: N^{15} *content in glycine.*
All values calculated on basis of 100 per cent in administered glycine.

acids, it was found that glycine contained 40 per cent and serine 20 per cent of the total isotopic nitrogen in the proteins from rat cytoplasm.

Experiments on hens. Table 3 and Fig. 2 give various values for ratios between N^{15} incorporation into the two amino acids glycine and serine (from proteins of different organs) on the one hand and the N^{15} incorporation into the polynucleotide purines guanine and adenine on the other. The specific ratios chosen were glycine / adenine, glycine / guanine, serine / adenine and serine / guanine. The figures demonstrate that when the amino acid shows an increase in N^{15} the purine compound does likewise.

Liver regeneration. Table 4 and Figs. 3, 4 and 5 summarize our findings in the experiments on regenerating liver. In the tables the ratio $\frac{E_{max}}{\gamma N/ml}$ is a test of purity with respect to freedom from foreign nitrogen [7]. Some of the values have been determined in duplicate (for separate animal groups) and in these cases the duplicate values have been recorded in the tables 3, 4 and 5.

It can be seen from the figures that the rate of synthesis of purines has a maximum at the 26-hour point (time of isotope administration 18—26 hours after hepatectomy). This maximum is especially high in the case of guanine from cell nuclei PNA [7]. The position of the maximum is the same for all the purines. We do not want to state that this would hold true if more frequent regeneration stages were measured in this region.

The curve for glycine represents the rate of synthesis of proteins; here the values are not as much above the "normal" incorporation of N^{15} in non-regenerating liver as in the case of the purines. The figures show a maximum situated beyond the purine peak at about the 56 hour point. The figures for N^{15} incorporation into the TCA-insoluble fractions from cytoplasm show the same maximum (See Table 5).

Table 4. Injection of glycine-N^{15} in rat. Regenerating liver.

Isolated substances	Cell nuclei				Cytoplasm	
	PNA Atom per cent excess N^{15}	E_{max} μg N/ml	DNA Atom per cent excess N^{15}	E_{max} μg N/ml	PNA Atom per cent excess N^{15}	E_{max} μg N/ml
			Regeneration during 11 hours			
Guanine	.782	.160	.053	.159	.283	.162
					.302	.155
Adenine	.488	.172	.006	.180	.165	.180
					.197	.175
Cytidine	.342	.285			.081	.287
	.476	.292				
Uridine	.534	.325				
	.589	.341			.155	.326
Thymine			.030	.270		
			.014	.264		
Cytosine			.020	—		
			.012	.223		
			Regeneration during 26 hours			
Guanine	1.722	.162	.641	.158	.637	.167
Adenine	(.868)	—	.209	.178	.251	.183
Cytidine	.503	.287			.224	.294
Uridine	.698	.340			.254	.332
Thymine			.085	.274		
Cytosine			—	—		
			Regeneration during 32 hours			
Guanine	1.441	.162	.171	.165	.587	.165
Adenine	.330	.178	.099	.169	.214	.180
Cytidine	.541	.291			.205	.308
Uridine	.665	.322			.228	.332
Thymine			.053	.264		
Cytosine			.078	.218		
			Regeneration during 56 hours			
Guanine	.833	.161	.146	.158	.279	.164
Adenine	.380	.180	.089	.172	.141	.182
Cytidine	.421	.288			.214	.291
Uridine	—				.143	.328

Table 4 continued.

	Regeneration during 170 hours					
Guanine	.225	.164	.071	.159	.086	—
	.344	.160			.083	.173
Adenine	.213	.174	.023	.176	.061	—
	.132	.175	.024	.179	.044	.183
Cytidine	.180	.290			.103	.293
					.053	.296
Uridine	.076	.332			.061	.329
					.115	.336
Thymine			.031	—		
			.012	.268		
Cytosine			.015	.231		

The curves for the pyrimidines are between glycine and the purines. For reasons outlined in the discussion we will not try to evaluate the pyrimidine values as a measure of polynucleotide synthesis.

The cytological findings are recorded in Fig. 6. This shows the changes in the optical cross-section area of the liver cell parts during regeneration. The measurements were made on 5μ sections stained with Ehrlich hematoxylin-eosin. The sections were made from pooled samples of the same livers as were used in the isotope experiments. The ratios between the nucleolar, nuclear and cytoplasmic areas were obtained by Chalkley's method for recording ratios of points indicated by ocular pointers in the plane of the microscope image [11,12]. Optics: Objective apochromat 90 x, num. ap. 1.35, ocular comp. 10 x. For each regeneration time a total of 2 500—3 000 loci were recorded.

The nuclear diameters were measured in 150—200 liver parenchymal cells at each regeneration stage, using an eye piece screw micrometer. By assuming

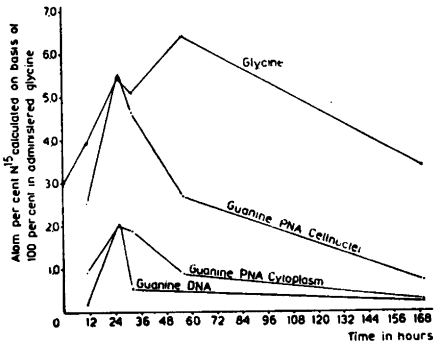
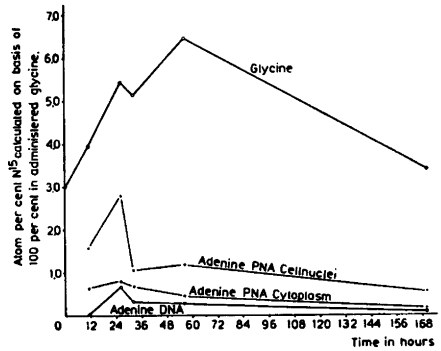


Fig. 3. The contents of N^{15} are calculated on basis of 100 per cent in administered glycine. The values for glycine (Table 5) are multiplied with 2/3 to reduce the height of the glycine curve.



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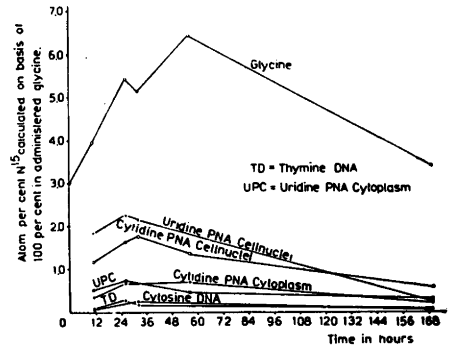

Fig. 5. The contents of N^{15} are calculated on basis of 100 per cent in administered glycine. The values for glycine (Table 5) are multiplied with 2/3 to reduce the height of the glycine curve.

Table 5. Injection of glycine N^{15} in rat. Regenerating liver.

Regeneration time in hours	Atom per cent excess N^{15}. The values are given in atom per cent N^{15} calculated on basis of 100 per cent in administered glycine.			
	Glycine	Serine	Hydrolysate of total protein	
			Cell nuclei	Cytoplasm
0	4.53	3.97	.741	.753
11	5.93	5.20	.757	.725
26	8.14	7.92	1.40	1.19
32	7.68	6.38	1.19	1.17
56	9.66	8.56	1.30	1.38
170	5.15	6.11	.712	.708

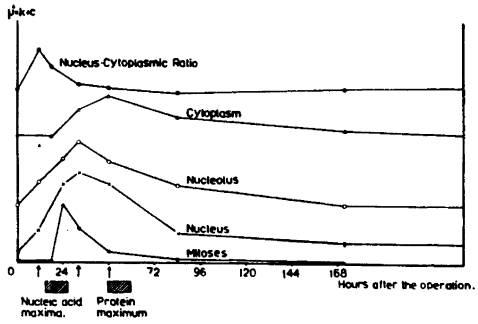

Fig. 6. Cytological changes during liver regeneration. To make data easily understood each curve is multiplied by an arbitrary factor and presented separately by the addition of a constant. The maximum values, expressed as percentage increase of the normal liver cell constituents, are as follows: Nucleolus 260, nucleus 160, cytoplasm 145 and nucleus-cytoplasmic ratio 130. The mitosis frequencies increased from less than 0.05 % in normal liver to 10 ± 0.32 % at 24 hours regeneration.

that the nuclei are spherical, the relative changes in the optical cross-section area of the cell parts can be easily calculated.

The mitosis frequencies were measured as a Poisson distribution using the above described optics. A total of 2 000 cells were counted at each regeneration stage.

The course of the cytological changes during liver regeneration, as illustrated in Fig. 6, does on the whole agree with the rather scanty earlier data [13]. It is interesting to note the coincidence of the maximum rate of polynucleotide synthesis with the maximum of mitosis frequency and with the maximum

Table 6.

Isotope	C^{13} Atom per cent excess	C^{14} Counts/min	N^{15} Atom per cent excess
I. Administered glycine	13.43	30 336	31.0
II. Isolated glycine	0.945	1 692	2.38
Excess in isolated glycine as % of administered glycine (Specific activity)	7.0	5.6	7.7

size of nuclei and nucleoli. Also it can be seen that during the period of maximum protein synthesis at regeneration times of 48—56 hours the above mentioned cytological quantities have already diminished. Further data on the liver cytology in relation to turnover values will be published elsewhere.

DISCUSSION

The investigation of the relationship between the turnover rates of two different cellular substances will naturally be dependent to a large extent on the precursor used. The ideal precursor should be directly incorporated into both of the actual substances without any intermediates, a situation which probably can be realized only in a very few cases.

In order to investigate to what degree glycine has been incorporated as an intact molecule, thrice-marked glycine was used in some experiments. The glycine contained C^{13} in the methyl-, C^{14} in the carboxyl- and N^{15} in the amino-group. The excess of isotopes was determined in a sample of glycine isolated from proteins.

The figures in Table 6 are the direct analytical values measured as excess of total carbon. No attempts have been made to degrade the glycine and determine the isotope figures in each carbon atom. The values show that the ratio between the excess of C^{13} and that of N^{15} is nearly the same in the glycine isolated from proteins as in the administered compound. The values for C^{14} show that the glycine incorporated into the protein molecule has undergone slight rearrangement involving rupture of carbon to carboxyl group. The analyses give no information, however, about a possible C^{13} content in the carboxyl group.

In any case these analyses show that the administered glycine has been directly incorporated into the proteins with only some slight intermediate turnover of the carboxyl group. The glycine can be regarded as fairly well distributed in the protein molecule, and we think that the isotope content of the glycine isolated from the proteins can be used as a measure of the protein turnover.

In the polynucleotides the incorporated glycine is probably situated mainly in position 4,5 and 7 of the purines. A great part of the glycine thus can be regarded as being a direct precursor for the purines. In the case of the pyrimidines, however, we know very little about the incorporation of glycine.

When glycine-N^{15} was used as tracer-precursor in rapidly regenerating bone marrow and non-regenerating liver in the same hen, a correlation could be observed between the isotope contents in guanine and adenine from the polynucleotides and the isotope contents in glycine from the proteins (Table

3, Fig. 2). Certainly the above-mentioned direct incorporation of glycine into both the purines and the proteins plays an important role in this correlation.

In spite of the wide deviations from a mean value we think that this correlation is not accidental but indicates a general tendency of simultaneous metabolic activity of polynucleotides and proteins. This connection might possibly signify a functional interaction of polynucleotide and protein turnover and should be considered along with the turnover rates found in the experiments on regenerating rat liver.

In these experiments the values in Table 4 and Figs. 3—5 show that the maximum turnover rates for all nitrogenous compounds in PNA in cell nuclei and in cytoplasm and in DNA appeared at about 30 hours after partial hepatectomy. The greatest increase in the amount of PNA and DNA per dry weight liver tissue appeared at the same time (Fig. 1), demonstrating the coincidence of high turnover rates with an increase of polynucleotides per dry weight of tissue.

The maximum turnover rate of glycine in the proteins appeared at approximately 30 hours later (60 hours after partial hepatectomy). All of the other amino acids also had a maximum at this point, indicating a common maximum of transamination. The regeneration curve and the curve for protein nitrogen (Fig. 1) demonstrate the well known fact that a protein formation takes place during regeneration. As we failed to determine the point of maximum regeneration during the eight-hour periods of isotope turnover, we can only state that glycine incorporation occured at a time when the total amount of proteins was increasing. We think that in a situation where a very rapid formation of proteins occurs this protein synthesis may have to be preceded by a rapid turnover and synthesis of polynucleotides. This would not necessarily exclude the possibility of some protein synthesis during stages of comparatively low turnover and synthesis of the polynucleotides if the concentration of polynucleotides was already sufficiently high.

The amount of food consumed during the time of regeneration may well influence the position of turnover maximum as indicated by some new experiments we have made. We are therefore continuing the investigation with controlled feeding starting four hours after the partial hepatectomy.

It has long been assumed that there is some fundamentally important interaction between polynucleotides in the cell structures and the synthesis of proteins. There seems to be no doubt about the rather sudden increase in the amounts of polynucleotides in phases of high growth activity. Chemical analysis — like those in the present work — and studies of absorption in the ultraviolet region of the spectrum, have clearly demonstrated the rapidity with which these changes take place [6]. The relationship of the protein synthesis to

these changes has, however, remained obscure. Attempts to correlate the rate of growth with the cellular polynucleotides by means of UV-absorption measurements, although very informative about the polynucleotide changes during different physiological conditions of the cell, cannot be considered conclusive with respect to the protein synthesis.

As has been pointed out, there is ample evidence that polynucleotides are synthetisized during stages of growth when proteins are formed. This was first shown for semen by Miescher in 1874, for embryonic liver by Masing in 1911 and more recently for rat liver regenerating after partial hepatectomy by Brues *et al.*, Novikoff and Potter [14-16] and others. Our experiments show a connection during growth between the turnover rates of polynucleotides and proteins. Furthermore they give a time schedule for these two synthetic processes. The linking mechanism, however, is quite unknown.

SUMMARY

Glycine marked with N^{15} was used as tracer-precursor in rapidly regenerating bone marrow and non-regenerating liver in the hen. A correlation could be demonstrated between the isotope contents in the glycine isolated from the proteins and the isotope contents in the purine bases from the polynucleotides.

Rat liver in different stages of regeneration after partial hepatectomy was analyzed for the content of N^{15} in the amino acids from the proteins and in the nitrogenous bases from the polynucleotides in cell nuclei and cytoplasm. Glycine labeled with N^{15} was again used as isotope precursor. Under the conditions of these experiments the maximum rate of polynucleotide turnover occured at about 30 hours after the operation and the maximum rate of protein turnover at about 60 hours.

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On the Distribution of Injected Radioactive Iron in Guinea Pigs and its Rate of Appearance in Some Hemoproteins and Ferritins

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Contents

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I. INTRODUCTION

During the last ten years knowledge about the metabolism of iron in mammals has been rapidly growing [1-3]. One of the reasons for this development is the improved knowledge about the individual iron-containing substances that are present in the body. Another reason is the availability of radioactive isotopes of iron. The pathway of iron in the mammalian organism can now be outlined [1]. For convenience it will be considered in its parts.

1. Absorption. It seems to be a well established fact that the mammalian organism will take up iron from the food only to the extent that there is a need for it in the body. Thus an increase in iron uptake occurs after bleeding and during pregnancy or rapid growth [4-12]. Granick [13] has shown that ferritin can be demonstrated in the intestinal mucosa a few hours after iron is given *per os* to an irondepleted animal. This rapid formation of ferritin seems to be demonstrable wherever iron is resorbed [13-18]. The mechanism of the formation of ferritin or its protein component, apoferritin, has not yet been elucidated.

2. Transport. Numerous investigations on the transport form of iron in the plasma have been published. However, since the experiments to be reported in this paper do not enter this problem, the question of the transport form of iron will be left aside. References are found in some monographs and reviews [19].

3. Storage. From a functional point of view the body iron can be divided into two groups: the physiologically active tissue iron and the storage iron. The hemoglobin iron occupies an interposition. Normally it acts as a tissue iron with a welldefined function, but in cases of need the blood is deprived of a part of its iron for the maintenance of a necessary level of the life essential parenchymal tissue iron. The stores are supplied from the breakdown of hemoglobin and other heme-containing substances plus — when there is a need for it — intestinal resorption.

A close relationship has been demonstrated between the content of iron in tissues and the occurrence of reticuloendothelial cells [20,21]. Accordingly, liver, spleen, and bone marrow constitute the main stores of iron [22-24]. The endocellular storage iron in these organs, "hemosiderin", is very probably ferritin. Several investigators [25,26] have arrived at the conclusion that iron recently deposited in the stores is utilized earlier than iron which has been stored for longer time, independently of origin. This means that when tagged iron is given to an animal, it is not mixed with the total stored iron but rather with only "the surface" layer.

Ferritin is found in most other organs [27]. Intraperitoneally injected hemoglobin is, to a certain extent, broken down and stored as ferritin in the kidneys [15,16].

4. Utilization. Iron is taken from the stores for the building-up of heme-pigments. When a red cell is "worn out", the iron is liberated and pooled in the stores. From this state it is re-utilized for the synthesis of new hemoglobin [26] and probably also other hemoproteins.

5. Excretion. Normally the body seems to have little or no ability to excrete iron [8, 28–32]. Loss of iron under pathological conditions occurs — in addition to losses by bleeding — *e. g.* during myoglobinuria after electrical shock [33] or blast injuries [34]. Parenterally administered cytochrome c is partly excreted via the kidneys [17, 18, 35, 36,]. There is also a small excretion of iron into the urine and the intestinal lumen during the first days following a parenteral administration of inorganic iron [4, 8, 32].

From this pattern it is obvious that the body tends to keep itself in iron balance by avoiding losses and by taking up iron only when there is a need for it. Thus, it is clear that sooner or later all iron-containing substances in the organism must come to an equilibrium with each other as regards the iron, so that if radioactive iron is administered to a normal animal in iron balancy, all organs or iron compounds that can be isolated must finally arrive at the same specific activity.

Most animal experiments with radioactive iron have aimed at a study of the distribution of the supplied isotope or to follow a certain compound, *e. g.* the fate of the iron which is liberated upon the breakdown of hemoglobin or the catabolic processes to which parenterally administered cytochrome c iron is subjected. The animals have often been bled before the experiments, kept on an iron-restricted diet or pre-treated in some other way in order to facilitate and accelerate the changes to be studied. In this paper the aim is to study the distribution of administered iron in the body and the rate of its incorporation into the different pigments under *conditions as normal as possible.* For that reason the animals were kept on a normal diet and their iron nutritional state, as indicated by the hemoglobin level, determined previously to the injections. We also used as small doses of iron as possible to avoid deviations from the normal state. Since animals in iron-balance only absorb tiny amounts of iron from the gut, parenteral administration by intraperitoneal injections had to be used. For reasons of convenience intraperitoneal were preferred to intravenous injections.

In addition to a general, detailed study of the distribution and incorporation of iron, there are some specific questions which this investigation was intended to answer.

The incorporation of iron into erythrocytes in anemia has been studied in man [37–39], dog [6, 38, 40], rat [40, 41], rabbit [42], and guinea pig [40], and during normal conditions in man [37–39], rat [12, 41], and dog [38]. Therefore it was of interest to

follow the radioactivity in the red cells of normal guinea pigs. Moreover it was desirable for our purposes to know the specific activity of the blood iron since with our technic considerable amounts of blood were found to remain in the organs after the post mortal perfusion. As will be seen in the sequel, the specific activities of the myoglobin preparations could be obtained only after corrections for the contaminating hemoglobin. Finally we wanted to compare the rates of incorporation of radioiron into hemoglobin and blood catalase.

In the case of catalase there was another interesting problem. Bonnichsen [43] had found that the protein components of blood and liver catalases in horse very probably were identical. It may also be recalled that while blood catalase contains four protohematins per molecule [44,45], it has never, except in the case of guinea pig liver catalase, been possible to obtain a liver catalase preparation with more than 3.6 protohematins per molecule [46–48]. Less hematin is frequently found. The liver catalases, however, sometimes contain bile pigment, which brings up the total number of tetrapyrrolic compounds per molecule to four [46]. These observations raised the question of whether the blood and liver catalases are formed independently of each other or whether catalase is synthesized exclusively together with the red blood cells and secondarily deposited and broken down in the liver [48].

Very few direct determinations seem to have been made to find out the life length or rate of formation of any one of the parenchymal hemoproteins, either as regards the complete molecule or a part thereof, *e. g.* the iron atom or the porphyrin nitrogen atoms. There are, however, some observations, which can give an idea about the order of magnitude of these properties. Greenstein *et al.* [49] and others [50] extended earlier studies [51] on the behaviour of some respiratory pigments in cancer-bearing animals. The liver catalase activity was found to decrease considerably within a short time. However, the manometric technic is unsuitable for the assay of catalase activity [52]. Moreover, the very high hydrogen peroxide concentrations employed in the experiments would very likely destroy hemo-proteins. Again, *if* there is a cancer induced decrease in catalase activity in tumor-bearing animals, it can be explained in several ways: a hampered synthesis, or an accelerated disintegration of catalase, or both. The influence of some inhibitory agent, emanating from the tumor, could also be considered. In fact, the existence of a catalase inhibitor in tumor tissue has recently been claimed [53], but the results have not yet been confirmed. As no information whatsoever is available concerning the normal life time of the catalase molecule, we considered it necessary to investigate this problem in order to obtain a basis for a discussion of pathological conditions.

Not very much seems to be known about the rate of incorporation of iron into cytochrome c in normal animals [54]. By feeding radioiron to anemic,

growing animals cytochrome c with as much as 80 % specific activity of the isotope has been obtained [18]. This highly active material was one of the tools used to demonstrate definitely that parenterally administered cytochrome c is not utilized unchanged as a respiratory catalyst by the cells.

Still less has been done in the case of myoglobin. A few suggestions on the rate of incorporation of isotopic iron have been reported; however, no details were given [54]. It can be presupposed from the remarkable independency of the myoglobin content of muscles in anemia, muscular inactivity, and infectious diseases [55] that its turnover rate could be expected to be low.

These problems also determined what species should be used for our experiments. We considered it to be preferable to take several small rather than one big animal for each experiment in order to nullify individual variations. In our preliminary series of experiments it proved difficult to prepare pure catalase from rat livers. The preparation of catalase from rat blood, where the hemoglobin crystals adsorbed the catalase very firmly, was still more difficult. From guinea pig liver pure catalase can be prepared easily and rapidly, and the use of this species also involves the advantage that all the catalase iron occurs in protohematin [46]. These facts were considered to be favorable enough to counterbalance the difficulty raised by the low myoglobin content of guinea pig muscles and the fact that the available guinea pigs, weighing 500—1 000 g, were not adult and thus not strictly in iron balance. A certain increase in weight with formation of new tissue thus occurred during the experiment period. This fact had to be taken into consideration for the compounds with comparatively slow formation.

II. EXPERIMENTS

A. The animals

Male and non-pregnant female guinea pigs weighing 500—1 000 g were kept on a daily diet of oats, turnips and hay and fed with carrots once a week. During a preliminary period of 2—4 months the blood hemoglobin was checked in samples taken by heart puncture from every tenth animal, chosen at random.

Series no. 1 showed the average value of 14.5 ± 1.2 g * hemoglobin per 100 ml blood before the injection. The animals in series 2 were not examined for their hemoglobin level immediately before the injections; from table 12 it is obvious, however, that they were not iron-deficient. Series 3 was controlled with respect to hemoglobin and number of red and white cells. The results two days before the injection of radioactive iron were:

Hemoglobin	15.7 ± 0.4 g/100 ml blood
Red cells	8.69 ± 0.27 millions/mm^3
White cells	18.000 ± 4.000 per mm^3

* In this paper numerical values are given with their probable errors.

It was concluded that the animals were healthy and not deficient in iron. We are indepted to Dr S. Paléus for carrying out the blood controls.

The injection of the radioactive ferric ammonium citrate was made intraperitoneally in the mornings. The dosage was 0.05 mg Fe per 100 g body weight. It did not cause any trouble to the animals.

After the time given in the tables about 15 animals for each experiment were killed by a blow on the neck. The aorta was opened below the heart, the blood collected and the animal perfused with saline (about 1 liter in 15—20 min) through the distal end until the liquid flew almost colorless from the proximal opening. The perfusion fluid was collected and the hemoglobin determined.

The livers were homogenized; in series 1 and 2 a small aliquot was taken for iron and activity determinations, another for ferritin preparation, and the main part for preparing catalase. The spleens were taken separately for the preparation of spleen ferritin.

About one tenth of the musculature was dissected from the hind legs, which were more effectively freed of hemoglobin by the perfusion than the fore leg muscles, and used for myoglobin preparation ("myoglobin muscle"). The rest of the musculature ("cytochrome muscle") was liberated together with the skeleton from the skin and the other organs ("rest of animals") and used for the preparation of cytochrome c. Various methods were tried to grind or homogenize the rests of the animals, but without success. Since it was inconvenient for technical reasons to hydrolyze all the 14—16 rests in each experiment, three of them were chosen at random for analysis. The result was taken as representing the whole group. In the series 3 only liver ferritin and catalase, hemoglobin, blood catalase and cytochrome c were investigated.

B. Preparation methods

a. Myoglobin

The hind leg muscles of 14—16 guinea-pigs, forming one group, were cut off and weighed. The amount of pooled muscle tissue for myoglobin preparation weighed 370 ± 24 g. An equal amount of distilled water was added and the mixture homogenized immediately. The homogenate was weighed and about 2 % of it was taken for iron and activity determinations. The rest was put in the cold room and left for 2 days. After 2 days it was centrifuged, the insoluble residue discarded, the extract neutralised, and 0.25 volumes of basic lead acetate (20 %) was added. The precipitate was centrifuged off, and the excess lead removed from the supernatant by the addition of disodiumphosphate until no further precipitate was formed. The pH was maintained at neutrality during this operation [56]. The precipitate was centrifuged off and the supernatant was dialysed in the cold against 70 % neutralised ammonium sulfate. The precipitate formed was centrifuged off and discarded. The supernatant was dialysed against 80 % saturated, neutral ammonium sulfate solution in the cold. The precipitate was centrifuged off and dissolved in distilled water. This precipitate was labelled "70—80". The supernatant was dialysed against 90 % saturated neutral ammonium sulfate in the cold. The precipitate was centrifuged off and dissolved in distilled water (fraction "80—90 %"). The supernatant was dialysed against saturated, neutral ammonium sulfate in the cold and the precipitate treated as above (fraction "90—100 %"). A sample of each of the three fractions (70—80, 80—90, 90—100 %) was suitably diluted with *M*/50 phosphate buffer, pH 7 and CO from the gas-tap bubbled through them for 2—3 minutes. Solid sodium dithionite was then added and the bubbling continued for a further 5 minutes. The

solution was then transferred to a 1 cm Beckman cuvette, the cuvette was covered and readings taken immediately.

Acid acetone (1 ml concentrated hydrochloric acid per 100 ml acetone) was added to the dissolved precipitate in a quantity of about 4 times their volume. The precipitate was filtered or centrifuged off and the acetone, containing the dissolved hemin, evaporated on a water bath. After the acetone had evaporated, the precipitated hemin was filtered off on a glass filter. Both the original vessel and the filter were washed three times with *M* HCl to remove foreign iron. The precipitated hemin was then dissolved from the walls of the vessel and the glass filter with *M*/10 NaOH. This solution of hemin in *M*/10 NaOH was taken for Fe analysis and plating.

The specific activities of the different myoglobin fractions are given in Tables 11 and 14 under the heading "uncorrected" and of hemoglobin in Tables 9 and 12.

In some cases, when centrifugation was incomplete, some of the precipitate remained in the supernatant after 100 % ammonium sulfate saturation. It was found that even in this case the hemin could be isolated by treatment with HCl-acetone and subsequent evaporation as described. In these cases it was treated as a separate fraction and labelled "> 100 %".

As can be seen from Tables 11 and 14 under the heading "uncorrected" the specific activity decreased with increasing ammonium sulfate saturation. This would be expected if the precipitated myoglobin still contained some contaminating hemoglobin. Since in ammonium sulfate fractionation hemoglobin precipitates before myoglobin, the quantity of the former should decrease as the concentration of ammonium sulfate increases. On the other hand, if the quantity of hemoglobin in the precipitates 70—80 %, 80—90 %, 80—100 % were known and also the amount of radioactive iron it contained, and this value subtracted from the figures in Table 7 and under the heading "uncorrected", then all fractions should give similar values, *i. e.* the content of radioactive iron in pure myoglobin. In order to arrive at this figure, the relative hemoglobin and myoglobin contents of the different fractions had to be determined. The first aim was to get pure, hemoglobinfree myoglobin from guinea-pig muscle. This was obtained by re-dissolving and re-precipitating the fraction coming out between 90—100 % ammonium sulfate saturation. Because of the small amount of material, crystallisation was not attempted. Different absorption spectra of this re-precipitated fraction were taken and all data obtained agreed with those given in the literature for crystalline myoglobin. The absorption peaks of the CO-compound in the visible were at 579 and 542 mμ, the Soret band at 423 mμ. The absorption peak of the ferromyoglobin was found at 434 mμ and after re-oxygenation at 412 mμ. The numerical value of the light absorption coefficient at the absorption maximum after re-oxygenation was 105 $\pm$ 3 % of that of the reduced compound. The hematin content was determined as pyridine-hemochromogen. Table 1 gives the extinction coefficients, ε, of the CO-compounds of 6 such preparations, calculated for a 10^{-3} molar solution in 1-cm cuvettes. It can of course not be proven that these preparations were absolutely free from hemoglobin. As a matter of fact it has never been proved beyond doubt that even crystalline myoglobin preparations so far obtained from different species have been absolutely free from hemoglobin. However, as all data obtained with our preparations agree fairly well with those given for crystalline myoglobin, it seems reasonable to suppose that, as far as evidence at present suggests, hemoglobin can only be present, if at all, in very small quantities.

Assuming the absorption coefficients, recorded in Table 1, to be those of pure CO-myoglobin, the quantity of hemoglobin and myoglobin in a given sample with known CO-

Table 1. Extinction coefficients of guinea pig Co-myoglobin and Co-hemoglobin. Molarity of protohemin (determined as pyridine hemochrome) = 10^{-3}. d = 1 cm. The values of ε for Co-myoglobin are means of determinations on six different preparations, those for Co-hemoglobin means of three different samples.

Wave length mμ	CO-myoglobin ε	CO-hemoglobin ε
590	5.68 ± .65	2.99 ± .08
584	10.38 ± .16	5.80 ± .03
580	12.50 ± .15	8.75 ± .02
578	12.66 ± .23	—
576	12.36 ± .22	12.09 ± .03
574	12.15 ± .13	13.24 ± .08
572	11.84 ± .08	14.20 ± .04
570	11.70 ± .11	14.68 ± .01
568	—	14.76 ± .01
566	11.07 ± .06	14.52 ± .01
562	10.60 ± .03	13.19 ± .03
558	10.71 ± .13	12.17 ± .04
554	12.07 ± .30	11.77 ± .12
550	13.66 ± .30	12.55 ± .03
546	14.52 ± .05	13.79 ± .02
544	14.70 ± .09	14.44 ± .09
542	14.78 ± .15	14.84 ± .03
540	14.54 ± .20	15.13 ± .05
538	—	15.04 ± .05
536	—	14.86 ± .08

spectrum can be calculated. This procedure is essentially the same as the one employed by de Duve [57] for the simultaneous determination of myoglobin and hemoglobin. Because of the greater error inherent in the present determinations (owing to the material not being crystalline) the contents of myoglobin and hemoglobin were determined at several wavelengths and from these average values were taken.

Three isosbestic points were found in the spectra of the CO compounds of myoglobin and hemoglobin; at 575.7, 554.5 and 542.3 mμ. The hemin content of the fraction in question was calculated from the absorptions at the three points and an average of these values was used for further calculations. The hemin content by the pyridine hemochromogen method was determined in a few cases only, because of the scarcity of the material and the ammonium sulfate content of the fractions. In the few analyses performed, the values found agreed well with those calculated as above.

To make our procedure clear, the complete record of one experiment is included in extenso. The following account is for a group of 15 guinea-pigs killed 3 weeks after the injection of radioactive iron (expt. nr 27). The muscle extract was treated and fractionated as described. The spectra of the CO compounds of the redissolved fractions "70—80",

"80—90" and "90—100 %" were taken. The optical densities of the three fractions at the isosbestic points, as read in the Beckman spectrophotometer, were:

Table 2.

Isosbestic point mμ	Optical density of fractions "70—80"	"80—90"	"90—100"
575.7	0.365	0.404	0.568
554.5	0.3465	0.366	0.495
542.3	0.434	0.473	0.660

The optical densities of both CO-hemoglobin and CO-myoglobin at the isosbestic points in a 10^{-3} molar concentration are:

Table 3.

Isosbestic point mμ	ε
575.7	12.30
554.5	11.86
542.3	14.78

From these values the hemin content of the above mentioned three fractions was calculated.

Table 4. Hemin content calculated from the absorption of the CO compounds at the 3 isosbestic points.

Calculated from mμ	Hemin content of fractions "70—80"	"80—90"	"90—100"
575.7	19.3	21.4	30.1
554.5	19.0	20.1	27.2
542.3	19.1	20.8	29.1
Average μg hemin per ml	19.1	20.8	28.8

Since the spectra of the CO compounds of the above fractions were determined and their hemin content known from the above calculations, their absorption coefficients for a 10^{-3} molar solution could be calculated for different wave-lengths. Five wave-lengths, 580, 572, 570, 566 and 562 mμ were chosen, because the absorption coefficients of CO hemoglobin and CO myoglobin differ greatly at these wave-lengths. From the CO spectra and the hemin content, the absorption coefficients at the above wave lengths in this particular experiment were found to be:

Table 5.

Wave length mμ	Absorption coefficient for a 10^{-3} molar solution of fractions "70—80"	"80—90"	"90—100"
580	10.68	11.37	12.53
572	13.23	12.58	12.06
570	13.34	12.53	11.83
566	12.82	12.16	11.32
562	11.94	11.42	10.53

The absorption coefficients for CO-myoglobin and CO-hemoglobin are given in Table 1. From this table and the absorption coefficients of the three fractions given above, the myoglobin and hemoglobin content of the three fractions was calculated.

Table 6.

Calculated from extinction value at mμ	Percentage myoglobin content of fractions "70—80"	"80—90"	"90—100"
580	51.5	70.0	101.0
572	41.0	68.5	91.0
570	45.0	72.0	96.0
566	49.0	68.0	92.5
562	48.5	68.5	107.0
Average	47.0	69.5	97.5

This means that fraction "70—80" contained 53 %, fraction "80—90" 30.5 % and fraction "90—100" 2.5 % hemoglobin.

From Table 7 it can be seen that the specific activities of these three fractions were:

0.155 % for fraction "70—80"
0.1045 % for fraction "80—90"
0.0519 % for fraction "90—100"

The hemoglobin prepared from these animals showed a specific activity of 0.2085 %.

Thus if fraction "70—80" contained 53 % hemoglobin, the activity corresponding to this amount of hemoglobin has to be subtracted from the value actually found for this fraction. If we carry out these subtractions for all the three fractions, we come to the following results:

Table 7.

	Fractions		
	"70—80"	"80—90"	"90—100"
Spec. activity of preparation	0.155 %	0.1045 %	0.0519 %
Spec. activity due to hemoglobin	0.110	0.0635	0.0052
Spec. activity due to myoglobin	0.045	0.041	0.047

As can be seen, the three fractions give comparable activities, as they naturally should if they contained only myoglobin and no hemoglobin. The average value of the three fractions: 0.044 is taken to be the specific activity of the iron in myoglobin. As stated, the absolute purity of the fractions after this subtraction is still questionable. However, it seems reasonable to suppose that they contain very little, if any, hemoglobin. The values corrected in the way described here are found in Tables 11 and 14 in the column "corrected". It can be seen that the values for the three fractions lie much closer to each other than they did before the corrections. The agreement is in some cases very good. In some, however, the differences are still fairly pronounced.

b. Cytochrome c

In some preliminary experiments we tried to dissect the muscles from the skeletons. This procedure was, however, found to be tedious, and considerable losses could not be avoided in the experiments to be described in this paper. Therefore the dissection was omitted. The skeletons with the muscles *in situ* (except the hind leg muscles which were taken for the preparation of myoglobin) together with the hearts were thus used as sources for the cytochrome preparations. The material was homogenized and worked up according to the method of Keilin and Hartree [58], slightly modified. In guinea pigs extraction with sulfuric acid gave much higher yield than with trichloroacetic acid. The homogenates were extracted overnight with 2.5 volumes of 0.15 *N* sulfuric acid, neutralized, centrifuged, and the residues re-extracted for two hours with two volumes of 0.2 *N* sulfuric acid. To the combined extracts were added 500 g of ammonium sulfate per liter. Next day the inert material was filtered off and the cytochrome c precipitated from the filtrate with 1/40 of its volume of 20 % trichloroacetic acid. This crude cytochrome c was dialyzed against 0.1 % ammonia until the sulfate reaction became negative. At this stage the iron content was 0.25—0.30 %, which, however, included some non-cytochrome iron. The following procedure was therefore employed for the specific isolation of the cytochrome c iron.

The cytochrome c was precipitated from its aqueous solution by the addition of acid acetone (1 ml 5 *N* sulphuric acid per 100 ml acetone), centrifuged, and the supernatant examined spectrophotometrically for the presence of a Soret band. The precipitate was dissolved in water and re-precipitated, the procedure being repeated until the supernatant became free from any specific absorption in the region 380—420 mμ. Five washings were found to be necessary. The final precipitate was dissolved in 20—50 ml of a silver sulfate

Table 8.

Mixture	Pipetted of stock solns.			Mixture contained		Yield of Fe		Net c. p. m.	c. p. m. per
no.	Hemoglob. ml	Ferritin ml	Cytochrome ml	Fe μg	c. p. m.	μg	% of cytochrome Fe		μg isolated Fe
I	1.0	1.0	5.0	484.9	3 773	24.8	53.3	2.18	0.088
II	1.0	1.0	2.5	461.7	3 773	16.8	72.1	1.45	0.086
III	0.5	0.1	5.0	162.5	989	34.6	74.4	1.96	0.057

solution (800 mg silver sulfate per 100 ml solution). Next, 0.2 volumes of glacial acetic acid were added and the mixture digested for 90 minutes at 70° [59]. The protein residue was then precipitated with 5 volumes of ice cold acid acetone and filtered off. The acetone solution was cautiously evaporated in a current of air with slight warming. To the remaining water-acetic acid solution 10 ml of butanol were added. The hemin solution was washed in a separatory funnel twice with water, twice with 0.1 *M* HCl, and twice again with water, each time with twofold volume. A small amount of the hemin usually went into the hydrochloric acid, from which it could be re-extracted with ether. Sometimes a small precipitate appeared at the alcohol-water interphase. The washed butanol solution was therefore always filtered down into a combustion tube. The butanol was evaporated and the remaining hemin combusted with sulfuric acid in the ordinary way.

The specificity of this isolation procedure was tested on the following mixtures, from which the cytochrome iron was isolated as described above.

Stock solutions: Cytochrome c: Cow heart cytochrome c, prepared according to Keilin and Hartree [60] and with an iron content of 0.41 %, was made up to a solution containing 9.3 μg iron per ml. This cytochrome c iron had no radioactivity.

Hemoglobin: Guinea pig blood from animals, previously injected with radioactive ferric ammonium citrate, was hemolyzed with water. The solution contained 180.4 μg iron per ml with 8.48 c. p. m. per μg iron.

Ferritin: Radioactive ferritin from guinea pig liver was isolated as described in this paper. The material had an iron content of 258.0 μg per ml with 8.62 c. p. m. per μg. iron.

The artificial mixtures, to which the isolation procedure was applied, were made up and analyzed as follows (Table 8). The isolated iron was thus about 100 times less active than either of the radioactive compounds in the mixture. Since no Soret band was detectable in the acetone washings, the activity could hardly derive from the hemoglobin. The purification of the cytochrome c hemin is based on its distribution between the alcohol and water phases, and the degree of contamination therefore depends on the starting material. In the crude cytochrome c preparations the ratios between the cytochrome and the non-cytochrome iron could hardly be as unfavorable as in the artificial mixtures. It must also, however, be taken into consideration that the bone marrow may contain some compounds that follow the cytochrome c hemin closely during the isolation procedure. Nothing is known, for instance, about the mode of linkage between the prosthetic group and the protein part in verdoperoxidase, except that no splitting will occur with acid acetone. The data obtained in table 8 were considered to give satisfactory evidence in favour of the efficiency of our isolation procedure for cytochrome c iron.

c. Liver catalase

The livers (280—375 g) were ground in a Turmix blender with twice their weight of water and the insoluble residue centrifuged down. The extract was shaken with one tenth of its volume of an ethanol-chloroform mixture (5 : 1) and clarified by centrifugation. The catalase was precipitated from the supernatant by the addition of ethanol to 70 % by volume at room temperature, centrifuged down, immediately dissolved in distilled water and dialyzed against water in order to remove the rest of the ethanol. Dilute acetic acid was added to give a pH of 4.0 and a precipitate removed by centrifugation. The volume of the supernatant which contained all of the catalase was now 20—30 ml; one and a half volumes of saturated ammonium sulfate solution was added (60 % saturation) and the precipitated catalase centrifuged down, dissolved in a few ml of water and dialyzed a few hours to remove most of the ammonium sulfate. The solution was checked spectroscopically for the absence of hemoglobin.

A tenfold volume of acid acetone (1 ml of conc. HCl in 1 000 ml of acetone) was added to the catalase solution. After a few minutes stirring the precipitated catalase protein was centrifuged down. The acetone was now evaporated by keeping the solution in a water bath at about 40° and blowing air on the surface. The hemin then precipitated out from the acid water phase. It was collected in a small centrifuge tube, washed several times with n/l HCl and water, rinsed over to a small glass filter, dissolved in a little n/10 NaOH and sucked off. The hematin solution was combusted with 1 ml of concentrated sulfuric acid, and aliquots taken for iron determination and electroplating.

Isotopic control of the purification procedure for liver catalase

1. 10 mg of Fe^{59} (3.7 million c. p. m.) were mixed in the blender with 192 g of guinea pig liver, and catalase iron was isolated according to the above described procedure. It was completely inactive.

2. 25 mg of ferritin (= 1.8 million c. p. m.) were mixed in the blender with 192 g of liver. The isolated catalase iron was inactive.

3. A guinea pig liver catalase solution was dialyzed against a solution of Fe^{59} — ammonium citrate solution over night. The isolated catalase hemin iron was inactive.

4. 1.2 mg of protohemin and 1.87 mg of Fe^{59} (0.69 million- c. p. m.) were dissolved in acetone-HCl, the acetone removed and the hemin isolated according to the above procedure. The hemin iron was inactive.

These experiments indicate that the isolation procedure for liver catalase was very satisfactory.

d. Preparation of ferritin from liver and spleen

The livers or the spleens were ground and extracted with twice their weight of phosphate buffer m/50, pH 6.8, centrifuged and the residue washed once with the same amount of buffer. The extract was diluted with water to four times its volume and heated to 80° for one minute. A large precipitate was removed by centrifugation and the supernatant saturated to 70 % with ammonium sulfate. The precipitate, containing all of the ferritin, was dissolved in water and re-precipitated with ammonium sulfate to 0.4 saturation. The precipitate contained almost pure ferritin and was taken for analysis.

e. Preparation of blood catalase

The washed, red blood cells were hemolyzed by the addition of a two-fold volume of water and left over night at + 4°. The hemoglobin was denatured by the Tsuchihashi procedure: per 100 ml of hemolyzate 10 ml of ethanol-chloroform 3 : 1 were added and the mixture shaken. The denatured hemoglobin was removed by centrifugation and the clear supernatant evaporated in vacuo to a small volume (15—20 ml).

The absence of hemoglobin was checked spectroscopically. The catalase was precipitated by ammonium sulfate to 0.6 saturation and the precipitate dialyzed against water. The hemin was split off from the protein component by acetone-HCl and the former treated as described above for the liver catalase.

C. Technical procedure for activity determination

a. Combustion of samples

The standard procedure was as follows: Aliquots or the whole material were heated on a free flame in glass tubes or flasks with 1 to 50 ml concentrated sulfuric acid. A few glass beads prevented excessive bumping. When the volatile solvents had evaporated, the heating was continued with addition of known, small amounts of perhydrol at suitable intervals. The combustion was regarded as complete when no brownish color reappeared within 15 minutes after the last addition of hydrogen peroxide. Since ferric sulfate seems to be highly insoluble in concentrated sulfuric acid, one volume of water was added and the solution boiled for another few seconds. The sample was then rinsed over with distilled water to a graduate flask or cylinder, and aliquots taken for iron determination and electroplating. If possible, two or three determinations were made on each sample in addition to the blank. The blank values generally amounted to 1.0—1.4 μg in a final volume of 10 ml, the major part of the iron in the blank values deriving from the sulfosalisylic acid.

b. The iron determinations

were made essentially according to the sulfosalisylic acid method by Lorber [61]. The extinction coefficient, $\log I_0/I$ in 1 cm layer is = 0.100 at 424 mμ for 1 μg of Fe^{+++} per ml, if 0.1 ml of 20 % sulfosalisylic acid solution is used for every 5 ml of the final volume.

c. Preparation of the samples for plating

In the case of the samples from cytochrome c hemin, hydrogen sulfide was passed into the acid solution in order to precipitate the silver ions remaining from the splitting. The silver sulfide was centrifuged off and washed with water; the washings were pooled with the sample. If the washing of the butanol solution had been properly made, no silver sulfide could generally be detected.

The following procedure was the same in all cases: 0.500 mg carrier-Fe was added as ferric ammonium sulfate, concentrated ammonia was added in excess and hydrogen sulfide bubbled through. When very little material was available, as for instance sometimes blood catalase iron, we saved the samples used for the iron determination by adding carrier-Fe and precipitating with hydrogen sulfide. The iron sulfide was centrifuged down and washed three times with distilled water. It was then dissolved in two drops of 2 *N* hydrochloric acid and the tube kept on a boiling water bath under a pressure air current. During this time one drop of 2 *N* HCl was added four times in order to ensure the com-

plete removal of hydrogen sulfide. The dry residue was dissolved in 1 ml of saturated ammonium oxalate solution and one drop of methyl red solution added as indicator for pH-control during the electroplating. In some samples, especially those containing the bones, a precipitate of calcium oxalate appeared and had to be removed after three hours by centrifugation and washing of the precipitate with saturated ammonium oxalate. This procedure is essential, since the calcium oxalate seriously interferes with the plating.

The samples were transferred to the plating vessels by rinsing with saturated ammonium oxalate solution into the final volume of around 5 ml. The plating vessels were designed essentially according to Vosburgh *et al.* [62], but had considerably smaller dimensions, inner diameter 10 mm, height 50 mm.

d. Plating

50 m ampères during 2.5 hours was found to be sufficient for nearly complete plating on the central area (diameter = 10 mm) of the copper discs. The 0.5 mg of iron on this area does not give any measurable self-absorption [62] of the radiation of Fe^{59} or Fe^{55}.

The completeness of the plating was checked in all cases either by quantitative iron determinations in the solutions after plating and in the washings from the sulfide precipitate, or by determining the iron that was dissolved by hydrochloric acid from the copper discs after the counting. Correction for lost iron was made, if necessary. It never exceeded 50 μg Fe, and was generally only a few μg.

e. Counting

We used small bell-shaped Geiger-Müller counting chambers with mica windows 0.9—1.5 mg/cm^2, 27 mm diameter, as described by Hevesy [2]. They were filled with alcohol (10 mm pressure) + argon (90 mm).

Two recording circuits were used during the course of this investigation. One was built by AB. LKB-produkter, Stockholm, Sweden (Scale of 64); the other was constructed by Mr. K. O. Särnesjö in this institute (Scale of 16).

The background was 2.0—2.9 c. p. m.

In general at least 1 000 counts per sample were taken, for very weak samples only 500. The probable error was calculated according to the usual formula

$$K = \left[\left(\frac{2/3\sqrt{N}}{T}\right)^2 + \left(\frac{2/3\sqrt{n}}{t}\right)^2\right]^{\frac{1}{2}}$$

$$E\,\% = 100 \times \frac{K}{\text{net count}}$$

where N = total counts of sample in time T
n = background counts in time t

D. Radioactive material

The series 1 and 2 were injected respectively on May 7 and July 1, 1949, with ferric ammonium citrate prepared from a sample of Fe^{59} + Fe^{55} from A. E. C., Oakridge, Tenn. It was stated to have shown 0.0054 mc/mg of Fe^{59} (April 13, 1949) and 0.0086 mc/mg Fe^{55}. The series 3 was injected on February 20, 1950, with a mixture of 97 % of this

Table 9. Distribution of iron

Expt. no.	A = 19 B = 20				A = 22 B = 23			
Days	7				14			
	Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotope Fe	Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotope Fe
Liver A	330	28.4	3.32	22.9	356	23.9	2.93	17.0
Liver B	280	20.5	3.57	17.7	419	26.6	2.38	15.3
Spleen A	10	3.8	0.23	0.2	8	3.3	0.25	0.2
Spleen B	8	2.8	0.23	0.2	12	3.9	0.27	0.3
Red blood cells A	250	127.6	0.16	4.8	201	102.6	0.33	8.2
Red blood cells B	208	106.1	0.13	3.4	188	95.7	0.38	8.8
Muscle for cytochrome A	2 900	259	0.09	5.7	2 550	88.5	0.17	3.6
Muscle for cytochrome B	2 700	270	0.09	5.9	2 750	85.6	0.24	5.0
Muscle for myoglobin A	305	4.8	0.19	0.2	265	4.5	0.21	0.2
Muscle for myoglobin B	297	4.1	0.17	0.2	344	5.0	0.19	0.2
Rest A	6 290	352	0.25	21.3	6 070	191	0.25	11.6
Rest B	5 100	160.5	0.37	14.4	5 440	224	0.13	7.0
Total A	10 085	775.6		55.1	9 550	413.8		40.8
Per animal	672	51.7			637	27.6		
Number of animals	A = 15 B = 15				A = 15 B = 15			
Total B	8 593	564.0		41.8	9 153	440.8		36.6
Per animal	573	37.6			610	29.4		

iron and 3 % of a highly potent Fe^{55} (0.4635 mc/mg, April 14, 1949). Our Geiger-Müller chamber could be determined from these data to count 15—25 % of the total radiation of Fe^{59} and 0.8 % of the radiation of Fe^{55}. In the series 1 and 2 about 98 % of the counts derived from Fe^{59} and the remainder from Fe^{55}; in the series 3, on the contrary, 10 % came from Fe^{59} and 90 % from Fe^{55} at the time the samples were counted.

For each series standard discs with suitable amounts of radioactive iron of the same mixture as was used for the injections were prepared. These discs were preserved until the series had been finished. Thus alterations in the counting equipment could be disclosed and corrected. Since our radioactive material consisted of a mixture of two isotopes, the decay of the radioactivity was corrected for by comparisons with the standard discs, and not by calculations from the half-lives of the isotopes.

III. DISCUSSION

A. Errors

Some of the data given in the Tables 4 and 15 may necessitate a comment on some possible source of errors.

and radioactivity (series 1).

A = 26 B = 27 21				A = 24 B = 25 28				A = 28 B = 29 35			
Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotope Fe	Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotope Fe	Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotope Fe
310	24.2	1.53	9.0	365	29.8	0.95	6.7	318	30.2	1.24	9.2
350	31.2	0.97	7.4	390	33.6	1.80	13.7	343	—	—	—
13	3.9	0.20	0.2	17	6.4	0.49	0.8	9	4.1	0.22	0.2
12	4.6	0.17	0.2	11	4.3	0.22	0.2	10	4.0	0.39	0.4
209	106.9	0.32	8.3	242	123.2	0.45	13.2	88	45.0	0.55	6.1
221	112.8	0.21	5.7	245	125.3	0.80	22.6	266	136	0.74	25.6
2 600	96.7	0.17	4.0	2 900	96.1	0.24	5.5	3 700	189	0.20	9.3
2 800	98.9	0.14	3.4	3 100	113.7	0.28	7.2	2 900	93.6	—	—
328	4.7	0.11	0.1	276	4.9	0.26	0.3	351	3.6	0.26	0.2
344	4.5	0.13	0.1	363	4.1	0.45	0.4	367	3.7	0.47	0.4
5 240	215	0.15	7.8	5 360	300	0.24	17.1	4 740	197	0.32	15.5
4 890	394	0.15	14.3	5 410	333	0.39	29.4	5 210	212	0.60	32.4
8 700	451.4		29.4	9 260	560.4		43.6	9 206	468.9		40.5
580	30.1			617	37.3			658	33.5		
A = 15 B = 15				A = 15 B = 15				A = 14 B = 14			
8 622	646.0		21.1	9 519	614.0		73.5	9 096			
575	43.0			635	40.9			649			

1. It can be seen in Tables 9 and 12 that the amount of hemoglobin iron obtained by the perfusion amounted in series 1 A—B and 2, as an average, to 7.7 mg per animal. Drabkin found the ratio between body mass and hemoglobin to be a consistent 12.7 g per kg body weight, for certain species examined by him (rat, dog. man, heifer, horse). On the assumption that this ratio is valid also in the case of guinea pig, the average amount of hemoglobin iron per animal in these series should be $12.7 \times 0.616 \times 0.34 \times 10^{-2} = 26.6$ mg. This demonstrates that only around 29 % of the blood had been washed out. It is to be observed that this calculation includes an error, since the total weights of the animals per experiment includes the calculated weight of the removed blood. This blood, however, has of course been replaced by saline, which remains in the bodies. The error is, however, not very large. If roughly one third of the blood had been removed from the bodies and replaced by saline, the error would increase the total weight of the animals by only three per cent. As an example of a very incomplete perfusion may be given experiment nr

28 (ser. 1 A, 35 days, Table 9), where 14 animals gave only 45 mg of hemoglobin iron. The major part of the blood obviously remained in the "musculature for cytochrome", which gave unusually high total iron values.

The remaining blood is thus to be taken into consideration when the values are to be interpreted.

2. In experiment nr 34 (ser. 2, 4 days, Table 12) the total iron is given as 464 mg for "musculature for cytochrome". This is 3—4 times more than was found in the corresponding fractions in the other experiments. The most likely explanation thereof is that some foreign iron has contaminated the material, *e. g.* a trace of metallic iron from a mill or a stand. In spite of all possible precautions an accident of this kind may of course happen during a series of experiments of this kind. This explanation is also supported by the low specific activity 0.13 %, only one third of what was found for the corresponding fractions in other experiments.

Contamination with even a few micrograms of foreign iron would of course influence the specific activities considerably in those cases where very small amounts of material were available, *e. g.* from blood catalase. Only 4.2—28.4 (mean 14.4) micrograms of iron from this substance could be obtained per experiment.

B. Rate of iron resorption via peritoneum

Granick and Hahn [63] observed that after intravenous injection into dogs of radioactive iron as ferric ammonium citrate, 41.3 and 60.0 per cent of the injected activity appeared in the liver ferritin after 1 and 2 hours respectively. It is known that the resorption of drugs and other substances given by peritoneal injection is very rapid. Therefore, an injected substance tends to reach a certain blood level at essentially the same rate, regardless of whether the substance is administered by intravenous or intraperitoneal injection. Our figures indicate, however, that the resorption of ferric iron, given as ferric ammonium citrate, must be regarded as comparatively slow. Maximum activity in the liver as well as in the liver ferritin were not reached until the second day after the injection, the values on the first day being considerably lower. It is remarkable that only part of the iron occuring in the liver after two days could be held back in the liver. The activity dropped markedly on the third day. After that time the values remain nearly constant for some days, whereupon they begin to decline to reach a level slightly above the equilibrium level.

Another evidence for the slow resorption via the peritoneum is given by the "rest" fraction (Table 12) which includes the intestine with the major part of peritoneum which on the second day had fallen to about 0.46 %, where it remained. On the first day the specific activity had its highest value, 0.81 %.

C. Recovery of injected iron

The recovery of the injected iron isotope in series 1 was 21.1—73.5 % and in series 2 was 74.5—91.3 %. No satisfactory account for the vanished iron can be given. The unavoidable losses of blood and other tissues at the sacrification were negligible. From series 1 and 2 it is evident that 35 days do not suffice to bring the various iron containing fractions in the body into isotopic equilibrium. From series 3 one can see that liver catalase and ferritin with their rapid rates, hemoglobin with its intermediate rate, and cytochrome c with its slow rate of incorporation tend to reach the same value after 59 days. That value is slightly above 0.50 %. The dilution factor for the injected isotopic iron is 0.84 % (= 0.05 × 100/5.97). If complete equilibration is assumed to be reached within two months, it would thus mean that only 60—65 % of the administered iron remained in the body. The possibility that a large part of the total iron had been lost somehow in series 1, which of course would lower the recovery, can be ruled out, since the total iron was the same in series 1 and 2 (Tables 9 and 12). In series 2 the recoveries were much higher. Feces and urine were not collected during the experiment, but we hesitate for several reasons to explain the losses by assuming excretion. There is no drift towards lower recoveries with increasing time. It seems unlikely that excessively high excretion took place during the first days after the injections, since small doses were given (0.8 % of the total body iron per animal) and no evidence for such high excretions can be found in the literature.

To determine the iron in the "rest" of the animals, 3 of the 14—16 bodies per experiment were randomly taken and hydrolysed (*cf.* p. 000). Since this iron fraction accounted for about half of the total iron, it is of course possible that the variations in per cent recovery can to a large extent be explained in this way.

In series 2, where a higher percentile recovery was found, the rate of incorporation of radioactive iron into all the isolated compounds was more rapid than in series 1. This finding is also difficult to explain. In fact, these discrepancies between series 1 and 2 led us to repeat the experiment with series 3.

Whatever may have been the course of these differences between series 1 and 2, this unknown effect did not influence the activity cycles of liver ferritin and liver catalase. They gave consistent values in all three series.

It has been reported for dogs [63] and rats [32] that the spleen plays a rather unimportant role in the iron metabolism. We found the same phenomenon in series 1, where the specific activity of the spleen iron remained at a rather constant and low level. Contrary to this, the spleen iron activity in series 2

Table 10. Per cent specific activity of

Expt. no.		A = 19 B = 20		A = 22 B = 23	
Days		7		14	
Spleen ferritin	A	0.22 ± .01	0.23 ± .01	0.31 ± .01	0.33 ± .01
	B	0.22 ± .01	0.23 ± .005	0.25 ± .01	0.27 ± .01
Liver ferritin	A	4.93 ± .09	4.80 ± .10	3.69 ± .08	3.70 ± .06
	B	2.70 ± .05	2.66 ± .07	3.48 ± .08	3.36 ± .06
Liver catalase	A	3.21 ± .07	3.13 ± .06	2.76 ± .06	2.89 ± .05
	B	2.83 ± .06	2.62 ± .06	3.35 ± .07	3.22 ± .05
Cytochrome c	A	0.077 ± .002	0.069 ± .002	0.067 ± .005	
	B	0.063 ± .004	0.071 ± .004	0.110 ± .003	

reached higher values and varied much more. Granick and Hahn [63] found that in a series of three dogs one obviously had a deranged mechanism for the conversion of intravenously administered iron (ferric ammonium citrate) to ferritin. They also supposed that the variations found in the ferritin contents of human livers could be explained by derangements of that probably enzymatic reaction. The amounts of ferritin, which we were able to prepare, varied from 0.4 to 9.2 mg in terms of iron. However, we are inclined to believe that these variations were due to preparative difficulties rather than to various contents in the organs.

D. Total iron and its distribution in guinea pig

Series 1 and 2 give information about the iron content and distribution in guinea pigs. The average values obtained from 226 animals in 15 groups were 5.97 mg of Fe per 100 g in series 1, 5.91 mg in series 2. The iron content of blood-free liver, equal to the difference between total iron and the hemoglobin-bound iron, was calculated in the following way: The iron in cytochrome, catalase and similar compounds was considered quantitatively negligible as compared to the iron in ferritin and hemoglobin. The latter was calculated according to the formula:

$$X = F \cdot \frac{b-c}{b-a}$$

iron in isolated compounds (series 1).

A = 26 B = 27				A = 24 B = 25				A = 28 B = 29			
21				28				35			
0.24 ± .01	0.22 ± .01	0.65 ± .01	0.58 ± .01	0.25 ± .01	0.24 ± .01
0.19 ± .01	0.17 ± .004	0.23 ± .01	0.21 ± .005	0.40 ± .01	0.38 ± .005
1.84 ± .04	1.68 ± .04	1.13 ± .03	1.14 ± .03	1.54 ± .03	—
1.35 ± .03	1.43 ± .03	2.15 ± .05	1.94 ± .04	1.68 ± .04	1.68 ± .04
2.74 ± .06	—	1.45 ± .03	1.37 ± .03	2.17 ± .06	1.86 ± .04
1.38 ± .02	—	2.29 ± .05	2.11 ± .05	1.70 ± .04	1.68 ± .04
0.099 ± .002	—	0.156 ± .003	—	0.207 ± .005	
0.064 ± .002		0.212 ± .005		0.285 ± .006	

where X = Hb—Fe in mg in the pooled livers from 14—16 animals
F = total iron » » » » » 14—16 »
a = specific activity of Hb—Fe
b = » » » liver ferritin iron
c = » » » total liver iron

An example may be given (exp. nr 35, ser. 2, 1 day, Tables 12 and 13):

$F = 33.2$ mg
$a = 0.09$ %
$b = (4.20 + 4.03) : 2 = 4.12$ %
$c = 3.72$ %

$$x = 33.2 \frac{4.12—3.72}{4.12—0.09} \text{ mg} = 3.3 \text{ mg}$$

Iron of the blood free organ = (33.2—3.3): 2.83 = 10.6 mg Fe per 100 g wet weight. In our incompletely perfused animals the hemoglobin iron amounted to 25.6 ± 2.0 % of the total iron in the bloodcontaining livers. The average value for iron on the blood free livers determined from series 1 and 2 was 6.4 ± 1.3 mg * per 100 g wet weight.

* In the calculation of the probable errors the values from the pooled organs from 14—16 animals were taken as individual values. The probable errors therefore refer to variations between the experiments, each of which includes 14—16 animals.

The "musculature for myoglobin" was probably more effectively perfused than the livers [32]. This was supported by the fact that the water extracts of muscle, submitted to fractionation with ammonium sulfate, turned out to contain very little hemoglobin which was of the same order of magnitude as myoglobin. In animals with high myoglobin content in their muscles, like seal, horse, beef, or man, the bulk of the tissue iron may be present in myoglobin. A calculation from some of the yields of myoglobin in the experiments disclosed that guinea pig muscles can not contain more than 0.03 % myoglobin of the fresh weight muscle, thus 10—20 times less than in man, even if due attention is given to incomplete extraction and losses during the preparation.

The total iron in the "musculature for myoglobin" was found to be 1.36 ± 0.05 mg per 100 g wet weight, calculated from series 1 and 2. To this quantity of iron myoglobin contributed around 0.1 mg, the hemoglobin not removed by perfusion about the same amount, and cytochrome c around 0.02 mg.

Roughly 1 mg of iron per 100 g of wet tissue is thus contained in components other than hemoglobin, myoglobin or cytochrome c. Since the specific activity of the iron in "musculature for myoglobin" was always higher than that of the iron in myoglobin and cytochrome c, and since the hemoglobin content was low, this 1 mg of iron must have had an activity slightly above the values presented here for "musculature for myoglobin". Thus there were considerable amounts of relatively highly active iron available for the synthesis of myoglobin. The fact that myoglobin, nevertheless, retained a very low specific activity for a long time (Table 11 and Fig. 1) is our main evidence for its slow formation and breakdown.

Table 11. Per cent specific activity of myoglobin fractions (series 1).

Fraction		7 days		14 days		21 days		28 days		35 days	
		Uncorr.	Corr.	Uncorr.	Corr.	Uncorr.	Corr.	Uncorr.	Corr.	Uncorr.	Corr.
»70— 80»	A	—	—	0.16	—	0.23	0.08	—	—	0.33	0.15
70— 80	B	—	—	0.10	0.00	0.16	0.05	0.17	0.00	0.48	0.19
80— 90	A	0.01	0.01	0.06	0.00	0.15	0.04	0.24	0.08	0.22	0.16
80— 90	B	0.05	0.04	0.09	0.02	0.10	0.04	—	—	0.34	0.26
90—100	A	—	—	0.07	0.01	0.07	0.06	0.14	0.10	0.17	0.17
90—100	B	—	—	0.08	0.03	0.05	0.05	0.17	0.11	0.29	0.28
> 100	A	—	—	—	—	—	—	0.11	0.07	—	—
> 100	B	—	0.04	—	—	—	—	0.15	0.09	—	—
Average	A		0.01		0.01		0.06		0.08		0.16
Average	B		0.04		0.02		0.05		0.07		0.24

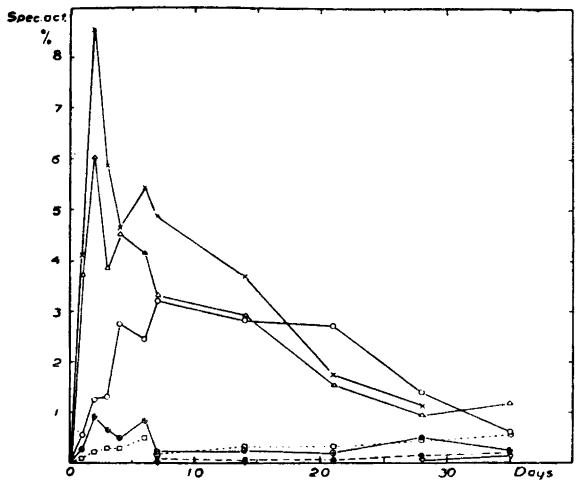

Fig. 1. Results of series 1 and 2.
——— × *Liver ferritin*
——— △ *Liver*
——— ○ *Liver catalase*
——— ⊕ *Spleen*
····· □ *Red blood cells*
– – – ● *Cytochrome c*
——— ⌓ *Myoglobin*

E. Rate of incorporation into various substances

a. Myoglobin. Specific activities significantly above zero are not achieved in myoglobin until after one month. At this time the above mentioned effect of the growth of the animals may already influence the results considerably. This means that the average life of the myoglobin molecule is probably still longer than would be estimated from our figures. On the other hand it must be born in mind that this does not necessarily apply to the myoglobin molecule as a whole. The protein part may be broken down and rebuilt again independently of the protohemin. Our results therefore only prove that the protohemin part of the molecule has a long lifetime. These observations correspond with the clinical observation mentioned in the introduction [55]. We would further like to draw attention to the fact that no rapid breakdown of protohemin in muscle tissue would be expected to occur since bile pigments do not normally appear in muscles in detectable amounts.

b. Cytochrome c. Cytochrome c takes up radioactive iron distinctly faster than the myoglobin does. In series 3 (Table 15) the activity increased steadily over the whole period of 59 days, but still at the end of the experiment it had not reached the equilibrium activity of around 0.5 %. In general the discussion on myoglobin is valid also for cytochrome c.

c. Hemoglobin and blood catalase. Conclusions regarding the average life time of the red blood cells can not be drawn from experiments with iron isotopes. We have not been able to find in the literature experiments on the life span of guinea pig erythrocytes. From our experiments, however, (*cf.* Figs. 1 and 2) it is evident that a comparatively rapid increase in specific activity

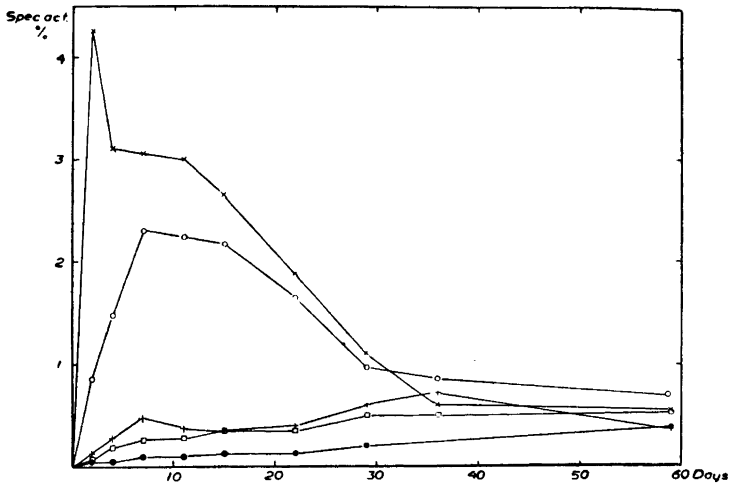

Fig. 2. Results of series 3.
× *Liver ferritin*
○ *Liver catalase*
\+ *Blood catalase*
□ *Red blood cells*
● *Cytochrome c*

occurs during the first week; the curve then flattens out more and more and approaches an equilibrium value asymptotically. The blood catalase activity gave rather scattered values owing to the technical difficulties encountered in preparing such minute enzyme quantities. In series 2 the activities of the blood catalase iron agreed reasonably well with the values for hemoglobin iron, with the exception of the three days value which came out lower for blood catalase. In series 3 the blood catalase activity was in most cases higher than the red blood cell activity. Because of the difficulties already mentioned in preparing sufficient amounts of blood catalase we can not, however, draw any other conclusions than that blood catalase is formed in the bone marrow simultaneously with the hemoglobin and probably takes its hemin iron from the same source as the hemoglobin.

d. Liver catalase, ferritin and blood catalase. As will be seen from Figs. 1 and 2 the specific activity of liver catalase iron follows the ferritin activity in a somewhat retarded cycle. After 4—5 days the catalase iron has reached half the activity of ferritin iron. The activity of the catalase later decreases, but as to be expected not quite as fast as the ferritin, so that the curves in series 3 intersect after 25 days. The average life time of catalase hematin can thus be

estimated to about 10 days, very much shorter than for myoglobin and cytochrome c. It is further obvious that the formation of liver catalase has nothing to do with that of blood catalase. They must be built up independently of each other in the liver and in the bone marrow. It is interesting to notice that these different organs are able to produce identical protein components, and it should be recalled that the liver acts as a hematopoietic organ during the fetal life.

e. Hemoproteins as possible precursors of stercobilin. It has been pointed out that a certain fraction ($\geq$ 11 %) of the stercobilin excreted by man cannot be derived from the hemoglobin metabolism. The possible role of catalase, cytochrome and myoglobin in the formation of that smaller fraction of stercobilin has been discussed.

Gray, Neuberger and Sneath [64] suggested that "the metabolism of porphyrins derived from myoglobin, catalase and possibly cytochrome c may account for the N^{15} content of the stercobilin excreted between the 30th and 80th day, but only for a small proportion of the high isotope content found at the initial period of the experiment". London, West, Shemin and Rittenberg [65] concluded that myoglobin could not be the sole source for the stercobilin not deriving from hemoglobin, but left the possibility open that myoglobin and the respiratory pigments might be the parent substance for that fraction of stercobilin.

From our experiments we are inclined to make the following comments.

Cytochrome c has a slow turnover number and can thus not participate in the "first" stercobilin peak. It may contribute to the "second" wave but owing to its low concentration only to a very restricted extent.

The blood catalase values of specific activity are quite close to those of hemoglobin. Any stercobilin deriving from blood catalase will therefore be included in the $\leq$ 89 % deriving from hemoglobin.

Liver catalase has a shorter life cycle than the two above mentioned substances. In man the N^{15} content of stercobilin has fallen from its initial high value to the minimum level on the 20th day. Thus liver catalase can contribute to the first "peak". However, from 500 g of guinea pig liver 700—800 mg catalase can be crystallized [46]. If the yield is assumed to be 1/3, and one guinea pig liver weighs 20 g, the total catalase content in one guinea pig liver is of the order of magnitude of 100 mg with the same hematin content as 25 mg of hemoglobin, corresponding to 0.06 ml blood. The guinea pig thus has roughly 300 times as much blood hematin as liver catalase hematin. The shorter life cycle of liver catalase as compared to hemoglobin is obviously still not short enough to give any appreciable contribution to the stercobilin production. Myoglobin is present in an amount of 1/5 of the hemoglobin in the human body [55]. The low turnover number, which from clinical observations can be supposed to be still lower in man than in guinea pig, diminishes the contri-

Table 12. Distribution of iron

Expt. no.	35				31			
Days	1				2			
	Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotopic Fe	Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotopic Fe
Liver	283	33.2	3.72	28.4	326	27.2	6.01	34.4
Spleen	13	5.1	0.26	0.3	11	2.5	0.92	0.5
Red blood cells	248	126.5	0.09	2.6	220	112.1	0.23	5.4
Muscle for cytochrome	2 700	140.8	0.45	14.6	3 000	115.5	0.56	13.6
Muscle for myoglobin	355	5.7	0.27	0.4	378	3.8	0.61	0.5
Rest	4 790	241.0	0.81	45.0	6 220	327.0	0.46	31.7
Total	8 389	552.3		91.3	10 155	588.1		86.1
Number of animals	15				16			
Total/number of animals	559	36.8			635	36.8		

Table 13. Per cent specific activity of isolated compounds (series 2).

Exp. No.	35	31	30	34	32
Days	1	2	3	4	6
Liver	0.51 ± .01	1.26 ± .03	1.29 ± .03	2.60 ± .06	2.46 ± .05
Catalase	0.55 ± .01	1.35 ± .03	1.31 ± .03	2.91 ± .05	2.47 ± .05
Liver	4.20 ± .06	8.53 ± .18	5.52 ± .10	4.64 ± .09	5.48 ± .10
Ferritin	4.03 ± .07	8.62 ± .13	6.20 ± .08	4.66 ± .06	5.41 ± .07
Spleen	0.37 ± .01	1.00 ± .02	0.80 ± .01	0.54 ± .01	0.84 ± .01
Ferritin	0.39 ± .01	1.00 ± .02	0.77 ± .01	0.62 ± .01	0.86 ± .01
Blood	0.07 ± .01	0.18 ± .01	0.17 ± .02	0.28 ± .01	0.46 ± .01
Catalase	0.09 ± .01			0.25 ± .01	0.44 ± .01
Cytochrome *c*	0.069 ± .002	0.122 ± .003 0.118 ± .003	0.116 ± .003 0.121 ± .003	0.102 ± .002	0.199 ± .004

and radioactivity (series 2).

30 3				34 4				32 6			
Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotopic Fe	Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotopic Fe	Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotopic Fe
311	25.3	3.85	20.5	299	33.7	4.52	32.1	356	26.4	4.16	24.7
13	3.38	0.65	0.5	13	5.0	0.49	0.5	12	2.3	0.85	0.4
272	138.7	0.29	8.5	251	128.0	0.27	7.3	292	149.0	0.50	16.8
3 000	150	0.36	11.4	3 000	464	0.13	12.7	3 000	124	0.53	14.8
435	4.9	0.52	0.5	408	7.2	0.27	0.4	405	4.5	0.49	0.5
6 120	308	0.51	33.1	4 830	278	0.49	28.7	5 940	210	0.52	24.5
10 151	630.3		74.5	8 801			81.7	10 005	516		81.7
16				16				15			
635	39.4			550				667	34.4		

bution of this important hematin fraction to the stercobilin production to a very low value. The initial peak in N^{15}-labelled stercobilin excretion can not derive from myoglobin metabolism.

The few experiments which we carried out with rats seemed to indicate that the liver catalase incorporated iron at about the same rate as in guinea pigs. If we compare this rate with the results of Greenstein *et al.* we find that the extremely rapid disappearance of catalase after implanting a liver tumor

Table 14. Per cent specific activity of myoglobin fractions (series 2).

Expt. no.	35		31		30		34		32	
Days	1		2		3		4		6	
	Uncorr.	Corr.	Uncorr.	Corr.	Uncorr.	Corr.	Uncorr.	Corr.	Uncorr.	Corr.
70— 80 %	0.05	0.00	0.17	0.04	0.12	0.00	0.10	0.00	0.28	0.08
80— 90 %	0.03	0.02	0.08	0.05	0.07	0.04	0.08	0.04	0.20	0.11
90—100 %	0.01	0.01	—	—	0.06	0.02	0.05	0.03	0.14	0.08
Average		0.01		0.05		0.02		0.02		0.09

Table 15. Per cent specific activity of

Expt. no.	36	37	38	39
Days	2	4	7	11
Liver	4.27 ± 0.09	3.12 ± 0.08	3.07 ± 0.07	2.93 ± 0.08
Ferritin	4.25 ± 0.09	3.07 ± 0.07	3.07 ± 0.07	3.04 ± 0.07
		3.10 ± 0.06		
Liver	0.87 ± 0.02	1.48 ± 0.03	2.40 ± 0.06	2.33 ± 0.06
Catalase	0.81 ± 0.03	1.50 ± 0.03	2.26 ± 0.05	2.22 ± 0.05
			2.29 ± 0.04	2.17 ± 0.04
Cytochrome	0.038 ± 0.002	0.053 ± 0.005	0.105 ± 0.004	0.117 ± 0.004
C	0.037 ± 0.003	0.066 ± 0.003	0.107 ± 0.005	0.121 ± 0.004
Red blood	0.072 ± 0.003	0.164 ± 0.005	0.233 ± 0.006	0.274 ± 0.007
Cells	0.078 ± 0.002	0.176 ±0.006	0.227 ± 0.005	0.297 ± 0.007
Blood	0.12 ± 0.02	0.32 ± 0.01	0.47 ± 0.01	0.38 ± 0.02
Catalase	0.09 ± 0.02	0.25 ± 0.01		

can not be caused only by an inhibition of the normal synthesis of catalase but must be due at least to a substantial part by an increased destruction. The normal formation rate is not high enough to account for re-establishing a normal catalase level in one or two days after the removal of the tumor. It seems possible, however, that the rate of catalase formation could increase during catalase deficiency in analogy with the increased formation of hemoglobin in anemia. This interesting problem is worth while of reinvestigating with tracer technic and more reliable catalase activity determinations.

SUMMARY

1. The total iron content of normal guinea pigs weighing 500—1 000 g was determined and found to be 5.95 mg per 100 g body weight. The iron contents of blood-free liver and muscle tissue were estimated to be 6.4 ± 1.3 and 1.26 ± 0.05 mg respectively per 100 g tissue wet weight.

2. Radioactive iron in amounts of 0.05 mg per 100 g body weight was given intraperitoneally as ferric ammonium citrate. The distribution of radioactive

iron in isolated compounds (series 3).

40 15	41 22	42 29	43 36	44 59
2.79 ± 0.07	2.21 ± 0.05	1.25 ± 0.04	0.61 ± 0.03	0.55 ± 0.03
2.66 ± 0.06	1.86 ± 0.05	1.14 ± 0.03	0.59 ± 0.02	0.54 ± 0.02
2.54 ± 0.05	1.67 ± 0.03	0.94 ± 0.02		
2.21 ± 0.06	1.64 ± 0.04	0.94 ± 0.05	0.86 ± 0.05	0.64 ± 0.04
2.11 ± 0.04	1.65 ± 0.04	1.00 ± 0.03	0.86 ± 0.02	0.77 ± 0.04
0.135 ± 0.004	0.117 ± 0.005	0.215 ± 0.005	—	0.389 ± 0.009
0.135 ± 0.004	0.117 ± 0.005	0.220 ± 0.005	—	0.392 ± 0.009
0.343 ± 0.008	0.348 ± 0.009	0.381 ± 0.009	0.368 ± 0.010	0.527 ± 0.010
0.333 ± 0.007	0.344 ± 0.008	0.404 ± 0.009	0.388 ± 0.008	0.523 ± 0.012
0.35 ± 0.02	0.42 ± 0.02	0.59 ± 0.02	0.71 ± 0.02	0.36 ± 0.01

iron in different organs and the specific activities of the iron in some hemoproteins and ferritins were determined after time intervals up to 59 days.

3. Liver ferritin exhibited a very high specific activity with a maximum after two days, followed by a drop to a plateau, where the values remained for about a week. The activities then decreased asymptotically to a final value.

4. The liver catalase iron activity followed the ferritin in a retarded cycle, indicating that the half life of liver catalase hematin in normal guinea pigs is around 4—5 days. The importance of this finding is discussed.

5. The hemoglobin incorporated radioactive iron comparatively fast. The blood catalase followed the hemoglobin in this respect, as far as could be judged from the rather uncertain determinations on the small quantities of catalase iron isolated. It can thus be said with certainty that liver and blood catalase are formed independently of each other.

6. Myoglobin incorporated iron extremely slowly, muscle cytochrome c somewhat faster. The significance of these results are discussed with regard to previous clinical observations on myoglobin and experimental work on bile pigment production.

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Investigations in Serum Copper

III. Coeruloplasmin as an Enzyme

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Coeruloplasmin, the dark blue copper containing protein present in mammalian plasma has been described by us in earlier papers [1, 2]. We have also shown that this copper proteide promotes the oxidation of *para*phenylene diamine (ppd).

In this paper we intend to show that coeruloplasmin must be regarded as an enzyme with an active group containing copper.

METHODS

Coeruloplasmin has been prepared according to a method described in an earlier paper [2]. The preparations used had a copper content of about 0.35 per cent. Copper has been determined with sodium diethyl dithiocarbamate after wet ashing and protein with the biuret method.

*Para*phenylene diamine (ppd) c. p. Coleman and Cell Co. has been used.

The enzymatic activity has been determined with the Warburg technique at 37° C. The consumption of oxygen per unit time, during the period of the most rapid oxidation, has been used as a measure for the velocity of the oxidation.

pH was measured with glass electrodes.

ENZYMATIC CHARACTER OF THE OXIDATION OF PPD BY COERULOPLASMIN

As ppd is a substance which is fairly instable in solutions it is always necessary to compare the action of a proposed enzyme on this substance with its spontaneous oxidation.

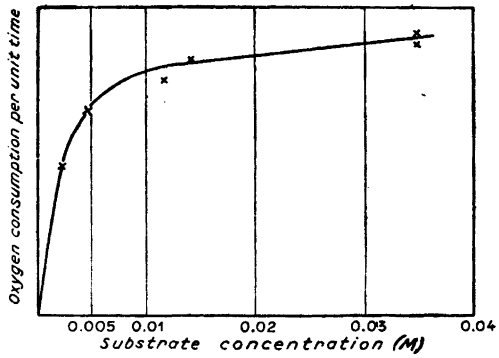

Fig. 1. Influence of substrate concentration on the activity of coeruloplasmin. The Michaelis constant as derived from the curve will be about $2.5 \cdot 10^{-3}$. The experiment has been performed at pH 6.0. The ppd was acidified with HCl. Enzyme concentration was $1.2 \cdot 10^{-6}$ M.

Under optimal conditions * at pH 6.0 the QO_2 at 37° C of the oxidation of ppd in the presence of coeruloplasmin amounts to about 1 500. Under the same conditions and in the presence of an equivalent amount of inorganic copper the O_2 consumption amounts to only about 3 per cent of that found in the presence of coeruloplasmin.

If albumine is added to the system ppd-inorganic copper the oxidation velocity is diminished. Albumin has no such effect on the system ppd-coeruloplasmin.

The relation between oxidation velocity and the concentration of substrate in the system ppd-coeruloplasmin is recorded in Fig. 1.

Variations of the oxygen in the atmosphere between 16 and 100 vol. per cent do not influence the oxidation velocity of ppd by coeruloplasmin.

These experiments seem to furnish conclusive proofs of the enzymatic character of the oxidation of ppd by coeruloplasmin.

There are also clear differences between the oxidation of ppd in the presence of coeruloplasmin on one hand and in the presence of inorganic copper on the other. As is well known the oxidation of ppd in the presence of inorganic copper proceeds much faster in slightly alkaline than in slightly acid solution. The optimal activity of coeruloplasmin, however, lies between pH 5 and 6.

When ppd is oxidized in the presence of copper there is an accumulation of H_2O_2. The amount of oxygen consumed amounts to 3 *M* O_2 per 2 *M* ppd.

By adding catalase we have been able to show that no H_2O_2 is accumulated when ppd is oxidized in the presence of coeruloplasmin. If catalase is present during the oxidation of ppd by coeruloplasmin this does not interfere with the

* Factors influencing the optimal conditions are discussed in paper 4 of this series /.

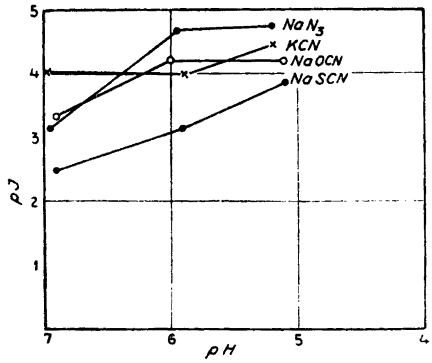

Fig. 2. Concentration of inhibitors which result in 50 per cent inhibition of coeruloplasmin at different pH:s. pI = −log of the molar concentration of the inhibitory substances. Enzyme concentration was kept at $1.9 \cdot 10^{-6}M$ ($7.6 \cdot 10^{-6}M$ Cu) and substrate concentration at $4.6 \cdot 10^{-3}M$. All points are results of a series of determinations performed at each pH with varying concentrations of inhibitor.

velocity of the reaction. The amount of oxygen consumed in this oxidation is 3 *M* O_2 per 4 *M* ppd. The same figures have been obtained by Graubard [4] and by Gregg and Miller [5] when ppd was oxidized by laccase from mushrooms.

DOES THE ACTIVE GROUP IN THE ENZYME CONTAIN COPPER?

If the copper is eliminated from coeruloplasmin by dialysis against KCN at pH 7 during 48 hours the resulting colorless preparation has lost its enzymatic activity. The remaining cyanide was of course eliminated by prolonged dialysis against distilled water before testing the activity. Neither the blue color nor the activity can be restored by adding copper to this preparation.

There is a proportional decrease in blue color and copper content if coeruloplasmin is dialyzed in acetate buffer at a pH below 5.

If a preparation which has lost some of its color by acidification for some hours to pH 5 is tested on its enzymatic activity there is a decrease in this activity proportional to the decrease in blue color.

From all these experiments it can be concluded that coeruloplasmin is an enzyme with copper in the active group and that its blue color is dependent on its copper content.

SUBSTRATE SPECIFICITY OF COERULOPLASMIN

Coeruloplasmin has greater activity against ppd than against other substrates hitherto tested. The activity of coeruloplasmin against other substrates which are generally oxidized by oxidases has been tested. These experiments have been performed in the presence of albumin or gelatin in order to depress the action of traces of heavy metals. The activity of coeruloplasmin against these substrates being considerably less than against ppd. With this precau-

tion we have been able to show that the following substances can act as substrates for coeruloplasmin: hydrochinone, catechol, pyrogallol, dopa, adrenaline and ascorbic acid. For all these substrates the optimum pH lies between 5 and 6. The experiments have also shown that it is doubtful whether monophenols and mono-amines are attacked at all.

INFLUENCE OF SOME INHIBITORS ON THE OXIDATION OF PPD BY COERULOPLASMIN

As early as 1944 [6] it was shown that the oxidation of ppd by a serum fraction containing copper was inhibited by KCN. We have now studied the inhibitory effect of KCN, NaN_3, KSCN and NaOCN * on the oxidation of ppd by pure coeruloplasmin. It is evident from Fig. 2 that all these substances inhibit the oxidation at fairly low concentrations especially on the acid side of pH 7. The activity of coeruloplasmin is also inhibited by sodium diethyl dithiocarbamate.

The effect of CO has also been studied. No inhibitory effect could be shown with a gas mixture containing 50—85 vol. per cent CO not even in complete darkness.

SUMMARY AND CONCLUSIONS

Our investigations have shown that coeruloplasmin is a true oxidase wich contains copper in its active group. For the following reasons we think that it should be classified as a laccase:

a. It is a blue copper proteide.

b. Its best substrate is *para*phenylene diamine (ppd).

It also acts on poly-phenols but probably not on monophenols and mono-amines.

c. It is inhibited by KCN, NaN_3, NaOCN, KSCN and sodium diethyl dithiocarbamate but not by CO.

Coeruloplasmin differs from laccases of plant origin in being less active. We have determined the optimal QO_2 at 37° C with ppd as substrate to 1 500.

* NaOCN was synthetisized according to Dupré and Schütz [7]. This preparation is completley free from cyanide.

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ACTA CHEMICA SCANDINAVICA 5 (1951) 481–484

Monolayers of Diastereoisomeric Long Chain Compounds. The Behaviour of (+)-2(L),9(L)-Dimethyltetracosanoic Acid, (+)-2(L),9(D)-Dimethyltetracosanoic Acid, and the Corresponding 7-Keto Acids

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Diastereoisomeric compounds usually exhibit large differences in the physical properties of their solid states. In the liquid state differences with regard to boiling point, refractive index etc. also exist, but in case of higher aliphatic compounds the differences in the liquid state are usually very small. It was of interest to study the properties of monolayers of diastereoisomeric long chain compounds in order to see whether differences in physical properties between diastereoisomers also exist in the two-dimensional states. The synthesis of the two dextrorotatory diastereoisomers of 2,9-dimethyltetracosanoic acid has recently been carried out [1] and the monolayer properties of these acids and the corresponding 7-keto acids will be described in the present communication.

That diastereoisomers may exhibit large differences in monolayer behaviour has been long known in case of sterols, for example the difference between cholestane-3-ol and *epi*-cholestane-3-ol [2], but no work appears previously to have been carried out with diastereoisomeric aliphatic compounds.

EXPERIMENTAL

Force-area curves were obtained of the acids spread on 0.01 *N* hydrochloric acid substrate and on a neutral substrate containing barium ions ($BaCl_2$ $0.3 \cdot 10^{-4}$ molar, $KHCO_3$ $4.0 \cdot 10^{-4}$ molar) with the aid of the recording mikrokator balance [3]. Spreading was effected from a solution in light petroleum (b. p. 60–80°) by means of the "Agla" micrometer syringe, the technique being the same as previously described [4].

The results are shown in Figs. 1–8. The force-area curve for one of the diastereoisomers was first recorded, the barrier moved back again (the camera is mechanically

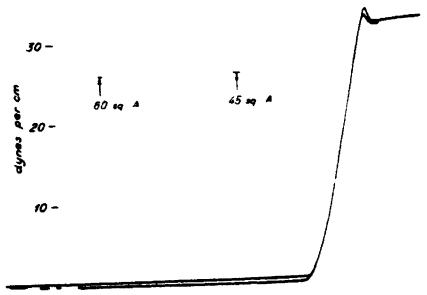

Fig. 1. Force-area curves for the two dextrorotatory diastereoisomers of 2,9-dimethyltetracosanoic acid on 0.01 N HCl substrate at a temperature of 4.0°.

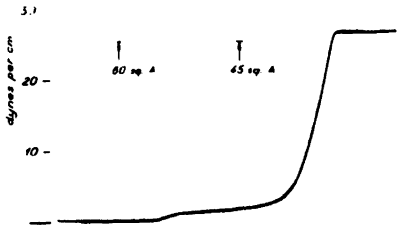

Fig. 2. Force-area curves for the two dextrorotatory diastereoisomers of 2,9-dimethyltetracosanoic acid on 0.01 N HCl substrate at 13.5°.

linked to the barrier carriage) and the surface cleaned. The second diastereoisomer was then spread and its force-area curve recorded on the same paper as the first one. The Figs. 1—6 show that the force-area curves for the (+)-2(L), 9(D)- and (+)2-(L), 9(L)-dimethyltetracosanoic acids are identical both on acid substrate and on a neutral substrate containing barium ions. At low temperatures the monolayers are of the liquid condensed type. On HCl at 4° (Fig. 1) the limiting area is 38 sq. Å, and the monolayers collapse at a pressure of 32.5 dynes/cm and an area of 31 sq. Å. At higher temperatures (Figs. 2 and 3), the monolayers are of the liquid expanded type, and the force-area curves show a transition of the myristic acid type. On barium substrate the monolayers are more condensed than on acid substrate, the limiting area at a temperature of 5° (Fig. 4) being 34 sq. Å. In this case the stability of the films is also greater, and collapse takes place at 27.5 sq. Å and a pressure of 48 dynes/cm. At higher temperature the mono-

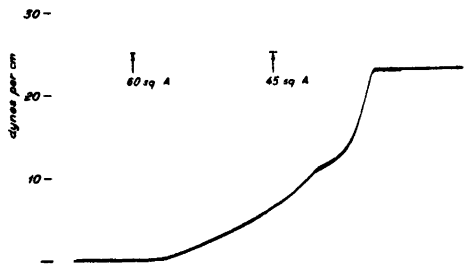

Fig. 3. Force-area curves for the two dextrorotatory diastereoisomers of 2,9-dimethyltetracosanoic acid on 0.01 N HCl substrate at 20.0°.

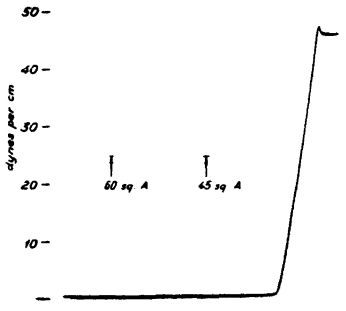

Fig. 4. Force-area curves for the two dextrorotatory diastereoisomers of 2,9-dimethyltetracosanoic acid on barium substrate at 5°.

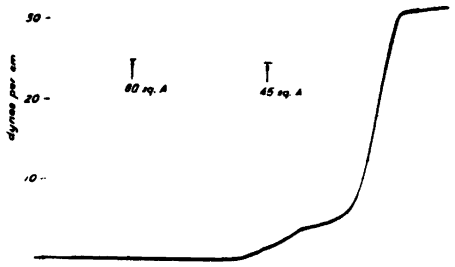

Fig. 5. Force-area curves for the two dextrorotatory diastereoisomers of 2,9-dimethyltetracosanoic acid on barium substrate at 20.0°.

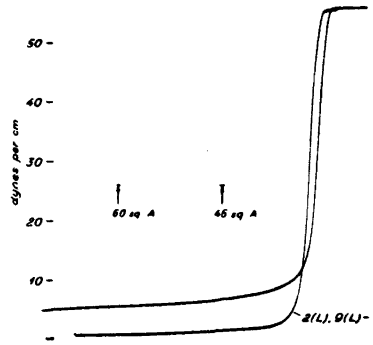

Fig. 6. Force-area curves for the stereoisomeric keto-acids on barium substrate at 5°.

layers are of the liquid expanded type (*cf.* Fig. 5). Mixtures containing the two stereoisomers in different proportions gave results identical with those of the pure compounds.

The diastereoisomeric 7-keto-acids, which are intermediates in the synthesis of the 2,9-dimethyltetracosanoic acids [1], differ, as would be expected, in the monolayer behaviour from the latter. On barium substrate at 5° (Fig. 6) force-area curves of the two diasteroisomers differ over the whole range, but both keto-acids give comparatively high pressures out to large areas. Although the force-area curves of the two diastereoisomers are not identical over any part of the curve, the collapse pressures are the same. At 20° the force-area curves for the keto-acids on barium substrate (Fig. 7) and on acid substrate (Fig. 8) are similar in appearance and of the liquid expanded type. The curves for the two diastereoisomers are identical over the expanded region, but after the transition to the condensed state sets in, the force-area curves begin to differ from each other.

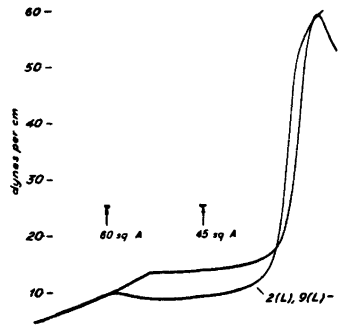

Fig. 7. Force-area curves for the stereoisomeric keto-acids on barium substrate at 20.0°.

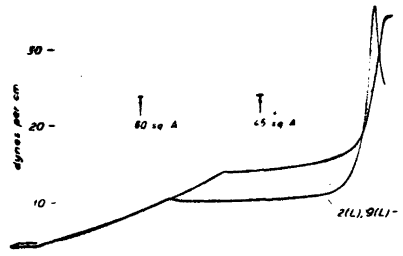

Fig. 8. Force-area curves for the stereoisomeric keto-acids on 0.01 N HCl substrate at 20.0°.

DISCUSSION

The identical monolayer behaviour of the two diastereoisomers of 2,9-dimethyltetracosanoic acid under the conditions investigated indicates that the monolayers are true liquids. The same applies to the keto-acids over the expanded region, but the fact that in this case the force-area curves become different as soon as the transition to the condensed state sets in, in our opinion indicates that the condensed monolayers are more organized than a liquid.

The results suggest that the nature of phase transitions in monolayers may in certain cases be studied with the use of diastereoisomers.

The curves in Figs. 1—3 also show the precision obtainable with the recording mikrokator balance. The largest differences found between the force-area curves of the two 2,9-dimethyltetracosanoic acids, both with regard to pressure and area values, are of the order of 0.3 %. This includes the weighing out of the acids, pipetting out the spreading solvent, the spreading by means of the Agla syringe, and the reproducibility of the balance in the area and pressure recordings. Impurities in the acids would of course also contribute to the error, and the results can therefore also be regarded as a check of the purity of the compounds used. It should be pointed out that the results in Figs. 1—8 have not been especially picked out from a series of recordings showing much greater variations. The three pairs of curves reproduced in Figs. 1—3 were obtained in strict succession, the temperature being changed after each double run.

SUMMARY

The monolayer behaviour of the two dextrorotatory diastereoisomers of 2,9-dimethyltetracosanoic acid has been studied. The acids give identical monolayer force-area curves. The corresponding 7-keto-acids, on the other hand, show identical force-area curves over the expanded region, but as soon as the transition to more condensed forms sets in, the force-area curves begin differ from each other.

We are indebted to Mrs. K. Nilsson for assistance in the experimental work, and to *Statens naturvetenskapliga forskningsråd* for financial support.

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ACTA CHEMICA SCANDINAVICA 5 (1951) 485–490

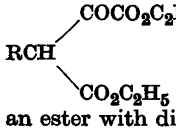
Short Communications

A Method for Condensations of Esters with Diethyl Oxalate

ARNE BRÄNDSTRÖM

Chemical Institute, University of Uppsala, Uppsala, Sweden

A general method for the preparation of α-ethoxalyl esters of the formula



is the condensation of an ester with diethyl oxalate using sodium ethoxide as a condensation agent [1,2]. As these esters readily undergo pyrolysis when heated to above about 150°, only the first relatively lowboiling esters have been prepared in a state of purity by distillation.

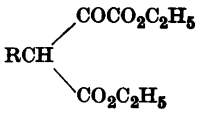
In the present investigation, where the reaction was forced by distilling off the alcohol formed using toluene as a solvent, good yields of rather pure esters with constant boiling points were obtained from esters which are not branched in the α- or β-positions. From the most simple ester branched in the β-position, ethyl *iso*-valerate, the yield was only about 20 % and from the highly branched ethyl *t*-butylacetate no condensation product could be obtained. All the esters prepared were fractionated at a pressure of 1–4 mm. At this pressure no appreciable decomposition of the ethoxalyl esters took place.

The ethoxalyl esters with at least one α-hydrogen atom are sufficiently strong acids to be titrated with standard sodium hydroxide solution, using a mixture of one part of thymol blue and three parts of phenolphthalein as an indicator. The somewhat high equivalent weights are probably due to a slight decomposition during the distillation.

Experimental. 0.25 mole of sodium ethoxide suspended in 250 ml of dry toluene was prepared from 5.75 g of sodium and 11.5 g of absolute alcohol in a three-necked round-bottomed flask, fitted with a dropping funnel, a mercury sealed stirrer, and a 30-cm Widmer column with a total reflux variable take off still head. The suspension was cooled in ice-water and a mixture of 0.25 mole of diethyl oxalate and 0.25 mole of the ester added. The flask was then heated with an electrical heating mantle and the alcohol formed carefully distilled off. When no more alcohol could be obtained, the flask with the deeply coloured solution was cooled in ice-water, and an ice cold solution of 0.25 mole of glacial acetic acid in 100 ml of water added as rapidly as possible. The mixture was stirred for five minutes and then transferred to a separatory funnel. Sufficient water was added to dissolve the salt eventually precipitated. The toluene layer was separated and the water layer extracted with ether. The combined ether and toluene layers were washed with water and then with a solution of sodium bicarbonate. After drying over a little anhydrous sodium sulphate, the ether and toluene were removed under reduced pressure, and the residue fractionated at a pressure of 1–4 mm.

The yields, boiling points and equivalent weights of the ethoxalyl esters are given in Table 1.

Table 1. Preparation of ethoxalyl esters



R	Yield %	B.p.	Equivalent weights Calc.	Found
Ethyl	60	104/4 mm	216.2	217.7
*Iso*propyl	20	100/2 mm	230.3	236.5
n-Propyl	63	99/1.5 mm	230.3	232.4
*Iso*butyl	57	102/1.5 mm	244.3	250.2
n-Butyl	61	116/2 mm	244.3	249.4

1. Adickes, F., and Andresen, G. *Ann.* **555** (1943) 48.
2. *Organic Syntheses* **II** (1943) 272.

Received March 12, 1951.

On the Action of Bacterial L-lysine Decarboxylase on Hydroxylysine

SVEN LINDSTEDT

Department of Physiological Chemistry, University of Lund, Lund, Sweden

Gale [1] has shown that various bacteria possess an enzyme capable of specifically decarboxylating L-lysine. He also tested the purified enzyme against different lysine derivatives and found it inactive in all cases except when hydroxylysine was the substrate. The hydroxylysine used was an impure preparation (85 % periodate-ammonia, $(\alpha)_D + 4.7$ in *N* HCl) that was decarboxylated to about 60 per cent although at a slower rate than lysine.

However, Zittle and Eldred [2] have claimed that L-lysine decarboxylase does not decarboxylate hydroxylysine. This question is obviously of importance for the quantitative determination of L-lysine with this enzyme.

Using pure hydroxylysine prepared according to Bergström and Lindstedt [3,4] we have reinvestigated this question. The results plotted in Fig. 1 show that hydroxylysine was decarboxylated to the same extent as lysine, although at a considerably slower rate. It is evident, therefore, that any hydroxylysine present will give an error in the determination of lysine with the usual preparation of bacterial decarboxylase as the carbon dioxide evolved represents the sum of L-lysine and hydroxylysine present.

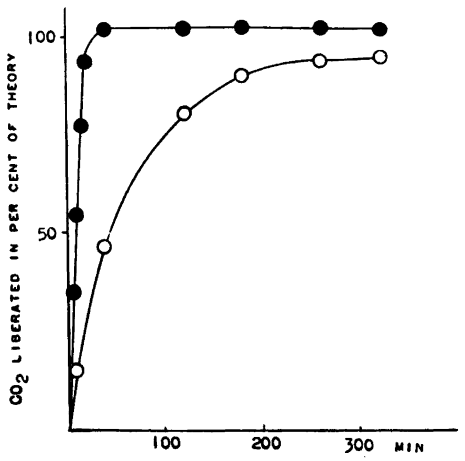


Fig. 1. ○ *hydroxylysine.* ● *lysine. The values are corrected for* CO_2 *retention in the solution.*

We have obtained one mole of carbon-dioxide per mole hydroxylysine on preparations from fish skin. The analytically pure samples from some commercial gelatines give less, possibly due to partial racemization in the manufacturing process. Synthetic hydroxylysine prepared according to Touster [5] was decarboxylated to 25 per cent while a synthetic L-hydroxylysine obtained from Dr. J. Weissiger showed 50 per cent decarboxylation. This work will be reported in detail in a subsequent publication.

Experimental. *Bacterium cadaveris* (strain no. 6578 Natural collection of type cultures, London) were grown and acetone dried as described by Zittle and Eldred. 5 μM of the amino acid in 0.5 ml water

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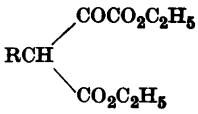

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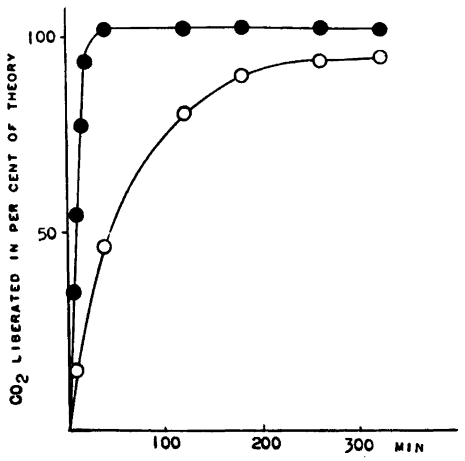

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My sincere thanks are due to Dr. Rune Grubb for assistance in culturing the bacteria.

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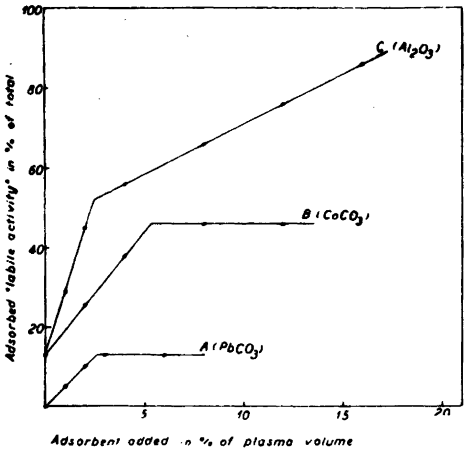

Fig. 1.

Curve A: effect of $PbCO_3$ on plasma treated with $SrCO_3$.
Curve B: effect of $CoCO_3$ on plasma treated with $SrCO_3$ and $PbCO_3$.
Curve C: effect of Al_2O_3 on plasma treated with $SrCO_3$ and $PbCO_3$.

The Complex Nature of the „Labile Factor" in Chicken Plasma and the Characterization of the Individual Components

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Department of Biology, Polytechnic Institute, Copenhagen, Denmark

During the study of the coagulation anomaly produced in chicks by vitamin K-deficiency and dicumarol poisoning, it has been demonstrated, in this laboratory, that these two coagulation anomalies are not identical [1-3].

Dicumarol causes depression of both prothrombin and the $\varkappa$-factor [2], while in vitamin K-deficiency low levels of both prothrombin and the δ-factor are found [3]. A common property of these three coagulation factors — prothrombin, $\varkappa$-factor and δ-factor — is their adsorbability by $BaCO_3$ and $SrCO_3$.

During these studies a rather sensitive method for determination of the labile activity of fresh plasma, *viz.* ability to restore the prothrombin time of stored oxalated chicken plasma, was developed using stored oxalated plasma as a substrate. It could be shown that adsorption with $BaCO_3$ or $SrCO_3$ sometimes, but not always, would reduce the labile activity of fresh plasma. The degree of reduction depends on the storage time of the plasma used as substrate and indicates a slow inactivation of the $\varkappa$-factor and possibly also of the δ-factor during storage.

The determination of the labile activity — after total removal of prothrombin, $\varkappa$-factor and δ-factor by $SrCO_3$, has now made it possible to compare the labile activity of different plasmas and to investigate in detail the effect of various adsorbents on the labile activity of fresh plasma.

It is regularly found that the labile activity of different fresh chicken plasmas may show great differences — depending on the diet and the length of time during

* Fellow 1951—52: Norwegian Research Council for Technical and Natural Sciences.

was shaken at 30° with 2.5 mg bacterial powder suspended in 2.5 ml of 0.2 m phosphate buffer at pH 6.0.

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2. Zittle, Z. D., and Eldred, N. R. *J. Biol. Chem.* **156** (1944) 401.
3. Bergström, S., and Lindstedt, S. *Arch. Biochem.* **26** (1950) 323.
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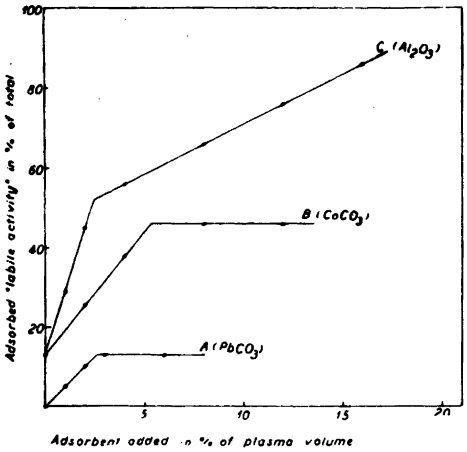

Fig. 1.

Curve A: effect of $PbCO_3$ on plasma treated with $SrCO_3$.
Curve B: effect of $CoCO_3$ on plasma treated with $SrCO_3$ and $PbCO_3$.
Curve C: effect of Al_2O_3 on plasma treated with $SrCO_3$ and $PbCO_3$.

The Complex Nature of the „Labile Factor" in Chicken Plasma and the Characterization of the Individual Components

ØYVIND SØRBYE*, INGER KRUSE and HENRIK DAM

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During the study of the coagulation anomaly produced in chicks by vitamin K-deficiency and dicumarol poisoning, it has been demonstrated, in this laboratory, that these two coagulation anomalies are not identical [1-3].

Dicumarol causes depression of both prothrombin and the $\varkappa$-factor [2], while in vitamin K-deficiency low levels of both prothrombin and the δ-factor are found [3]. A common property of these three coagulation factors — prothrombin, $\varkappa$-factor and δ-factor — is their adsorbability by $BaCO_3$ and $SrCO_3$.

During these studies a rather sensitive method for determination of the labile activity of fresh plasma, *viz.* ability to restore the prothrombin time of stored oxalated chicken plasma, was developed using stored oxalated plasma as a substrate. It could be shown that adsorption with $BaCO_3$ or $SrCO_3$ sometimes, but not always, would reduce the labile activity of fresh plasma. The degree of reduction depends on the storage time of the plasma used as substrate and indicates a slow inactivation of the $\varkappa$-factor and possibly also of the δ-factor during storage.

The determination of the labile activity — after total removal of prothrombin, $\varkappa$-factor and δ-factor by $SrCO_3$, has now made it possible to compare the labile activity of different plasmas and to investigate in detail the effect of various adsorbents on the labile activity of fresh plasma.

It is regularly found that the labile activity of different fresh chicken plasmas may show great differences — depending on the diet and the length of time during

* Fellow 1951—52: Norwegian Research Council for Technical and Natural Sciences.

which the chickens have received a certain diet.

The introduction of adsorption procedures show clearly that the labile activity of fresh plasma cannot be explained by the assumption of only one labile factor.

$CoCO_3$ added to plasma in increasing quantities causes only partial reduction of the labile activity — and the adsorption curve (curve B) indicates the adsorption of one factor with labile activity — the $CoCO_3$-factor.

Al_2O_3 will give quite another type of adsorption curve, which seems to be composed of two straight lines (curve C). It indicates the adsorption of probably two factors with labile activity. Both $Cr(OH)_3$ and $ZnCO_3$ give the same type of adsorption curve as Al_2O_3 does, indicating their similarity in adsorption properties.

Other experiments have shown that the two Al_2O_3-factors responsible for the broken-line adsorption curve are not adsorbed by $CoCO_3$ and that the $CoCO_3$-factor is not adsorbed by Al_2O_3, $Cr(OH)_3$ or $ZnCO_3$ in the quantities studied so far.

The full details of the Al_2O_3-curve in the range approaching 100 % adsorption are still an object of further study.

By testing other adsorbents we became aware that $PbCO_3$ would remove a coagulation factor from $SrCO_3$-treated plasma which seems to be of a more stable nature than the factors mentioned above and is not adsorbed by Al_2O_3 or $CoCO_3$. The adsorption of this $PbCO_3$-factor can be studied by the same technique when stored plasma from chicks deficient in this factor is used as substrate (curve A).

It is not yet evident which of the labile factors mentioned above eventually corresponds to factor V of Owren, plasma Ac-globulin of Seegers or labile factor of Quick, but selective adsorption analysis of the kind described seems to offer a convenient method for classification of the components of the chick coagulation mechanism not adsorbed by $SrCO_3$ or $BaCO_3$. The selective adsorption also offers a method for preparation of the different factors. It has been found possible to elute the adsorbed factors from $CoCO_3$ and $ZnCO_3$ by elution with phosphate buffers of suitable pH and molarity.

In the opinion of the authors this adsorption analysis offers a method for quantitative estimation of the different adsorbable factors. The minimum amount of adsorbent, necessary for full adsorption of each component has been interpreted as a measure of the amount present.

Thus we have been able to start a search for the dietary principles which seem to determine in what amount each factor will be present in the chicken plasma. Such studies are in progress.

1. Dam, H., and Søndergaard, E. *Biochim. et Biophys. Acta* **2** (1948) 409.
2. Sørbye, Ø., Kruse, I., and Dam, H. *Acta Chem. Scand.* **4** (1950) 549.
3. Sørbye, Ø., Kruse, I., and Dam, H. *Acta Chem. Scand.* **4** (1950) 831.

Received May 5, 1951.

A Note on the Food Sparing Effect of Liver Extracts on Adult Rats

GUNNAR ÅGREN

Institute of Medical Chemistry, University of Uppsala, Uppsala, Sweden

In a recent paper on the food sparing effect of liver extracts on growing rats [1] it was reported that a similar effect could not be observed on adult animals. A second series on nearly full-grown rats has now been carried out with the same negative result.

The animals were the same female rats as used in the last experiments on growing animals [1], and the experimental procedures were the same as described for the second series in that paper. At the beginning of the present experiments the weight of the

which the chickens have received a certain diet.

The introduction of adsorption procedures show clearly that the labile activity of fresh plasma cannot be explained by the assumption of only one labile factor.

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rats was about 180 g. The experimental time was 15 days. Liver extract was added to the bread in amounts calculated to give each animal the equivalent of 0.3 ml of extract, when the daily food consumption was about 18 g. The results are given in Table 1.

Table 1. Growth of rats on a mixed diet given as mouse bread fortified with liver extracts.

Group	No. of animals	Average daily food consumption	Average daily weight gain
Liver	15	18.4 + 0.18 *	0.49 ± 0.009*
Control	15	18.2 ± 0.15	0.48 ± 0.008

* The values in these columns are the means and the standard error.

There was no significant difference in the daily weight gain or in the daily food consumption between the liver and the control group.

There is an additional difference between the effect of the liver extract in growing and adult animals. In previous series [1-3] on growing animals it was observed that the rats which received only the commercial mouse bread showed a comparatively poor fur development. Animals which were given liver extracts were observed to have a more dense, lustrous underfur. In the previous and the present series of experiments on adult, or nearly full-grown animals, the liver extract had no obvious effect on the fur development.

The investigation was supported by a grant for medical research from the Swedish Medical Research Council. The technical assistance of Mr. T. Persson is greatfully acknowledged.

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6-Methyl-1,4-naphthaquinone Produced by *Marasmius graminum*

GERD BENDZ

Chemical Institute, University of Uppsala, Sweden

It has been reported earlier, that a red crystalline substance active against *Staphylococcus aureus* has been isolated from the metabolism solution of *Marasmius graminum* [1,2]. After further purification by steam distillation and repeated recrystallizations from petroleum ether the active principle was obtained as red needles m. p. 87–88° C. The molecular weight estimated by the Rast method indicated, that the red crystals could be a methylnaphthaquinone. This was supported by other facts such as the absorption spectrum and the analyses data for 2,4-dinitrophenylhydrazone. Degradation of the red compound resulted in trimellitic acid which suggested that it might have been 6-methyl-1,4-naphthaquinone [3].

Diene condensation of isoprene and *p*-benzoquinone, followed by isomerization and oxidation gave 6-methyl-1,4-naphthaquinone, which after recrystallization from dilute acetic acid melted at 90–91° C. The melting point of a mixture of 6-methyl-1,4-naphthaquinone with the red substance was 87–88° C. The ultra-violet absorption curves of the two compounds were practically identical in the region of 2 400–2 600 Å.

For further purification of the red material several chromatographic methods were tried and finally a complete separation of a yellow substance from a minor quantity of a dark red one was accomplished by using an acid-washed alumina column. Evaporation of a yellow-green fraction of the percolate left a biologically active, crystalline residue which was

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further purified and recrystallized from dilute acetic acid. It yielded golden, yellow needles m. p. 90—91° C. There was no depression of the melting point when these yellow crystals were mixed with 6-methyl-1,4-naphthaquinone.

$C_{11}H_8O_2$	Calc.	C 76.73	H 4.68
	Found	» 76.39	» 4.50

A detailed report will be published later.

This investigation has been supported financially by the Swedish Natural Science Research Council whose support is gratefully acknowledged.

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Some Studies on Azo Dyes. II

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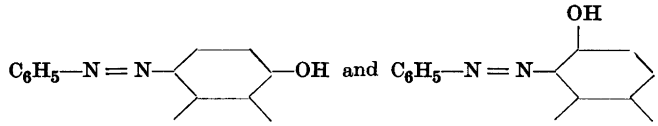
In a previous publication [1] the author discussed the effect of an *o*-methyl group, *o*- and *m*-carboxyl groups, and combinations of the two on the absorption spectra of 4-phenylazo-phenol and 1-phenylazo-naphthol-(2). It was found that hydrogen bond formation in dyes containing an *o*-carboxyl group stabilized the hydrazone form and caused a displacement of the azo-hydrazone equilibrium toward the right. Some quantitative values for the relative amount of hydrazone form in the β-naphthol dyes were given.

The purpose of the present paper was to examine the effect of linearly annulated benzene nuclei on the absorption spectra of *o*- and *p*-hydroxy azo dyes.

For this purpose, the two isomeric series of dyes



with the auxochrome contained in a benzene, naphthalene, and anthracene nucleus, were synthesized and examined. The six corresponding O—CH_3 derivatives were also prepared.

THE ABSORPTION CURVES OF THE DYES

Fig. 1 shows the absorption curves of 2-phenylazo-phenol and its O-methyl ether. It can be seen that the hydroxy compound shows a double maximum (325 and 373 mμ). The methoxy compound, however, shows only a single maximum at 322 mμ.

Fig. 2 shows the curves of 1-phenylazo-naphthol-(2) and its O-methyl ether. In the visible region this hydroxy compound also shows a double

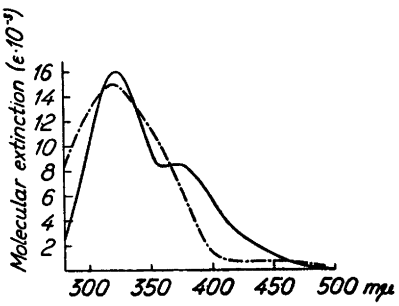


Fig. 1. Absorption curves of o-phenol dyes.
——— *2-phenylazo-phenol*
—·—·— *2-phenylazo-phenol, methyl ether*

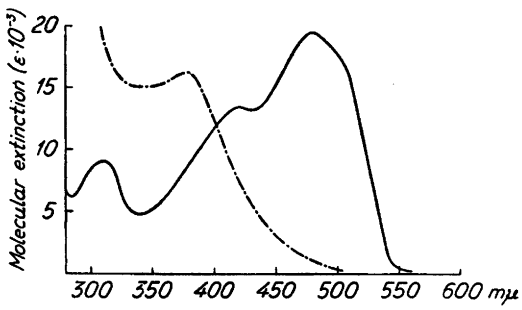


Fig. 2. Absorption curves of β-naphthol dyes.
——— *1-phenylazo-naphthol-(2)*
—·—·— *1-phenylazo-naphthol-(2), methyl ether*

maximum (422 and 480 mμ). The methoxy compound shows only a single maximum at 380 mμ.

Fig. 3 shows the curve of 1-phenylazo-anthrol-(2). The hydroxy compound, in this case, shows only a single, almost symmetrical maximum (511 mμ), in the visible region. The O—CH_3 derivative could not be obtained in a pure crystalline state, but spectrophotometric studies on the methylation product indicated that this derivative shows an absorption band at approximately 435 mμ. (As indicated in the figure.)

Fig. 4 shows the absorption curves of 4-phenylazo-phenol and its O-methyl ether. It can be seen that both of these show only a single, almost symmetrical maximum (349 and 343 mμ respectively) in the region examined.

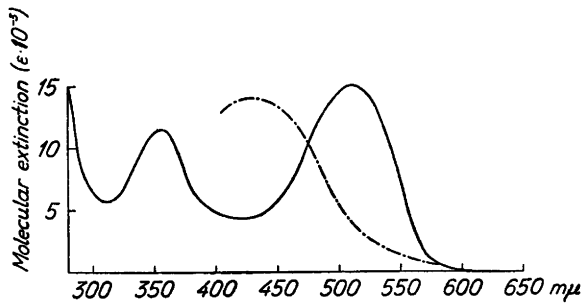


Fig. 3. Absorption curves of β-anthrol dyes.
——— *1-phenylazo-anthrol-(2)*
—·—·— *approximate absorption band of 1-phenylazo-anthrol-(2), methyl ether*

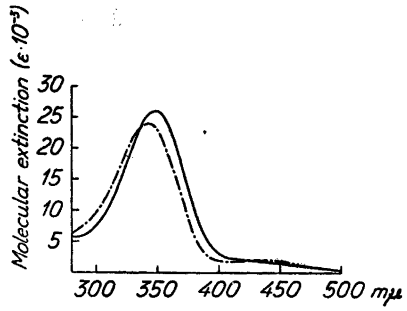


Fig. 4. Absorption curves of p-phenol dyes.
——— *4-phenylazo-phenol*
—·—·— *4-phenylazo-phenol, methyl ether*

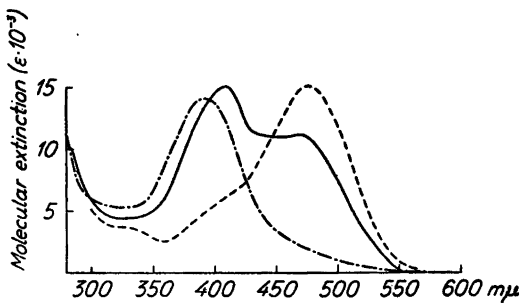


Fig. 5. Absorption curves of α-naphthol dyes.
——— *4-phenylazo-naphthol-(1)*
—·—·— *4-phenylazo-naphthol-(1), methyl ether*
— — — *4-phenylazo-naphthol-(1) in acetic acid*

Fig. 5 shows the curves of 4-phenylazo-naphthol-(1) in alcohol and glacial acetic acid and its O-methyl ether in alcohol. The hydroxy compound (in alcohol) shows a double maximum (410 and 470 mμ), while in acetic acid it shows only a single maximum (475 mμ). The O-methyl derivative shows only a single maximum at 392 mμ.

Fig. 6 shows the curves of 4-phenylazo-anthrol-(1) and its O-methyl ether. In the visible range both of these dyes show only a single, almost symmetrical maximum at 506 and 445 mμ, respectively.

All of the measurements were made in 96 % alcohol, except as noted. The extinction values of the O—CH_3 derivatives have been computed so as to best show their relationship to the corresponding dye.

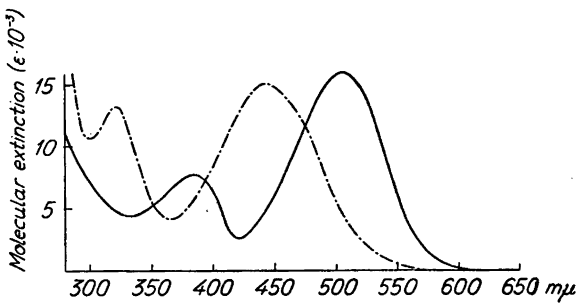


Fig. 6. Absorption curves of α-anthrol dyes.
——— *4-phenylazo-anthrol-(1)*
—·—·— *4-phenylazo-anthrol-(1), methyl ether*

The location of the absorption maxima are summarized in Table 1.

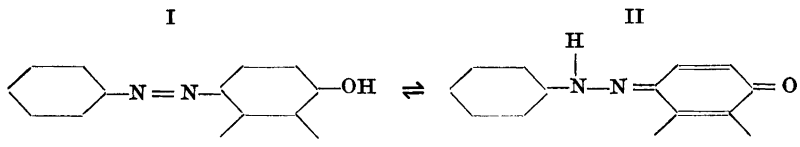
Table 1. The location of the absorption maxima.

Dye	Max. (mμ)
4-Phenylazo-phenol	349
4-Phenylazo-phenol, methyl ether	343
2-Phenylazo-phenol	325 373
2-Phenylazo-phenol, methyl ether	322
4-Phenylazo-napthol-(1)	410 470
4-Phenylazo-naphthol-(1) (HOAc sol.)	475
4-Phenylazo-naphthol-(1), methyl ether	392
α-Naphthoquinone-methylphenyl-hydrazone *	466
1-Phenylazo-naphthol-(2)	422 480
1-Phenylazo-naphthol-(2), methyl ether	380
4-Phenylazo-anthrol-(1)	506
4-Phenylazo-anthrol-(1), methyl ether	442
1-Phenylazo-anthrol-(2)	511
1-Phenylazo-anthrol-(2), methyl ether	435

* in benzene sol., Kuhn and Bär [2]

AZO AND HYDRAZONE BANDS

The azo-hydrazone tautomerism is a form of prototropy, where a proton migrates between an azo N atom and a phenolic O atom.



If this mobile proton is replaced by a methyl group attached to either the O or N atoms, the resultant methyl derivative cannot exhibit tautomerism and should indicate the approximate location of the absorption band of the corresponding form of the actual dye. That is to say, the O—CH_3 derivatives should indicate the absorption band of the azo form (I) and the N—CH_3 derivative that of the hydrazone form (II).

Kuhn and Bär [2], in their studies on this tautomerism, made use of this fact and studied the *o*- and *p*-phenol and naphthol dyes and some of their O and N derivatives. The present author has synthesized and examined the remaining O—CH_3 derivatives of these dyes and also the *o* and *p*-anthrol dyes and their O—CH_3 derivatives. The results tend to confirm Kuhn and Bär's theory.

4-Phenylazo-phenol shows only a single maximum which agrees very closely with that of its O—CH_3 derivative. For this reason it is assumed to exist almost completely in the azo form. The *ortho* isomer (2-phenylazo-phenol), however, shows a double maximum. The band which is similar to the O—CH_3 derivative is assumed to be due to the azo form while the band at longer wave length is assumed to be due to the hydrazone form.

Both of the naphthol dyes show a double maximum. The maxima at shorter wave lengths agree fairly well with the O—CH_3 derivatives, and are therefore assumed to be due to the azo form. The maximum at the longer wave length, in the case of the *α*-naphthol dye, agrees fairly well with both the N—CH_3 derivative and with the curve of the dye in acetic acid (in which Kuhn and Bär [2] state that the dye exists mainly in the hydrazone form). For this reason these maxima are assumed to be due to the hydrazone form.

The two anthrol dyes show only a single maximum in the visible range which differs widely from that of their O—CH_3 derivatives. For this reason

Table 2. The location of the azo and hydrazone bands.

Dye	Azo band (mμ)	Hydrazone band (mμ)
p-Series		
4-Phenylazo-phenol	343	...
4-Phenylazo-naphthol-(1)	392	475
4-Phenylazo-anthrol-(1)	442	506
o-Series		
2-Phenylazo-phenol	322	385
1-Phenylazo-naphthol-(2)	380	480
1-Phenylazo-anthrol-(2)	435	511

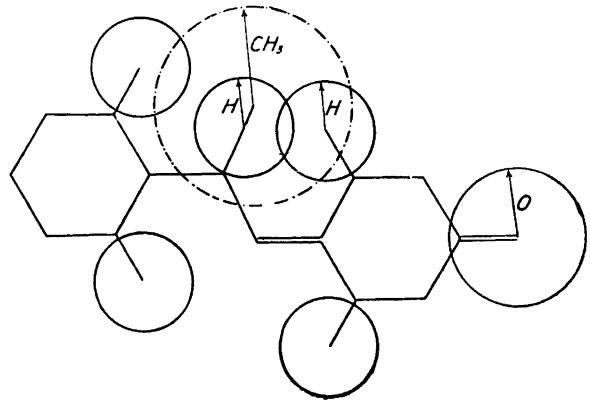


Fig. 7. Schematic diagram of hydrazone form of p-hydroxy azo dye, drawn to scale. The circles are the van der Waal radii of the atoms concerned.

the dyes are assumed to exist almost completely in the hydrazone form absorbing at the longer wave length.

On this basis, the following approximate values can be assigned for the absorption bands of the azo and hydrazone forms of the dyes.

THE CONFIGURATION OF THE N- AND O-METHYL DERIVATIVES OF THE DYES

In the foregoing it was stated that the N—CH_3 and O—CH_3 derivatives of the dyes should indicate the approximate positions of the hydrazone and azo bands of the corresponding dye. The validity of the use of these derivatives, however, should be further discussed.

The general similarity between the absorption curves of hydroxy and methoxy compounds, due to the "cushioning" effect of the oxygen atom, is well established [3]. Less is known regarding the use of N-methyl derivatives. It would be expected, however, that the replacement of an N—H group by a N—CH_3 group would not cause any appreciable shift in the location of the absorption band.

The question also arises, however, whether or not there would be any steric effect caused by the replacement of a relatively small hydrogen atom by a twice as large methyl group.

Resonance in the azo dyes, giving all of the bonds partial double bond character, tends to make the entire molecule coplanar [4]. In *cis* azo benzene, however, steric effects cause the phenyl groups to be rotated by approximately 50° [5]. This steric effect can be predicted by drawing the *cis* azo benzene

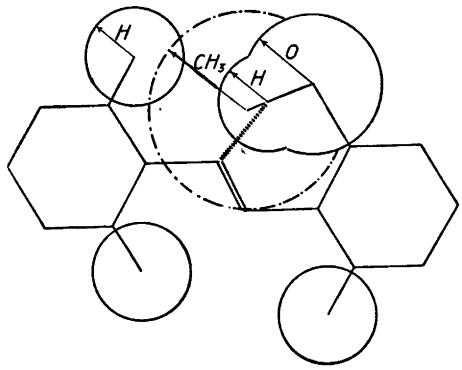


Fig. 8. Schematic diagram of azo form of o-hydroxy azo dye, drawn to scale.

molecule to scale with the use of 1.0 Å for the van der Waals radius for hydrogen [4].

In the *p*-hydroxy dye series, the replacement of the hydroxyl group by a methoxy group would obviously not cause any steric interference on the configuration of the molecule.

If the above-mentioned technique is applied to the hydrazone form of the *p*-hydroxy dye (using a van der Waal radius of 1.0 Å for hydrogen, 1.4 Å for oxygen, and 2.0 Å for the methyl group), Fig. 7 is obtained. It can be seen from this figure that the H attached to the hydrazone grouping overlaps only slightly the adjacent H atom. That is to say, there would be little or no steric interference towards the coplanarity of the molecule. If this H is replaced by a CH_3 group (dot-dash circle), however, it can be seen that the overlapping is nearly complete. This means that the N—CH_3 group would tend to prevent the coplanar configuration of the N—H form.

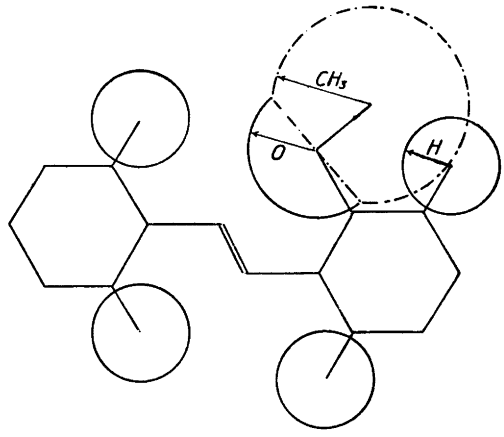


Fig. 9. Schematic diagram of alternative azo form of o-hydroxy azo dye, drawn to scale.

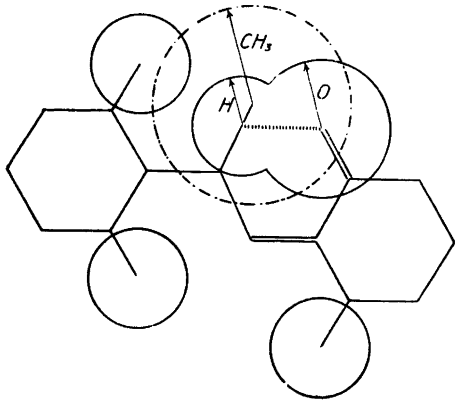


Fig. 10. Schematic diagram of hydrazone form of o-hydroxy azo dye, drawn to scale.

A similar drawing for the azo form of the *o*-hydroxy dye, containing an O—H · · · N hydrogen bond, is shown in Fig. 8. It can be seen that the OH group causes no steric interference and the molecule would be coplanar. If this H atom is replaced by a CH_3 group (dot-dash curve), it overlaps the center of the N atom, to which it is not bound, and thereby tends to prevent coplanarity. In this case, however, there is free rotation about the C—OH bond and the strain could be relieved by such a rotation rather than by a rotation of the phenyl groups. If the C—OCH_3 rotates by 180°, the configuration shown in Fig. 9 is obtained. Here also the CH_3 group overlaps considerably the *o*-hydrogen atom. It will be noticed, however, that a rotation of 90° removes all steric interference and allows the phenyl groups to remain coplanar for both the OH and OCH_3 forms.

A similar drawing for the hydrazone form of the *o*-hydroxy dye, containing an N—H · · · O hydrogen bond, is shown in Fig. 10. Here also, as in the case of the hydrazone form of the *p*-hydroxy dye, the replacement of the N—H by a N—CH_3 group causes strong steric interference and would tend to prevent the coplanar configuration of the N—H form.

Thus it would appear as if the OCH_3 derivative of the *p*-hydroxy dyes can maintain the same configuration as the OH form, while the OCH_3 derivative of the *o*-hydroxy dyes would be rotated by 90° around the C—OCH_3 bond. It would also appear as if the N—CH_3 derivatives of both *o*- and *p*-hydroxy dyes cannot achieve the coplanar configuration of the N—H form.

How much effect these changes would have on the visible absorption spectrum is not known. The close similarity between the OH and OCH_3 curves for the *o*- and *p*-phenol dyes tends to indicate that the effect is slight. In the only case where the N—CH_3 derivative can be compared (α-naphtho-

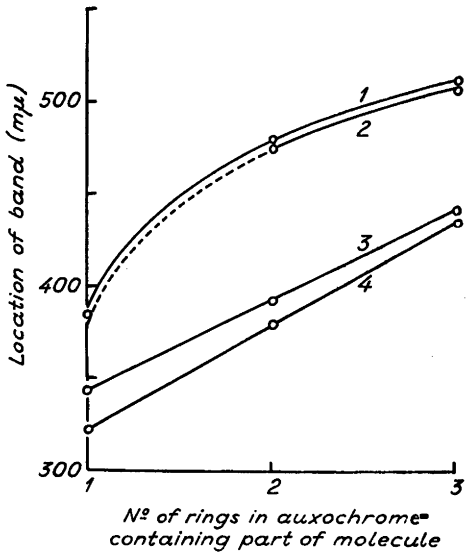


Fig. 11. Effect of linearly annulated benzene nuclei on the location of the azo and hydrazone bands.

Curve 1. hydrazone band of o-hydroxy dye
Curve 2. hydrazone band of p-hydroxy dye
Curve 3. azo band of p-hydroxy dye
Curve 4. azo band of o-hydroxy dye

quinone-methylphenyl-hydrazone), it agrees fairly well with the value for the hydrazone form (see Tables 1 and 2) and thereby indicates that, in this case also, the effect of the change in configuration has little effect on the visible spectrum.

THE EFFECT OF LINEARLY ANNULATED BENZENE NUCLEI ON THE AZO AND HYDRAZONE BANDS

If the values for the location of the azo and hydrazone bands, shown in Table 2, are plotted against the number of rings in the auxochrome- containing part of the molecule, the curves shown in Fig. 11 are obtained.

In this Fig., curves 1 and 2 show the effect of linearly annulated benzene nuclei on the location of the hydrazone band of *o* and *p*-hydroxy azo dyes, respectively. It can be seen that the hydrazone band of the *o*-hydroxy dye is located at slightly longer wave lengths than that of its isomer; however, the difference is very small. This fact can be verified by comparing the curves of the two anthrol dyes (Figs. 3 and 6) as well as the β-naphthol dye in alcohol (Fig. 2) and the α-naphthol dye in acetic acid (Fig. 5), in both of which the hydrazone form predominates. No value for the hydrazone band of the 4-phenylazophenol dye could be obtained. An extrapolation of curve 2 (as shown by the dotted line) would, however, tend to indicate that the hydrazone band of this dye should be located at approx. 375 mμ.

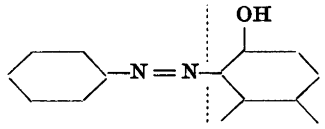
Curves 3 and 4 show the effect of linearly annulated benzene nuclei on the azo bands of *p*- and *o*-hydroxy azo dyes, respectively. It will be noticed that in this case, in contrast to the hydrazone bands, the azo bands of the *p*-hydroxy dyes are located at longer wave lengths than the *ortho* isomers. This difference between the location of the azo bands of the isomeric dyes seems to decrease as the number of annulated rings increases.

The similarity between curves 1 and 2 indicates that the annulation of benzene nuclei to the hydrazone form of the dye has approx. the same effect on both the *ortho* and *para* isomers. In the same way, the similarity between curves 3 and 4 indicates that the azo bands of both isomers are similarly affected by linear annulation. On the other hand, linear annulation does not have the same effect on the azo and hydrazone bands of dyes in the same series. This is shown by the dissimilarity in the curves 1 & 4 and 2 & 3. That is to say, the annulation of one benzene nucleus to the *ortho* phenol dye causes a much greater bathochromic effect on the hydrazone band than on the azo band. The same is true in the *para* series.

The linearity of the bathochromic effect on the azo bands (curves 3 and 4) is striking. Lewis and Calvin [6] have found a similar linear relationship between the absorption max. and the number of conjugated double bonds between chromophors in a series of carbocyanines. This they explained on the basis of resonance in the conjugated chain.

In the case of the azo dyes, the auxochrome and chromophor are also separated by a resonating group, *i. e.*, a benzene, naphthalene, or anthracene group. It is very possible that these act in a similar manner as the resonating chain in the carbocyanines. It is the author's purpose to discuss qualitatively the influence of linearly annulated benzene nuclei on the hydrazone and azo bands of azo dyes in terms of enhanced resonance possibilities.

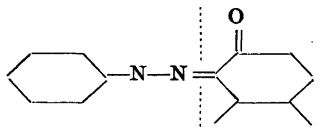
The linear relationship in curves 3 and 4 may be explained in the following way:



The resonance in the left-hand portion of all the dyes (in the azo form) may be considered to be approximately constant, and therefore only the resonance in the auxochromic part need be discussed. The resonance energies of benzene, naphthalene, and anthracene are shown in Table 3. It can be seen that there is an approximately linear relationship between the resonance energies of these three structures, within the fairly large limit of accuracy of

the energy values. The addition of the approximately constant resonance energies of the phenylazo and hydroxyl groups would not affect the linearity of the resonance energies. Thus it can be seen that both the location of the azo band and the resonance energies are linear functions of the number of annulated benzene nuclei.

The hydrazone bands, as can be seen from curves 1 and 2, (Fig. 11) are not linear functions of the number of linearly annulated rings. This non-linearity can, however, be explained in exactly the same manner as was used for the azo bands.



As before, the resonance in the left-hand part of the molecule may be considered constant. An approximate value of the relative resonance energies of the quinoid structures (right-hand part) can be obtained by comparing the 1,2-benzo-, naphtho-, and anthraquinones. The resonance energies of these substances are shown in Table 3. It must be noted that the accuracy of these values is undoubtedly not too great. Their general relationship is, however, supported by other facts. The very low resonance in the benzoquinone structure, for example, is shown by the interatomic distances. The C—C value for this substance is 1.50 Å [7], which corresponds to 92 % single bond character [4], and the C=C value is 1.32 [7], which corresponds to 100 % double bond character [4], showing that the resonance is slight. The increase in resonance energy is also manifested in the reduction in redox-potential (see Table 4).

It can be seen from Table 3 that the linear annulation of a benzene nucleus to 1,2-benzoquinone causes a relatively large increase in resonance energy (approx. 50 kcal/mole) whereas the annulation of another nucleus has a much smaller effect (approx. 30 kcal/mole). This non-linear increase in resonance energy is similar to the increase in the hydrazone band (curves 1 and 2, Fig. 11), in which a large bathochromic effect is noted on the annulation of one benzene nucleus and a much smaller bathochromic effect on the annulation of an additional nucleus.

Thus, once again, a parallel relationship is found to exist between the increase in resonance energy and the shift in the location of the hydrazone band.

Although the above discussion was based on the *o*-hydroxy dyes, the same results are obtained using the *p*-hydroxy dyes (*cf.* resonance energies of the 1,4-quinones, Table 3).

Table 3. The resonance energies of the benzoid and quinoid structures.

Substance	Resonance energy kcal/mole	Diff.
Benzene	41[1]	
		34
Naphthalene	75[2]	
		30
Anthracene	105[2]	
1,2-Benzoquinone	10[3]	
		50
1,2-Naphthoquinone	60[3]	
		30
1,2-Anthraquinone	90[3]	
1,4-Benzoquinone	15[3]	
		47
1,4-Naphthoquinone	62[3]	
		33
1,4-Anthraquinone	95[3]	

1. Dewar, M. J. S. *The electronic theory of organic chemistry*, Oxford (1949).
2. Pauling, L. *The nature of the chemical bond*, Ithaca, N. Y. (1948).
3. Calculated from data given by Berliner, E. *J. Am. Chem. Soc.* 68 (1946) 49.
(The values for the anthraquinones were obtained by using the redox-potentials, see Table 4, and the curve given by Berliner.)

AZO-HYDRAZONE EQUILIBRIUM

The author has previously discussed [1] the effect of hydrogen bond formation on the azo-hydrazone equilibrium in azo dyes. The effect of linearly annulated benzene nuclei on this equilibrium has now been examined.

It was found that as the number of linearly annulated rings, in the auxochrome-containing part of the molecule, increased, the relative amount of hydrazone form increased, until in the anthrol dyes the equilibrium was apparently completely displaced in favor of the hydrazone form.

In the above-mentioned paper [1], the author discussed the breakdown of the absorption spectrum of 1-phenylazo-naphthol-(2) and various derivatives into the component azo and hydrazone bands. The use of the value $\frac{\varepsilon_H}{\varepsilon_A + \varepsilon_H}$ as indicative of the relative amount of hydrazone form was also discussed.

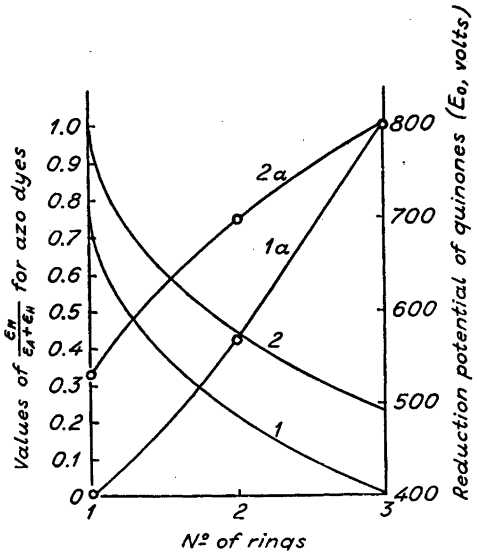


Fig. 12.

Using the values given in Table 2 as indicative of the approximate location of the hydrazone and azo bands, the double maxima curves of 2-phenylazo-phenol and 4-phenylazo-naphthol-(1) can also be broken down into their component azo and hydrazone bands and the values of $\frac{\varepsilon_H}{\varepsilon_A + \varepsilon_H}$ calculated. It was found that 2-phenylazo-phenol gave a value of approx. 0.32 for $\frac{\varepsilon_H}{\varepsilon_A + \varepsilon_H}$ and 4-phenylazo-naphthol-(1) a value of approx. 0.42. The value for 1-phenylazo-naphthol-(2), previously found, is approx. 0.75. As previously discussed [1], if the extinction values for the pure azo and hydrazone forms are similar, then the above values times one hundred are the approximate percent hydrazone form.

The nearly perfect symmetry of the 4-phenylazo-phenol curve and its close similarity to the curve of its O—CH_3 derivative (see Table 1 and Fig. 4) tend to indicate that this dye exists completely in the azo form, *i. e.*, $\frac{\varepsilon_H}{\varepsilon_A + \varepsilon_H} \cong 0$.

The maxima in the visible spectrum for both 4-phenylazo-anthrol-(1) and 1-phenylazo-anthrol-(2) are also almost perfectly symmetrical but differ widely from those of their O—CH_3 derivatives. For these reasons, it appears as if these dyes exist almost completely in the hydrazone form, *i. e.*, $\frac{\varepsilon_H}{\varepsilon_A + \varepsilon_H} \cong 1$.

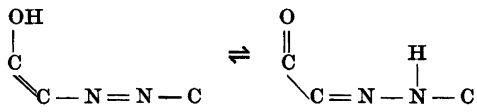
If these values of $\frac{\varepsilon_H}{\varepsilon_A + \varepsilon_H}$ are plotted against the number of rings in the auxochrome-containing part of the molecule, *i. e.*, in the phenolic component, the curves 1 a and 2 a in Fig. 12 are obtained. This shows that as the number of rings in the phenolic component of *p*-hydroxy azo dyes increases from 1 to 3 the relative amount of hydrazone form increases from 0 to 1.0. In the *o*-hydroxy dyes, the same increase in the number of rings causes the amount of hydrazone form to increase from approx. 0.3 to 1.0.

One cause of this shift in the azo-hydrazone equilibrium would be expected to be a change in the relative stability of the two forms. Branch and Calvin [8] have calculated that the hydrazone form of the triad system:

$$H-C-N=N \rightleftharpoons C=N-N-H$$

is more stable by approximately 9 kcal./mole.

A system such as:



which occurs in *o*-hydroxy dyes, gives a value of 432 kcal./mole for the summation of bond energies for the azo form and 456 kcal./mole for the hydrazone form. That is, the hydrazone form, neglecting resonance effects, is more stable by approx. 24 kcal./mole. Thus it can be seen that the hydrazone grouping is, in general, more stable than the azo grouping.

Also the stability of the resulting quinoid structure in the hydrazone form would be expected to be of importance. Table 4 shows the reduction potentials of the 1,2- and 1,4- quinones. Curves 1 and 2 in Fig. 12 show the plot of these values. It can be seen that as the stability of the quinoid structure increases (*i. e.*, as the reduction potential decreases) the relative amount of the hydrazone form of the dye (which contains the quinoid structure) increases.

On this basis, the curves 1 a and 2 a in Fig. 12 could be explained in the following way. Although the hydrazone grouping is, in itself, always more stable than the azo grouping, the relative instability of the quinoid structure in the phenol dyes counteracts this effect and causes an equilibrium to be established which favors the azo form. In the naphthol dyes, which contain the relatively more stable 1,2- or 1,4- naphthoquinone structures, the equilibrium is displaced further toward the hydrazone form. In the anthrol dyes, the anthraquinone structures are so stable that the equilibrium is displaced completely in favor of the hydrazone form.

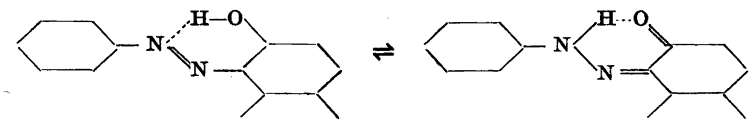
Table 4. Reduction potentials of quinones (25°).

Quinone	E_0 volts	
	Aq. sol.	Alc. sol.
1,4-Benzoquinone	0.699	0.715 [1]
1,2 »	0.794 [2]	....
1,4-Naphthoquinone	0.470 [3]	0.484 [4]
1,2 »	0.555 [4]	0.576 [2]
1,4-Anthraquinone	.....	0.401 [5]
1,2 »	.....	0.490 [6]

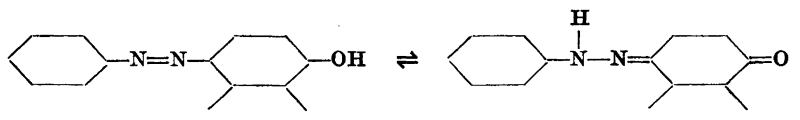
[1] Fieser, L. F. *J. Am. Chem. Soc.* **52** (1930) 4915.
[2] Fieser, L. F., and Peters, M. A. *Ibid.* **53** (1931) 793.
[3] La Mer, V. K., and Baker, L. E. *Ibid.* **44** (1922) 1954.
[4] Fieser, L. F., and Fieser, M. *Ibid.* **56** (1934) 1565.
[5] Fieser, L. F. *Ibid.* **50** (1928) 465.
[6] Conant, J. B., and Fieser, L. F. *Ibid.* **46** (1924) 1858.

It will be noticed, that the 1,4-quinone structure is always more stable than the isomeric 1,2-quinone (Table 4). On the basis of the above reasoning, one would expect that the *p*-hydroxy dyes would therefore show a larger amount of hydrazone form than the corresponding *o*-hydroxy dyes. This, however, is not the case. The *o*-phenol and *β*-naphthol dyes both show a larger $\frac{\varepsilon_H}{\varepsilon_A + \varepsilon_H}$ value than the isomeric *p*-phenol and *α*-naphthol dyes (Fig. 12). This fact would tend to indicate that there is another factor which influences the position of the azo-hydrazone equilibrium.

In the *o*-hydroxy dyes the tautomeric equilibrium is between the following two structures:

I 

whereas that in the *p*-hydroxy dyes is between the structures:

II 

In I, the change between the azo and hydrazone form is very easily accomplished by a slight displacement of the proton and a simultaneous rearrangement of the electrons. This undoubtedly intramolecular mechanism should allow for a very mobile tautomerism [9].

The equilibrium II, which is accompanied by a relatively large displacement of the proton, must proceed by some other mechanism and would not be expected to be as mobile as I. It is possible that II involves an intermolecular mechanism since it shows a great dependence on solvent, whereas I is almost independent of solvent [2].

Thus the relatively greater amount of hydrazone form in the *o*-hydroxy dyes, in spite of the lower stability of its 1,2-quinoid structure, can be ascribed to some factor dependent on the mechanism or mobility of the tautomerism. This point will be further discussed in a later publication.

EXPERIMENTAL

β-Anthrol. Sodium anthraquinone-(2)-sulfonate was reduced according to Liebermann [10]. Recrystallization from boiling water yielded almost colorless crystals, which were dried at 165° to remove all water of crystallization. Yield approx. 65 %.

$C_{14}H_9SO_3Na$ (280.27)	Calc.	S	11.44
	Found	»	11.55

The resultant sodium anthracene-(2)-sulfonate was fused with KOH according to Lagodzinski [11]. Recrystallization from benzene yielded light yellow-tan crystals. The yield of crude β-anthrol was approx. quantitative. M. p. 253—254° (lit. 255°).

$C_{14}H_{10}O$ (194.22)	Calc.	C	86.57	H	5.19
	Found	»	86.43	»	5.06

α-Anthrol. Sodium anthraquinone-(1)-sulfonate was reduced according to Schmidt [12] and the resulting sodium anthracene-(1)-sulfonate was fused with KOH according to Dienel [13].

Recrystallization from aqueous alcohol yielded yellow-tan crystals. M. p. 150—151° (lit. 150—153°).

$C_{14}H_{10}O$ (194.22)	Calc.	C	86.57	H	5.19
	Found	»	86.48	»	5.08

4-Phenylazo-phenol. The preparation of this dye was given in detail in a previous publication [1].

It was obtained as yellow-brown crystals from aqueous alcohol. M. p. 151—152° (lit. 152, 154°).

$C_{12}H_{10}N_2O$ (198.22)	Calc.	N	14.13
	Found	»	13.97

4-Phenylazo-phenol, methyl ether. This was prepared from the above with diazomethane according to Smith [14]. Two recrystallizations from petroleum ether yielded orange-red plates. M. p. 52—53° (lit. 53°).

4-Phenylazo-naphthol-(1). This was prepared in the usual manner [1] from α-naphthol and benzene diazonium chloride.

Two recrystallizations from benzene yielded dark-violet crystals. M. p. 205—206° (lit. 206°).

$C_{16}H_{12}N_2O$ (248.27)	Calc.	N	11.31
	Found	»	11.34

4-Phenylazo-naphthol-(1), methyl ether. This was prepared from the above by means of dimethyl sulfate according to Charrier [15].

Recrystallization from aqueous alcohol yielded red-orange crystals. M. p. 82—83° (lit. 83°).

4-Phenylazo-anthrol-(1). This was prepared according to Sircar [16]. However, it could not be obtained in a crystalline form, as claimed by the above author, but precipitated from benzene as a dark-violet powder.

$C_{20}H_{14}N_2O$ (298.33)	Calc.	N	9.40
	Found	»	9.60

4-Phenylazo-anthrol-(1), methyl ether. 1 g of 4-phenylazo-anthrol-(1) was added to 100 ml of aqueous NaOH (5 g NaOH per 100 ml water). 5 ml of dimethyl sulfate were added and the mixture was refluxed on a water bath for several hours. It was then poured into a large excess of water and extracted several times with large amounts of ether. The ether was dried over Na_2SO_4 and evaporated. The resultant red powder was recrystallized first from 96 % alcohol and then from benzene yielding fine red crystals. M. p. 140—141°.

$C_{21}H_{16}N_2O$ (312.36)	Calc.	OCH_3	9.93
	Found	»	9.98

This compound is not previously reported in the literature.

2-Phenylazo-phenol. This was prepared according to Bamberger [17]. Recrystallization from ether and alcohol-water yielded fine orange needles. M. p. 81—82° (lit. 82.5—83°).

2-Phenylazo-phenol, methyl ether. This was also prepared according to Bamberger [18] from *o*-anisidine and nitrosobenzene. The orange oil obtained, however, did not crystallize even after standing several months *in vacuo* at 0°. A methoxyl determination on the orange oil, however, showed the substance to be quite pure.

Calc.	OCH_3	14.62
Found	»	14.89

1-Phenylazo-naphthol-(2). The preparation of this dye was given in detail in a previous publication [1].

It was obtained as red-orange needles from ether. M. p. 131—132° (lit. 128.5°).

$C_{16}H_{12}N_2O$ (248.27)	Calc.	N	11.31
	Found	»	11.28

1-Phenylazo-naphthol-(2), methyl ether. The above dye was methylated according to Charrier [15] with dimethyl sulfate.

Recrystallization from aqueous alcohol yielded beautiful red plates. M. p. 61—62° (lit. 62°).

1-Phenylazo-anthrol-(2). This dye was prepared in the same manner as the isomeric *a*-anthrol dye.

Two recrystallizations from 96 % alcohol yielded fine red-orange needles. M. p. 199° (decomposition).

$C_{20}H_{14}N_2O$ (298.33)	Calc.	N	9.40
	Found	»	9.61

The isolation of this dye has not previously been reported.

1-Phenylazo-anthrol-(2), methyl ether. Attempts were made to methylate the above dye with dimethyl sulfate in aqueous KOH and with methyl iodide and silver oxide. In neither case could the absolutely pure crystalline substance be obtained.

SUMMARY

The effect of linearly annulated benzene nuclei on the isomeric *o*- and *p*-hydroxy azo dyes has been examined. The absorption curves of the phenolic dyes and their methyl ethers are given. The location of the azo and hydrazone bands of each of the dyes is discussed, as well as the configuration of the O—CH_3 and N—CH_3 derivatives.

The shift in the location of the azo and hydrazone bands caused by linear annulation is explained on the basis of resonance. It was found that the azo band showed a linear increase while the hydrazone band did not.

The location of the azo-hydrazone equilibrium is explained on the basis of three factors:

1. The relatively greater stability of the hydrazone grouping.
2. The relative stability of the quinoid structure in the hydrazone form.
3. A factor depending on the mechanism or mobility of the tautomerism.

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A Rapid Colorimetric Method for the Assay of Xylocaine * and the Determination of some other Organic Bases, using Ammonium Reineckate

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The new local anaesthetic α-diethylamino-2,6-acetoxylidide, known as Xylocaine or Lidocaine, has acquired an ever increasing application since it was first synthesised by Löfgren [1,2] and Lundqvist [1], and there is now a great need for a rapid and certain method for a quantitative estimation of the substance in pharmaceutical preparations. The existing procedure, involving solvent extraction of the Xylocaine and subsequent titration, is troublesome and time-consuming, and we therefore began as early as 1948 to experiment with an alternative colorimetric method.

Many organic bases are known to give precipitates with ammonium reineckate, ($[Cr\begin{matrix}(SCN)_4\\(NH_3)_2\end{matrix}]NH_4$), and the reaction has been utilised for quantitative estimations by Canbäck [3], Steigner and Hippenmeyer [4], Brandelin, Slifer and Pankratz [5] and others. It has now been found that under controlled conditions Xylocaine also is readily and quantitatively precipitated by ammonium reineckate, and by colorimetric measurement of an acetone solution of the precipitate an accurate estimation of the base can be achieved.

PROCEDURE

A known volume (not more than 10 ml) of solution containing approximately 0.1 mg-equiv. (25 mg) of Xylocaine is acidified to pH 2 or a little lower with hydrochloric acid, and 5 ml of a saturated aqueous solution of ammonium reineckate is then *slowly* added dropwise with vigorous stirring at room temperature; the addition should extend over at least one minute. The mixture is then allowed to stand for a period; this should

* Regd. trade mark.

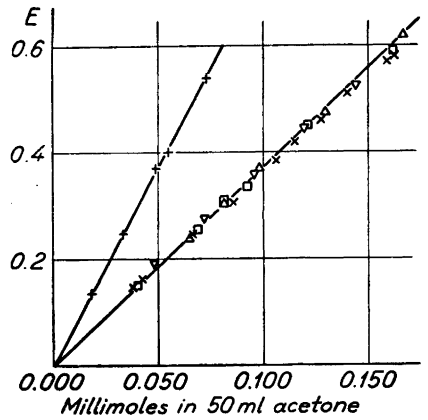

Fig. 1. Extinction-concentration curves (Brunius colorimeter filter S 53, 2 cm cell).
Reineckates of
\+ *procaine*
□ *aniline*
▽ *LL 31*
× *Xylocaine*
△ *"Thiergan"*

be not less than 5—10 minutes, but may be prolonged to as much as 24 hours without adverse effect. The precipitate is collected by suction on a small filter and washed with water (two portions of 2 ml); when drained it is dissolved in acetone whilst still on the filter and the resulting solution transferred to a volumetric flask and diluted to 50 ml. The extinction value of the solution is then measured, either on a Brunius type colorimeter * using a S53 filter and a 20 mm cell or on an EEL type ** instrument with filter 624 and the appropriate round cell. The extinction value is directly proportional to the amount of Xylocaine present (see Fig. 1), and a previously prepared standard curve or a conversion factor gives the actual weight of base in the sample of solution taken.

If the concentration of Xylocaine in the solution to be examined is less than 3 mg/ml, the determination can be carried out on a larger volume than 10 ml such that the amount of base taken is again of the order of 25 mg, provided that the volume of ammonium reineckate reagent used is also increased by 5 ml for every additional 30 ml of the Xylocaine solution. In such cases the mixture should be allowed to stand for an hour or more before filtration of the precipitate.

The saturated ammonium reineckate solution should be freshly prepared each day; it is conveniently made by mechanically shaking 1.5 g of the salt with 100 ml of water at room temperature for 15 minutes and then filtering. The salt used should be of good quality it deteriorates slowly on prolonged storage and may then give rise to low results in the Xylocaine estimation (a sample of the salt which had been stored for ten years gave values 14 % lower than those given by a fresh sample).

The acetone employed should be free from ester impurities, which may cause extraneous coloration of the solution of precipitated reineckate; the water-content has little influence on the results of the estimation provided that it is below 10 %. Suitable tests for the purity of the solvent have been described by Merck (*Prüfung der chemischen Reagenzien auf Reinheit*, Darmstadt (1939) p. 1), and when these are satisfactorily met the extinction value of the acetone solution at a wavelength of 530 mμ remains unchanged for at least 5 hours at room temperature.

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* A single-cell photoelectric colorimeter constructed by Professor Brunius, State Institute for Public Health, Stockholm, Sweden.

** Evans Electroselenium Ltd., Harlow, Essex, England.

APPLICABILITY OF THE METHOD TO BASES OTHER THAN XYLOCAINE

Although Xylocaine reineckate is practically insoluble in water and in the ammonium reineckate reagent, the solubilities of the reineckates of other bases vary considerably (see Table 1); the method is therefore not of universal applicability and for the present must be tested in each individual case. The procedure described above has been found to give good results for the estimations of β-diethylaminoethyl 4-aminobenzoate (procaine), diethylamino acetomesidide ("LL31"), and 10-[β-(dimethylamino)-propyl]-phenthiazine ("Thiergan"). With aniline and diethylaminoethanol it was found possible to obtain quantitative results by using a modified procedure. A more concentrated solution of the base was employed, containing about 0.05 mg-equiv. per ml; 2 ml of this was precipitated at 0° with 5 ml of reagent, and the mixture allowed to stand at that temperature for a few hours before filtration. Finally, the precipitate was washed with ice-cold water (two portions of 2 ml). The reineckates of α-(3,4-dihydroxyphenyl)-β-methyl-aminoethanol (adrenaline), 1-phenyl-2-aminopropane (phenopromine), diethylamine, diethanolamine and guanidine all proved to be far too soluble to give quantitative results. (This

Table 1. Losses of some freshly precipitated reineckates in different washing procedures. Precipitates (corresponding to approx. 0.12 mg.-equiv. base) washed on filter, extinction values measured in acetone solution. Differences between values so obtained and theoretical values corresponding to amount of base taken given as % of latter.

Washing agent	Temp. °C	Reineckate of				
		Aniline	"Thiergan"	"LL 31"	Xylocaine	Procaine
	20	48	10	8	8	25
50 ml dist.	10	40	5	5	10	23
water	3	21	2	6	8	17
	20	5	3	0	0	0
2 × 2 ml dist.	3	1	0	0	0	0
water	0	0	0	0	0	0
50 ml 0.15 % ammonium reineckate solution	20	63	0	0	0	17
followed by 1 ml dist. water	3	15	0	0	0	—

fact has rendered it possible to accurately estimate Xylocaine in pharmaceutical preparations such as "Umbradil Viskös" and "Isofen", in which diethanolamine and diethylamine are also present. Such solutions, however, must be specially treated before the estimation can be carried out).

It is clear that the applicability of the reineckate method is not governed merely by the strength or type of the base concerned. Thus, among the substances which give quantitative precipitates under the specified conditions, are five aromatic compounds having pK_a values between 9 and 4.5, but adrenaline, which has a pK_a value in this range (approx. 7), is not precipitated quantitatively and 1-phenyl-2-amino-propane (pK_a approx. 10) gives only a negligible precipitate. Among the aliphatic bases investigated only one, diethylaminoethanol (pK_a approx. 10), gives a quantitative result and that only under modified conditions.

Extinction values for the reineckates of all the bases which have been investigated are given in Table 2.

SOME GENERAL OBSERVATIONS ON THE METHOD

The reineckate method has been tested at first hand in rapid routine analyses of preparations which apart from Xylocaine itself contain only sodium chloride, methyl *p*-hydroxy-benzoate and small amounts of adrenaline (the latter does not interfere owing to its very small concentration and to the solubility of its reineckate). A statistical calculation of the accuracy of the method has been made from data obtained in analyses on twelve solutions of "Xylocaine-Exadrin 2 %", in which the Xylocaine content was also independently estimated by the older extraction method. The ratio of corresponding colorimetrically and titrimetrically determined values was 1 ± 0.03, the average ratio being 1.003. The standard deviation for individual ratios $\left(\sqrt{\frac{\Sigma\, \Delta^2}{n}}\right)$ was $\pm$ 2 %; this figure includes the experimental errors of both methods. In the case of nine samples of "Umbradil Viskös" solutions "B" and "H", colorimetric determinations gave values of 100 $\pm$ 2 %. The standard deviation here was $\pm$ 1.6 %, which represents variations not only in the estimations themselves but also in the pre-treatment of the solutions. A group of 120 arbitrarily chosen solutions of "Xylocaine-Exadrin 2 %" were analysed by eight different persons; the average, the maximum, and the minimum values obtained were respectively 20.2, 21.0 and 19.2 mg/ml, and the standard deviation of the individual values was $\pm$ 2 % (this includes possible inaccuracies in the preparation of the solutions).

Table 2. Summary of results for reineckates investigated. pK_a values of base components are approximated to one place of decimals without consideration of the difference between thermodynamic and stoichiometric constants. Values in parentheses are calculated from original potentiometric titration results; other values are taken from Löfgren [2] or Landolt-Börnstein "Physikalisch-Chemische Tabellen". Extinction values measured according to standard procedure and calculated on the amount of base in the original solution.

Reineckate of	Remarks	pK_a	$E^{1\text{ g mol/l}}_{1\text{ cm}}$ 526 mμ	$E^{1\text{ g equiv/l}}_{1\text{ cm}}$ 526 mμ	$E^{1\text{ g equiv/l}}_{1\text{ cm}}$ 395 mμ
Ammonium	Salt recryst. 3 times from water. Extinction constant after second and third recrystallisations.	9.3	106.0	106.0	85.5
Aniline	Freshly distilled aniline precipitated in ether as hydrochloride and recryst. twice. Nitrogen content (Dumas) taken to correspond to % aniline.	4.6	107.5	107.5	88.5
"Thiergan"	100 % pure	(7.8)	105.5	105.5	85.0
Diethylamino-acetomesidide ("LL 31")	100 % pure m.p. 46—48°	7.9	106.0	106.0	81.9
Xylocaine	100 % pure m.p. 68—70°.	7.9	106.3	106.3	85.3
Procaine	Quality prescribed by Swedish Pharmacopeia Ed. XI. Purity (determined by titration) 100 %.	8.9	210.0	105.0	85.0
Diethylamino-ethanol	Titration indicated 96 % purity. The preparate was probably not entirely homogeneous.	(10.1)	104	104	84.5
1-Phenyl-2-aminopropane	Quality prescribed by Swedish Pharmacopeia Ed. XI. Purity (determined by titration) 100 %.	10.0	91	No quantitative precipitation with ammonium-reineckate	
Adrenaline	»	(8.9)	89		

Diethylamine	Purity (determined by titration) 100 %	12.0	—	No precipitate	
Diethanol-amine	»	7.9	—		
Guanidine	»	rather strong base	—		
Average over the first six estimations				106.0 ± 0.4	85.1 ± 0.9

In the case of the other bases to which the reineckate method is applicable no large series of analyses have been carried out; the distribution of the points on the standard curve in Fig. 1 suggests, however, that the accuracy of the estimation is of the same order as that for Xylocaine.

The sensitivity of the method is mainly determined by the minimum extinction value which can be accurately measured. On the Brunius colorimeter this is approximately 0.15, and when the standard procedure is followed and the colorimetric measurements are made in a 2 cm cell this corresponds to about 0.04 mg-equiv. of base. Smaller amounts of base may however be estimated by increasing the cell thickness and diminishing the amount of acetone used to dissolve the reineckate precipitate; thus, employing a 3 cm cell and only 10 ml of solvent instead of 50 ml, the minimum that can be estimated is approximately 0.005 mg-equiv. (1.2 mg Xylocaine). The sensitivity is limited not by the absorption of the reineckate precipitate in acetone solution but rather by the solubility of the precipitate in the aqueous media employed in the estimation.

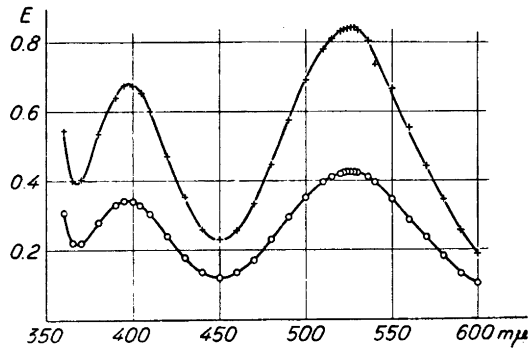

Fig. 2. Extinction-wavelength curves (Beckman Spectrophotometer DU, 0.004 M solutions, 1 cm cell).

\+ *Reineckate of procaine*

O *Reineckates of aniline, LL 31, Xylocaine, and "Thiergan"*

Table 3. Values of $10^3 \cdot E_{1\ cm}^{0.004\ M}$ *for some reineckates in acetone solution (determined on Beckman DU spectrophotometer).*

Slit no.	Wave-length mμ	Reineckate of							
		Procaine	Xylocaine	"LL 31"	"Thiergan"	Aniline	Ammonium *	Ammonium **	Ammonium ***
0.2	500	690	347	346	349	360	348	350	349
»	510	778	391	391	393	403	389	391	390
»	520	831	417	418	416	425	415	422	417
»	526	840	425	424	422	430	418	426	424
»	530	832	425	422	420	428	415	422	418
»	540	733	397	397	394	400	388	393	390
»	560	552	289	292	290	289	274	281	275

* Recrystallised once.
** » twice.
*** » three times.

The color given in acetone solution by reineckates of organic bases is, as has been pointed out by Canbäck[3], due to the presence of the reineckate group. In equimolar solutions, therefore, the intensity of the color is independent of the actual base concerned. We found that the quantity $E_1^{1\ \text{g-equiv/l}}_{\text{cm}}$ has a value of 85 ± 1 at 395 mμ and 106.1 ± 0.2 at 526 mμ. This fact has been verified for 0.004 *M* solutions of ammonium, aniline, diethylaminoacetomesidide, diethylaminoethanol and 10-[β-(dimethylamino)-propyl]- phenthiazine reineckates, all of which give the same curve, namely the lower one in Fig. 2 (the readings were taken on a Beckman DU spectrophotometer with a 1 cm cell). The degree of coincidence is also clearly seen from the extinction values in the wavelenth region of 500—560 mμ, given in Table 3. This generalisation is, of course, only true if the base combines with a single reineckate residue. Procaine, for example, combines with two such residues, one at the primary and one at the tertiary nitrogen atom, and the resulting extinction value is therefore twice as great as that given by an equivalent amount of any of the other bases investigated. It should however be noted that the basicity of a substance with respect to reineckate is not always the same as when determined by titrimetric methods; thus procaine although it forms a di-reineckate, behaves only as a monobasic compound on titration.

This latter fact renders it possible to estimate individually the components of a binary mixture of bases, one of which exhibits identical, the other different

Table 4. Analyses of solutions containing mixtures of Xylocaine and procaine hydrochlorides. Values given as % of total base-hydrochloride present.

Amount taken		Amount found		
% Xylocaine HCl	% Procaine HCl	% Xylocaine HCl	% Procaine HCl	% Total
11.3	88.7	13.8	87.2	101.0
46.7	53.3	46.7	52.4	99.1
91.2	8.8	89.6	9.8	99.4

equivalent weights in the two cases, by carrying out both colorimetric and titrimetric determinations. The accuracy is however poor if one of the bases is present in large excess compared with the other. In Table 4 are included the results of three such estimations on mixtures of Xylocaine and procaine (in order to increase the accuracy in these cases, a Beckman DU spectrophotometer was employed). Similarly it is possible to follow the decomposition of procaine into diethylaminoethanol and *p*-aminobenzoic acid, since the basicities of these compounds with respect to reineckate are two, one and zero respectively. In cases where the equivalent weight of a base cannot be directly determined titrimetrically owing to the precence of another base, the reineckate method may provide an alternative if the accompanying base does not give an insoluble reineckate. Thus Xylocaine may be determined in the presence of diethylamine, and procaine may be estimated in the presence of caffeine provided the pH is adjusted fairly exactly to 2 before precipitation. Procaine in procaine penicillin can also easily be determined with reineckate.

SUMMARY

A rapid method for the estimation of Xylocaine by means of ammonium reineckate is described. It is based on spectrophotometric measurement of the colour produced in acetone solution by the reineckate ion. In routine analysis the standard deviation of the results of individual estimations does not exceed $\pm$ 2 % and under favourable conditions it can be reduced to $\pm$ 1 %. The method is also applicable to many other organic bases and is under certain conditions superior to the ordinary titrimetric procedure. By combining the latter with the colorimetric method it is possible to estimate separately the constituents of some binary mixtures of bases.

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Aryloxyesters. The Reacitivity of the α-Hydrogen and the Carbonyl Carbon towards Basic Reagents

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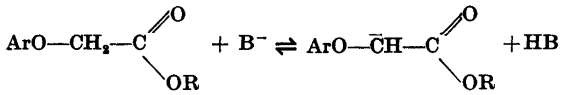
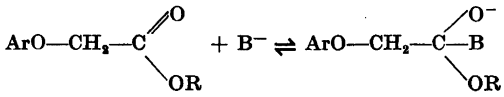
The two ways in which a base B^- is able to react with a carbonyl compound, *e. g.* an aryloxyacetic ester, may be represented by the two equations:

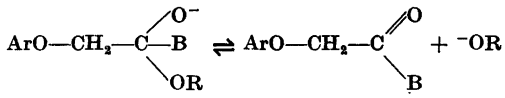


I



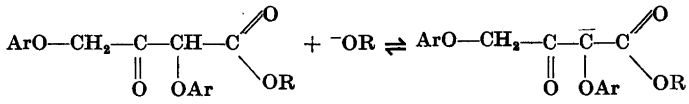
II

The reaction product of equation II may be stabilized by splitting off an alkoxide ion:



III

If the anion formed according to I acts as B^- in II, condensation takes place, and the β-ketoester formed will be stabilized by donating a proton to the stronger base, the alkoxide ion:



IV

In a previous paper [1] the behaviour of alkoxy and aryloxy ketones and esters in acylation processes was discussed, and the view was expressed that the alkoxy group activates the carbonyl carbon relatively more than the α-hydrogen towards basic reagents. The case of aryloxy esters, however, was not thoroughly studied, and, in view of the importance that many of the corresponding acids and some of the esters have recently acquired, a study was made of the acylation of certain of these esters.

The aryloxy group seems to activate the α-hydrogen as well as the carbonyl carbon, but, as in the alkoxy esters, the carbonyl carbon is relatively more active than the α-hydrogen when compared with the corresponding unsubstituted esters. All attempts to acylate ethyl phenoxyacetate (and ethyl 2,4-dichlorophenoxyacetate) with phenyl benzoate or phenyl propionate * using sodium amide [3] as a condensing agent, or with benzoyl chloride or acetyl chloride using potassium triphenylmethide [1] failed completely, the self-condensation product exclusively being obtained. This was also the case when ethyl phenoxyacetate was added to the stirred and previously refluxed mixture of ethyl benzoate and sodium ethoxide [4] and even when ethyl phenoxyacetate and sodium were added in small portions to heated ethyl benzoate over a period of three hours [5]. Self-condensation also was the only result of an attempt to propionylate t-butyl phenoxyacetate, in which the carbonyl group should be less active than in the ethyl ester **.

That the α-hydrogen also is active was shown by an attempt to use ethyl phenoxyacetate as an acylating agent towards ethyl propionate and t-butyl acetate. Again mainly self-condensation of the phenoxy ester took place. Ketones, however, appear to be successfully acylated by phenoxy esters. Walther [8] acylated acetophenone (and phenylacetonitrile) with ethyl phenoxyacetate using sodium ethoxide, but no yields are given. Henecka [9] recently reported the acylation of acetone in 30 % yield with methyl phenoxyacetate using pulverized sodium. In the present work acetophenone was acylated with ethyl phenoxyacetate using sodium amide in ether suspension [10] the formation of the anion of acetophenone being indicated by the copious evolution

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* Phenyl esters are more active acylating agents than are ethyl esters [2,1]; the reaction according to equation III is facilitated, since the phenoxide ion is a weaker base than the alkoxide ion.

** Apparently the more active esters ethyl formate or ethyl oxalate[4,6] must be used in order to effect acylation by the usual procedures. A preliminary experiment was carried out condensing ethyl phenoxyacetate with benzaldehyde, the carbonyl group of which is much more active than those of the esters. Ethyl α-phenoxycinnamate, m. p. 47—48° and α-phenoxycinnamic acid, m. p. 181—182° (reported 184° [7]) were obtained and identified. This reaction will be further studied.

of ammonia before adding the ester. A 60 % yield was obtained. ω-Methoxyacetophenone, however, was not acylated by ethyl phenoxyacetate under similar conditions, but this ketone was previously shown to be rather unreactive [1].

Generally the self-condensations of phenoxy esters were satisfactory (Table 1). In most cases diisopropylaminomagnesium bromide [1,11] was used as a condensing agent. Some of the acylation-attempts may be considered as self-condensations since they gave excellent yields as such, viz. those of ethyl phenoxyacetate, ethyl 2,4-dichlorophenoxyacetate and t-butyl phenoxyacetate, using sodium amide.

Ethyl 2,4,6-trichlorophenoxyacetate gives a notably low yield (28 %) of self-condensation using diisopropylaminomagnesium bromide, and when sodium amide is used, no self-condensation occurs, the ester being almost completely converted into the amide (according to equations II and III). Similar results were obtained with ethyl 2-methyl-4-chlorophenoxyacetate. The t-butyl ester of 2,4,6-trichlorophenoxyacetic acid, however, gives a good yield (51 %) of the corresponding acetoacetic ester, using diisopropylaminomagnesium bromide. These results may perhaps be explained by a steric effect of the large 2,4,6-trichlorophenoxy group. (This effect is also shown by the 2-methyl-4-chlorophenoxy group, but, apparently, not by the 2,4-dichlorophenoxy group.) In the case of the ethyl ester, the attack of the base on the α-hydrogen should be hindered; the carbonyl group, therefore, will react first with the base, the yield of self-condensation product consequently being reduced. In the case of the t-butyl ester the steric effect of the trichlorophenoxy group should be counterbalanced by the t-butoxy group, which will delay the attack on the carbonyl carbon.

The t-butyl ester of phenoxyacetic acid also gave a good yield of self-condensation product. In the attempt to propionylate this ester, distillation *in vacuo* of the condensation product was tried, but destruction took place. The yellow distillate, after standing for several days, crystallized and was identified as α,γ-diphenoxyacetone, apparently the decarboxylation product of the self-condensation product.

The self-condensations of α-aryloxypropionic esters were complicated by the fact that the resulting β-ketoesters have no α-hydrogen and, accordingly, do not form any sodium derivative, so difficulties arose in separating them from the unchanged esters. Equation IV is not valid for α-substituted propionic esters and, accordingly, the yields by the acylations of these esters are generally lower than by the corresponding acetic esters. In the case of both α-phenoxypropionic and α-(2,4-dichlorophenoxy)propionic ester [1], however, probably self-condensation occurred and no carbonyl carbon attack by the

condensing base was observed (no nitrogen was found by the sodium fusion test). In order to obtain a crystalline product, ethyl α-(2,4,6-trichlorophenoxy)-propionate was prepared and self-condensed in the usual way. As in the case of the acetic ester, the yield was rather low, but the β-ketoester was obtained pure in a 22 % yield.

EXPERIMENTAL

All melting and boiling points are uncorrected.

Ethyl 2,4-dichlorophenoxyacetate, b. p. 147° at 1 mm (reported 149—155° at 5 mm [13]) was obtained in 66 % yield from 2,4-dichlorophenoxyacetic acid, m. p. 138.5—139.5° (reported 139—140° [14] and 138° [15]) by refluxing the ethanolic solution with a small amount of concentrated sulfuric acid.

Similarly *ethyl 2-methyl-4-chlorophenoxyacetate*, b. p. 158—160° at 10 mm was obtained in 82 % yield from the corresponding acid. This 2-methyl-4-chlorophenoxyacetic acid, m. p. 113—115° (reported 119—120° [14]) was obtained by four recrystallizations from benzene of the technical product. Alkaline hydrolysis of the ester:

$C_{11}H_{13}O_3Cl$ (228.67) Equiv. wt. found 228.5

The remaining ethyl esters were prepared according to the following general procedure: One equivalent of the corresponding phenol in ethanolic solution was added to a solution of one equivalent of sodium in ethanol. While this was gently refluxing, one equivalent of ethyl chloroacetate in ethanol was added from a dropping funnel. After refluxing on a water-bath (preferably with stirring to avoid bumping) for 3—4 hours, generally the equivalent amount of sodium chloride had separated. Most of the excess of ethanol was distilled on the water-bath, the residue cooled and poured into water. Ether was added, the water layer extracted with ether, the combined ether layers washed with ice-cold 2 *N* sodium hydroxide solution, and dried over anhydrous potassium carbonate. The ether was distilled and the residue distilled *in vacuo*.

Ethyl phenoxyacetate, b. p. 250—251° (reported 250° [16]), yield 65 %. Alkaline hydrolysis:

$C_{10}H_{12}O_3$ (180.20) Equiv. wt. found 179.1

Ethyl 2,4,6-trichlorophenoxyacetate, m. p. 41.5—42.5° (reported 33—34° [12]). On pouring the reaction mixture into water, the ester separated in solid form. After the above-mentioned treatment of the ether solution with sodium hydroxide and evaporation of the ether, the ester was recrystallized from ethanol into beautiful white crystals. Yield 80 %; alkaline hydrolysis:

$C_{10}H_9O_3Cl_3$ (283.54) Equiv. wt. found 283.6

On acidification, 2,4,6-trichlorophenoxyacetic acid was isolated and recrystallized from benzene, m. p. 177—178° (reported 177—178° [12] and 190° [14]).

Ethyl β-naphthoxyacetate, b. p. 170—190° at 10 mm, recrystallized from ethanol, m. p. 49—51° (reported 48—49° [17]), light bluish crystals, yield 70 %. Alkaline hydrolysis:

$C_{14}H_{14}O_3$ (230.5) Equiv. wt. found 229.0

Ethyl α-(2,4,6-trichlorophenoxy)propionate, b. p. 143° at 1 mm, 189° at 25 mm, m. p. 22°. 80 % yield from ethyl α-bromopropionate, only 30 % yield from ethyl α-chloropropionate.

$C_{11}H_{11}O_3Cl_3$ (297.57)	Calc.	Cl	35.75
	Found	»	35.64

Alkaline hydrolysis: Equiv. wt. found 299.0
On acidification after the alkaline hydrolysis α-(2,4,6-trichlorophenoxy)-propionic acid was isolated and recrystallized from benzene, m. p. 112—113° (reported 115—116° [14]).

t-Butyl phenoxyacetate. The general procedure [18] of treating the acid chloride with t-butyl alcohol in the presence of dimethylaniline was followed. Yield 55 %, b. p. 110—113° at 1 mm (attempted distillation at 15 mm resulted in destruction). Alkaline hydrolysis:

$C_{12}H_{16}O_3$ (208.25) Equiv. wt. found 207.1

t-Butyl 2,4,6-trichlorophenoxyacetate. Attempts to prepare this ester according to the above-mentioned general procedure for t-butyl esters failed: The acid was obtained by alkaline hydrolysis of the ethyl ester and was recrystallized from benzene, m. p. 177—178°. By treatment of the acid with excess thionyl chloride on a steam-bath, 2,4,6-trichlorophenoxyacetyl chloride was obtained in 83 % yield, b. p. 160—165° at 11 mm, m. p. 59° (reported 55—56° [12]).

$C_8H_4O_2Cl_4$ (273.99)	Calc.	Cl	51.78
	Found	»	51.99

From the acid chloride, t-butyl alcohol and dimethylaniline, however, only the free acid could be obtained *. Instead the t-butyl ester was prepared from t-butyl chloroacetate [19] and potassium 2,4,6-trichlorophenolate (an attempt using the sodium phenolate failed) in t-butyl alcohol solution. The procedure was analogous to the above-described method for ethyl aryloxyacetates; only the final distillation was omitted. The residue, after evaporation of the ether, was heated *in vacuo* at 100° by which treatment a small amount of t-butyl chloroacetate was removed. On cooling in ice, the residue crystallized but melted again on standing at room temperature. The residue was mixed with methanol and this solution cooled in dry-ice. The crystals were filtered on a Buchner funnel, the funnel being cooled by surrounding it with dry-ice, and washed with cooled methanol. The white crystals melted on standing. To remove the methanol, the product was heated *in vacuo* on a boiling water bath, dry benzene added and again removed by distillation *in vacuo*. This benzene treatment was repeated, and the residue finally heated *in vacuo* at 100° for two hours. The yield of crude product on a 0.5 mole scale was 75 %. Alkaline hydrolysis:

$C_{12}H_{13}O_3Cl_3$ (311.59) Equiv. wt. found 307.1

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* This was also the case in an attempt to prepare t-butyl 2,4-dichlorophenoxyacetate. 2,4-Dichlorophenoxyacetyl chloride was obtained in 89 % yield, b. p. 135° at 15 mm, m. p. 24°.

$C_8H_5O_2Cl_3$ (239.49)	Calc.	Cl	44.41
	Found	»	44.32

Attempted acylations of ethyl phenoxyacetate and ethyl 2,4-dichlorophenoxyacetate with phenyl benzoate or phenyl propionate using sodium amide. Several experiments were carried out. Although the relative quantities of sodium amide, ethyl phenoxyacetate and acylating ester were varied [3] the general course remained the same. Generally 0.2 mol of sodium amide was employed. To the stirred suspension of sodium amide [10] in ether was added ethyl phenoxyacetate. After a few minutes evolution of ammonia began, indicating the formation of the anion of the ester. The acylating ester was added and the reaction mixture refluxed for two to three hours * then poured onto ice and concentrated hydrochloric acid added until acidic reaction. A white precipitate, insoluble in both water and ether, was filtered, washed with ether and water and recrystallized from ethanol; white crystals, m. p. 140°. This compound, apparently, is α,γ-diphenoxyacetoacetamide. The yield was 25—30 %.

$C_{16}H_{15}O_4N$ (285.29)	Calc.	C	67.35	H	5.30	N	4.91
	Found	»	67.44	»	5.03	»	4.87

The filtrate which consisted of a water and an ether layer was separated and the water layer extracted with ether. The combined ether extracts were shaken with 2 *N* sodium hydroxide solution, by which treatment in most cases a white solid precipitated. In some cases the ether layer, after shaking with sodium hydroxide, had to stand overnight in the ice-box, or it was necessary to evaporate the ether in order to effect crystallization. The yield of the sodium derivative of ethyl α,γ-diphenoxyacetoacetate was 40 % of the theoretical, the over-all yield of self-condensation products being 65—70 %. After filtration and washing, the sodium derivative was converted into the free β-ketoester by shaking with ether and 4 *N* hydrochloric acid. After washing the ether solution with saturated sodium bicarbonate solution and water and drying over anhydrous sodium sulfate, the solvent was evaporated and the free β-ketoester was obtained as an almost colorless oil, which, however, decomposed, when distillation *in vacuo* at 0.5 mm was attempted. With 2,4-dinitrophenylhydrazine a yellow hydrazone was obtained, m. p. 159—160°.

$C_{24}H_{22}O_8N_4$ (494.46)	Calc.	C	58.29	H	4.48	N	11.33
	Found	»	58.20	»	4.59	»	11.33

With ethyl 2,4-dichlorophenoxyacetate an 87 % yield of the sodium derivative of the β-ketoester was obtained directly from the reaction mixture by pouring onto ice. The sodium derivative was converted into the free ethyl α,γ-di(2,4-dichlorophenoxy)acetoacetate which crystallized and was recrystallized from ethanol, white crystalls, m. p. 96—98°, yield 60 %.

$C_{18}H_{14}O_5Cl_4$ (452.12)	Calc.	C	47.82	H	3.12	Cl	31.37
	Found	»	47.64	»	3.19	»	31.35

* In some experiments the acylating ester was added immediately after the phenoxy ester or the two esters were added in mixture; no difference in results was observed. During the addition of the acylating ester the ether refluxed, indicating that some reaction took place. The acylating phenyl esters were converted into the corresponding ethyl esters (see ref. 1, note 18). In one experiment with ethyl phenoxyacetate the reaction time was reduced to thirty minutes. No acetoacetamide was formed, but 48 % of the self-condensation product was obtained.

Attempted propionylation of t-butyl phenoxyacetate with phenyl propionate using sodium amide. The procedure was as described above; equivalent amounts (0.2 mole) of sodium amide, t-butyl phenoxyacetate (41.6 g) and phenyl propionate (30 g) were used except for a 10 % excess of sodium amide. The reaction mixture was poured onto ice and filtered. The white precipitate of the sodium derivative, after washing and drying, weighed 28 g (77 % yield of self-condensation). The free β-ketoester was obtained in the usual way (see above). Attempted distillation *in vacuo* resulted in decomposition. One fraction (7 g) of yellow distillate, after standing for six days, had crystallized and was recrystallized from ethanol, white needles, m. p. 59—60°. It was identified as α,γ-diphenoxyacetone:

$C_{15}H_{14}O_3$ (242.26)	Calc.	C	74.36	H	5.82
	Found	»	73.98	»	5.53

It formed a yellow 2,4-dinitrophenylhydrazone, m.p. 125-126°:

$C_{21}H_{18}O_6N_4$ (422.39)	Calc.	N	13.27
	Found	»	13.25

α,γ-Diphenoxyacetone, which apparently is not previously reported in the literature, was further identified in the following way: Glycerol-α,γ-diphenylether, m. p. 81—82° (reported 82° [20]), which was obtained in 75 % yield by refluxing one equivalent of glyceroldichlorohydrin with an ethanolic solution of two equivalents of each of sodium and phenol, was oxidized with potassium dichromate in sulfuric acid for two hours on a steam-bath. The reaction mixture was extracted with ether and the ether distilled. From the residue, after fractional crystallization, by which most of the glyceroldiphenylether was recovered, a small amount of a substance was isolated which was found to be identical with the decomposition product of the condensation product of t-butyl phenoxyacetate: m. p. 59—60°, mixed m. p. 59—60°, dinitrophenylhydrazone m. p. 125—126°.

Self-condensations (Table 1). Most of the esters were self-condensed by means of diisopropylaminomagnesium bromide according to the general procedure previously described [1,11]. The self-condensation of ethyl phenoxyacetate and of methyl 2,4-dichlorophenoxyacetate are previously described [1]. Generally the β-ketoesters were obtained first as sodium derivatives when the ether extract of the reaction mixture was treated with sodium hydroxide solution (see above). These were converted into the free β-ketoesters which were obtained as almost colorless oils which could not be distilled.

Table 1.

Ester	Yield of α,γ-diaryloxyacetoacetic ester %
Ethyl phenoxyacetate [1]	63
Methyl 2,4-dichlorophenoxyacetate [1]	65
Ethyl 2,4-dichlorophenoxyacetate	60
t-Butyl phenoxyacetate	55
Ethyl 2-methyl-4-chlorophenoxyacetate	21
Ethyl β-naphthoxyacetate	61
Ethyl 2,4,6-trichlorophenoxyacetate	28
t-Butyl 2,4,6-trichlorophenoxyacetate	51
Ethyl α-(2,4,6-trichlorophenoxy)propionate	22

1. Ethyl phenoxyacetate and *ethyl 2,4-dichlorophenoxyacetate.* Several acylation-attempts using other bases (see above) resulted in self-condensation. By attempted benzoylation of ethyl phenoxyacetate with ethyl benzoate using sodium ethoxide [4] or sodium [5] self-condensation was effected in 60 and 55 % respectively. The 2,4-dinitrophenylhydrazone is described above.

2. t-Butyl phenoxyacetate. The corresponding β-ketoester did crystallize and could be recrystallized from ethanol, m. p. 57—58°.

$C_{20}H_{22}O_5$ (342.38)	Calc.	C	70.15	H	6.48
	Found	»	69.88	»	6.27

The dinitrophenylhydrazone has the m. p. 143—144°.

3. Ethyl 2-methyl-4-chlorophenoxyacetate was self-condensed by diisopropylaminomagnesium bromide, the product being an oil, which was not purified. When sodium amide was used, the ester was converted into the amide, which was recrystallized from ethanol, m. p. 150—151°.

$C_9H_{10}O_2NCl$ (199.64)	Calc.	N	7.02	Cl	17.76
	Found	»	6.98	»	17.78

4. Ethyl β-naphthoxyacetate. The self-condensation product was an oil, which was not purified.

5. Ethyl 2,4,6-trichlorophenoxyacetate. The β-ketoester was obtained as white crystals (from ethanol). Rather surprisingly, the melting point was as low as 77—78°.

$C_{18}H_{12}O_5Cl_6$ (521.02)	Calc.	C	41.49	H	2.32	Cl	40.83
	Found	»	41.29	»	2.55	»	40.86

When sodium amide was used as a condensing agent the ester was converted into the amide in 70 % yield, m. p. 195—196° (from ethanol) (reported 195—196° [12]).

$C_8H_6O_2NCl_3$ (254.51)	Calc.	N	5.50	Cl	41.79
	Found	»	5.38	»	41.56

6. t-Butyl 2,4,6-trichlorophenoxyacetate. The β-ketoester was obtained as white crystals (from ethanol), m. p. 129—131°.

$C_{20}H_{16}O_5Cl_6$ (549.07)	Calc.	Cl	38.75
	Found	»	38.77

7. Ethyl α-(2,4,6-trichlorophenoxy)propionate. The treatment with sodium hydroxide was not applied and the β-ketoester crystallized directly, after evaporation of the ether from the ether extract of the acidified reaction mixture [1]. Ethyl α,γ-dimethyl-α,γ-di-(2,4,6-trichlorophenoxy)acetoacetate, white crystals (from ethanol), m. p. 116—117°. A

mixed melting point with α-(2,4,6-trichlorophenoxy)propionic acid, m. p. 112—113° (reported 115—116° [14]) was found to be 84°.

$C_{20}H_{16}O_5Cl_6$ (549.07)	Calc.	Cl	38.75
	Found	»	38.42

Acylation of acetophenone with ethyl phenoxyacetate using sodium amide. The general procedure given by Adams and Hauser [10] was followed except that equivalent amounts (0.2 mole) of each of sodium amide (10 % excess), the ketone (24 g) and the ester (36 g) were used. The reaction mixture was poured onto ice and the sodium derivative of the β-diketone was filtered and washed, and converted into the free β-diketone by shaking with ether and hydrochloric acid. The alkaline water layer from the filtrate, after washing with ether, was acidified and more β-diketone was obtained. After recrystallizations from ethanol and ligroin (b. p. 90—110°) in total 31 g (60 %) of α-phenoxy-γ-benzoylacetone was obtained, m. p. 81—82° (reported 79—80° [8]).

$C_{16}H_{14}O_3$ (254.37)	Calc.	C	75.58	H	5.55
	Found	»	75.63	»	5.52

SUMMARY

The behaviour of α-aryloxy esters in condensation processes brought about by basic reagents has been studied. These esters are shown to be extremely apt to self-condensation, and a number of such condensations have been effected, while mixed condensations between other esters and aryloxy esters for this reason have been unsuccessful. The influence of the aryloxy group is discussed.

The preparations of a number of aryloxy esters and certain other aryloxy compounds are given.

The acylation of acetophenone with ethyl phenoxyacetate to give phenoxybenzoylacetone is described.

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Some Analytical Evidence for the Purity of Proteus Flagella Protein

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The preparation of flagella from *Proteus vulgaris* in a highly purified state has been described previously [1,2] and the properties of the flagella so obtained have been investigated in various ways (for references see Weibull [3]). It has been shown that the flagella consist almost entirely of protein material. A maximum of 0.7—0.8 % fatty material, less than 0.2 % carbohydrate and 1 % ashes are found in the flagellar preparations. There is evidence that these non protein constituents may consist of impurities from the culture medium or from damaged bacterial cells.

As to the possible occurrence of impurities of a protein nature, there are hardly any reliable criteria of purity available for particles of this size and shape besides chemical analyses. Electrophoresis and ultracentrifugation are insufficent, since even rather crude preparations of varying chemical composition show homogeneity in these tests, probably because particles of different kinds are associated and do not migrate freely. Electron microscopical observations are not quite reliable since material of a size at the limit of the resolution power of the microscope may be confused with the background structure [3].

Some chemical analyses have already been performed on the flagella [3]. Additional determinations of the constituents of the flagellar protein, namely amino acids and amide nitrogen, have now been made, in order to find out whether reproducible values could be obtained in all cases. Valuable information has been obtained as to both the nature of the flagellar protein and its homogeneity.

EXPERIMENTAL

Amide nitrogen was determined according to the method quoted by Rees [4].

For the amino acid analyses, the electrodialysis method of Theorell and Åkeson [5] was used with some modifications. The protein was hydrolyzed for 30 hours at 100°

(boiling water bath) with 6 N hydrochloric acid. After the hydrolysis, dilute sulphuric acid, equivalent to the nitrogen present in the hydrolysate, was added, and the hydrochloric acid was removed by repeated evaporation. The electrodialysis was performed in a three compartment cell of perspex of about 50 ml capacity. The cathode membrane was a cellophan sheet and the anode membrane consisted of parchment (goatskin) soaked in 2 % gelatin and hardened with formaldehyde. The cellophan was chosen because histidine did not migrate quantitatively into the cathode compartment with other membranes. The goatskin was treated with gelatin in order to make it completely water tight. Distilled water was introduced into the electrode compartments, the protein hydrolysate into the middle compartment and the electrical circuit was closed. The current reached a limiting value (about 0.5 mA at 220 V) in about 90 min. The ammonia in the cathode compartment was then determined by distillation [5], the residue neutralized with sulphuric acid to pH 5.7 and reelectrodialyzed twice. The determination of the basic amino acids was then performed according to Macpherson [6]. — The contents of the anode department were neutralized with ammonia and reelectrodialyzed. To get a quantitative migration of the acid amino acids into the anode compartment it was found necessary to replace the anolyte by distilled water towards the end of the electrodialysis and then let it proceed for another 30 min. This was done in all dialyses with acid amino acids present. In this way only 1.5 % of these acids were found in the neutral amino acid fraction, and a corresponding correction was made.

Glutamic and aspartic acid were determined in the final anolyte by separation on an ion exchange column (Dowex 50) according to a method worked out by Drake at this Institute [7]. By this method the ratio between the amounts of these two acids in a mixture of them is obtained.

Tryptophan and cystine (+ cysteine) were determined according to methods used earlier [3]. Tryptophan was also determined according to the method of Bates [8]. For the determination of cystine hydrolysis was interrupted after 6 hours in order to avoid decomposition of this acid [9]. It may be mentioned, however, that even complete hydrolysis occurred without any humin formation. Only a slight yellow colour was noted.

In an earlier paper [10] it was mentioned that the untreated flagella were decomposed during electrodialysis. This was apparently due to the fact that the electrodialysis was performed with two membranes of parchment paper. On account of electroosmosis the pH of the middle compartment drops below the stability limit of the flagella [1]. When, however, the unhydrolyzed flagella were electrodialyzed in the apparatus described above, the pH dropped to a final value of 4.8. At this pH the flagella are rather stable. In this way it was found that 0.5—1.5 % of the total nitrogen present in the flagella was ammonia, which migrate into the cathode compartment. This ammonia is not included in the figures given below, since it most likely originates from the ammonium sulphate used during the purification of the flagella.

RESULTS AND DISCUSSION

Tables 1 and 2 give the analytical data obtained from six different preparations of flagella from *Proteus vulgaris*.

Table 1.

Prep.	basic amino acids = I	neutral amino acids = II	acid amino acids = III	Amide, hydr. = IV	Amide, Rees = V	Argi-nine = VI	Lysine = VII	Histi-dine = VIII	Recov-ery I +II + III +IV
1	20.55	66.54		—	—	11.23	9.21	0.11	
2	20.87	46.64	20.14	11.62	10.91	11.02	9.71	0.14	99.27
3	20.02	47.34	20.04	11.61	10.94	10.87	9.07	0.08	98.99
4	20.43	47.44	19.78	11.43	10.92	11.21	9.11	0.11	99.02
Ave-rage:	20.47	47.14	19.99	11.55	10.92	11.08	9.28	0.11	99.07

All figures are given as % N in the specified substances (specific N)/total N. Amide hydr. = amide as found in the protein hydrolysate (30 hrs, 100°, 6 *N* HCl). Amide, Rees = amide nitrogen as determined according to Rees [4].

Table 2.

Prep.	aspartic acid	glutamic acid
5	12.58	7.41
6	12.36	7.64
6	12.30	7.70
Average:	12.41	7.58

The figures are given as % specific N/total N on the assumption that 19.99 % of the total N refers to the acid amino acids (*cf.* Table 1).

Since the individual figures given above are obtained by combining at least two determinations (total N and specific N), the accuracy of which is about ± 1 %, the deviations found for different flagellar preparations can be judged to be within the experimental errors. The difference between the values for amide nitrogen (amide hydr. and amide Rees) is due to decomposition of serine and threonine in the former case [4]. The correct value is the latter (amide, Rees).

Some analytical data concerning the amount of tryptophan and cystine (+ cysteine) have been given earlier [2]. By using greater amount of material

(cystine) and a new analytical method for tryptophan, it has now been found that less than 0.02 % cystine + cysteine and about 0.02 % tryptophan (spectrophotometric determinations as described earlier [2]; the method of Bates [8] has given still lower results) are present in the flagella.

The analytical data given above, together with earlier determinations of tyrosine and methionine [2] clearly show that the flagella consist of a protein with constant chemical composition. The fact that there is practically no histidine, tryptophan or cystine in this protein is a striking feature.

By means of X-ray analysis it has been found that the flagella belong to the keratin-myosin group of fibrous proteins [11]. Therefore, it may be of interest to compare the chemical composition of the flagella with that of keratin and myosin.

Keratins are characterized by a high content of cystine, about 7—11 % of the total N [12]. Myosin contains 1 % cystine-N/total N. — Furthermore keratins contain 2—3 % lysine N/total N, myosin 12 %. In both these cases the flagellar protein is more like myosin (9.3 % lysine-N, no cystine).

However, myosin is characterized by a high content of free acid + base groups in the side chains: 34 % of the total number of amino acid residues are of this kind [13]. From the analytical data given above the corresponding value for the flagella is 22 % *i. e.* considerably lower.

Furthermore, it is clear that the flagellar protein in other respects differs from both keratin and myosin, *e. g.* because of the absence of tryptophan, histidine (and cystine) and the glutamic: aspartic acid ratio.

The low content of tryptophan, histidine and cystine can be used as a criterium of purity for the flagellar preparations. Most other proteins contain some of these acids and then in amounts corresponding to, in terms of total nitrogen, at least 1 % cystine and tryptophan or 3 % histidine nitrogen. This is especially true for the protein and peptide material in the culture medium used for the growth of the *Proteus* bacteria (meat extract and enzymatic digests of meat), from where possible contaminations may originate. From the analytical figures given above it is obvious that such protein can only occur in a concentration of about 2 % (from cystine and tryptophan determinations) or 4 % (histidine) of the total protein present. In this connection it may be mentioned that the histidine values may be too high: the colorimetric test gave a yellowish colour not identical with the pure histidine colour.

Concerning the different tests for purity used so far (traces of non-protein material or of some amino acids, reproducible values of other amino acids, "crystallisation" tests [12] *etc.*) it has been found that the most sensitive test by far is a determination of light absorption in the ultraviolet. The values for the tyrosine and tryptophan contents obtained are very reproducible for carefully

purified preparations but much higher for crude preparations or for preparations of doubtful purity according to other tests.

In order to find out whether some material from the culture medium is adsorbed or carried down with the flagella during the ultracentrifugations in the purification process [1], pure flagella in the form of ultracentrifuge pellets were weighed and resuspended in the diluted broth, used for the growth of the bacteria. The suspended flagella were again centrifuged down and the nitrogen content of the broth determined before and after the centrifugation.

By determination of the volume of the pellets by weighing and by determination of the volume of flagellar protein (from N-analysis and specific volume) the degree of dilution of the broth during the centrifugation process could be calculated and compared with the value found experimentally. The known fact, that proteins carry about 10 times their own volume of bound water with them in ultracentrifugation experiments was taken into concideration [13]. Also the small amount of nitrogen left in the supernatent liquid when the flagella sediment in pure buffer was determined and subtracted from the values found for the broth after the centrifugations. Table 3 shows the results.

Table 3.

mg N in pellets	10 X volume of flagella in the pellets, ml	Volume of pellets, ml	Free water in pellets ml	Nitrogen in broth, mg		
				before centr.	after centr.	calc.
1.72	0.08	0.27	0.19	1.800	1.716	1.745
1.72	0.08	0.27	0.19	1.800	1.725	1.745

Table 3 shows that a very small, if any, adsorption effect is found in these experiments. It must then be concluded that possible impurities (at the most a few per cent) consist of particles from autolyzed or damaged bacterial cells. These particles may be of a size that makes them follow the flagella during the ultracentrifugation process. Such particles are also occasionally found in electron micrographs [1].

SUMMARY

The flagella from the bacterium *Proteus vulgaris* are found to consist of a protein of constant chemical composition. The following values are found as % of total N: 10.9 % amide-N, 11.1 % arginine-N, 9.3 % lysine-N, 12.4 aspartic acid-N, 7.6 glutamic acid-N, 0.83 % tyrosine-N (earlier determinations [2]) and 47.1 % N from neutral amino acids.

According to analyses for tryptophan, cystine and histidine at most a few per cent foreign protein material is present in the flagellar preparations. By earlier determinations it had been found that a maximum of 2 % non protein material (ashes, fatty material and carbohydrate) is present.

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Phenol and Cresols in Coal Tar from Coals Carbonized at 800° and 900° C*

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Phenol was discovered in coal tar by Runge [1] in 1834 and was isolated [2] in 1841 as pure phenol by distilling the tar, extracting with caustic soda, acidifying the extract, and fractionating the tar acids. Later *o*-cresol was isolated [3] through careful fractional distillation of the tar-acid mixture. The *m*- and *p*-cresols distilled within a boiling range of about 1.5° C and had to be separated by other means. Raschig [4] fractionated the tar-acid mixture to obtain pure *o*-cresol, sulfonated the mixture of *m*- and *p*-cresols, and treated the cresol-sulfonic acids with superheated steam at 120°–130° C to hydrolyze the *m*-cresol sulfonic acid; the *p*-isomer was not affected at that temperature. He also attempted to make use of the different crystallizabilities of the *m*- and *p*-cresol sulfonic acids [5]. When treated with sodium bisulfate and sodium pyrosulfate at 100°–110° C or with sulfuric acid (90 %) at 20° C, only the *m*-cresol was sulfonated [6]. Hoffmann-La Roche [7] treated the *meta-para* mixture with concentrated sulfuric acid at 40°–100° C, when *m*-cresol and part of the p-cresol was sulfonated, and precipitated the *m*-cresol sulfonic acid by diluting with water. The same sort of separation may be carried out by means of 80 % sulfuric acid at 35° C and 35 mm pressure [8].

If the mixture is treated with sulfurylchloride and chlorine, only *m*-cresol is chlorinated and *p*-cresol and chloro-*m*-cresol may be separated by distillation [9]. Other processes for meta-para separation made use of oxalic acid [10], urea [11], and acetyl-, benzoyl-, benzidine-, and glycolic acid derivatives [12]. Efforts were made also to separate phenol mixtures by use of calcium salts [13], by separating into petroleum soluble and petroleum insoluble fractions [14], and by fractionated decomposition of the phenolates [15].

More recently, the oxalic acid method was adapted to commercial use [16] and the separation by means of an alkali salt of benzenesulfonic acid was studied by Bettelheim [17]. Other recently developed methods concern fractional crystallization of tar-acid phenolates from petroleum [18] and separation by alkylation [19].

In 1937 Bahr and Wiedeking [20] investigated the various analytical methods proposed for the determination of phenol and cresols in mixtures. Quantitative results could not be

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obtained by fractional hydrolysis of sulfonic acids. By nitrating the phenol mixture, *m*-cresol could be determined fairly well as the trinitro derivative, while the other phenols were destroyed. Under certain circumstances, the determination of freezing points gave accurate criteria.

Harrison [21] proposed a colorimetric method for the determination of phenol and *m*-cresol, using the Spekker photoelectric absorptiometer for the comparison of colors, and Savitt [22] recently proposed a colorimetric method for analyzing mixtures of *m*- and *p*-cresols. Paris and Vial [23] analyzed a mixture of phenol and cresol by determining the temperature at which clouding takes place when a known weight of sample is mixed with a known weight of water.

Several investigations have been carried out to determine the quantities of different phenols present in coal tar. Weiss and Downs [24] determined the constituents of coke-oven tar and found 0.7 % phenol and 1.5 % phenol homologs, calculated on dry tar. In two tar samples from typical byproduct coke-oven installations 0.449 and 0.861 % phenol and 0.735 and 1.822 % cresols, calculated on dry tar, have been found commercially recoverable [25]. Brückner [26] separated the cresols and xylenols in coal tar quantitatively and found cresols in the following proportions: Ortho, 35 %, meta, 37 %, and para, 28 %.

At the U. S. Bureau of Mines Experiment Station in Bruceton, Pa., extensive work has been carried out on phenols from coal hydrogenation by Woolfolk, Golumbic, Friedel, Orchin, and Storch [27]. The tar acids were subjected to distillation and refractive indexes and densities of the fractions were determined. It was shown that careful interpretation of the data on physical constants made it possible to select those fractions which were rich in any desired phenol known to occur in the oil. A study of the distribution of various phenols between cyclohexane and a buffered aqueous phase led not only to the development of rules and formulas relating partition coefficient to ionization constant but also to a more complete understandig of the factors governing the extractibility of the phenols. The differences in partition coefficients of isomeric phenols permitted application of the countercurrent distribution method for their separation and determination of purity. The infrared spectra of many phenols of known constitution were determined, and this information was applied to the qualitative and quantitative estimation of the composition of most of the tar-acid fractions. The ultraviolet absorption spectra were determined and the more classical methods such as crystallization and preparation of derivatives also were used.

Most of the early work has been carried out on tar samples, obtained under not strictly defined conditions. Later, the carbonization of a great number of American coals was carried out under strictly defined conditions by Fieldner and Davis [28] and their coworkers [29]. The Bureau of Mines-American Gas Association Method [30] was used and tests were carried out at temperatures from 500°—1 000° C on coals of different rank. They found that the tar-acid

yields decrease slowly between 500° and 700° C and rapidly thereafter. For instance, from one coal the tar-acid yield was 15.5 % at 500° C, 13.2 % at 700 C, 7.4 % at 800° C, 4.0 % at 900° C, 2.15 % at 1 000° C, and 0.9 % at 1 100° C, calculated on dry tar.

The purpose of the present investigation was to determine the distribution of lower phenols in coal tar obtained from carbonizing coals of different rank at 800° and 900° C.

Table 1 gives the origin and partial chemical analyses of the five coals studied.

Table 1. Origin and chemical analyses of coals.

Coal no.	Origin	Dry, ash-free percentage						
		Volatile matter	Fixed carbon	Hydrogen	Carbon	Nitrogen	Oxygen	Sulfur
381	Elkhorn no. 1 bed, no. 28 mine, Floyd County, Kentucky	43.2	56.8	5.7	83.4	1.6	8.4	0.9
382	Elkhorn no. 2 bed, Turner no. 5 mine, Floyd County, Kentucky	43.6	56.4	5.7	82.6	1.6	8.6	1.5
383	Leatherwood bed, Leatherwood mine, Perry County, Kentucky	40.5	59.5	5.5	84.1	1.8	8.0	0.6
a 368	Pittsburgh bed, Jamison no. 9 mine, Marian County, W. Va.	39.7	60.3	5.6	84.8	1.7	6.9	1.0
413	Upper Freeport bed, Bull Run no. 1 mine, Preston County, W. Va.	31.2	68.8	5.2	87.5	1.7	4.4	1.2

EXPERIMENTAL PROCEDURE

Tars from carbonization tests at 800° and 900° C in the Bureau of Mines-American Gas Association (BM—AGA) standard equipment [30] were separated mechanically from the liquor, dehydrated, and distilled according to the procedure described in Bureau of Mines Bulletin 344 [31]. The oil was shaken with 20 % H_2SO_4, saturated with K_2SO_4, to extract the tar bases, and then three times with 10 % NaOH or until the NaOH solutions were colorless. The NaOH solution containing the tar acids, was extracted with benzene to remove oil, and was then heated until all benzene was removed. After cooling, the

solution was acidified with 20 % H_2SO_4, saturated with K_2SO_4. During this procedure, the solution was cooled to prevent rise of temperature. After separating, the Na_2SO_4 solution was extracted with benzene and rejected. The water from the dehydration of the tar was also extracted with benzene as well as the H_2SO_4 solution used for removing the tar bases, and all benzene solutions were combined, extracted with NaOH, *etc.* The wet acid mixture thus obtained was fractionated at ordinary pressure to remove water and high boiling residue.

RESULTS

Table 2 shows the yields of tar-acid mixtures for the boiling range 180—230° C (at 760 mm pressure), calculated as percentages of dry tar and of the coal on the dry and ash-free basis.

Table 2. Yield of crude tar acids from boiling range 180°—230° C (760 mm pressure), expressed as percentages by weight of dry tar and of dry, ash-free coal.

Sample number	Tar acids, percent by weight of	
	Dry tar	Dry, ash-free coal
381, 800° C	6.9	0.6
381, 900° C	2.8	.2
382, 800° C	8.3	.7
382, 900° C	3.7	.3
383, 800° C	7.1	.6
383, 900° C	2.8	.2
a 368, 800° C	7.7	.8
a 368, 900° C	3.4	.3
413, 800° C	4.3	.3
413, 900° C	2.1	.1

The samples were then refractionated at approximately 20 theoretical-plate efficiency at 40 mm pressure and analyzed by the infrared spectrometer at the Bureau of Mines, Bruceton Station. In order to get enough quantities for the vacuum distillations it was necessary to combine the samples from tests 381—383.

Table 3 summarizes the composition of the phenol-cresol fractions and Table 4 shows the yields of phenol and cresols on the dry-tar and dry, ash-free coal bases.

Table 3. Composition of phenol-cresol fractions.

Sample number	Percent of phenol-cresol fraction				
	Phenol	*o*-Cresol	*m*-Cresol	*p*-Cresol	Total
381—383, 800° C	33.9	19.5	27.7	18.9	100.0
381—383, 900° C	35.2	18.0	29.2	17.6	100.0
368, 800° C	33.6	21.5	27.0	17.8	100.0
368, 900° C	34.7	21.5	27.9	15.9	100.0
413, 800° C	28.4	21.3	33.5	16.9	100.1
413, 900° C	26.3	23.0	33.5	17.2	100.0

Table 4. Yield of phenols, expressed as percentages by weight of dry tar and of dry, ash-free coal.

Sample number	Phenols, percent by weight							
	Basis of dry tar				Basis of dry, ash-free coal			
	Phenol	*o*-Cresol	*m*-Cresol	*p*-Cresol	Phenol	*o*-Cresol	*m*-Cresol	*p*-Cresol
381—383, 800° C *	1.8	1.0	1.4	1.0	0.15	0.08	0.12	0.08
381—383, 900° C *	.7	.3	.5	.3	.06	0.02	0.04	.02
a 368, 800° C	1.7	1.0	1.3	.9	.17	.10	.13	.09
a 368, 900° C	.7	.4	.6	.3	.06	.03	.05	.03
413, 800° C	.7	.5	.8	.4	.05	.04	.06	.03
413, 900° C	.3	.3	.4	.2	.02	.02	.03	.01

* Based on average tar yield.

SUMMARY

Tar samples from BM—AGA tests at 800° and 900° C of different American coals have been investigated for their contents of phenol and cresols. The tars were dehydrated and extracted and the tar-acid mixtures were fractionated and analyzed by the infrared spectrometer. The contents of phenol and of each cresol isomer was calculated on the dry tar and dry, ash-free coal.

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The Breakdown of the High Molecular Reserve Proteins of Peas during Germination

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The protein breakdown in different seeds during germination has been studied for about 150 years. The most fundamental experiments were done by Pfeffer, Schulze and Prianischnikow. For a review of their work and other references Chibnall's monograph [1] on this subject is recommended, where also a large number of references can be found.

In most experiments described by earlier authors the most important question concerned itself with the amino acids formed in the young plant. The amino acids have been identified and their concentrations have been determined at different stages during germination. These old determinations are probably inaccurate. Many experiments have been performed in order to determine the large amounts of asparagine, and in certain cases glutamine, which are accumulated in the shoots during germination. These amides probably store all nitrogen which can not be used immediately by the plant. The leguminosae plants have a very high protein content, and have therefore been investigated most. These experiments have given many important contributions to the understanding and knowledge of the nitrogen metabolism in the plants.

The present study of the protein breakdown has been undertaken from another point of view. Instead of studying the breakdown products of the reserve proteins in the seed, the amounts of unaltered proteins have been determined at different stages during the germination. This has been possible with the help of the new methods for the study of high molecular substances which have been worked out during the last decades *i.e.* ultracentrifugation and electrophoresis.

Earlier investigations have shown that seeds of peas, *Pisum sativum*, contain two well-defined globulins, vicilin and legumin. Osborne [2] was the

first one to prepare these two globulins. The methods used by Osborne, however, do not give complete separation of the two globulins. By using simple precipitation methods, the present author succeeded in separating the two globulins completely from each other. The two resulting proteins were homogeneous in the ultracentrifuge and electrophoresis [3,4]. The molecular weight for vicilin was determined to be 186 000 with an iso-electric point at pH 5.5. For legumin the corresponding values were 331 000 and pH 4.8. Vicilin and legumin are also different in their chemical composition. Osborne and Campbell [5] showed that legumin contains considerably more sulfur than vicilin, and the present author has shown that the tryptophan content of legumin is about four times higher than that of vicilin. A third high-molecular protein fraction can be obtained from seeds of peas. This fraction is still very poorly defined, but it is called the albumin fraction in this paper because it is soluble in water. Vicilin and legumin are insoluble in water but soluble in dilute salt solutions. If a solution containing albumin, vicilin and legumin is dialysed against water, the two globulins are precipitated and the albumin remains in solution. The albumin fraction is, however, not homogeneous. Ultracentrifugation has shown that it contains at least two components. The albumin fraction is called legumelin by Osborne and Campbell [5].

The experiments described in this paper were performed in order to determine at which stage during the germination and at which speed the breakdown of the reserve proteins occurred.

EXPERIMENTAL

A. The treatment of the seed material before extraction

For the present experiments ripe, air-dried seeds from yellow peas, field variety "Torsdagsärt II" from the Royal Agricultural College in Uppsala were used. The seeds were placed in water overnight in order to swell and were then allowed to germinate in Petri dishes between wet filter papers. After 5 days, when the roots were about 3—4 centimeters long, the seeds were placed in a nutrient solution on a metal gauze in such a way that the roots were dipping into the solution but the seeds themselves were above the surface. The nutrient solution had the following composition: 1 g · $Ca(NO_3)_2$, 1 g · KNO_3, 1 g · $MgSO_4 \cdot 7\ H_2O$, 1 g · KH_2PO_4, 0.5 g · KCl and 5 drops 5 % $FeCl_3$ to 7 l H_2O. This nutrient solution was used in order to get germination conditions as normal as possible. It is very difficult to grow peas under sterile conditions, but the experiment

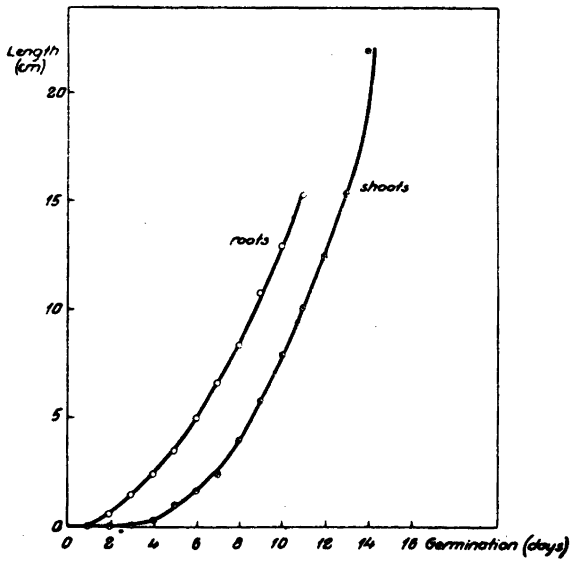


Fig. 1. The lengths in cm of the roots and the shoots at different stages of the germination. Each value in the figure is the average of 25 determinations.

was discarded if moulds could be seen on the seeds. The time of germination was counted from the day when the seeds were first placed in water to swell. Prianischnikow started to count one day later when the seeds had been placed between wet filter papers [6]. After different time intervals the germination was interrupted, the material was washed with water and finely ground with distilled water in a Waring blendor for 6 minutes. The product obtained was frozen down to —16° C and dried in vacuum over $CaSO_4$ in the frozen state. The dry material consisted of a fine powder. Kjeldahl nitrogen determinations on each preparation showed that the material was rather homogeneous. The water content of each preparation was determined by heating to 105° C for 20 hours. The results of the nitrogen determinations are shown in Table 1. Fig. 1 shows the length of the roots and the shoots at different stages of germination.

The advantage of the method described above is that it is possible to use a known amount of the substance for each extraction experiment, and the homogeneity and water content of the material is also known. Before this method was worked out the material was extracted without previous drying and the same number of seeds or plants were used for each experiment. This method, which has been used by many authors on this subject is, however, of too low accuracy for investigations of this kind.

Table 1. Determinations of nitrogen content in dried preparations of peas, germinated for different times. The values are average values from three determinations with a maximum deviation from the mean of ± 2 %.

Time of germination days	Nitrogen content of the dry weight %
1	4.43
2	4.13
3	4.41
4	4.29
5	4.31
6	4.43
7	4.52
8	4.61
9	4.51
10	4.82
12	5.65
15	6.32
19	6.55

B. Determination of the best extraction conditions

In order to determine the best extraction conditions, some experiments with different extraction solutions and different extraction times were done. In all experiments the temperature was + 4° C which is convenient inasmuch as it reduces the effect of any proteolytic enzymes. The risk of bacterial growth is also lower at this temperature. Previous experiments with the pure globulins have shown that the extraction should be done at a pH value close to 7 because legumin is insoluble in the pH range 4—6 [3].

Two (2.0) grams of macerated, frozen and dried seeds, germinated for one day, were extracted with 100 ml of different extraction solutions at + 4° C with stirring. At different time intervals 3 ml were withdrawn with a pipette, centrifuged at 3 500 r.p.m. (radius 5 cm) for 5 minutes. The nitrogen content of this solution was then determined according to Kjeldahl. The results are shown in Table 2.

From Table 2 it can be seen that 0.2 *M* NaCl, buffered to pH 7.0 with 0.03 Na_2HPO_4 and 0.02 NaH_2PO_4 is as effective as a buffer with much higher ionic strength. This buffer solution will be referred to as "standard buffer" in this paper and was used in all experiments here described. From Table 2 it can be seen that water is almost as effective as standard buffer in the first stage of the extraction, but after prolonged extraction the nitrogen content of the

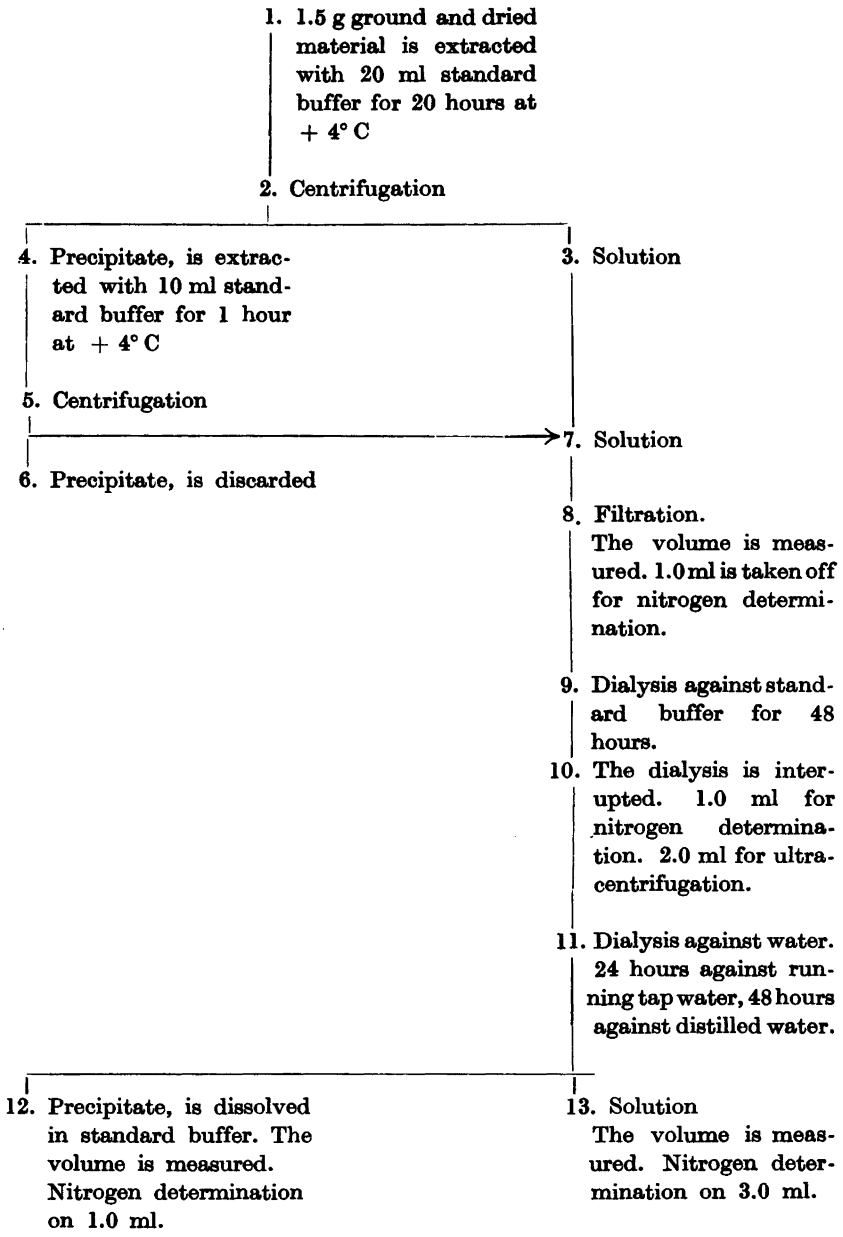
Table 2. Determination of the best extraction conditions of nitrogen compounds from seeds of peas at pH 7. Temperature + 4° C. 2.0 g dried seed material and 100 ml extraction solution were used for each extraction experiment.

Extraction solution	Nitrogen content in extract after different extraction times mg N/ml				
	10 min.	60 min.	3 hours	9 hours	24 hours
H_2O	0.636	0.615	0.580	0.539	0.537
0.2 *M* NaCl 0.03 *M* Na_2HPO_4 0.02 *M* NaH_2PO_4	0.654	0.662	0.686	0.690	0.703
1 *M* NaCl 0.06 *M* Na_2HPO_4 0.04 *M* Na NaH_2PO_4	0.647	0.662	0.688	0.691	0.703

water extract decreases. According to definition globulins should not be soluble in water. The fact that the globulins can be extracted with water may be explained by assuming protective colloids in the seeds. If a water extract from pea seeds is dialysed against water for about one hour or is stored for a few days, the globulins are precipitated. This effect can be seen from the values in Table 2.

C. Preparation methods used

One and a half grams (1.50 g) of the dried material were weighed into an Erlenmeyer flask of 100 ml and one drop of toluene was added. The extraction was carried out for 20 hours with 20 ml standard buffer at + 4° C with stirring. The extraction was interrupted and after centrifugation the precipitate in the centrifuge tube was again extracted for 1 hour with another 10 ml standard buffer. After centrifugation the two extracts were mixed and filtered through a glass filter (Jena G 2) and the volume was measured. 1.0 ml was taken off for nitrogen determination. In the final calculations corrections were made for all volumes taken off for nitrogen determinations and ultracentrifugations. The extract was then dialyzed in a thin cellophan bag against 1 000 ml of standard buffer. The dialysis was continued for 48 hours and the standard buffer outside the bag was changed every twelve hours. After the dialysis was finished the volume was measured and 1.0 ml was taken off for nitrogen determination and 2.0 ml for ultracentrifugation. The remaining solution was then dialysed for 24 hours against running tap water (+ 8° C), immediately followed by dialysis against distilled water for 48 hours at + 4° C. By this procedure a dense precipitate of globulins was obtained, which was centrifuged down. The volume of the supernatant solution was measured, and nitrogen determination was carried out on 3.0 ml of the solution. The precipitate was dissolved in standard buffer, the volume measured and the nitrogen content of 1.0 ml of the solution determined. The fractionation scheme is shown below.



With this extraction method only 70—80 % of the total nitrogen in the seeds is extracted. If larger volumes are used the yield is higher but the solutions are then too dilute for investigation in the ultracentrifuge and the losses

by dialysis against water are larger because small amounts of vicilin remains in the supernatant even after prolonged dialysis. The nitrogen remaining in the extracted material is probably bound in the cells in such a way that it can not diffuse out through the cell walls during the extraction. This nitrogen could possibly be extracted by Miller and Chibnalls ether-water method [7] but previous experiments on the purified globulins have shown that these proteins are denatured by ether. Thus the ether extraction is not suitable in this special case. The preparation method used here seems to give very little denaturation of the proteins. The experiments described have been performed in order to study the breakdown of the globulins, and it has been impossible to isolate any products of globular character from the leaves, stems and roots.

In the following discussion the numbering of the fractionation scheme will be used. At no. 8 an extract with a maximal amount of extractable nitrogen will be obtained. After the dialysis at no. 9 all low-molecular nitrogen has dialysed out through the bag, and at no. 10 the sum of the globulin N and albumin N is obtained. This nitrogen can be called protein nitrogen, even if the expression is not exactly correct but the error is very small. In this paper the nitrogen determined at no. 10 is called non-dialysable N. The amount of dialysable nitrogen (low-molecular N) is obtained by taking the difference between the determinations at no. 8 and no. 10. The ultracentrifugation at no. 10 gives the relative amounts of albumin, vicilin and legumin according to what will be said below. The dialysis at no. 11 separates the albumins from the globulins. The amount of albumin N is determined at no. 13 and the globulin N at no. 12.

The following example may be given:

One and a half grams (1.50 g) of the dried material, germinated for 4 days, were extracted according to the fractionating scheme above. At no. 8 the volume was 24.0 ml, and the nitrogen determinations gave the value 1.00 mg N/0.5 ml. Thus 48.0 mg N had been extracted. After dialysis against standard buffer according to no. 9, at no. 10, 23.0 ml solution containing 0.697 mg N/0.5 ml were obtained. Thus, after correcting for the solution taken off for nitrogen determination at no. 9, 33.5 mg non-dialysable nitrogen had been obtained. After dialysis against water according to no. 11 and dissolving of the precipitate at no. 12 the following values were obtained. The precipitate was dissolved in 34.0 ml and this solution contained 0.586 mg N per 1.0 ml. Thus 19.9 mg globulin N could be found. After correction for the volumes which had been taken off (1 ml at no. 8 and 3 ml at no. 10) $\frac{19.9 \times 24}{20} = 23.9$ mg globulin N are obtained. The volume of the supernatant solution at no. 13 was 28.0 ml, since the volume increases during the dialysis to the saltfree state. It contained 0.657 mg N/3.0 ml. Thus 6.1 mg albumin N were found, which value after correction increases to 7.4 mg albumin N.

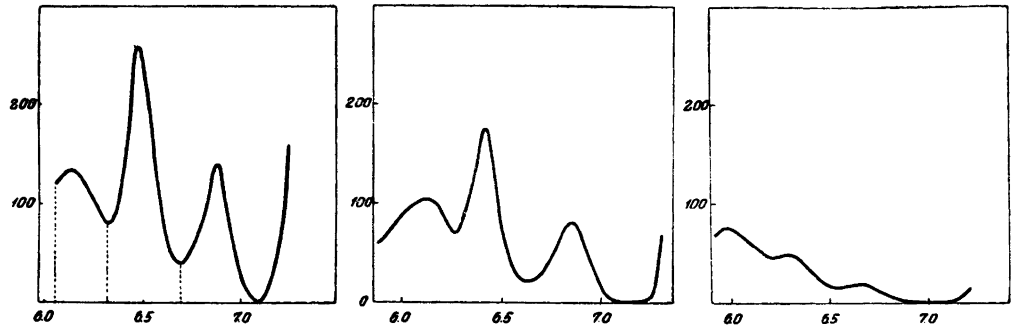


Fig. 2. Ultracentrifugation diagrams of NaCl-extracts of pH 7 from seeds of peas, germinated for 1 day (left), 9 days (centre) and 15 days (right). The extracts had been dialyzed for 48 hours against the extraction buffer. The exposures are taken 45 minutes after full speed at 65 000 r.p.m. (300 000 × g). The same scale distance (80 mm) has been used. The areas have been measured in the way indicated on the diagram to the left. In the diagrams the abscissae represent the distance from the centre of rotation (cm), the ordinates represent the scale-line displacement (Z in μ).

The ultracentrifugation * at no. 10 showed that the solution, 24.0 ml, contained 0.15 % (g/100 ml) albumin, 0.34 % vicilin and 0.24 % legumin. Thus 36.0 mg albumin, 81.6 mg vicilin and 57.6 mg legumin were found in the solution. By dividing these values with the factor 6.25, the values above correspond to 5.8 mg albumin-N, 13.0 mg vicilin-N and 9.2 mg legumin-N, or totally 22.2 mg globulin-N. If these values are compared with those obtained from the nitrogen determinations, it is clear that the ultracentrifugation method gives lower values. The sedimentation diagrams are shown in Fig. 2.

As can be seen from Fig. 2, three main peaks are obtained, albumin, vicilin and legumin. The albumin peak is divided into two peaks after about 100 minutes at 65 000 r.p.m. The sedimentation constants of these peaks are about 1 S and 4 S. In some diagrams a new component, sedimenting still faster than legumin, can be seen. Its sedimentation constant is about 19 S. It is an association product of legumin. When a solution containing purified legumin is heated to 90° C large amounts of this component are formed. In some cases legumin has been quantitatively transformed into this new component. Solutions containing pure legumin in high concentrations also show this high-molecular component in the ultracentrifuge.

For information about the ultracentrifugation method see Svedberg and Pedersen's monograph on the subject [8].

D. Results from the nitrogen determinations

The values obtained from the nitrogen determinations are shown in Table 3 and Fig. 3.

Table 3. Nitrogen distribution in NaCl-extracts from seeds of peas, germinated for different times. In all experiments 1.50 g dried material has been extracted.

Days of germination	Tot. N in 1.50 g dried material mg		Extractable N mg	% of total N	Nondialysable N (protein N) mg	% of total N	Dialysable N mg	% of total N	Globulin N mg	% of total N	Albumin N mg	% of total N
1.	64.6		49.8		37.2		12.6		26.0		6.4	
			48.1		37.5		10.7		26.2		7.1	
			45.3		36.1		9.2		28.4		8.5	
			52.4		39.7		12.7		28.5		8.6	
			47.5		37.3		10.2		26.5		8.2	
		Av:	48.6	75	37.6	58	11.1	17	27.1	42	7.8	12
2.	55.6		46.8		38.8		8.0		26.4		7.3	
			44.2		35.7		8.5		25.8		8.4	
			44.7		40.3		4.4		25.2		10.7	
			45.6		34.5		11.1		25.9		8.1	
			45.2		36.6		8.6		25.1		10.7	
		Av:	45.3	82	37.2	67	8.1	15	25.7	46	9.0	16
3.	61.2		44.9		36.5		8.4		26.2		7.2	
			44.2		36.2		8.0		24.7		8.5	
		Av:	44.6	73	36.4	59	8.2	13	25.5	42	7.9	13
4.	59.8		48.0		33.5		14.8		23.9		7.4	
			49.7		33.0		16.7		26.0		6.5	
		Av:	48.9	82	33.3	56	15.8	26	25.0	42	7.0	12
5.	58.8		48.4		31.9		16.5		22.6		7.8	
			48.8		29.6		19.2		22.4		7.0	
			41.4		29.3		12.1		21.5		7.8	
			49.6		33.7		15.9		25.6		7.4	
			46.6		34.2		12.4		24.6		7.9	
		Av:	47.0	80	31.7	54	15.2	26	23.3	40	7.6	13
6.	61.0		45.2		28.0		17.2		24.8		7.1	
			45.5		30.9		14.6		22.4		7.5	
			46.7		31.9		14.8		21.4		5.4	
			48.9		32.4		16.5		22.4		6.7	
		Av:	46.6	76	30.8	50	15.8	26	22.8	37	6.7	11

Table 3 (cont.)

7.	63.0	43.3		28.3		15.0		20.6		6.1	
		51.8		29.7		22.1		22.2		7.3	
		47.6		31.0		16.6		20.1		6.8	
		44.6		28.0		16.6		19.2		7.0	
		39.9		26.3		13.6		19.5		7.0	
	Av:	45.4	72	28.7	46	16.8	27	20.3	32	6.8	11
8.	63.2	44.9		18.8		16.1		13.4		5.4	
		44.1		20.5		23.6		13.4		5.6	
		46.6		22.1		24.5		12.7		6.7	
		47.2		23.7		23.5		15.1		8.0	
	Av:	45.7	72	21.3	34	21.9	35	13.7	22	6.4	10
9.	60.8	44.7		20.9		23.8		13.9		5.8	
		53.6		24.3		29.3		13.8		6.0	
	Av:	49.2	81	22.6	37	26.6	44	13.9	23	5.9	10
10.	65.7	46.3		20.3		26.0		10.9		6.7	
		41.8		18.4		23.4		9.2		6.4	
	Av:	44.1	67	19.4	30	24.7	38	10.1	15	6.6	10
12.	75.5	41.0		15.3		25.7		9.4		4.8	
		38.0		12.8		25.2		7.4		4.9	
		49.2		15.7		33.5		7.2		6.7	
	Av:	42.7	57	14.6	19	28.1	37	8.0	11	5.5	7
15.	86.6	24.2		6.2		18.0		2.5		2.5	
		36.3		8.3		28.0		2.9		3.4	
	Av:	30.3	35	7.3	8	23.0	27	2.7	3	3.0	3
19.	87.4	35.7		8.8		26.9		3.8		3.2	
		34.6		7.9		26.7		3.2		2.1	
	Av:	35.2	40	8.4	10	26.8	31	3.5	4	2.7	3

It can be seen from Table 3 that the amount of extractable N varies strongly in the different preparations. The amount of non-dialysable N has been determined with a maximum deviation from the average of $\pm$ 14 %. The deviation in the determinations of globulin N is lower than $\pm$ 10 % with one exception. For the preparation germinated for 12 days the deviation is $\pm$ 17 %. The albumin N has been determined with a maximum deviation of $\pm$ 20 % from the average. The determinations are more difficult to carry out and the accuracy is lower the longer the time of germination. This depends mainly on two things, first the protein concentration decreases and second the extracts contain a great deal of breakdown products which together with the

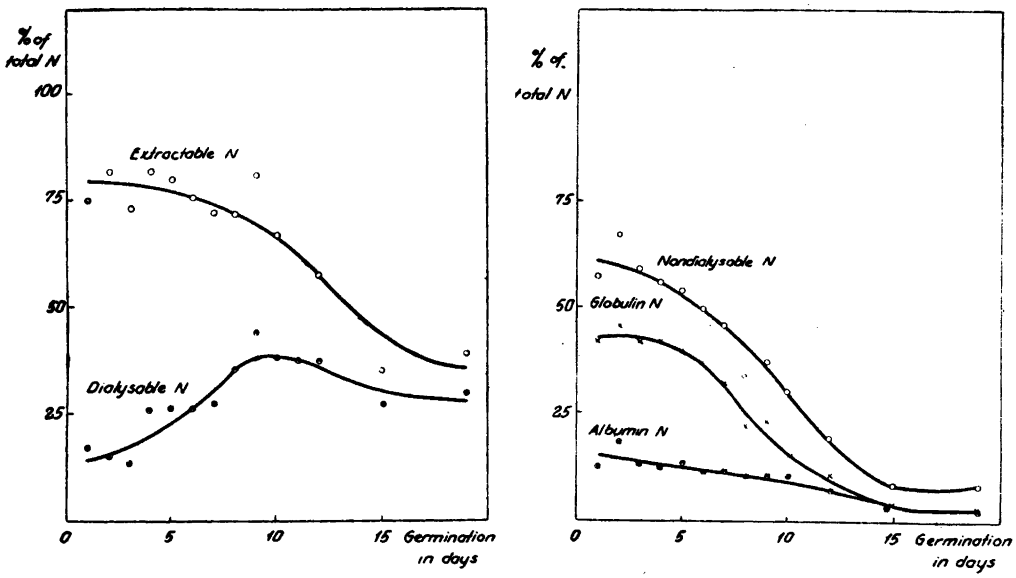


Fig. 3. Nitrogen distribution in peas at different stages of germination.

chlorophyll and other products formed during the germination lower the accuracy of the determinations. As can be seen from Table 3 only 70—80 % of the total nitrogen in the seeds can be extracted. If less nitrogen is extracted the extracts contain relatively more of low molecular products. This is shown by the following experiment.

One and a half grams of the dried material, germinated for three days, were extracted with 25 ml standard buffer without subsequent washing of the precipitate according to nos. 4—6 in the fractionating scheme. By this method only 60—65 % of the total nitrogen can be extracted. Three experiments were carried out. The ratios $\frac{\text{Dialysable N}}{\text{Non-dialysable N}}$ obtained were 0.58, 0.49 and 0.48 respectively when 64, 63 and 60 % of the total nitrogen were extracted. From Table 3 this ratio after three days is 0.22, when 73 % of total nitrogen was extracted.

Thus the low-molecular nitrogen is more easily extracted than the high-molecular nitrogen, and it is important to extract as much nitrogen as possible in order to get a true picture of the nitrogen distribution in the seeds.

E. Results from the ultracentrifuge experiments

As was mentioned in connection with the preparation method, determinations of concentrations from sedimentation diagrams give lower values than

those obtained with other methods, *e. g.* determination of the nitrogen content and refractive index of the solution. In some cases the values obtained from the sedimentation diagrams are only half of those obtained from the nitrogen determination. This depends on the fact that relatively large amounts of low-molecular material are present and part of these substances do not sediment in the ultracentrifuge. Quantitatively the values from the centrifuge diagrams can not be directly compared with those obtained with other methods. The sedimentation diagrams, however, can give an idea of the relative amounts of the different protein components. It is, however, important to remember that the concentration obtained from centrifugation diagrams of the faster sedimenting component always is too low. This effect has been discussed by several authors [9—11] but it is of little importance at concentrations lower than one percent. In our case the solutions contain three components, two of which, vicilin and legumin, are always in about the same relative concentrations. When determining the relative amounts of vicilin and legumin from the ultracentrifuge diagrams the error caused by the effect mentioned above should be of about the same magnitude all the time.

In order to compare the relative concentrations of vicilin and legumin, ultracentrifuge diagrams taken 45 minutes after full speed at 65 000 r.p.m. were used. The areas were measured in the way indicated in Fig. 2. The errors in these determinations are large and increase with germination of the seeds. The longer the germination has been going on the more non-sedimenting products are present in the solutions in comparison with the amounts of vicilin and legumin. This fact can be clearly seen from Fig. 2. The ratio vicilin/legumin was determined after 1, 2, 3, 5, 6, 7, 9 and 12 days of germination. In all cases the average values of this ratio from the different determinations were between 1.1 and 1.5. After what has been said about the errors in these determinations, it is impossible from these measurements to detect any definite differences in the breakdown velocities of the two globulins. According to these experiments vicilin and legumin are broken down with the same speed during the germination. The absolute amounts of albumin, vicilin and legumin determined from sedimentation diagrams gave breakdown curves of the same form as the curves obtained from nitrogen determinations.

DISCUSSION

From Table 3 some important conclusions can be drawn. The amount of extractable nitrogen shows, even if the errors in these determinations are large, a clear tendency to decrease as the germination is going on. According to Table 3 the new plant does not lose any nitrogen during the germination.

During the last stage of the germination it takes up nitrogen from the nutrient solution. Thus, when the total nitrogen in the plant increases while the extractable nitrogen decreases, the nitrogen in the developed plant must be present in a different form than in the seeds. The proteins of the leaves are synthesized when the new plant grows up and according to Chibnall [12] these proteins cannot be easily extracted without previous cytolysis. According to Table 3 the amount of extractable nitrogen begins to decrease after about 6 days of germination, or exactly at the time (Fig. 1) when the new plant begins to grow and green leaves are formed. Thus a clear dependence of the amount of extractable nitrogen on growing can be seen. The breakdown curve of the non-dialysable nitrogen, or protein nitrogen, shows a maximum rate of breakdown after 8—10 days of germination. A similar curve was obtained by Prianischnikow [13] with a maximum breakdown after 9 days of germination. The globulin nitrogen, however, is broken down in a still more interesting way. During the first stage of the germination, about the first five days, globulin is broken down very slowly. After 5—6 days the breakdown of the globulins increases, and after 15 days practically no globulin remains. A comparison of this curve (Fig. 3) with Fig. 1 shows that the breakdown of the globulins is most intense at the time when the new plant is growing. Thus the conclusion can be drawn that the globulins, which amount to 70 % of the total protein content of the seeds, are used for the synthesis of the proteins in the new leaves.

The albumin nitrogen is slowly broken down with constant speed during the germination. The fact that albumin is broken down more slowly than globulin can be seen clearly from the ultracentrifuge diagrams in Fig. 2. The role of the albumin during germination is not clear. The author has two theories on this subject. Firstly albumin can be an intermediate product in the breakdown of the high-molecular globulins to amino acids. Secondly, and more probably, the albumin is broken down quite independently of the globulins, and the breakdown products from the albumin fraction serve other purposes than the breakdown products from the globulins.

As can be seen from Table 3, it is difficult to determine the amount of dialysable (low-molecular) nitrogen with satisfactory accuracy. It can be seen, however, that during the first 10 days of the germination the amount of low-molecular nitrogen increases at about the same rate as the breakdown of the globulins and the albumin, during which of course amino acids and other low-molecular nitrogen products are formed. After ten days the amount of low-molecular nitrogen decreases, because this nitrogen is probably being used by the plant for the synthesis of new proteins. From the results of these experiments, it is clear that the protein breakdown is most effective after 5—10 days'

germination. Thus, the proteolytic enzymes are most active at this stage. Very little is known about the proteolytic enzymes from seeds of plants belonging to the *Leguminosae*. The present author has isolated a protein fraction with proteolytic activity by extracting overnight macerated pea seeds, germinated for 8 days with water or 50 % glycerol. By dialysis of the extracts against water and precipitation of the supernatant solution from the dialysis with $(NH_4)_2SO_4$, the enzyme can be concentrated and purified. A still more active enzyme preparation can be obtained by extracting unripe seeds with water and subsequent dialysis of the extract, by which procedure the globulins are precipitated. The proteolytic activity follows the albumin fraction. These investigations will be described in a later paper.

SUMMARY

1. The breakdown of albumins and globulins in seeds of peas has been studied during germination.

2. From the ripe seeds, 70—80 % of total N can be extracted at pH 7 with 0.2 M NaCl. After ten days' germination less nitrogen can be extracted.

3. The breakdown curve of the globulins shows a pronounced maximum between 5—10 days' of germination which is the period when the new plant begins to form leaves. Vicilin and legumin are broken down with the same speed.

4. The breakdown of the albumins is slow and occurs at a constant rate.

5. A protein fraction with proteolytic activity has been isolated and purified. The enzyme follows the albumin fraction.

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The Corrosion of Silver by Potassium Cyanide Solutions and Oxygen

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During the last century numerous investigations have been made concerning the dissolution of silver in potassium cyanide solutions, chiefly with a view to the utilization of the reaction in industry. The fact that oxygen is a necessary factor in this reaction was for the first time pointed out by Elsner [1] (1846) and later on confirmed through experiments made by Bodländer [2] (1896), who investigated the corresponding reaction with gold. Elsner's experiments made it probable that the reaction was as follows:

$$4\ Ag + 8\ KCN + O_2 + 2\ H_2O = 4\ KAg(CN)_2 + 4\ KOH \qquad (I)$$

Bodländer admitted the correctness of this equation as an overall reaction, but found hydrogen peroxide as an intermediate:

$$2\ Ag + 4\ KCN + O_2 + 2\ H_2O = 2\ KAg(CN)_2 + H_2O_2 + 2\ KOH \qquad (II)$$

The hydrogen peroxide reacts with silver and potassium cyanide:

$$2\ Ag + 4\ KCN + H_2O_2 = 2\ KAg(CN)_2 + 2\ KOH \qquad (III)$$

By adding equations (II) and (III) we obtain the overall reaction (I).

Raschig [3] (1928) has dealt with these problems in a paper giving a comprehensive review of former investigations. He explains the mechanism of the reaction by the assumption that silver gives off positive ions to the solution, itself obtaining a negative charge. Simultaneously oxygen is reduced to O_2^{--}. The silver ions combine with the cyanide ions, while the O_2^{--} ions combine with hydrogen ions from the water to form hydrogen peroxide.

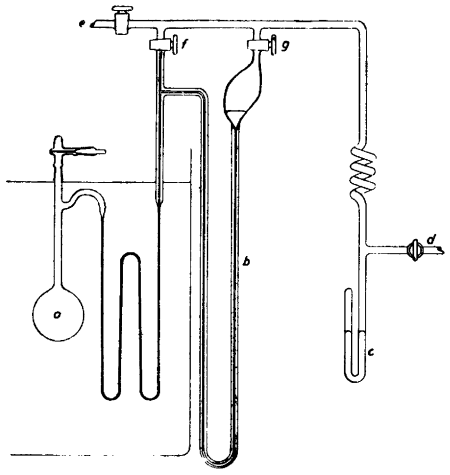

Fig. 1. Brønsted shaking apparatus. a) reaction flask, b) manometer, c) manometer, d) stopcock to gasometer containing oxygen, e) stopcock to vacuum pump.

With the object of investigating the kinetics, the rate of the reaction has been followed by continuously measuring the oxygen absorption in a system of silver, oxygen and potassium cyanide solution in which there is a deficit of the latter.

TECHNIQUE

The experiments were carried out in a Brønsted shaking apparatus (Fig. 1). By means of a capillary tube the reaction flask (a) was connected to the manometer (b) which in turn was connected to the closed manometer (c). At (d) the apparatus was in communication with a gas holder containing oxygen and at (e) with a vacuum pump. The flask (a) was closed by means of a cap with an additional ground glass joint so that by turning this additional joint it was possible to raise and lower a sheet of silver during the experiment. The volume of the closed system, consisting of flask with cap and tubes up to the manometer, was 71 ml.

Into the flask was poured 11.5 ml of a solution the concentration of which as regards potassium cyanide (kalium cyanatum Merck) varied, but the normality of which with regard to sodium hydroxide was always $N/10$. The cyanide concentration was on beforehand determined by titration with silver nitrate according to Liebig's method in the presence of ammonia and potassium iodide.

Above the solution a sheet of silver was fixed (pure silver containing 1 000 parts per 1 000 silver, the fineness being determined with an accuracy of 0.02 per cent by the Government Assay Office which has supplied the silver for the

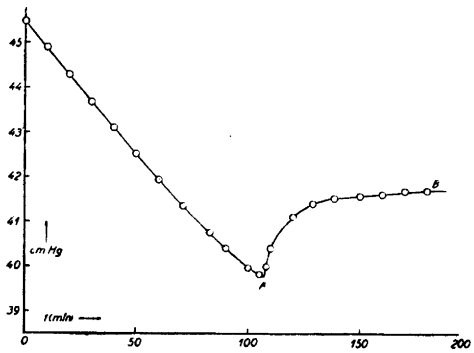

Fig. 2. Absorption of oxygen in a system of silver, oxygen and a solution of potassium cyanide at 25° C. During the experiment are consumed 9.821 · 10^{-4} Mol potassium cyanide, 4.829 · 10^{-4} equiv. of silver, 1.220 · 10^{-4} oxygen to B, 1.826 · 10^{-4} Mol oxygen to A.

experiments). When no other statement is made, the sheet used was 40 mm long by about 7 mm wide and 0.6—0.8 mm thick. The apparatus was evacuated, the stopcocks f, g, and e being kept open. When the pumping had proceeded for one minute, stopcock f was closed, so that water vapour was removed from the rest of the system, and now the stopcocks g and e were closed. Oxygen was now passed from the gas holder into the flask. This oxygen was evacuated, fresh oxygen was admitted through stopcocks d and f, and the shaking apparatus was started. When the shaking had lasted for three minutes and the solution was saturated with oxygen, the oxygen pressure was read, this value being considered to correspond to the time zero; the silver sheet was lowered into the solution and readings were made at suitable intervals. When the oxygen pressure had become constant, the experiment was discontinued and the silver sheet was rinsed in water and weighed after having been left to dry on filter paper. The rate of the oxygen absorption was highly dependent on the speed at which the apparatus was shaken. The maximum rate of absorption was ascertained to occur at an average speed of 200 shakings per minute with an amplitude of 1.9 cm. By altering the speed to 220 or 180 shakings per minute it was possible to alter the slope of the curve in Fig. 2 considerably. The apparatus was therefore fitted with a speed regulator and the speed was kept constant at 200 shakings per minute.

In certain cases the experiment has, however, been discontinued before the oxygen pressure had become constant, in order to determine not only the weight of the silver sheet, but also the content of hydrogen peroxide by titration with potassium permanganate in a solution acidified with sulphuric acid.

Experiments have also been made to ascertain the conditions prevailing when silver was acted upon by a potassium cyanide solution to which hydrogen peroxide (Perhydrol Merck) had been added.

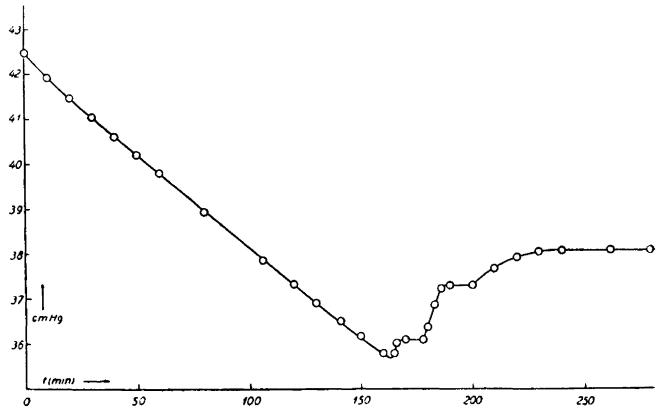

Fig. 3. Absorption of oxygen in a system of silver, oxygen and a solution of potassium cyanide at 25° C. The silver plate is raised and lowered twice during the experiment. $10.736 \cdot 10^{-4}$ Mol potassium cyanide, $5.256 \cdot 10^{-4}$ equiv. of silver, $1.386 \cdot 10^{-4}$ Mol oxygen are consumed.

EXPERIMENTAL RESULTS

1. Fig. 2 represents one of the approximately 50 experiments in which the experiment has been continued until the oxygen pressure above the solution had become constant. The trend of the curve was always as shown in the figure. During the first part of the experiment the absorption took place at an almost constant rate, followed by a sudden rise at the time corresponding to the point *A* in the figure, whereupon the pressure became constant. A determination of the amount of oxygen absorbed calculated on the basis of the difference between the initial and final pressure and the weight of the dissolved silver shows, when correlated to the amount of cyanide used, agreement with Elsner's overall reaction. (During the experiment there was usually a loss of cyanide amounting to 1—2 per cent, which may perhaps be due to hydrolysis of cyanide into formic acid, a small quantity of formic acid having been determined in the mixture $CN^- + H_2O_2$).

2. The trend of the curve obtained when the silver sheet was periodically liftet out of the solution beginning immediately when the rise in pressure had commenced, *i. e.* when the point *A* (Fig. 2) had been passed, will appear from Fig. 3. The rise in pressure stopped when the silver sheet was no longer in the solution, but began again as soon as the sheet was lowered into the solution. The presence of the silver sheet in the solution is thus necessary to produce the rise in pressure.

3. Other experiments were broken off at a time corresponding to point A in Fig. 2. By titration of the solution large quantities of hydrogen peroxide

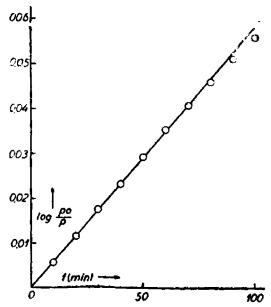

Fig. 6. log p_0/p plotted against time. Results from experiment Fig. 2.

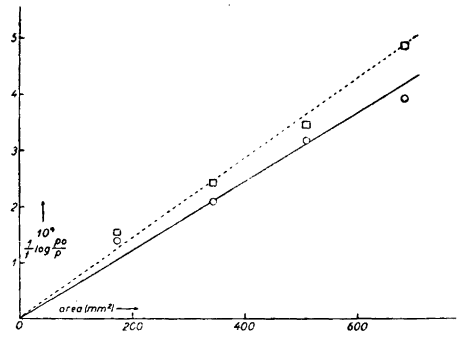

Fig. 7. The velocity constant plotted against the area of the silver plate (15° C and 25° C). The initial concentration of cyanide is 0.043 and of hydroxyl 0.1.

were found to be present. When the silver sheet was again lowered into the solution, an evolution of gas on the sheet was clearly visible. When this evolution of gas had stopped, the solution did not contain any hydrogen peroxide. The sudden increase in pressure at A (Fig. 2) is obviously due to the catalytic decomposition of the hydrogen peroxide on the silver sheet.

4. The rate of absorption proved to be independent of the initial cyanide concentration within the range examined, where c_{CN^-} varied between 0.05 and 0.2, while it is dependent on the oxygen pressure, the reaction being approximately of the first order with regard to oxygen (see Fig. 6). Four experiments in which the initial oxygen pressure was 45, 29, 15, and 10 cm Hg, respectively, showed that the gradients of the tangent at $t = 0$ of the curve obtained by plotting log p_0/p against t were 0.000591, 0.000634, 0.000634, and 0.000644, respectively.

5. The rate at which the oxygen absorption took place was further dependent on the area of the surface of the silver sheet, this surface being assumed to be plane. In the course of four experiments this area was varied in the ratios of 1, 2, 3, and 4, the area of sheet 1 being 174 mm², that of 2 : 345 mm², that of 3 : 510 mm², and that of 4 : 686 mm². The initial concentrations of cyanide and hydroxyl ions were the same through all the experiments. It will be seen from Fig. 7 that the rate of oxygen absorption is proportional to the area of the surface.

A similar experimental series made at 15° C has been represented in the same figure. The ratio k_{25}/k_{15} is, as will appear from the figure, equal to 1.17. From the constants the activation energy of the reaction has been calculated to be 3.2 kg cal. according to the formula:

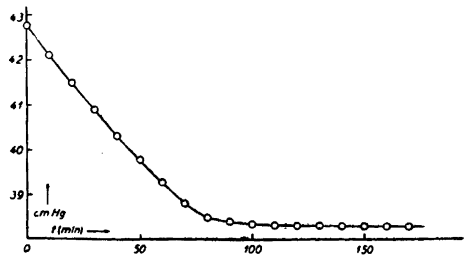

Fig. 4. Absorption of oxygen in a system of silver, oxygen and a solution of potassium cyanide at 25° C. During the experiment are consumed 6.152 · 10^{-4} Mol potassium cyanide, 3.041 · 10^{-4} equiv. of silver, 1.438 · 10^{-4} Mol oxygen. 1.324 · 10^{-4} Mol hydrogen peroxide is formed.

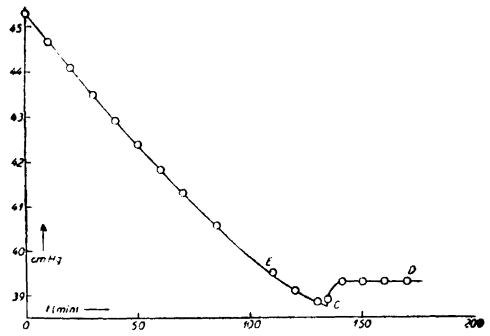

Fig. 5. Absorption of oxygen in a system of silver, oxygen and a solution of potassium cyanide at 25° C. During the experiment are consumed 9,821 · 10^{-4} Mol potassium cyanide, 4,848 · 10^{-4} equiv. of silver, 1,896 · 10^{-4} Mol oxygen to D, 2.056 · 10^{-4} Mol oxygen to C. 1.355 · 10^{-4} Mol hydrogen peroxide is formed.

$$E\ (1/298—1/288) = R\ \ln\ k_{25}/k_{15}$$

where R is the gas constant, k_{25} and k_{15} the velocity constants for the monomolecular reaction at 25° C and at 15° C, respectively, while 298 and 288 are the absolute temperatures.

6. In all the above mentioned experiments the silver sheet had previously been etched in a potassium cyanide solution in the presence of air and then rinsed in water to give the sheet a white, crystalline appearance.

By altering the preliminary treatment of the silver sheet, the surface of the latter was altered, and the experiments with such sheets produced oxygen-absorption curves of an altered appearance as regards the last part of the curve. When thus the silver sheet preparatory to the experiment was kept immersed in a mixture of potassium cyanide and hydrogen peroxide with a large excess of hydrogen peroxide, it lost its crystalline appearance and became perfectly bright, while at the same time an evolution of oxygen took place on the sheet. When such a sheet, after rinsing and drying, was lowered into the potassium cyanide solution, the oxygen absorption curve would exhibit a trend as the one represented in Fig. 4, the curve being readily reproducible. When in the experiment the initial concentration of cyanide ions was greater, a slight break would occur in the curve (Fig. 5). After having maintained a constant oxygen pressure for some time, the experiment was discontinued,

the silver sheet was weighed, and the hydrogen peroxide content in the solution was determined. It will be seen from the explanations beneath the illustrations that the quantities of silver and cyanide were equivalent. The quantity of oxygen absorbed corresponded in the experiment illustrated in Fig. 4 to 93.5 per cent of the quantity consumed according to equation (II). It will be seen from the curves that during the first part of the experiment the oxygen absorption occurred at the same rate as in the other experiments, while it may be seen from the final horizontal part of the curve that hydrogen peroxide was not, or practically not, decomposed catalytically on the plate.

7. To ascertain whether the primary reaction proceeds according to the equation:

$$2\ Ag + O_2 = 2\ Ag^+ + {}^-O{-}O^- \qquad \text{(IV)}$$

the quantity of oxygen absorbed, the silver dissolved and the content of hydrogen peroxide in the solution were determined simultaneously during five experiments which were discontinued after periods of 10, 20, 30, 40, and 120 minutes, respectively.

Table 1. Simultaneous determinations of silver dissolved, oxygen absorbed, and hydrogen peroxide formed, at 25° C.

(1)	(2)	(3)	(4)	(5)	(6)
Min.	Equiv. Ag dissolved $\times$ ½ $\times 10^5$	Mol. O_2 absorbed $\times 10^5$	Mol. "H_2O_2" formed $\times 10^5$	(2)−(4)	$\frac{(2)+(4)}{2} - (3)$
10	1.48	1.45	1.43	0.05	0.00
20	3.38	3.07	3.09	0.29	0.16
30	4.68	4.17	4.00	0.68	0.17
40	6.21	5.40	5.51	0.70	0.46
120	18.03	16.00	14.76	3.27	0.40

At the beginning of the experiment the quantity of silver dissolved was equivalent to the quantity of hydrogen peroxide formed, which indicates that the course of the reaction was as described by equation (IV). As the experiment proceeded, the difference between dissolved silver and hydrogen peroxide content gradually increased (Table 1, column 5). It is obvious that the hydrogen peroxide formed took part in the process of dissolution of the silver. It will be seen from equations (II) and (III) that half of the dissolved silver in equivalents (Table 1, column 2) + the quantity of hydrogen peroxide formed,

in moles (column 4) should be equal to twice the quantity of oxygen consumed, in moles (column 3). The results found (column 6) do not agree completely with this, but this is possibly due to errors in the hydrogen peroxide titration, which was difficult to carry out on account of the small amounts of standard solution used, or more probably to the formation of small quantities of formic acid which also consumes permanganate, for which reason the H_2O_2 content is given in inverted commas in the table.

8. The question now arises whether free cyanide ions are present at the time when the decomposition of the hydrogen peroxide commences. To obtain an answer to this question, a number of experiments were made which were broken off at the time corresponding to point A (Fig. 2). The silver sheet was weighed, replaced in the apparatus, which was evacuated. The silver sheet was again lowered into the liquid, and the decomposition process was allowed to proceed to completion. The silver sheet showed a further loss in weight equal to 5 to 10 per cent of the total loss, a fact which indicates that cyanide ions must be present in the solution at the moment when the decomposition commences. A comparison between the experiment illustrated in Fig. 2, in which decomposition took place, and that of Fig. 5, in which practically no decomposition occurred, also shows that in cases where there is no decomposition, the primary absorption of oxygen is greater which in turn means that silver was dissolved during the time corresponding to the last sloping part of the curve, *i. e.* from E to C. The quantity of silver dissolved after the discontinuation of the experiment at constant oxygen pressure was the same in the two cases in which the initial concentration of cyanide ions was the same.

9. For further elucidation of the process of dissolution of the silver sheets, additional experiments have been made, not in the Brønsted apparatus, but in a test tube into which oxygen at constant pressure was passed. The silver sheet was suspended from a scale of an analytical balance and immersed in the solution in the test tube. The sheet was frequently weighed during the process of solution, the test tube with the reagent solution being for this purpose replaced by a test tube with water. The curve representing the relationship between loss in weight and time is given in Fig. 8. At the beginning the order of the reaction is zero, corresponding to the fact that the oxygen pressure is kept constant. At the time *A* an evolution of oxygen was observed to begin in the solution. As shown above, the solution at this time still contained small quantities of free cyanide ions which could dissolve silver. The explanation of the increased rate of absorption will be discussed later.

10. We have already in connection with the process of dissolution: oxygen, potassium cyanide and silver, seen that the hydrogen peroxide formed must take part in the dissolution of the silver. This assumption was confirmed by

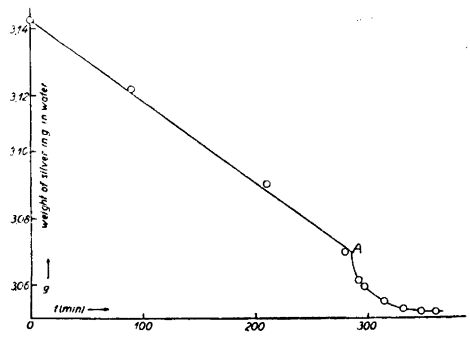

Fig. 8. Loss in weight of the silver plate plotted against time in a system of silver, oxygen and a solution of potassium cyanide at an oxygen pressure of 1 atm. at 25° C.

direct experiments with hydrogen peroxide, potassium cyanide and silver. The experiments were made in test tubes. Potassium cyanide solution free from oxygen, and hydrogen peroxide solution were poured into a test tube, and the mixture was aerated by means of nitrogen which was freed from oxygen by passing through a furnace in which reduced copper was held at 300° C. The test tube was placed in a thermostat below the analytical balance from which the silver sheet was suspended, immersed in the liquid, and the silver sheet was weighed periodically. In the course of a few hours a small quantity of silver was dissolved, *e. g.*, in a cyanide concentration of 0.05 and a hydrogen peroxide concentration of 0.02 a quantity of 5 mg silver was dissolved in the course of three hours, while no catalytic decomposition of the hydrogen peroxide was observed on the sheet. It proved difficult to reproduce these experiments, but small amounts of silver were dissolved in all cases.

11. Experiments have further been made in the Brønsted apparatus with hydrogen peroxide, potassium cyanide and a sheet of silver, in which the rise in pressure due to the oxygen evolution was measured and the silver sheet was weighed after the conclusion of the experiment. It also proved difficult to reproduce these experiments, due to difficulties in obtaining identical silver sheets. The course of the experiment was dependent on the concentrations of hydrogen peroxide and potassium cyanide and on the nature of the surface of the silver. A curve belonging to either of three different classes of curves was, however, always obtained. First, an almost horizontal curve in the cases in which there was a great excess of potassium cyanide as compared to the hydrogen peroxide concentration, refer equation (III). The second case is illustrated in curve I, Fig. 9. Here there is a great excess of hydrogen peroxide as compared to potassium cyanide. In this case hydrogen peroxide is immediately decomposed while silver is at the same time dissolved. Finally the course of the experiment when the hydrogen peroxide surplus is smaller is

illustrated by curve II. In this case the decomposition of hydrogen peroxide will only set in when a fairly long time has elapsed. The quantity of silver dissolved was smaller than the quantity equivalent to the amount of cyanide added. This seems to indicate an interaction between cyanide and hydrogen peroxide.

12. A number of experiments on this problem on the whole confirmed Masson's [4] experiments. According to Masson the following reactions take pl ace:

$$KCN + H_2O_2 = KCNO + H_2O \quad (1)$$

$$KCN + 2\ H_2O = HCOOK + NH_3 \quad (2)$$

four fifth of the cyanide being consumed according to equation (1), one fifth according to equation (2).

When 0.2 molar solutions of potassium cyanide and hydrogen peroxide were mixed, they reacted on each other. In the course of four hours practically all cyanide had been consumed to form potassium cyanate which was partly hydrolysed into carbonate; about 10 per cent of the cyanide was converted into formic acid, the quantity of which was determined by addition of mercuric chloride to the solution to which on beforehand acetic acid and sodium chloride had been added; the mercurous chloride formed was determined by iodometry. It was also ascertained whether the solution contained any nitrous oxide (by combustion analysis in a Christiansen [5] gas-analysis apparatus) or any nitrite or nitrate (according to Blom [6]), but the results were negative.

DISCUSSION

It has been shown in the preceding that the velocity of the reaction in the system $Ag + KCN + O_2$ is:

1. of the 1st order with respect to the oxygen pressure,
2. independent of the cyanide ion concentration,
3. proportional to the area of the surface of silver, the latter being considered to be a plane,
4. dependent on the speed at which the shaking is effected (the trend of the curve in fig. 8 may be explained with a view to this fact, as the decomposition of the hydrogen peroxide on the surface of the silver results in a more vigorous agitation of the liquid around the silver, which in turn results in a greater speed of dissolution).
5. Finally the temperature coefficient of the reaction is small.

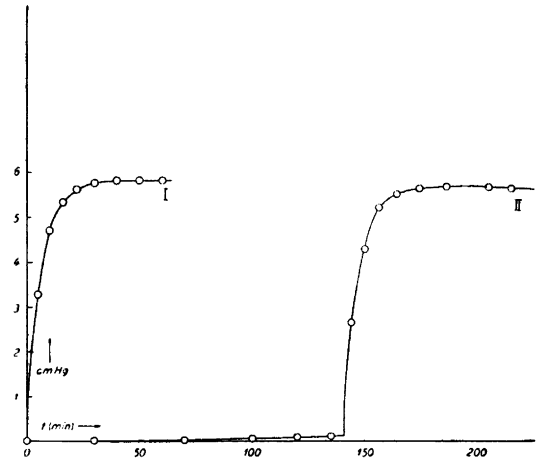

Fig. 9. Oxygen pressure in a system of silver, hydrogen peroxide and a solution of potassium cyanide at 25° C. During the experiment are consumed I. *$1.700 \cdot 10^{-4}$ Mol potassium cyanide, $0.834 \cdot 10^{-4}$ equiv. of silver, $4.163 \cdot 10^{-4}$ Mol hydrogen peroxide, $1.861 \cdot 10^{-4}$ Mol oxygen evolved.* II. *$4.242 \cdot 10^{-4}$ Mol potassium cyanide, $2.086 \cdot 10^{-4}$ equiv. of silver, $4.830 \cdot 10^{-4}$ Mol hydrogen peroxide, $1.839 \cdot 10^{-4}$ Mol oxygen evolved.*

The mechanism of the reaction may be illustrated as follows:

$$O_2\text{gas} \rightleftharpoons O_2\text{diss.} \qquad \pm 1$$

$$O_2\text{diss.} + Ag_{\text{solid}} \rightarrow X| \qquad 2$$

where the reaction $\pm$ 1 represents the solution of oxygen in water and reaction 2 the fact that the dissolved oxygen diffuses to the silver sheet and reacts with the latter.

On the condition that the concentration of dissolved oxygen is stationary the following expression is obtained for the determination of the velocity of the reaction of the flow s:

$$c_{o_2}\text{gas}/s = 1/k_1 + k_{-1}/k_1 k_2 q, \qquad \text{(Christiansen}^7\text{)}$$

where q is the area of the surface of the silver. As $k = aq$ (a is a constant), it is only the second term which will have to be taken into account.

$k_1/k_{-1} = K_1 = c_d/c_g$, *i. e.* Ostwald's coefficient of absorption (c_d is the concentration of oxygen in the solution, c_g the concentration over the solution). Hence $s = K_1 k_2\ qcg$.

This expression is in agreement with the one which according to the experiments apply for the flow found:

$s = kqcg$, *i. e.* it is reaction 2 which determines the velocity.

If the velocity is determined by a flow of oxygen molecules through a diffusion layer δ at the silver sheet, the expression for the flow is according to Nernst [8] $s = Dq/\delta \times c_d$, where D is the coefficient of diffusion. The concentration of oxygen molecules on the silver has been taken to equal zero. This equation is also in agreement with the equation of the reaction examined (Fig. 6).

The ratio of $Dq/\delta \times K_1$ at 15° C and at 25° C is equal to 1.11, as D is proportional to T/η according to Stoke-Einstein's law, η is the viscosity and T the absolute temperature. The ratio between the velocities found is 1.17, which means that the velocity is obviously determined by a diffusion.

During the reaction there is thus a flow of oxygen molecules towards the silver, the latter is subjected to autoxidation and dissolved through the action of the cyanide ions present. In the course of the reaction cyanide ions obviously prevent a catalytic decomposition on the surface of the silver of the hydrogen peroxide formed. In certain cases the decomposition is prevented completely (Fig. 4), in others (Fig. 2) the decomposition commences when a certain low cyanide ion concentration has been reached, dependent on the properties of the silver surface. An explanation of this phenomenon may perhaps be found in the fact that by treatment with a mixture of cyanide ions and hydrogen peroxide the surface area is reduced considerably and all crystalline edges eliminated, so that the sheet is more readily kept covered by cyanide ions, which are adsorbed by the sheet in some way or other.

SUMMARY

Solution of silver in a potassium cyanide solution to which oxygen is being added proceeds according to the equation:

$$2\ Ag + O_2 = 2\ Ag^+ + O_2^{--}$$

The hydrogen peroxide formed is decomposed on the silver due to a catalytic effect when the cyanide ion concentration is small. The cyanide ion concentration at which this decomposition begins depends on the preliminary treatment

to which the silver sheet has been subjected. The hydrogen peroxide formed takes part in the process of dissolution according to the equation:

$$2\ Ag + H_2O_2 + 4\ CN^- = 2\ Ag(CN)_2^- + 2\ OH^-$$

The process of dissolution is of the 1st order with regard to the oxygen pressure and has an activation energy of 3.2 kg cals from 15°C to 25°C. The "activation energy" of the quantity $T/\eta \times K$, where η is the viscosity, is 1.8 kg cals. This seems to indicate that the velocity of the reaction is determined by a diffusion of oxygen molecules towards the silver.

The author wishes to express her gratitude to the Head of the Department of Physical Chemistry, Professor, dr. phil. J. A. Christiansen for his valuable advice and help.

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A Spectro-photometric Study of Aqueous Molybdate Solutions

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In a recent review [1] of the polymolybdates, the author discussed a theory concerning the ionic conditions present in aqueous solutions. That discussion was based on experimental evidence of different kinds, but especially on diffusion measurements and conductometry. The results were not quite conclusive, however, and the present investigation has been carried out in order to give a more definite confirmation of the theory developed.

Spectro-photometric methods have earlier been used to study the stepwise formation of mononuclear weak complexes. This problem has been attacked very successfully by Scandinavian chemists (J. Bjerrum [2], Olerup [3], Fronaeus [4]), who have worked out mathematically clear and experimentally suitable methods. The polynuclear complexes are too complicated, however, to permit such a stringent treatment. But if only strong complexes are considered, the following simplified method can be used.

MODE OF CALCULATION

Earlier spectro-photometric works on molybdate solutions have been carried out by Jander [5] and Carpéni [6], who measured extinction curves for different acidities and found that the curves are shifted towards longer wave lengths on acidification. Fig. 1 showing the curves of molybdate solutions with pH 0 and 14, has been given by Carpéni [6]. An absorption edge occurs towards shorter wave lengths where, consequently, no measurements can be made. On the horizontal parts of the curves ($\lambda > 3400$ Å), however, the assumption of a constant molar extinction coefficient will still be correct.

This affords a possibility of measuring the effect of the addition of mineral acids to molybdate solutions on their light absorption properties. It is still

better, however, to calculate the effect of a given consumption of H^+ ions by the molybdate solutions as will be discussed here.

The following notation is used:

Extinction coefficient ε, defined by $\varepsilon \cdot 1 = {}^{10}\log \frac{I}{I_c}$

Concentration of added, strong monobasic acid S.

Concentration of remaining H^+ ions h.

The extinction for $S = 0$ $(\varepsilon_{MoO_4^{2-}})$ ε'

$\triangle \varepsilon = \varepsilon - \varepsilon'$ the increase of ε caused by the H^+ consumption $S - h$.

$\frac{\triangle \varepsilon}{S-h}$ is then the effect on the extinction of one mole of consumed H^+.

Plotting $\frac{\triangle \varepsilon}{S-h} = f(S-h)$ vs. $S-h$ more or less complicated curves are obtained:

If one type of ions is predominant at the first stages of acidification, the curve will start as a straight horizontal line. If, then, a second type of ion were formed with no interference between the two ionic species, the curve would continue as a new straight line. There is always an overlap, however, which will give rise to S-shaped curves instead of straight lines. The greater the difference in the $S-h$ values at which complexes are formed, the straighter the lines will appear. In the polymolybdate case the interference is sufficiently small to permit a consideration of the curves as almost straight lines.

As to the magnitude of ε, Jander [5] claimed that a larger value of ε (at a given concentration) indicates a larger complex. This assumption seems rather reasonable for this type of complexes, but it is of course only a hypothesis.

EXPERIMENTAL METHODS

Extinction measurements. A Beckmann Spectro-photometer Model DU, with an hydrogen lamp and 10 mm quartz cells, was used for the optical measurements. As no thermostate device was available, there was always a continous rise with time in the temperature of the cell compartment, and therefore the investigations were carried out ½ – 1 ½ hour after the lamp had been started, when the corresponding rise of the ε values was never more than 2 %. For one series of solutions the values obtained with the hydrogen lamp were compared with those obtained with a tungsten lamp and a blue filter, and they were found to be identical within the limit of experimental error. This was governed in most cases by the temperature drift error but greater errors appear, certainly at very high and very low extinction values which are undoubtedly very inaccurate.

In the wave length region used (3500 – 3900 Å) only the molybdate ions contribute to the extinction; the exclusion of the effect of H^+, Na^+ and Cl^- ions is very important (*cf.* the conductometry).

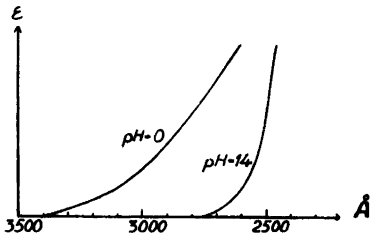

Fig. 1. The extinction curves of molybdate solutions with pH 0 and 14.

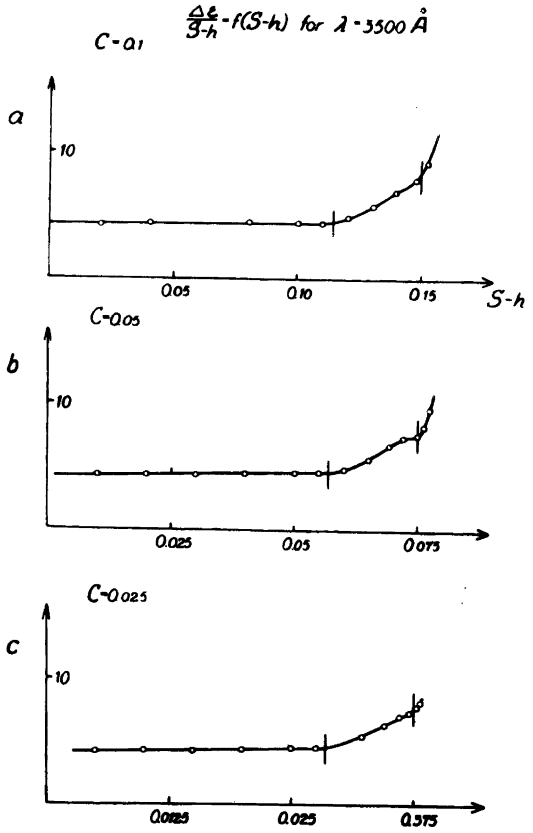

Fig. 2. $\frac{\Delta \varepsilon}{S-h} = f\ (S-h)$ *for* $\lambda = 3500$ *Å at different concentrations*

a) C = 0.100
b) C = 0.050
c) C = 0.025

Concentration of remaining H^+ions. The measurement of h can only be carried out with a large salt excess giving ionic strengths to the molybdate solutions almost identical with those of the acids used for comparison. All solutions were therefore 2-molar in NaCl.

These measurements were carried out on a battery-supplied tube potentiometer (Ermi, Stockholm, described by Blomgren [7]) with a glass electrode and a saturated calomel electrode. The *emf* values of HCl solutions with different concentrations were measured simultaneously with the molybdate solutions. It was then possible to deduce the h values consistent with a measured *emf* for the molybdate solutions from the *emf* values obtained with the standard HCl solutions. As the performance of the glass electrode was not quite constant the *emf* values in each series (*v. infra*) of molybdates and standard HCl solutions were measured in a sequence determined by their magnitudes. (This was made possible by a preliminary pH measurement of the molybdate solutions.) The *emf* values were measured with an accuracy of at least 2 mV, which corresponds to a largest error in h of 5 %. Standard solutions of HCl more dilute than 0.001-molar can not be used because of their low buffering capacity.

The best accuracy would have been obtained if all measurements had been made at the same constant temperature, but there are indications, that this is not necessary for our

purpose. Potentiometric measurements at 20° C and 25° C give the same values of h, and the variation of ε due to the temperature differences does not exceed 2 %.

Procedure. Varying amounts of HCl and H_2O were added to a number of molybdate solutions so that solutions with identical total concentrations (C) of Na_2MoO_4 were obtained. (They were also 2-molar in NaCl.) The only varying factor in each series was thus S (HCl added). h and ε were then measured for the different solutions and the function $\frac{\Delta \varepsilon}{S-h} = f(S-h)$ was calculated. This procedure was repeated for different concentrations of Na_2MoO_4. It was found that no appreciable contraction took place during the preparations.

In one series $HClO_4$ was used instead of HCl with the same result.

Concentration $C > 0.4$ lead to formation of solid polymolybdates, and for $C < 0.01$ the inaccuracy of $S-h$ increases rapidly. The concentration range investigated was therefore $0.01 < C < 0.4$.

RESULTS

The experimental data are given in Table 1. In Fig. 2 the function $\frac{\Delta \varepsilon}{S-h} = (S-h)$ is shown for some series.

Three curves (Fig. 2) all show one straight practically horizontal line, continued by another slightly S-shaped line. They show also a third part after a second break.

The two breaks indicate the existence of the ions $Mo_7O_{24}^{6-}$ and $Mo_8O_{26}^{4-}$ in the solutions according to the equations:

$$7MoO_4^{2-} + 8H^+ = Mo_7O_{24}^{6} + 4H_2O$$
$$8MoO_4^{2-} + 12H^+ = Mo_8O_{26}^{4} + 6H_2O$$

as is shown by the agreement of the experimental with the calculated discontinuities (*cf.* Fig. 2) at $\frac{S-h}{C} = \frac{8}{7}$ and $\frac{S-h}{C} = \frac{12}{8}$. (The existence of $Mo_7O_{24}^{6-}$ and $Mo_8O_{26}^{4-}$ has also been discussed in ref. 1.)

The slight S-shape can easily be interpreted as due to a formation of small amounts of $Mo_8O_{26}^{4-}$ before all MoO_4^{2-} ions are transformed into $Mo_7O_{24}^{6-}$. This explanation is also supported by the very small but clear positive inclination of the initial lines (Table 2).

The third kind of ion formed after $Mo_8O_{26}^{4-}$ may be still larger than $Mo_8O_{26}^{4-}$ (as assumed in ref. 1) and it is even formed for MoO_4^{2-} concentrations as low as 0.005-molar (as indicated by a continued rise in ε on addition of sufficient HCl; the values are not given in Table 1).

Table 1. Results of the spectro-photometric measurements (Each series gives the mean values of two measured series).

S	$S-h$	ε_{3500}	ε_{3600}	ε_{3700}	ε_{3800}	ε_{3900}
			$C = 0.4$			
0.000	0.000	0.011	0.010	0.009	0.009	0.008
0.400	0.400	1.756	0.516	0.162	0.053	0.021
0.440	0.440	2.004	0.611	0.188	0.061	0.023
0.480	0.480	—	0.766	0.277	0.108	0.048
0.520	0.520	—	1.227	0.476	0.197	0.092
0.560	0.559	—	1.870	0.763	0.323	0.147
0.600	0.596	—	—	1.004	0.455	0.217
0.640	0.619	—	—	1.686	0.699	0.300
			$C = 0.2$			
0.000	0.000	0.008	0.007	0.007	0.007	0.007
0.040	0.040	0.177	0.053	0.018	—	—
0.200	0.200	0.912	0.263	0.081	0.028	0.012
0.220	0.220	1.014	0.294	0.092	0.033	0.014
0.240	0.240	1.336	0.396	0.148	0.061	0.029
0.260	0.260	—	0.574	0.232	0.102	0.050
0.280	0.279	—	0.832	0.335	0.145	0.069
0.300	0.296	—	1.003	0.410	0.179	0.086
0.320	0.310	—	1.669	0.633	0.261	0.114
0.340	0.322	—	—	1.051	0.409	0.153
0.360	0.332	—	—	1.334	0.465	0.173
			$C = 0.1$			
0.000	0.000	0.007	0.006	0.006	0.006	0.006
0.020	0.020	0.090	0.030	0.014	—	—
0.040	0.040	0.180	0.055	0.021	0.010	—
0.080	0.080	0.368	0.108	0.036	0.014	0.008
0.100	0.100	0.449	0.135	0.044	0.017	0.009
0.110	0.110	0.509	0.155	0.051	0.020	0.011
0.120	0.120	0.598	0.216	0.087	0.040	0.023
0.130	0.130	0.775	0.300	0.129	0.062	0.036
0.140	0.139	0.992	0.393	0.166	0.079	0.044
0.150	0.148	1.190	0.460	0.195	0.091	0.046
0.160	0.152	1.437	0.575	0.239	0.108	0.056
0.170	0.157	—	0.883	0.348	0.143	0.062
0.180	0.162	—	1.258	0.485	0.193	0.083
0.190	0.166	—	1.613	0.612	0.231	0.094
0.200	0.169	—	—	0.685	0.258	0.102

S	$S-h$	ε_{3500}	ε_{3600}	ε_{3700}	ε_{3800}	ε_{3900}
			$C = 0.05$			
0.000	0.000	0.006	0.006	0.006	0.005	
0.010	0.010	0.046	0.018	—	—	
0.020	0.020	0.095	0.031	0.013	—	
0.030	0.030	0.136	0.042	0.017	0.008	
0.040	0.040	0.185	0.056	0.021	0.010	
0.050	0.050	0.235	0.072	0.026	0.011	
0.055	0.055	0.256	0.081	0.029	0.012	
0.060	0.060	0.295	0.102	0.041	0.019	
0.065	0.065	0.378	0.145	0.063	0.029	
0.070	0.069	0.477	0.183	0.078	0.037	
0.075	0.072	0.543	0.209	0.086	0.040	
0.080	0.075	0.581	0.225	0.094	0.043	
0.085	0.076	0.628	0.243	0.100	0.046	
0.090	0.077	0.754	0.296	0.122	0.053	
			$C = 0.025$			
	0.0000	0.0000	0.005	0.004	0.003	
	0.0050	0.0050	0.026	0.010	0.004	
	0.0100	0.0100	0.049	0.017	0.007	
	0.0150	0.0150	0.070	0.025	0.010	
	0.0200	0.0200	0.093	0.030	0.011	
	0.0250	0.0250	0.116	0.036	0.013	
	0.0275	0.0275	0.127	0.040	0.016	
	0.0300	0.0299	0.144	0.050	0.020	
	0.0325	0.0323	0.183	0.072	0.026	
	0.0350	0.0344	0.227	0.093	0.039	
	0.0375	0.0361	0.261	0.104	0.045	
	0.0400	0.0369	0.279	0.111	0.048	
	0.0425	0.0372	0.288	0.114	0.050	
	0.0450	0.03755	0.299	0.119	0.051	
	0.0475	0.03765	0.304	0.122	0.053	
			$C = 0.01$			
	0.0000	0.0000	0.009	0.009	0.008	
	0.0020	0.0020	0.017	0.011	0.009	
	0.0040	0.0040	0.026	0.013	0.009	
	0.0060	0.0060	0.034	0.016	0.010	
	0.0080	0.0080	0.043	0.019	0.011	
	0.0100	0.0100	0.053	0.022	0.012	
	0.0110	0.0110	0.058	0.025	0.013	
	0.0120	0.0119	0.065	0.028	0.015	
	0.0130	0.0128	0.080	0.035	0.020	
	0.0140	0.0136	0.094	0.043	0.024	
	0.0150	0.0142	0.106	0.047	0.026	
	0.0160	0.0147	0.113	0.051	0.028	
	0.0170	0.0150	0.119	0.053	0.029	
	0.0180	0.01505	0.123	0.054	0.030	
	0.0190	0.0151	0.125	0.057	—	

Table 2. $f\ (S - h)$ for $\lambda = 3600$ Å.

	Concentrations			
$\frac{S-h}{C}$	0.025	0.05	0.1	0.2
0.200	1.2	1.2	1.2	1.2
1.000	1.3	1.3	1.3	1.3

One further fact may be noted in this connection; the $Mo_8O_{26}^{4-}$ break is obtained at $\frac{S-h}{C} = 1.5$, where $\frac{S}{C} \geq 1.5$ and not at $\frac{S}{C} = 1.5$. This shift is not so evident in conductometric measurements because it is then compensated by rapid increase of the amount of unconsumed H^+ ions already for $\frac{S}{C} < 1.5$. A rounded break near the correct position is therefore obtained (*cf.* ref. 1).

In the previous discussion of the ionic conditions, one question was left unanswered, *viz.* whether there is a dissociation of the $Mo_8O_{26}^{4-}$ ions to $Mo_4O_{13}^{2-}$ ions in dilute solutions.

The discussion of this question may be preceded by a study of the identity of the $Mo_7O_{24}^{6-}$ ions at different concentrations. For comparison, the values of $f\ (S-h)$ for $\frac{S-h}{C} = 1.0$ are given in Table 3. Evidently there is a complete constancy of $f\ (S-h)$ within the limits of experimental error in the concentration range 0.01—0.4.

A similar discussion of $Mo_8O_{26}^{4-}$ is more difficult because of the increasing inaccuracy of $S - h$ in that region. In Table 4 a comparison is made between $f\ (S-h)$ values for $\frac{S-h}{C} = 1.5$ interpolated for different concentrations from the measurements. It is evident that the results are not so conclusive in

Table 3. $f\ (S - h)$ for $\frac{S-h}{C} = 1.000$.

	Concentrations					
λÅ	0.01	0.025	0.05	0.1	0.2	0.4
3500	4.4	4.5	4.6	4.4	4.5	4.4
3600	—	1.3	1.3	1.3	1.3	1.3
3700	—	—	—	0.37	0.37	0.38

Table 4. $f\ (S-h)$ for $\frac{S-h}{C} = 1.500$.

	Concentrations					
λÅ	0.010	0.025	0.05	0.1	0.2	0.4
3600	2.9	3.0	3.1	3.2	3.5	—
3700	1.3	1.3	1.2	1.3	1.4	1.7
3800	—	—	0.52	0.58	0.60	0.76
3900	—	—	—	0.27	0.28	0.36

this case. The occurrence of larger values at higher concentrations can be explained by two different assumptions: (i) that there is a dissociation of $Mo_8O_{26}^{4-}$ on dilution, or (ii) that complexes larger than $Mo_8O_{26}^{4-}$ are formed already before the $Mo_8O_{26}^{4-}$ break at high concentrations.

The problem must be considered more critically before any final decision is made. It has been concluded that $Mo_8O_{26}^{4-}$ ions are present in concentrated solutions (*cf.* ref. 1). A dissociation $Mo_8O_{26}^{4-} \rightarrow 2Mo_4O_{13}^{2-}$ could be reconciled with the experimental data only if the degree of dissociation were constant for $C \leq 0.1$ (*cf.* Table 4), and as dissociation increases with dilution, this is only possible if there is a complete dissociation into $Mo_4O_{13}^{2-}$ ions in this concentration range. This assumption can be disproved by reference to the results of Jander's diffusion measurements. These show definitely that acidification of 0.1-molar Na_2MoO_4 solutions leads to the formation of complexes with increasing molecular weights [5]. Thus the ions existing at the concentrations $C \leq 0.1$ cannot be $Mo_4O_{13}^{2-}$.

The only alternative is that $Mo_8O_{26}^{4-}$ ions are formed after the $Mo_7O_{24}^{6-}$ break. The slight S-shape of the curves is then explained by the formation of small amounts of $Mo_8O_{26}^{4-}$ together with $Mo_7O_{24}^{6-}$ before the break. At higher concentrations ($C > 0.1$) larger ions are formed together with $Mo_8O_{26}^{4-}$ before the $Mo_8O_{26}^{4-}$ break. This formation has not yet started at the acidification stage $\frac{S-h}{C} = 1.25$ (Table 5) where $f\ (S-h)$ has a constant value independent of the concentrations.

This theory is in agreement with Dumanski's theories[8] about the formation of colloidal ions in molybdate solutions as well as with Jander's results [5].

Table 5. $f\ (S-h)$ for $\frac{S-h}{C} = 1.250$.

	Concentrations					
	0.01	0.025	0.05	0.1	0.2	0.4
3600 Å	1.9	1.9	2.0	2.0	1.9	2.0

Table 6. Spectro-photometric measurements of salt free solutions.

S	ε_{3500}	ε_{3600}	ε_{3700}	S	3500	ε_{3600}
	$C = 0.1$				$C = 0.025$	
0.000	0.008	0.008	0.008	0.0000	0.007	0.007
0.020	0.099	0.035	0.015	0.0050	0.027	0.013
0.040	0.184	0.060	0.023	0.0100	0.048	0.021
0.060	0.277	0.088	0.032	0.0150	0.073	0.029
0.080	0.357	0.123	0.044	0.0200	0.100	0.039
0.100	0.467	0.158	0.057	0.0250	0.134	0.053
0.110	0.516	0.179	0.068	0.0275	0.152	0.063
	$C = 0.05$				$C = 0.01$	
0.000	0.007	0.007	0.007	0.0000	0.007	
0.010	0.049	0.019	0.009	0.0020	0.013	
0.020	0.093	0.033	0.014	0.0040	0.024	
0.030	0.137	0.048	0.018	0.0060	0.037	
0.040	0.183	0.065	0.027	0.0080	0.047	
0.050	0.236	0.087	0.035	0.0100	0.062	
0.055	0.265	0.100	0.041	0.0110	0.066	

COMPLEMENTARY INVESTIGATION OF SALT-FREE SOLUTIONS

The method of measuring h and thus $S - h$, can not be applied to solutions which do not contain a salt excess, but as long as the h values are very small compared with S ($S - h \sim S$) the same procedure can be used. This is true for $\frac{S}{C}$ values below 1.1 and therefore such solutions have been investigated. The results are given in Table 6 and Fig. 3. (It may be noted that the molar extinction coefficient of $Mo_7O_{24}^{6-}$ is almost independent of the large difference in ionic strength.)

From Fig. 3 it is easily seen that for low concentrations ($C < 0.05$) there is an increasing formation of $Mo_8O_{26}^{4-}$ together with $Mo_7O_{24}^{6-}$ in the early stages of the acidification (an increasing positiv inclination of the line).

A comprehensive description of the reactions which take place on acidification of molybdate solutions of different concentrations and ionic strengths has thus been obtained. It remains to determine the nature of the large ions formed on strong acidification. Probably a structure determination of an „octo" or „decamolybdate" would provide the answer.

It can not be neglected that also other polymolybdate ions may exist in very small amounts or under special conditions.

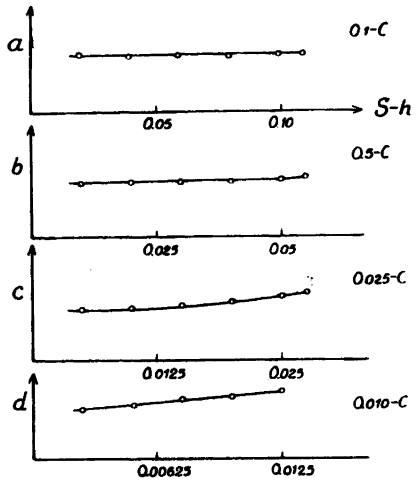

Fig. 3. $\frac{\Delta \varepsilon}{S} = f\ (S)$ *for* $\lambda = 3500$ *Å at different concentrations.*

a) $C = 0.100$
b) $C = 0.050$
c) $C = 0.025$
d) $C = 0.010$

SUMMARY

A spectro-photometric method has been devised for studying the formation of polymolybdate ions. The measurements give strong evidence to the assumption that the reactions taking place upon acidification are $MoO_4^{2-} \rightarrow Mo_7O_{24}^{6-} \rightarrow Mo_8O_{26}^{4-} \rightarrow$ larger complexes.

Finally I wish to thank Miss Britta Svensson for valuable help throughout the investigation. The work has been financially supported by the Swedish Natural Science Research Council, whose support is gratefully acknowledged.

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On the Cerium Dioxide — Uranium Dioxide System and „Uranium Cerium Blue"

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Hofmann and Höschele [1] obtained dark blue, cubic crystals of a cerium uranium oxide by heating a mixture of cerous sulphate and uranyl sulphate with an excess of anhydrous magnesium chloride in a covered crucible at a high temperature. The composition, which varied somewhat for different preparations, was approximately $2CeO_2 \cdot UO_2$. The authors concluded that, owing to the intense colour of this product, which is quite different from those of the pure dioxides, this so-called uranium cerium blue should be considered as a definite chemical compound. By precipitating a solution of cerous nitrate and uranyl nitrate with excess ammonia the same authors obtained a yellow precipitate that turned deep blue after a while. The composition of this substance was approximately $2CeO_2 \cdot UO_2 \cdot 2H_2O$.

Cerium dioxide and uranium dioxide both crystallize with a lattice of fluorite type and the difference between their lattice constants is quite small. From this point of view, the occurrence in the cerium dioxide — uranium dioxide system of an intermediate phase did not seem very likely and we thus decided to study this matter. Intimate mixtures of the two oxides were heated in evacuated silica tubes at about 1000° C for several days. The products thus obtained were in the form of a dark blue or blue-black powder. They were investigated by taking X-ray powder photographs in Guinier focusing cameras (with monochromatized Cu-$K\alpha$ radiation), and in high angle Phragmén-Hägg focusing cameras (using Cu-K radiation).

For recrystallized cerium dioxide the lattice constant was found to be 5.412 ± 0.002 Å * in agreement with values recently reported by Harwood [2]

* All dimensions are referred to the wave lengths of Cu-$K\alpha_1$, $K\alpha_2$, and $K\beta$ radiation equal to 1.54050, 1.54434, and 1.39217 Å respectively.

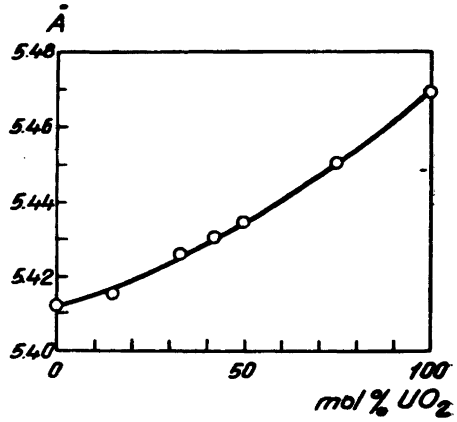

Fig. 1. Lattice constants for the system cerium dioxide — uranium dioxide.

(5.411 ± 0.002 Å) and Mc Cullough [3] (5.411 ± 0.001 Å). For uranium dioxide a value of a equal to 5.469 ± 0.002 Å was obtained. Rundle and his co-workers [4] have recently given an axial length of 5.4581 ± 0.0005 Å, however, without stating whether this value is expressed in kX or in true Å units. (If the former is the case this value will correspond to 5.4691 Å.)

The powder photographs showed the intermediate products to be homogeneous and to have the CaF_2-type lattice. No extra lines indicating a superstructure were visible even in amply exposed photographs. The lattice constants for the various preparations are represented in Fig. 1. Cerium dioxide and uranium dioxide evidently form a continuous series of solid solutions, as does cerium dioxide with thorium dioxide [5] and with praseodymium dioxide [3].

A sample of "uranium cerium blue" obtained by heating cerous nitrate and uranyl nitrate with anhydrous magnesium chloride gave powder photographs only containing the lines corresponding to a lattice of fluorite type with a equal to 5.44 Å. According to Fig. 1 this would indicate the composition of the specimen to be approximately $Ce_{0.4}U_{0.6}O_2$ (no chemical analysis was made). A preparation of "uranium cerium blue", that had been obtained from solution and dried at 120° C gave similar powder photographs but the lines were diffuse. After heating for six hours at about 950 °C in a stream of nitrogen the sample had lost all its water (~ 8 %) and recrystallized. The powder lines of the product appeared at unaltered positions but had now grown rather sharp. The lattice constant of 5.45 Å corresponds to the approximate composition $Ce_{0.25}U_{0.75}O_2$. It is thus obvious that these products of "uranium cerium blue" are not a definite chemical compound but are of variable composition and members of a continuous series of solid solutions $(Ce,U)O_2$ of fluorite type lattice.

SUMMARY

"Uranium cerium blue" is of variable composition and belongs to the continuous series of solid solutions formed by the isomorphous dioxides of uranium and cerium.

The investigation has been carried out in connection with studies on oxide systems, financially supported by the Swedish Natural Science Research Council.

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Studies on the Vanadium Pentoxide — Molybdenum Trioxide System. I. The Relation between the Crystal Structures of the two Oxides

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The crystal structure of vanadium pentoxide has been investigated by Ketelaar [1] and, quite recently, by Byström, Wilhelmi, and Brotzen [2]. The atomic arrangement derived by the latter authors differs from that given by the former by being centro-symmetrical (space-group *Pmnm* instead of *Pmn*) and introduces considerable adjustments especially of the oxygen parameters, leading to oxygen-oxygen distances of plausible lengths throughout.

Byström and his co-workers favoured the centro-symmetrical space-group after examining the symmetry of etch-figures on crystals of the substance. Mr. Erik Blomgren and Mr. Johannes Bæcklund of this Institute have kindly tested a specimen of vanadium pentoxide for piezoelectricity, but in spite of using very sensitive methods no such effect could be detected. This negative result may also be considered as an argument in favour of the centro-symmetrical arrangement, although the possibility of the crystal lattice lacking a centre of symmetry cannot be definitely excluded in this way.

According to the new structural scheme the vanadium atoms of the lattice have five adjacent oxygen atoms at distances of 1.54, 1.77, 1.88 (two atoms), and 2.02 Å. If a sixth oxygen atom, situated 2.81 Å from the vanadium atom, is included in the coordination polyhedron, this will form a very distorted VO_6 octahedron (*cf.* Fig. 1a and b). Byström and his co-workers, however, prefer to exclude this remote oxygen atom and consider the lattice as built up of trigonal VO_5 bipyramids.

The vanadium-oxygen distances of 1.8—2.0 Å agree fairly well with those derived by Aebi [3] for the VO_6 octahedra of $V_{12}O_{26}$ (1.85—2.50 Å), by Brandt [4] for the VO_4 tetrahedra of $CrVO_4$ (1.72 and 1.80 Å), and by Sundberg and Sillén [5] for the VO_4 tetrahedra of KUO_2VO_4 (1.7 Å). The close similarity

between the short vanadium-oxygen distance of 1.54 Å and that obtained by Palmer [6] from electron diffraction studies on $VOCl_3$ (1.56 Å) is remarkable, but the conditions in the two compounds are fairly different and hardly allow of a direct comparison.

We have carried out a calculation of the *ab* projection of the electron density function of vanadium pentoxide, on the basis of carefully estimated intensity data obtained from zero layer line Weissenberg photographs of a minute crystal rotated around (001) and using multiple film technique. This projection is centro-symmetrical irrespective of the symmetry being *Pmn* or *Pmnm*. The parameter values of the vanadium atom and of the oxygen atom in question, obtained in this way, agree with those reported by Byström and his coworkers. Provided that the actual space-group is *Pmnm*, which is most probably the case, the corresponding interatomic distances given by these authors are thus fully confirmed.

The crystal structure of molybdenum trioxide is well known through the work of Braekken [7] and N. Wooster [8]. A determination of accurate atomic positions has recently been carried out at this Institute [9]. The structure is built up of distorted MoO_6 octahedra, joined by sharing edges to form zigzag shaped rows. The rows are mutually connected by corners to form layers, which are placed on top of each other without having atoms in common (*cf.* Fig. 1c and d).

If the lattice of vanadium pentoxide is considered to be built up of VO_6 octahedra (*v. supra*), it is obvious that there exists a close relationship between this structure and that of molybdenum trioxide (*cf.* Fig. 1). The former compound may also be described as containing zigzag shaped rows, running parallel to the *c* axis, composed of VO_6 octahedra sharing edges (*cf.* the double chains of VO_4 tetrahedra suggested by Machatschki [10]). The rows are mutually connected to form layers of infinite extension normal to the *a* axis, by having corners of the octahedra in common. Contrary to what is the case in molybdenum trioxide, the layers in the vanadium pentoxide structure are also mutually joined by octahedra sharing corners to form a three-dimensional network. Geometrically, an idealized arrangement of octahedra corresponding to the lattice of molybdenum trioxide may be transformed into that of vanadium pentoxide by giving every second layer of the structure a translation of $^1/_2\ (a + c)$.

That this relationship between the two oxide structures is not merely a formal, geometrical one is strikingly demonstrated by the ability of the vanadium pentoxide lattice to take up considerable quantities of molybdenum trioxide in solid solution [11]. Vanadium atoms are then statistically replaced by molybdenum atoms, the correct number of metal atom positions being left

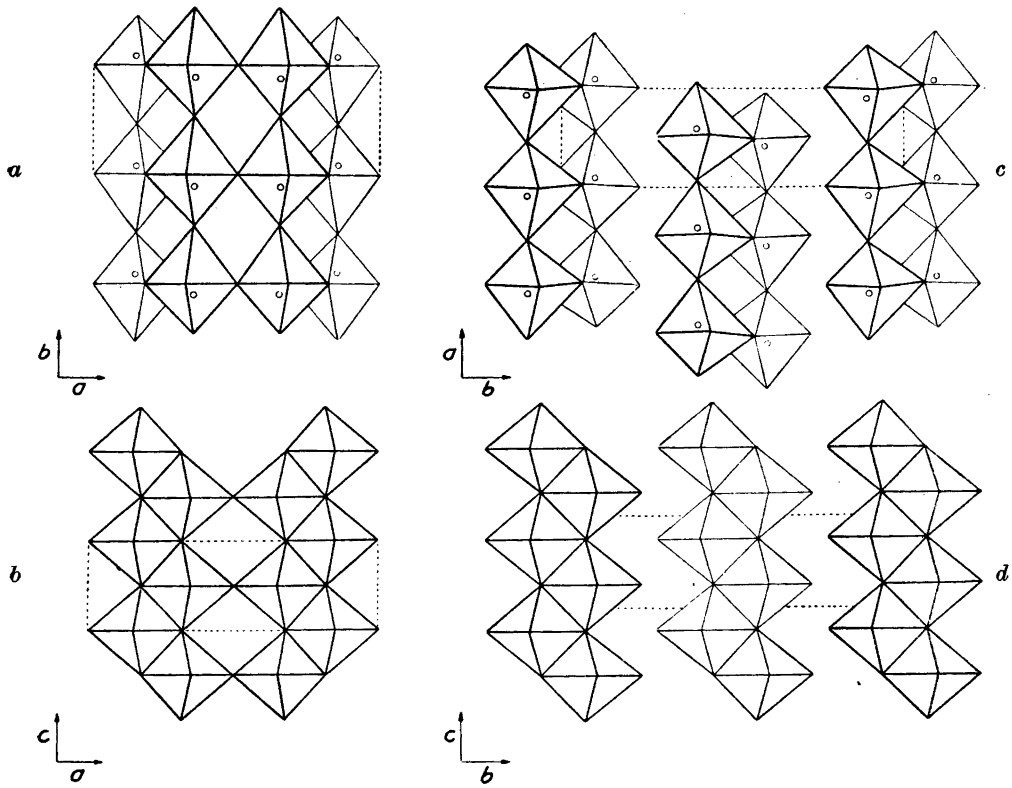

Fig. 1. The crystal structures of vanadium pentoxide (a, b) and molybdenum trioxide (c, d) represented in terms of the octahedra, which are obtained by joining the centres of the oxygen atoms coordinated with each metal atom. Octahedra indicated by heavy lines are situated at a higher level than those drawn with thin lines. In a) and c) the positions of the metal atoms within the octahedra are marked with small rings.

vacant in order to maintain electroneutrality. At about 700° C, 17 % of the vanadium atoms of vanadium pentoxide may be substituted in this manner. It is probable that the abundance of vacant metal atom positions causes the phase to break down at a higher molybdenum content. In contrast the solubility in vanadium pentoxide of tungsten trioxide crystallizing with a deformed ReO_3-structure, is negligible if any [12]. Similarly, tungsten trioxide is not soluble in molybdenum trioxide [13].

With increasing content of molybdenum atoms in the vanadium pentoxide phase there is a continuous increase of the lengths of the *a* and *c* axes, while the *b* axis steadily diminishes. This decrease of the distance between adjacent

rows of the structure evidently implies that the metal-oxygen polyhedra gradually change towards a more pronounced octahedral arrangement (*cf.* Fig. 1). Full data on this subject will be published in the near future.

A solubility of vanadium pentoxide in the molybdenum trioxide lattice would imply either the insertion of vanadium and oxygen atoms in interstices in the lattice or substitution of molybdenum atoms by vanadium atoms, the latter alternative being accompanied by the appearance of holes in the oxygen lattice of the original structure in order to maintain electroneutrality. However, addition of extra atoms or subtraction of oxygen atoms is highly improbable in this case for crystallochemical reasons. This supposition has been verified by experimental evidence which has shown that vanadium pentoxide is insoluble in the molybdenum trioxide phase [11].

SUMMARY

It is pointed out that there exists a remarkable relationship between the crystal structures of vanadium pentoxide and molybdenum trioxide. This kinship is manifested in an extended solubility of molybdenum trioxide in the vanadium pentoxide phase. The changes of the unit cell dimensions accompanying this process indicate that the metal-oxygen polyhedra of this phase — be they considered as triangular bipyramids of MeO_5 with an additional, remote oxygen atom or as highly distorted MoO_6 octahedra — are gradually modified towards a more regular octahedral arrangement.

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Studies on the Vanadium Pentoxide — Molybdenum Trioxide System. II *. Phase Analysis

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Rieck [1] has shown by means of thermal analysis that molybdenum trioxide and tungsten trioxide do not give solid solutions with each other. As these two metals have the same atomic radii the lack of extended homogeneity ranges of the trioxide phases must be connected with the different crystal structures of these substances **. It has recently been pointed out that there exists a remarkable relationship between the structures of vanadium pentoxide and molybdenum trioxide [3]. We thus found it of interest to examine the systems of vanadium pentoxide with molybdenum trioxide and also with tungsten trioxide. This paper will give an account of the results of a phase analysis performed for the former system.

Weighed mixtures of vanadium pentoxide and molybdenum trioxide (both of *puriss.* grade) were heated in evacuated silica tubes (for one day at about 700° C) or in porcelain crucibles (for one day at 600° C and several days at 650° C). No analysis was thought necessary for the former preparations. The latter, however, suffered a slight loss of weight during the heat treatment. The vanadium content of these preparations was determined by permanganate titration after reduction to the quadrivalent state by means of sulphur dioxide, which does not affect molybdic acid in a sulphuric acid solution [4]. Both vanadic and molybdic acids are reduced by cadmium (to V^{2+} and Mo^{3+}) and it was thus possible to find the combined content of vanadium and molybdenum by permanganate titration after percolating a sulphuric acid solution of the sample through a cadmium reducer [4,5].

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* I. *Acta Chem. Scand.* 5 (1951) 581.

** An intermediate phase of the approximate composition $MoWO_6$ has previously been shown to exist in the system molybdenum trioxide — tungsten trioxide [2]. An attempt to determine the crystal structure of this compound is in progress.

Table 1. Results of the phase analysis.

Composition of the preparation		Appearance of the preparation	Phases observed	Unit cell dimensions in Å (true Å units [7])			Density observed
Mol fraction of MoO_3 u	Fraction of V atoms substituted in the V_2O_5 phase x			a	b	c	
0	0	Brownish yellow powder	V_2O_5	11.505 ± ± 0.01	4.373 ± ± 0.003	3.564 ± ± 0.003	3.37
0.075	0.045	Greyish black crystal powder	V_2O_5				3.40
0.145	0.09	»	V_2O_5	11.55	4.33	3.575	3.46
0.215	0.14	»	V_2O_5	11.60	4.29	3.595	3.54
0.275		»	V_2O_5 + I	11.63	4.26	3.61	
0.36		»	I(+V_2O_5)				
0.44		»	I				
0.50		»	I				3.73
0.57		»	I				
0.70		Mixture of black and colourless crystals	I(+MoO_3)				
0.82		»	MoO_3 + I	3.95	13.85	3.69	
1		Almost colourless (slightly greenish) crystals	MoO_3	3.966	13.85	3.696[8]	

The phase analysis was performed by taking X-ray powder photographs of the various preparations in Guinier focusing cameras (with monochromatized Cu-$K\alpha$ radiation). The photographic film was covered by a thin aluminium

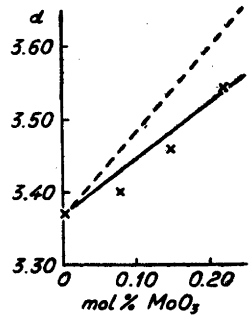

Fig. 1. Comparison between the observed densities (x) of solid solutions of molybdenum trioxide in the vanadium pentoxide phase and calculated densities corresponding to a) substitution of n *vanadium atoms by* 5n/6 *molybdenum atoms (full line) and b) substitution of vanadium atoms by the same number of molybdenum atoms and addition of oxygen atoms to the lattice in order to maintain electroneutrality (dashed line). (The latter alternative is very improbable from a crystallographic point of view.)*

foil (10 μ) in order to reduce the background due to the fluorescence of the vanadium. Powder photographs taken in high angle Phragmén-Hägg focusing cameras with Cr-K radiation were used for the precision determination of the lattice constants. The results of the phase analysis are summarized in Table 1. Three phases have been found to occur in this system at the aforementioned temperature.

The vanadium pentoxide phase shows a considerably extended homogeneity range. The lattice dimensions obtained for the pure substance are in close agreement with those recently reported by Byström, Wilhelmi, and Brotzen [6]. The variations of the unit cell dimensions show the homogeneity range of the phase to extend to a mol fraction of molybdenum trioxide, u, approximately equal to 0.25.

In order to elucidate the way in which molybdenum trioxide is taken up by the vanadium pentoxide lattice the densities of the various preparations were determined (Table 1). Fig. 1 shows the good agreement between the experimentally obtained values and those derived assuming that a number, n, of vanadium atoms are substituted by $5n/6$ molybdenum atoms. Electroneutrality is thus maintained by the appearance of vacant metal atom positions ($n/6$) accompanying the entrance of the molybdenum atoms. The mol fraction of 0.25 for the maximum solubility of molybdenum trioxide in the vanadium pentoxide phase evidently corresponds to about 17 per cent. of the vanadium atoms being substituted in this way. No extra lines which would indicate an ordered distribution of the molybdenum atoms could be observed in the powder photographs. The considerable solubility of molybdenum trioxide in the vanadium pentoxide phase is obviously dependent on the relationship between the crystal structures of these two oxides [3]. The changes of the coordination conditions accompanying the solubility process have also been previously discussed [3].

Powder photographs of the preparation with u equal to 0.36 showed traces of the vanadium pentoxide phase, together with the lines of an intermediate phase (I of Table 1). The latter appeared alone in photographs of preparations with u values around 0.5, but when u had reached 0.70 lines of molybdenum trioxide also became visible. The homogeneity range of the intermediate phase must thus be situated somewhere between the limits u equal to 0.36 and 0.70, and is probably rather narrow as no considerable variations in the positions of the reflections have been observed in the various photographs. The composition of the compound is likely to correspond to u equal to 0.5, *i. e.* to be V_2MoO_8.

The reflections of molybdenum trioxide occur at the same positions in the photographs of the pure substance and in those of specimens also containing the intermediate compound. The homogeneity range of the molybdenum trioxide phase in this system is evidently very narrow. This lack of solubility must imply that the molybdenum trioxide lattice offers a resistance to the introduction of extra atoms or the removal of oxygen atoms (the latter alternative being required by a hypothetical substitution of sexavalent molybdenum atoms by quinquevalent vanadium atoms). This stability of the molybdenum trioxide lattice appears very natural from the crystallochemical point of view.

It is interesting to note that all the intermediate preparations have a greyish-black colour in contrast to those of the pure oxides. This may be connected with the existence of a state of resonance between the vanadium and molybdenum atoms. Rough quantitative measurements of the electric conductivity of powder specimens of a few intermediate products carried out by Mr. Lars Kihlborg have given very low values.

SUMMARY

A phase analysis of the vanadium pentoxide — molybdenum trioxide system carried out on the basis of X-ray powder photographs of preparations obtained at 650°—700° C has revealed the existence of an intermediate phase having the probable composition V_2MoO_8.

The vanadium pentoxide phase shows an extended homogeneity range, the solid solutions being represented by the formula $V_{2(1-x)}Mo_{5x/3}O_5$ with the maximum value of x approximately equal to 0.17.

No signs indicating an extended homogeneity range of the molybdenum trioxide phase were observed.

This investigation has been financially supported by the Swedish Natural Science Research Council, whose assistance is gratefully acknowledged.

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Fungus Pigments

I. Cinnabarin, a Colouring Matter from *Trametes Cinnabarina* Jacq.

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Trametes cinnabarina Jacq., a red coloured fungus growing on decaying wood, particularily mountain-ash, was investigated some sixty years ago by Zopf[1], who obtained therefrom a red crystalline substance for which he proposed the name xanthotrametin. Zopf extracted the fungus with absolute alcohol and evaporated the extract *in vacuo*. Treatment of the residue with ether removed a yellow substance, leaving xanthotrametin undissolved.

When the fungus is extracted with acetone, a compound is obtained, which can be purified by recrystallisation from quinoline or nitrobenzene, but which is almost insoluble in water and the common organic solvents. It thus differs markedly from xanthotrametin, which is said to be soluble in water and alcohol[1], and the name *cinnabarin* is proposed for it.

Due to the scarcity of the fungus, the amount of cinnabarin which has been available for investigation has been very limited, but the compound exhibits some interesting properties and it is the purpose of this communication to give a short account of the, admittedly incomplete, studies so far carried out.

Cinnabarin has no melting point but decomposes gradually on heating above 300 °. For this reason its purity is difficult to assess and analysis have given somewhat varying results, which, however, seem to indicate either $(C_{14}H_{10}O_5N_2)_n$ or $(C_{20}H_{17}O_7N_3)_n$. It contains neither methoxyl nor C-methyl groups. Due to the low solubility of cinnabarin it has not been possible to carry out any molecular weight determination, but analysis and molecular weight determinations on some of its derivatives, described below, show that the simple formula $C_{14}H_{10}O_5N_2$ is the most probable.

Although a number of nitrogen containing pigments have been isolated from bacteria [2], phomazarin, a derivative of aza-antraquinone [3,4], isolated

by Kögl and Sparenburg [5] from *Phoma terrestris* appears to be the only one previously obtained from a higher fungus.

Reference has already been made to the low solubility of cinnabarin in organic solvents. On the other hand it dissolves very readily in conc. sulphuric and nitric acids and less readily in conc. hydrochloric acid, forming in all cases deep red coloured solutions which give a red amorphous precipitate on dilution. This appears to be mostly unchanged cinnabarin, but there are indications that some decomposition also takes place. It has not been possible to prepare any stable salts of cinnabarin.

Cinnabarin is easily soluble in dilute sodium hydroxide and sodium carbonate solutions in the cold, and in sodium hydrogen carbonate solution on warming. The colour in sodium hydroxide is violet at first but soon changes to a dull red. It is not possible to regenerate the cinnabarin from the alkaline solution.

When cinnabarin is heated with dilute sodium hydroxide one molecule of ammonia is evolved, and the alkaline solution gives, after acidification, one equivalent of volatile acid. The nature of this acid has not been determined, and all attempts to obtain any identifiable products from the remaining solution have been fruitless.

Attempts to prepare derivatives of cinnabarin have usually met with little success. Acetylation with acetic anhydride under various conditions has invariably led to dark resinous material. With diazomethane the starting material was recovered unchanged. Although methylation with dimethyl sulphate in alkaline solution gave only resinous material, methylation with dimethyl sulphate and potassium carbonate in acetone has given, in low yield, a red, beautifully crystalline substance. However the analysis of this compound, which is easily soluble in ether, alcohol and acetone, does not correspond with any reasonable formula. The carbon, hydrogen and methoxyl values fit well for a formula $C_{16}H_{14}O_5N_2$, with one methoxyl group, but the nitrogen value is far too high. The relationship of this compound to cinnabarin is thus obscure.

Reductive acetylation yields two different products depending on the conditions. Using zinc and acetic anhydride in the presence of a small amount of pyridine, a yellow, neutral compound is obtained. This compound, m. p. 200—202 °, is only sparingly soluble in alcohol but readily soluble in chloroform giving a solution which shows a strong green fluorescence. Analyses correspond with the formula $C_{18}H_{16}O_6N_2$ and the compound is provisionally named cinnabarin leucoacetate B.

When, on the other hand, the reductive acetylation is carried out in the presence of acetic acid there is formed, in addition to leucoacetate B, another

substance. The latter is also yellow, but is strongly acidic, being easily soluble in sodium hydrogen carbonate. It melts at 213—214 °, is sparingly soluble in chloroform but readily soluble in alcohol, corresponds to the formula $C_{16}H_{16}O_6N_2$, and is termed cinnabarin leucoacetate A.

The reduction leading to the leucoaceate A has been difficult to duplicate. In one experiment a fair yield of both compounds was obtained, but other experiments gave only very small amounts of the leuco derivatives. Lack of material has prevented any thorough study of the conditions for optimum yields.

Both leucoacetates give ammonia on alkaline hydrolysis. Leucoacetate B gives nearly one molecule, but with leucoacetate A no quantitative determination has been made. Leucoacetate B gives, further, three equivalents of volatile acids.

On catalytic hydrogenation leucoacetate B takes up one molecule of hydrogen. The colour of the yellow solution does not change noticeably during hydrogenation, but the product, which has not been isolated, is extremely sensitive. The colour changes to an orange red, which is quite different from the original light yellow, as soon as air is admitted to the hydrogenation vessel.

The most important of all the reactions of the two leucoacetates is that when leucoacetate A is treated with acetic anhydride and a drop of pyridine it is converted into leucoacetate B. This is not however a simple acetylation as leucoacetate B differs from leucoacetate A only by two carbon atoms, *i. e.*, acetylation has been accompanied by the simultaneous removal of an extra molecule of water. The disappearance of the acidity when leucoacetate A is converted into leucoacetate B may be due to acetylation of a strongly acidic phenolic hydroxyl group, or to the formation of a lactone from a carboxyl group. This last mentioned possibility would also account for the loss of one molecule of water.

The facts presented above are clearly too meagre to allow any discussion on possible structures for cinnabarin. The following points may however be stressed: 1) The extremely low solubility and the high stability towards heat of a compound with such a comparatively low molecular weight, as corresponds to the formula $C_{14}H_{10}O_5N_2$ seem to indicate a zwitter ionic structure; 2) It contains two nitrogen atoms, one of which is removable as ammonia. As the molecule, however, suffers deep-seated decomposition during the alkaline hydrolysis is it difficult to draw any conclusions regarding the nature of this nitrogen atom. Likewise the origin of the equivalent of volatile acid which is formed in the alkaline hydrolysis is obscure; 3) The reductive acetylation suggests the presence of a quinonoid system, a hypothesis which is supported by the fact that cinnabarin is reduced by sodium hyposulphite.

It must, however, be borne in mind that neither of the two acetylation products are normal leucoacetates. Leucoacetate A can be regarded as a monoacetyltetrahydrocinnabarin and leucoacetate B as diacetylanhydrotetrahydrocinnabarin, whereas a normal leucoacetate would be a derivative of dihydrocinnabarin. There must, therefore, be some group which is responsible for the uptake of the second molecule of hydrogen. The formation of three equivalents of volatile acids in the hydrolysis of leucoacetate B, while cinnabarin itself gives only one equivalent, supports the formulation of leucoacetate B as a diacetylderivative; 4) Leucoacetate B contains one double bond which can be hydrogenated catalytically. The reaction which takes place when the hydrogenation product comes in contact with air is evidently not a simple dehydrogenation back to leucoacetate B.

This investigation will be continued as soon as more fungus material becomes available.

EXPERIMENTAL

Isolation of cinnabarin

The fungus material was collected on Omberg, Sweden. 360 G of the finely ground, air dried, material was extracted with acetone in a Soxhlet apparatus. The brown precipitate which formed (10 g) was filtered off and freed from waxy contaminants by extraction with ether. The crude cinnabarin thus obtained was dissolved in boiling quinoline, the solution filtered hot, allowed to cool to about 100°, and a large amount of alcohol added. The product, 2.3 g (0.65 %), separated in the form of small glistening leaflets. It was found advisable to carry out this purification with small portions at a time and the material thus obtained was used for the reactions described in this paper.

For analysis it was further purified by recrystallisation from a mixture of nitrobenzene and anisole. It decomposed, without melting, above 300°.

$C_{14}H_{10}O_5N_2$	Calc.	C	58.72	H	3.53	N	9.79
$C_{20}H_{17}O_7N_3$	»	»	58.66	»	3.70	»	10.27
	Found	»	57.69	»	3.93	»	9.66
	»	»	58.98	»	3.52	»	10.17
	»	»	59.42	»	3.58		

Methylation of cinnabarin

A mixture of cinnabarin (100 mg) suspended in dry acetone (50 ml) to which was added potassium carbonate (2 g) and dimethyl sulphate (1 ml) was boiled for twenty hours, the solvent removed *in vacuo*, and water added. After twelve hours the brown powder (30 mg) was filtered off and purified by recrystallisation from very dilute acetone, forming long, dark red needles of m. p. 183—185°.

$C_{15}H_{11}O_4N_2(OCH_3)$	Calc.	C	61.12	H	4.50	N	8.91	OCH_3	9.88	Mol. wt. 314
	Found	»	61.35	»	4.57	»	11.22	»	9.74	» (Rast) 338

Hydrolysis of cinnabarin

Cinnabarin was warmed with 2 *N* sodium hydroxide solution in a slow stream of nitrogen, the exit gases being passed through dilute hydrochloric acid. This was evaporated to dryness, leaving a white crystalline residue, which sublimed without melting, gave a strong smell of ammonia when treated with sodium hydroxide, and gave a negative test for alkylaminohydrochlorides [6].

In a quantitative experiment 62.8 mg (0.22 mmole) of cinnabarin was hydrolysed in the same way, but the exit gases were passed through 15 ml of 0.0515 *N* sulphuric acid. After 10 hours the sulphuric acid required 10.1 ml of 0.055 *N* sodium hydroxide for neutralisation, corresponding to an absorption of 0.216 mmole of ammonia.

The alkaline solution remaining after the hydrolysis was acidified with sulphuric acid and steam distilled. The distillate consumed on titration 3.86 ml of 0.055 *N* sodium hydroxide, corresponding to 0.212 m.equiv. of volatile acid.

The acidic solution containing the nonvolatile products was extracted with ether in a continuous extractor but only a small amount of brown resinous material was obtained.

Reductive acetylation of cinnabarin

1. Cinnabarin (200 mg) was suspended in acetic anhydride (5 ml) containing a few drops of pyridine. Zinc powder was added in small portions and the reaction mixture gently warmed. The cinnabarin gradually dissolved giving a yellow solution, which was finally boiled for some minutes. Unreacted zinc was filtered off and washed with hot acetic anhydride, and the filtrate was poured into water and set aside overnight. The yellow precipitate that was formed was taken up in chloroform and the aqueous solution extracted with additional portions of chloroform. The combined chloroform extracts were washed with sodium hydrogen carbonate and evaporated, leaving a residue (150 mg) which crystallised immediately on the addition of alcohol. Purification by chromatography on aluminium oxide, and recrystallisation from alcohol containing a small amount of chloroform, gave cinnabarin leucoacetate B as yellow needles, melting at 198—200°.

$C_{18}H_{16}O_6N_2$	Calc.	C 60.65	H 4.54	N 7.86	Mol.wt.		356
	Found	» 60.00	» 4.14	» 7.65	»	(Rast)	360

2. Cinnabarin (200 mg) was reduced and worked up in the same way as above, except that a few drops of acetic acid was added to the reaction mixture. In this case the sodium hydrogen carbonate washings of the chloroform solution acquired a bright red colour. Acidification of the sodium hydrogen carbonate solution gave a yellow precipitate of cinnabarin leucoacetate A which was recrystallised from alcohol and formed yellow micro-crystals of m. p. 213—214°.

$C_{16}H_{16}O_6N_2$	Calc.	C 57.80	H 4.87	N 8.43	Mol.wt.		332
	Found	» 57.63	» 4.93	» 8.42	»	(Rast)	330

From the chloroform solution, leucoacetate B was obtained as before.

Upon acetylation leucoacetate A gives a substance with m. p. 200—202°, which does not depress the m. p. of leucoacetate B.

Hydrolysis of leucoacetate B

Leucoacetate B (97.3 mg, 0.268 mmole) was hydrolysed as described for cinnabarin. The ammonia evolved was absorbed in 15 ml of 0.0515 *N* sulphuric acid, which, after the reaction, required 9.58 ml of 0.055 *N* sodium hydroxide for neutralisation, corresponding to 0.245 mmole of ammonia.

The alkaline hydrolysate was acidified with sulphuric acid and steam distilled. The distillate consumed 14.77 ml of 0.055 *N* sodium hydroxide corresponding to 0.81 m.equiv. of volatile acids.

Hydrogenation of leucoacetate B

Cinnabarin leucoacetate B (13.9 mg) was hydrogenated in acetic acid in the presence of hydrogenated PtO_2-catalyst. The uptake of hydrogen amounted to 0.86 ml (Calculated for one molecule of hydrogen, 0.875 ml). When the yellow solution was exposed to air, its colour immediately changed to an orange red.

SUMMARY

A nitrogen containing pigment, for which the name cinnabarin is proposed, has been isolated from the fungus *Trametes cinnabarina* Jacq. Some of its reactions are described.

The author wishes to express his thanks to Prof. H. Erdtman and Mrs. G. Aulin-Erdtman, Stockholm, who have collected most of the fungus material used in this investigation. The analyses were carried out by W. Kirsten, Upsala, and K. Salo, Helsingfors.

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Utilization of Ion Exchangers in Analytical Chemistry. XVIII

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DETERMINATION OF THE DEGREE OF SUBSTITUTION OF CELLULOSE XANTHATE IN VISCOSE

The possibility of separating high molecular anions from low molecular anions by anion exchangers has been demonstrated in several publications [1–4]. This separation is based on the fact that only the low molecular ions can penetrate into the narrow net-work structure of the ion exchanger and be adsorbed. These investigations have been performed by means of anion exchangers of the weakly basic type which can be used only in acid or neutral media. Recently anion exchange resins of strongly basic type (quaternary ammonium bases) have become available and these resins may be used also in alkaline medium. As a great number of high molecular acids are insoluble in water, or in certain cases, decompose in acid or neutral medium but give stable solutions in alkaline medium, it is of great interest to investigate if this type of anion exchanger can be used for a quantitative separation of high molecular anions from low molecular in alkaline medium.

As an example, the separation of cellulose xanthate from the by-products formed in the preparation of viscose (trithiocarbonate and sulphide) has been studied in the present paper. Based on this principle a new method for the determination of the degree of substitution (D. S.) of cellulose xanthate solutions (viscose) has been investigated.

A great number of methods have been used for the determination of the D. S. of cellulose xanthate in viscose. Most of the older methods are based on the reaction between cellulose xanthate and iodine which results in the formation of disulphide [5]. A number of modifications have been suggested, but the accuracy of all these methods leaves a great deal to be desired.

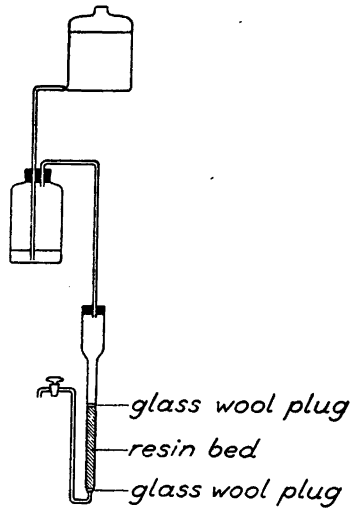

Fig. 1. Ion exchange apparatus.

A well-established method is the precipitation of cellulose xanthate with diethylchloroacetamide according to Fink, Stahn, and Matthes [6]. An insoluble, stable derivative is formed, the nitrogen content of which is determined according to Kjeldahl, and the D. S. calculated from the equivalence between nitrogen and xanthate sulphur. The method gives good reproducibility but the diethylchloroacetamide is an expensive and hazardous chemical.

More recently a volumetric method has been proposed by Barthélemy and Williams [7]. By boiling with sodium zincate solution the sulphur-containing products of viscose (cellulose xanthate, sulphide, and thiocarbonate) are transformed quantitatively into zinc sulphide, which is determined by iodometric titration. In another sample the cellulose xanthate is salted out with a saturated sodium chloride solution and the sulphur content in the filtrate is determined. The difference between the two sulphur determinations corresponds to the xanthate sulphur. The procedure is rather simple and rapid but errors may occur due to incomplete salting out and coprecipitation of trithiocarbonate and sulphide. For most purposes the method may be considered as satisfactory.

PREPARATION OF THE ION EXCHANGE COLUMN

The anion exchange resins Dowex 1 and Amberlite IRA-400 were sieved to obtain the particle size 0.36—0.85 mm (air dried). The columns were of ordinary type [8]. The diameter and height were 9 and 150 mm respectively. The flow-rate was kept at a proper level by means of an apparatus which is shown in Fig. 1.

The ion exchanger was transformed into the hydroxyl form by passing 500 ml. 1.5 *N* sodium hydroxide through a column and subsequent washing with distilled water (about 200 ml) until sodium hydroxide could not be detected in the effluent with phenolphthalein. The total time used for these operations was about 2 hours.

After the passage of the diluted technical viscose the columns were emptied and the resin from several columns collected in a beaker and treated with 4 *N* hydrochloric acid. The resin was then collected in a funnel and regenerated with sodium hydroxide as described above. This is less time-consuming than separate regeneration of each column. The treatment with hydrochloric acid was made in a beaker and not directly in the columns because gas bubbles were obtained which would have caused channeling in the columns.

PROCEDURE FOR DETERMINATION OF D. S. OF CELLULOSE XANTHATE

About 3 g of technical viscose is used in each experiment. The viscose is diluted with 250 ml ice-cooled distilled water and kept cool during the subsequent percolation through the ion exchange column. If highest accuracy is required the ion exchange column should also be cooled during the experiments. This is especially necessary at very high degrees of substitution of the cellulose xanthate. Afterwards the column is washed with 100 ml water at the same flow-rate as during the adsorption step (5—8 ml/min).

The combined filtrate and washing water is collected in a 500-ml Erlenmeyer flask containing 10 ml of a sodium zincate solution, prepared according to Barthélemy and Williams [7]. The mixture is boiled for 30 minutes and the flask then ice-cooled. After cooling the contents of the flask is washed into a 600-ml beaker containing a cold solution of 50 ml of 1 *M* sulphuric acid and 30 ml of 0.1 *N* iodine. Immediately thereafter the mixture is transferred quantitatively back to the Erlenmeyer flask. After 20 minutes the iodine not consumed is titrated with a 0.1 *N* solution of sodium thiosulphate with starch as indicator. The cellulose content in the viscose is determined according to Jentgen[9] on a separate sample. The D. S. is calculated according to the following formula.

$$\text{D. S.} = \frac{0.0405 \cdot (\text{ml } I_2 \cdot n_I - \text{ml } Na_2S_2O_3 \cdot n_t)}{\text{g cellulose in the viscose sample}}$$

n_I and n_t are the normalities of the iodine and thiosulphate solutions respectively.

RESULTS AND NOTES ON PROCEDURE

On passing the diluted viscose solution through the anion exchange column the upper layer of column was coloured red. About one day after the adsorption had been performed the layer below the red-coloured one was slightly green. After some time (about 14 days) the colour of both layers had changed to brown. The height of the column was chosen so that the lowest part of the column had the same colour after the adsorption step as before.

The first experiments were performed to study if the retention of thiocarbonate and sulphide in the column was quantitative. The ice-cooled filtra-

tes from the column were immediately titrated potentiometrically with 0.1 *N* mercuric chloride [10], [11]. Sulphide and thiocarbonate could not be detected showing that a quantitative adsorption had been obtained.

Separate experiments were made in order to find out whether the cellulose passes the column quantitatively. 7.9 g technical viscose was diluted with distilled water to 250 ml in a volumetric flask. Two aliquots of 50 ml each were diluted to the same concentration as in the other experiments and filtered through two ion exchange columns (Dowex 1), and after washing with 100 ml distilled water the filtrates were neutralized with hydrochloric acid with methyl red as indicator. After evaporating most of the water ethyl alcohol was added and the precipitated cellulose filtered off on glass filters, washed with water, carbon disulphide, and water again. The cellulose was then dried at 100—105° C for six hours and the crucibles were weighed after cooling in a desiccator. Two other aliquots of 50 ml each were neutralized directly and the cellulose content was determined as described above. The results presented in Table 1 indicate that the cellulose xanthate remains quantitatively in the solution.

Table 1. Determination of cellulose content.

Cellulose content of diluted viscose g/50 ml	
Direct determination	After passage of the ion exchanger
0.1106	0.1110
0.1119	0.1100

The procedure described in the previous section has been tested using three viscoses of different ripeness and two different anion exchange resins, Dowex 1 and Amberlite IRA-400. In order to study the efficiency of the regeneration some experiments were also performed with resins which had previously been used for viscose, and regenerated as described above. The results have been compared with parallel determinations of the D. S. according to Fink, Stahn, and Matthes [6], as well as Barthélemy and Williams [7], *i. e.* without use of ion exchangers. The results may be seen from Table 2.

Furthermore, experiments have been performed with direct addition of 0.1 *N* iodine solution (20 ml) to the effluent from the column after neutralization with acetic acid. Excess iodine was back-titrated with sodium thiosulphate [5]. The method does not give good results. The iodometric titration

has earlier been criticized by many investigators for inaccuracy, and this is confirmed by our own experience.

Table 2. D. S. of cellulose xanthate determined by different methods.

Method	Viscose A		Viscose B		Viscose C	
Proposed ion exchange method:						
Dowex 1	37.3	38.0	27.9	27.4	21.7	21.2
Dowex 1, regenerated	37.6		27.3		21.1	
Amberlite IRA-400	37.7	38.1	27.3	27.9	21.7	21.6
Amberlite IRA-400, regenerated	37.2		27.9		21.6	
Fink, Stahn, and Matthes	35.0		24.6		21.6	
Barthélemy and Williams	37.4	36.4	25.8	23.9	22.0	22.4

The influence of the flow-rate and the temperature at which the experiment is performed has been investigated using a fresh viscose sample. The resin Dowex 1 was used in the experiments and the obtained results are presented in Table 3.

The results indicate that a flow-rate of 5—8 ml/min is necessary in order to prevent decomposition of the cellulose xanthate during the experiment. For the same reason the ice-cooling cannot be excluded.

Table 3. D. S. of cellulose xanthate determined at different flow-rates and temperatures.

Temp.	Flow-rate ml/min	Viscose 1	Viscose 2
The viscose and the ion exchange column ice-cooled	8		45.9
	5		45.8
	2		45.0
The viscose ice-cooled	5	49.0	45.5
	2	45.7	44.1
Room temp.	5	46.1	44.3
	2	43.6	42.3

As a complement, resin beds of different heights have been investigated as well as the influence of an addition of sodium sulphite. The results presented in Table 4 were made using a fresh viscose sample. The resin Dowex 1 was used in these experiments. The experiments were in other respects made according to the procedure described in the previous section. As can be seen from the Table a height of 80 mm is sufficient for complete adsorption under the conditions in question. Furthermore it is obvious that sulphite ions do not interfere with the determination.

Table 4. D. S. of cellulose xanthate determined under different conditions.

Height of resin bed mm	Addition of Na_2SO_3 g	D. S.
150	0.105	49.6
150	—	49.4
80	0.112	49.6
80	—	49.1
50	—	52.1

DISCUSSION

The experiments presented above show that the sulphur-containing by-products present in viscose (trithiocarbonate and sulphide) are quantitatively retained in a column filled with an anion exchange resin of the strongly basic type, whereas the cellulose passes the column. In the purified cellulose xanthate solution obtained the degree of substitution can be easily determined in a reproducible manner according to the zincate method. The method can be applied to viscoses containing sulphite.

The figures obtained are slightly higher than for corresponding analyses performed on the same viscose according to Fink, Stahn, and Matthes [6]. As the adsorption of by-products in the ion exchange column has been shown to be complete there is no reason to believe that the ion exchange method should give too high values. Therefore the conclusion must be drawn that the method to Fink, Stahn, and Matthes [6] gives values which are slightly lower than the correct value.

A comparison between the proposed method and the method devised by Barthélemy and Williams [7] shows that rather good agreement has been obtained. The ion exchange method is, however, superior not only from the theoretical point of view but gives also better reproducibility. The procedure is simple and rapid, the time required for the determination being about two hours. Several analyses can be performed simultaneously.

SUMMARY

In the present paper a new method for the determination of the degree of substitution of cellulose xanthate solutions (viscose) has been investigated. The method is based on the possibility to separate low molecular anions from high molecular anions by anion exchangers. The procedure is simple and rapid and gives more accurate values than other methods for the same determination.

Statens Tekniska Forskningsråd has supported the research financially, which support is gratefully acknowledged.

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Treatment of Spruce Lignin with Sulphite Solutions at pH 4—9*

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According to Hägglund [1] the dissolution of lignin during the sulphite process takes place in two steps. The first step involves the sulphonation of the lignin, the other a hydrogen catalysed splitting (probably a hydrolysis) of linkages binding the lignin in the wood.

In order to study further these two steps, extracted wood powder (spruce, *Picea abies* (L.) Karst.) has been heated at 135° with sulphite solutions, for times varying from 1 to 24 hours, and at pHs varying from 4 to 9. (All pH values reported in this paper are determined at room temperature.) The sulphite treated wood powders were then analysed for lignin and sulphur.

The methoxyl content of the wood powders has been used as a measure of the lignin content. Eighty-nine percent of the methoxyl groups of the extracted wood are found in the Klason lignin. If no methoxyl groups are split off from the lignin during the isolation of Klason lignin, eleven percent of the methoxyl groups are bound to the carbohydrates (*cf.* Hägglund and Sandelin [2]). The methoxyl estimation consequently gives values for the lignin content which are too high. For this reason, in some cases, spectrophotometric determinations of the amounts of dissolved lignin have been used as a complement to the methoxyl determinations.

Sulphur has been determined by a modification of the Grote-Krekeler method [3], which allows a more rapid analysis of samples containing sodium salts.

The discussion of these experiments has been divided into two parts. The first deals with the sulphonation of the undissolved lignin, and the second with the splitting of the linkages binding lignin in the wood.

* Part 48 on the chemistry of the sulphite process by E. Hägglund and coworkers. Part 47 *Acta Chem. Scand.* 4 (1950) 971.

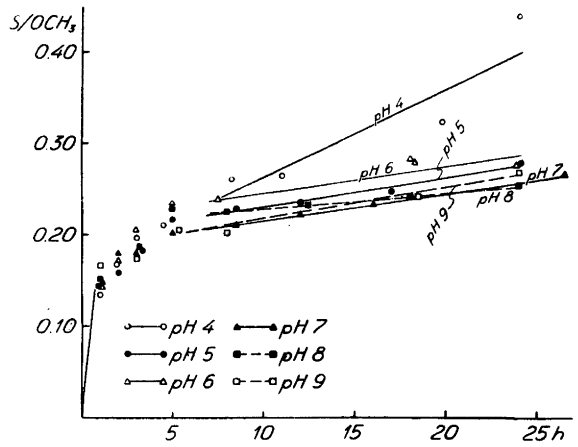


Fig. 1. The ratio of sulphur atoms per methoxyl group of the sulphite treated wood as a function of heating time at 135° for sulphite solutions of different pH.

THE SULPHONATION OF UNDISSOLVED LIGNIN

Hägglund and Johnson [4] heated wood powder with an almost neutral sulphite solution (pH 6) at 135°. The greater part of the lignin remained in the wood after this treatment. After 30 hours heating the wood contained 0.33 sulphur atom per methoxyl group. Most of the sulphur (0.22 atom per methoxyl group) was taken up during the first 5 hours. If the heating was continued for longer than 30 hours no more sulphur was taken up.

From these results Hägglund and Johnson concluded that the sulphonation of lignin takes place in two steps. The lignin is first rapidly sulphonated until it contains 1 sulphur atom per 4 methoxyl groups. A considerably slower sulphonation then follows.

The results of the present investigation show that the wood takes up 0.25—0.28 sulphur atom per methoxyl group during 24 hours heating at 135° with a sulphite solution having a pH between 5 and 9 (see Fig. 1). At pH 4, 0.44 sulphur atom per methoxyl group have been taken up after 24 hours heating, but under these conditions the greater part of the lignin is dissolved. This value can, therefore, not be compared with those obtained at pH 5—9 because, in those cases, not more than a third of the lignin is dissolved during 24 hours heating (see below).

A considerable part of the sulphur (0.13—0.17 sulphur atom per methoxyl group) is bound during the first hour. If the final portions of the curves in Fig. 1 are extrapolated to time 0, it is found that 0.18—0.22 sulphur atom per methoxyl group is rapidly taken up.

When wood is heated with sulphite solution, part of the sulphur may be bound as loosely bound sulphite, *i. e.* sulphite which can be liberated by alkali at room temperature. (This sulphite is mainly bound to carbonyl groups [5]). When the sulphonated wood powder was treated with alkali, the sulphur content was reduced by 0—0.03 sulphur atom per methoxyl group. The formation of loosely bound sulphite is consequently of little quantitative importance when wood reacts with sulphite solutions of pH 4—9.

In order to investigate the initial rapid sulphur uptake, the lignin was isolated as hydrochloric acid lignin (by Richtzenhain's method [6]) from wood powders which had been obtained by heating the wood for one hour with the sulphite solutions. As mentioned above, these woods contained 0.13—0.17 sulphur atom per methoxyl group. The hydrochloric acid lignin preparations contained 0.12—0.16 sulphur atom per methoxyl group (see Table 1), and this shows that the rapid sulphur uptake during the first hour of heating is due to the sulphonation of lignin. (Vanillyl alcohol is the only model substance hitherto studied which reacts as rapidly as lignin under these conditions [7]).

Table 1. The preparation of hydrochloric acid lignin from wood powders treated with sulphite solutions of different pH at 135° for 1 hour.

pH of sulph. solut.	Yield of hydrochloric acid lignin		Analysis of the hydrochloric acid lignin			Ratio S/OCH_3 in the wood
	Weight %	Methoxyl %	Sulphur %	Methoxyl %	S/OCH_3	
4	27.2	75	1.63	13.5	0.12	0.13
5	26.4	73	1.72	13.0	0.13	0.15
6	25.3	74	1.87	13.8	0.13	0.14
7	24.7	73	2.18	14.5	0.15	0.15
8	25.1	72	2.41	14.4	0.16	0.15
9	24.9	75	2.18	14.7	0.14	0.17

From a sulphonated wood powder, prepared by heating wood with sulphite solution at pH 5.2 and 135°, containing about 0.3 sulphur atom per methoxyl group, a large part of the lignin could be removed by heating in acid solutions (see Scheme 1). The dissolved lignin ("low sulphonated lignin" = LSL) contained about 0.3 sulphur atom per methoxyl group [8]. Thus the slower sulphonation during which the sulphur content increases from 0.18—0.22 0.25—0.28 sulphur atom per methoxyl group is probably due to the sulphonation of lignin.

Note added in proof (Mai 12, 1951). Mikawa and co-workers (private communication) have recently investigated the sulphonation of *Picea jezoensis* and *P. densiflora* at 135° and pH 6. They too assumed that this sulphonation is a two-step reaction — a rapid sulphonation followed by a slower one. The groups which react rapidly they called Groups X and those which react slowly Groups Z. Erdtman had earlier called all the groups which are sulphonated at pH 6 Groups A.

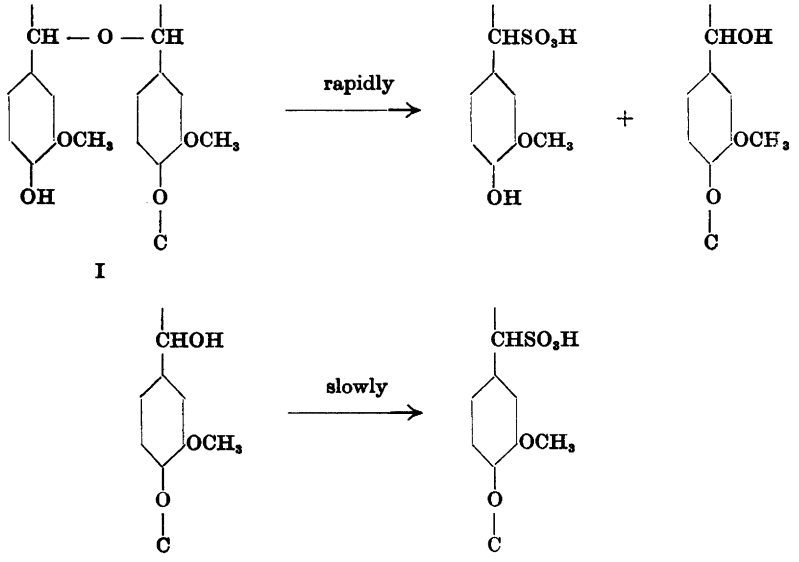
In this paper it has been stated that vanillyl alcohol is the only model substance investigated which is sulphonated as rapidly as Groups X. However, Lindgren and Saedén have recently found that pinoresinol also reacts as rapidly as Groups X with neutral sulphite solutions. Thus Groups X may be *p*-hydroxyl-benzyl alcohol groups (as in vanillyl alcohol) or *p*-hydroxyl-benzyl ether groups (as in pinoresinol).

As the Groups Z are sulphonated at a rate which is of the same order of magnitud as the rate of sulphonation of model substances containing *p*-alkoxy-benzyl alcohol groups, Groups Z may be groups of this type.

Groups X and Z can be combined in a simple way to one type of group, having the formula I. During sulphonation at pH 6, the phenol activated part of such a group is rapidly sulphonated. In this process a *p*-alkoxy-benzyl alcohol group is formed, and this group then is sulphonated slowly under these conditions.

If all the Groups X and Z (= Groups A) have the formula I, the lignin must contain the same amounts of Groups X as Groups Z. Mikawa and co-workers found this to be the case in the lignins of the spruces they investigated. On the other hand in this paper it is assumed that the lignin of *Picea abies* contains more Groups X than Groups Z. This may be explained by assuming that some of Groups X of *P. abies* lignin are etherified by other Groups X or by hydroxyl groups which can not be sulphonated.

In Part 49 of this series, it is shown that the groups in lignin which are sulphonated at pH about 1.5—2 but not at pH 6 (Groups B) may be benzylether groups and thus all the lignin groups which are responsible for the sulphonation reactions may be benzyl ether groups.



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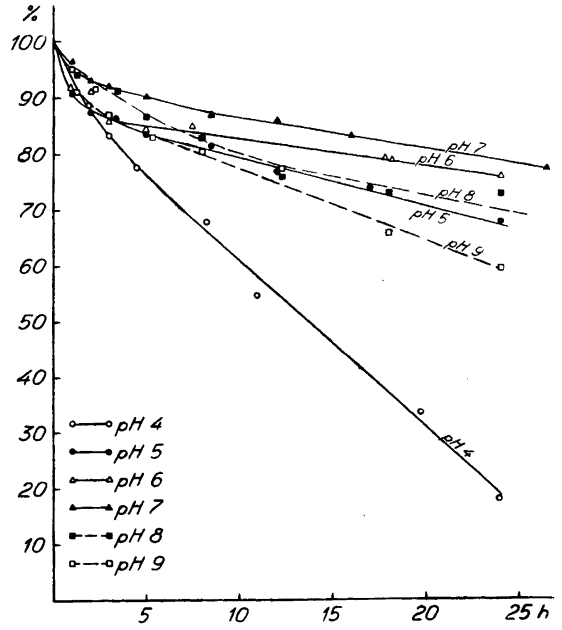


Fig. 2. The number of methoxyl groups of the sulphite treated wood (expressed as the percentage of the number of methoxyl groups of the original wood) as a function of the heating time at 135° for sulphite solutions of different pH.

THE SPLITTING OF THE LINKAGES BINDING LIGNIN IN THE WOOD

In Fig. 2 the number of methoxyl groups in the sulphite treated wood powder, expressed as the percentage of the number of methoxyl groups in the original wood powder, has been plotted as a function of the heating time for sulphite solutions of different pH.

Table 2. The number of lignin-bound methoxyl groups (expressed as the percentage of the number of lignin-bound methoxyl groups in extracted wood) dissolved by treating the wood with sulphite solutions of varying pH at 135° for 4 hours.

pH of sulphite solut.	4.9	5.3	5.8	6.2	6.7	8.0
Amount of dissolv. lignin, %	16.5	17.1	12.0	11.4	14.7	17.6

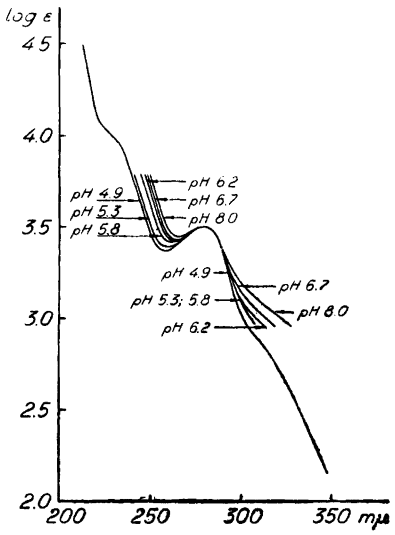


Fig. 3. The ultra-violet absorption curves of the lignin which is dissolved by heating the wood with sulphite solutions of pH 4.9 to 8.0 at 135° for 4 hours. The curves have been displaced along the ordinate so that the maximum at 280 mμ lies at log ε = 3.5.

The first, rapid dissolution of methoxyl at pH 6 and 7 (see Fig. 2) is due to dissolution of lignin. This was shown by spectrophotometric determinations of the amount of lignin which has passed into solution after 4 hours heating (see Table 2). Thus, only 11—15 percent of the lignin is rapidly dissolved by sulphite solutions at pH 6—7, the bulk remaining in the wood.

Fig. 3 shows the absorption curves of the different solutions obtained by these treatments. The form of the curves varies in a regular way with the pH of the sulphite solutions.

A large part of the lignin of the sulphite treated wood is dissolved if the wood is heated with acids [4,8] (see Scheme 1). When buffered solutions of varying pH are employed, the rate at which the lignin dissolves increases with the hydrogen ion concentration [9]. This shows that the bulk of the lignin is bound by linkages the splitting of which is catalysed by hydrogen ions (Häggglund [1]).

The small fraction of the lignin which is rapidly dissolved at pH 6—7 cannot be bound in this way. This fraction has neither been isolated nor studied.

Some investigations have previously been carried out on a similar fraction the "low molecular ligninsulphonic acid" (= LMLA, see Scheme 1). This fraction is dissolved when wood is heated at 135° with a sulphite solution of pH 5.2 [10], and it comprises about one third of the lignin of the wood.

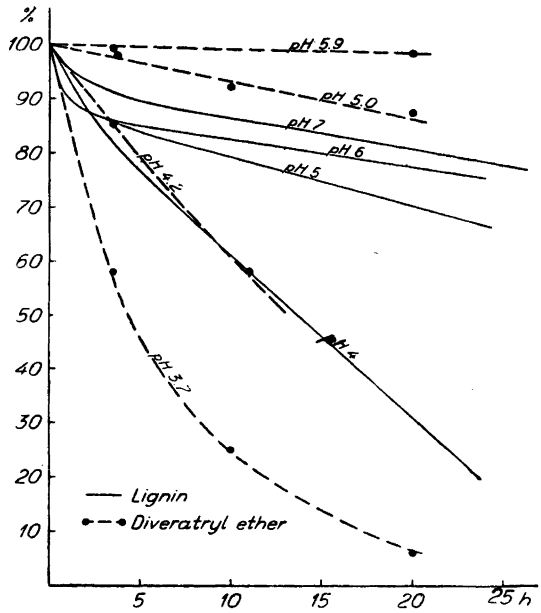


Fig. 4. The amount of diveratryl ether recovered, after heating at 135° with sulphite solutions, as a function of the heating time at different pH. For comparison, the curves of Fig. 2 for the dissolution of lignin are plotted in the figure.

LMLA seems to be sulphonated to a higher extent (probably about 0.5 sulphur atom per methoxyl group) than the fraction of lignin which remains in the wood (0.3 sulphur atom per methoxyl group), and the molecular weight is lower than that of the LSL [10].

As mentioned above, at pH 6—7, the linkages which bind the bulk of the lignin are not split to an appreciable extent. The fact that the lignin is dissolved more quickly and in greater amounts at lower pH implies a splitting of these linkages.

The rate-determining factor for the dissolution of the bulk of the lignin at a pH just below 6 is therefore, probably, the rate of the splitting of these linkages. The pH range seems to extend to at least pH 4 because the rate of dissolution of the lignin in the pH range 4—6 is strongly pH dependent, indicating that the rate is determined by a hydrogen ion catalysed reaction.

Heating with alkaline solutions dissolves the lignin more quickly and to greater extent than heating with sulphite solutions of pH 6—7 (see Fig. 2). This cannot of course be explained by the hydrogen ion catalysed reaction.

Many investigators have assumed that lignin is bound in the wood by acetal linkages because of the acid-labile nature of such linkages [11, 12]. On the

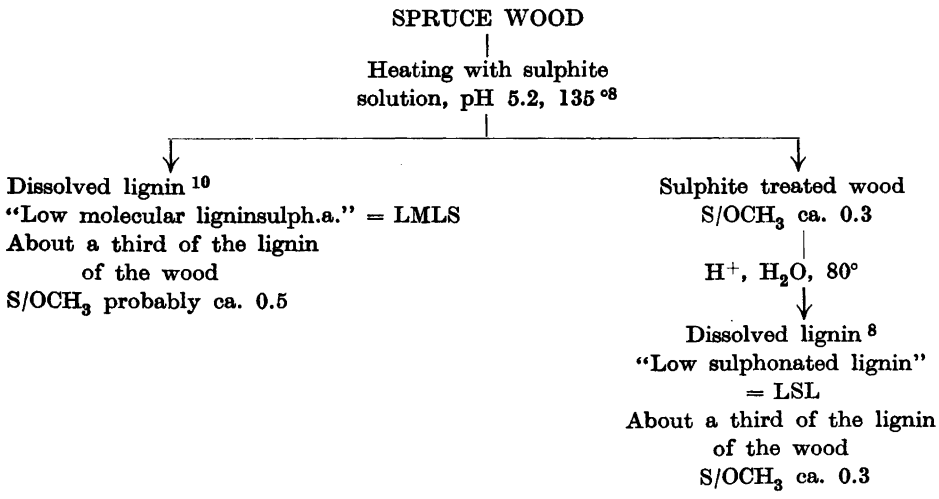
other hand Holmberg [13] has assumed that these linkages are of the benzyl ether type. Such ethers are hydrolysed much more easily than ordinary ethers.

In a recent communication [14] the sulphonation of diveratryl ether (3,3', 4,4'-tetramethoxy-dibenzyl ether) was studied at different pHs. It was found that diveratryl ether disappears from the reaction mixture at a higher rate than the sulphonic acid is formed. This indicates that the sulphonation proceeds via an intermediate product, most probably veratryl alcohol. In Fig. 4 the amount of recovered diveratryl ether is plotted as a function of the time of heating, at different acidities of the sulphite solution. The figure shows that the rate of hydrolysis of the ether linkage is rather similar to the rate of solution of the lignin using sulphite solutions of a pH lower than 7. This indicates that alkoxy-benzyl alkyl ether linkages may be responsible for binding the bulk of the lignin in wood*.

The mechanism of the dissolution of lignin in alkaline sulphite solutions cannot be studied using diveratryl ether as a model substance, as this ether is not hydrolysed by this reagent.

From the above it may be concluded that lignin in the native state is built up of units containing a number of C_9-monomers. The units are coupled together or with carbohydrate molecules by means of benzyl ether linkages.

Scheme 1. Preparations obtained by treating spruce wood with sulphite solution at pH 5.2 and 135°.



* The above mentioned experiments with pinoresinol show that the fraction which is easily dissolved at pH 6—7 may be bound with *p*-hydroxy-benzyl ether linkages (added in proof).

This hypothesis is supported by the fact that several lignans contain this type of grouping. Further, since p-methoxy-benzyl alcohols undergo etherification even at room temperature [14,15] these linkages could be formed by direct etherification of benzyl alcohol groups in a protolignin.

It is probable that the lignin units just mentioned are built up from only a few C_9-monomers, since lignin preparations isolated by a mild method have a molecular weight of the order of 1 000 [16]; corresponding to about 5 C_9-monomers.

In a recent paper Stumpf and Freudenberg [17] reported that they have succeeded in dissolving the lignin of spruce wood under very mild conditions. They found no indications of a chemical linkage between the lignin and the carbohydrates of the wood, and considered that the union must be by hydrogen bonding.

EXPERIMENTAL

Sulphur determination

The sample of sulphite treated wood powder (0.7 g) weighed into a platinum boat was combusted in the usual way in a Grote-Krekeler apparatus [3]. The sulphuric acid formed was determined by titration with 0.1 N NaOH.

The sodium content of the wood powder caused the formation of ash containing sodium sulphate and sodium carbonate. The amount of sodium carbonate in the ash was determined by titration with 0.1 N HCl (bromophenol blue as indicator), and the remainder was assumed to be sodium sulphate. The amount of sulphur in the sample thus becomes the sum of the amounts of sulphur in the sodium sulphate and the sulphuric acid.

The values obtained for the amount of sulphur in the ash were checked for certain samples by precipitating the sulphate in the titrated solution as $BaSO_4$. The difference between the determination were less than 1 % — an accuracy sufficiently great for this investigation.

Treatment of the wood powder with sulphite solutions

The wood powder (spruce, *Picea abies*) was extracted for 24 hours with ethanol-benzene (1 : 1). The extracted wood powder contained 4.99 % methoxyl and 28.8 % Klason lignin having a methoxyl content of 15.5 %.

The sulphite solutions were prepared by dissolving sulphur dioxide and sodium hydroxide in water in such amounts that the total content of sulphur dioxide was 5 %, and the pH of the solution had the desired value (4, 5, 6, 7, 8, or 9).

The extracted wood powder (25 g) was mixed with the sulphite solution (300 ml) in an acid-resistant steel autoclave, which was then rotated for the desired time in an oil bath at 135°.

The wood powder was then filtered off and the pH of the solution was determined. The product was thoroughly washed (several days) with water until the filtrate did not contain sulphite or sulphate ions, and determinations were then made of the yield (Fig. 5), the methoxyl content (Fig. 6) and the sulphur content (Fig. 7).

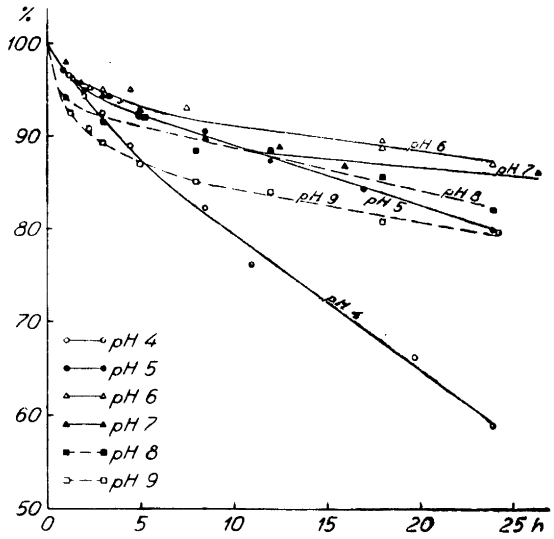


Fig. 5. The yield of sulphite treated wood as a function of the heating time at 135° for sulphite solutions of different pH.

Because of the formation of sulphonic acid groups, the pH of the solutions decreases during the heating periods, the change being greatest for solutions whose pH initial was 4, 5, and 9 because of the low buffer capacity of sulphite solutions at these pHs. At pH 4 it decreased after 24 hours to 3.3; at pH 5, to 4.3; at pH 6, to 5.8; at pH 7, to 6.7; at pH 8, to 7.9; and at pH 9, to 8.5.

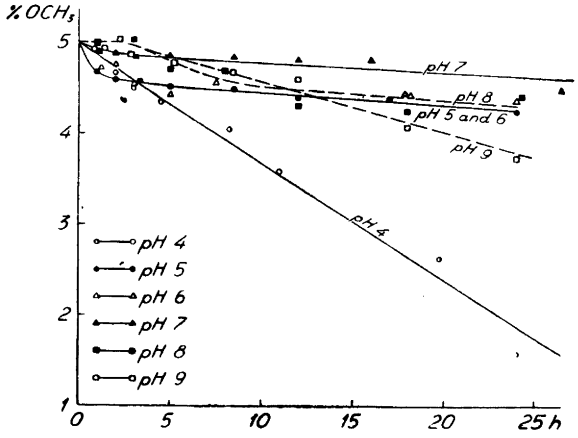


Fig. 6. The methoxyl content of the sulphite treated wood as a function of the heating time at 135° for sulphite solutions of different pH.

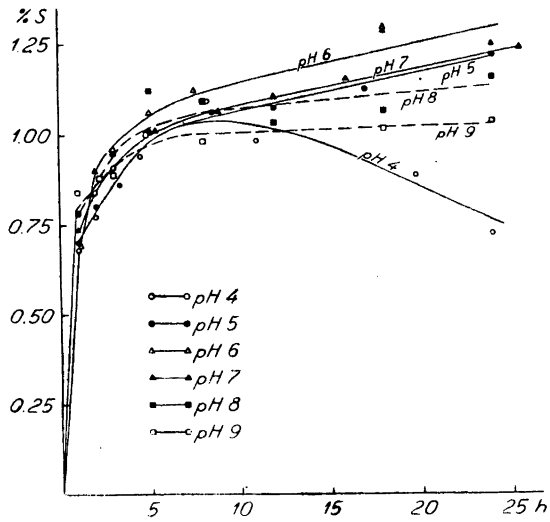


Fig. 7. The sulphur content of the sulphite treated wood as a function of the heating time at 135° for sulphite solutions of different pH.

Spectrophotometric determination of dissolved lignin-sulphonic acid

Wood powder (10 g) was treated with sulphite solution (300 ml, pH 4.9—8.0) as described above, for 4 hours at 135°. The powder was filtered off and washed with sufficient water to give a total volume of 1 000 ml. The resulting solutions were then diluted 10 times. The ultra-violet absorption of these solutions was determined using a Beckman spectrophotometer and the content of lignin-bound methoxyl in the solutions was calculated assuming that $\log \varepsilon = 3.5$ [18] (concentration measured in equivalents of methoxyl per litre) for a ligninsulphonic acid at the absorption maximum at 280 mμ. In Table 2 is given the number of dissolved lignin-bound methoxyl groups as a percentage of the number of lignin-bound methoxyl groups in the original wood powder. Fig. 3 shows the ultra-violet absorption curves of the different solutions.

Alkali treatment of the sulphonated wood powders

Sulphonated wood powder (1.5 g of the preparations which had been sulphonated for 2 and 24 hours) was treated with a NaOH solution (25 ml, 5 g NaOH per litre). The mixture was allowed to stand 4 hours at room temperature, then the powder was collected and washed with water. The yield of wood powder was quantitative. The methoxyl content was determined on one sample: very little (about 4 %) of the methoxyl groups in the sulphonated wood had been dissolved by the alkali treatment. For powders which had been treated with sulphite solutions of pH 7 or less, the sulphur contents decreased 0.11—0.17 % (absolute). For pHs greater than 7, the decreases were 0.01—0.04 %.

Treatment of the sulphonated wood powders with fuming hydrochloric acid by Richtzenhain's method[6]

Sulphonated wood powder (5 g of the preparations which had been sulphonated for 1 hour) was allowed to stand with fuming hydrochloric acid (saturated at − 6°) at − 4° for 24 hours with occasional shaking. The insoluble product was filtered off and washed with concentrated hydrochloric acid (s. g. 1.18) until the filtrate was colourless and did not give any turbidity when diluted with water, then washed with water, and with saturated sodium bicarbonate solution, and finally again with water. The preparation was analysed for sulphur and methoxyl. The results are given in Table 1.

SUMMARY

1. Extracted wood powder (spruce, *Picea abies*) had been treated at 135° with sulphite solutions, for times varying from 1 to 24 hours, and at pHs varying from 4 to 9.

2. Only a small fraction of the lignin is dissolved at pH 6—7. This part seems to be bound by weaker linkages than the remainder.

3. The dissolution of the bulk of the lignin with sulphite solutions of different pH closely parallels the hydrolysis of the dibenzyl ether linkage of diveratryl ether.

4. The lignin which is not dissolved, is sulphonated very rapidly to a sulphur content of 0.18—0.22 sulphur atom per methoxyl group. A slower sulphonation then follows. After 24 hours the wood contains 0.25—0.28 sulphur atom per methoxyl group.

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Group B in Lignin *

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Wood cannot be properly delignified by heating with normal sulphite cooking acid (pH 1—2) in the presence of reactive phenols such as resorcinol, β-naphthol, or pinosylvin. However, if the wood is first heated with sulphite solution with pH higher than about 4, delignification takes place during a subsequent normal sulphite cook at pH 1—2 even in the presence of such a phenol [1].

To explain this Erdtman [1] assumed that lignin contains two groups possessing the following properties:

Group A 1: is sulphonated by acid, neutral and weakly alkaline sulphite solutions.

2: reacts at a higher rate with sulphite than with the phenols at a high pH.

3: reacts at a higher rate with the phenols than with sulphite at a low pH.

Group B 1: is sulphonated by sulphite solutions only at a low pH.

2: reacts, at all pH values, more slowly with phenols than with sulphite.

3: forms the linkage binding lignin in the wood. If the linkage is split, Group B is transformed into another group, termed *Group B'* (Erdtman [2]).

Since the sulphonation of all benzyl alcohols and benzyl ethers investigated is inhibited by phenols at a low pH and the inhibition decreases with increasing pH [3–5], these substances exhibit the properties 2 and 3 in Group A above.

Of the same reason, benzyl alcohols and benzyl ethers do not possess property 2 in the scheme for the reactions of Group B.

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* Part 49 on the chemistry of the sulphite process by E. Hägglund and co-workers. Part 48, *Acta Chem. Scand.* 5 (1951) 603.

That lignin is dissolved from sulphite pretreated wood despite of the presence of a reactive phenol depends on two factors. Firstly, the linkages [6] binding the lignin are split, and, secondly, the lignin product formed is soluble in water. Since the product is soluble, Erdtman [1] assumed that no condensation with phenols takes place and that Group B has the property described under Point 2. However, condensation products between ligninsulphonic acids and phenols can be soluble in water. Erdtman [7] has shown that if a "low sulphonated lignin" (= LSL) is heated with a normal sulphite cooking acid in the presence of resorcinol, the lignin product formed is soluble in water, despite the fact that the analyses show that it is a condensation product of the ligninsulphonic acid and resorcinol.

In order to investigate further the reactions of Group B in this respect, a LSL was heated with sulphite solutions of pH 1.2 and 2.3 in the presence of β-naphthol. Such a ligninsulphonic acid is obtained from wood which has been heated with sulphite solution of pH 5.2 at 135°, by dissolving the lignin by mild acid treatment [7]. Since Group A is sulphonated during the first step, the re-sulphonation of the LSL depends on Group B′. The LSL used contained 0.32 sulphur atom per methoxyl group. It was sulphonated (pH 1.1, 135°, 6 hours) to 0.52 sulphur atom per methoxyl group (Table 1).

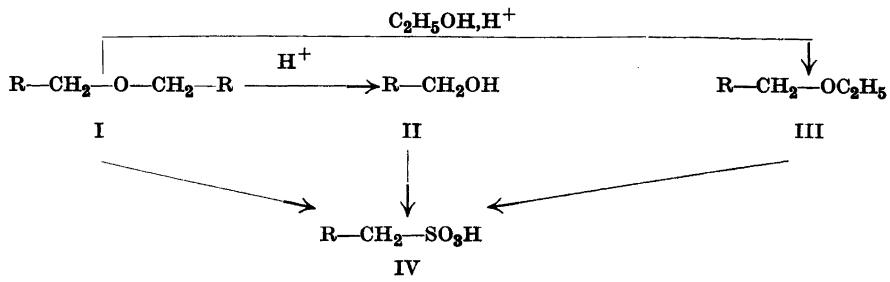
Table 1. The degree of sulphonation and condensation with β-naphthol at pH 1.2 and 2.3

Sulph. solution		Analyses of preparations obtained		
pH	β-Naphthol with + without −	S/OCH_3	C/OCH_3	Condensed β-naphthol per OCH_3 *
	LSL	0.35	10.4	—
1.2	+	0.35	13.4	0.30
2.3	+	0.42	11.0	0.06
1.1	−	0.52	—	—

* Calc. from the ratio C/OCH_3.

The addition of β-naphthol completely prevented sulphonation of LSL at pH 1.2 (Table 1). The C/OCH_3 ratio showed that β-naphthol had combined with the lignin in the ratio of 0.30 mole af β-naphthol per methoxyl group of lignin. At pH 2.3, Group B′ is partly sulphonated despite the presence of β-naphthol. Thus, *in the competitive reactions with sulphite and phenols Group B′ reacts as a benzyl alcohol or benzyl ether group.*

Fig. 1. Diveratryl ether as a lignin model.



R = 3.4-dimethoxy-phenyl-

The preparations obtained were soluble in water. For that reason the dissolution of the lignin from sulphite treated wood by a normal sulphite cooking acid in the presence of a reactive phenol is probably due to the splitting of the linkages which bind the lignin in the wood, and to the formation of a soluble condensation product of the ligninsulphonic acid and the phenol. The pretreatment with a sulphite solution of high pH results in sulphonation to such a degree that the phenol condensation product obtained in the second stage becomes water soluble.

Apart from the competitive reactions between sulphite and phenol, the following is known about the Group B and B′.

(i) The acetylation of ligninsulphonic acids of varying sulphur content has shown that the hydroxyl content decreases on sulphonation. For that reason Group B′ is evidently a hydroxyl group [8].

(ii) If sulphite treated wood is heated with ethanolic hydrochloric acid, ethoxyl groups are introduced into the ligninsulphonic acid. If these ethylated ligninsulphonic acids are then re-sulphonated, the ethoxyl groups are eliminated as ethanol. This reaction sequence can be most easily explained by assuming that ethylation of lignin takes place at the same group as the sulphonation — that is Group B [9].

(iii) Benzyl aryl ether groups can be sulphonated [10,11], thereby giving rise to phenolic groups, but as ligninsulphonic acids contain only few phenolic groups the sulphonation of lignin may not to any large extent be due to such groups [12–14]. For that reason Group B may not be a benzyl aryl ether group.[12]

Points 1 and 3 as well as (i)—(iii) are satisfied, at least in a qualitative sense, by the model substance diveratryl ether [5] (I, the Fig. 1). On heating with a normal sulphite cooking acid at 135°, this is rapidly transformed into 3,4-dimethoxy-toluene-ω-sulphonic acid (IV). At pH 5 it is sulphonated only slowly, and at pH 6 and higher, no reaction takes place. The rate of hydrolysis

of the dibenzyl ether linkage of diveratryl ether by sulphite solutions of varying pH is rather similar to the rate of dissolution of lignin in the same reagents [15]. Diveratryl ether reacts with ethanolic hydrochloric acid to give veratryl ethyl ether (III): the latter reacts with sulphite cooking acid, with the elimination of the ethoxyl group and formation of the sulphonic acid IV [5] (cf. Holmberg's theory for the formation of ethanol lignin [16]).

Consequently, there is much evidence in favour of the view that Group B is a *para-* (or *ortho-*) alkoxy-benzyl ether group. From that follows that Group B′ is the corresponding substituted benzyl alcohol.

EXPERIMENTAL

(Microanalyses by W. Kirsten)

All values are calculated on a salt free basis. The pH determinations are carried out at room temperature.

1) 1.39 g of a "low sulphonated lignin" ($C_9H_{8.3}O_{2.6}(OCH_3)_{0.95}(SO_3H)_{0.33}$) dissolved in a sulphite solution (50 ml, 5 % SO_2, 0.8 % NaOH, pH 1.2). β-Naphthol (0.43 g) was added and the mixture was then heated for 6 hours at 135° in a rotating stainless steel tube.

The solution was extracted with benzene (3 × 25 ml), and the extract dried over anhydrous sodium sulphate, and evaporated. A red oil (0.26 g) was obtained which on vacuum sublimation yielded α-naphthol (0.14 g).

The aqueous solution was dialysed with water until free from sulphate and sulphite ions, and then evaporated to dryness in *vacuo*. The residue weighed 0.86 g.

2) The second experiment was carried out as above, but with the variation that the pH of the sulphite solution was 2.3.

3) The third experiment was carried out as the first one but with the variations that no β-naphthol was added and the extraction with benzene was omitted.

The results are shown in Tables 1 and 2.

Table 2. The yield and analyses of the lignin preparations obtained.

pH	β-Naphthol with + without −	Recovered β-naphthol g	Lignin preparations obtained				
			Amount g	Yield of OCH_3, %	S, %	OCH_3, %	C, %
	LSL		—	—	4.97	13.8	56.0 *
1.2	+	0.19	1.03	63	4.30	11.8	61.4
2.3	+	0.33	1.15	80	5.77	13.4	57.1
1.1	−	—	0.86	60	7.17	13.3	—

* H 5.28 %.

SUMMARY

1. A "low sulphonated lignin" has been treated at 135° with sulphite solutions of pH 1.2 and 2.2 in the presence of β-naphthol.

2. It is suggested that Group B is an *ortho-* or *para*-alkoxybenzyl alkyl ether group.

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The Thiocyanation of Retene

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Only few cases of thiocyanation of polynuclear hydrocarbons are described in the literature [1], and no examples of thiocyanation of simple phenanthrene derivatives are known.

Since the bromination and the chlorination of retene (1-methyl-7-*iso*propylphenanthrene) always lead to very complex mixtures of halogen derivatives [2], and since it is known that the thiocyanation reaction, although very similar to halogenation, is more specific [3], we have found it interesting to investigate the thiocyanation of this hydrocarbon.

In our experiments the thiocyanation was carried out using solutions of free thiocyanogen in different solvents under various conditions, and we found that when chloroform, carbon tetrachloride, carbon disulphide, glacial acetic acid and ether were used, no thiocyanation took place. We found, however, that dry benzene was a suitable solvent, and that illumination from a mercury lamp was of great importance. The optimal temperature was found to be about 40° C.

The main reaction product was obtained in a yield of about 70 % of the theoretical as white crystals, and the analysis showed it to be a mono thiocyanoretene.

When treated with ethanol in the presence of sodium ethoxide it gave a thiourethane, while morpholine gave a thiourea derivative and hydrazines substituted thiosemicarbazides. All these reactions show that the reaction product is an *iso* thiocyanoretene.

When the compound was heated with a mixture of ethanol and conc. hydrochloric acid, hydrogen thiocyanate was liberated, and a white crystalline precipitate was formed. This new compound which was free from nitrogen and sulphur, was sparingly soluble in most organic solvents, and the melting point was rather high (245 ° C).

Analysis showed it to be a hydrocarbon, and we found that the molecular weight was about three times the molecular weight of retene.

This result is similar to that of Wood and Fieser working with thiocyanomethyl cholanthrene [1].

From this reaction it was natural to assume that an unsaturated hydrocarbon was formed as an intermediate product, but we did not succeed in isolating such a compound from the reaction mixture, and we did not find it possible to depolymerize the trimeric hydrocarbon into a dehydroretene by heating since all attempts in that direction led to the formation of retene and a mixture of unknown hydrocarbons.

It was possible, however, to prepare a highly unsaturated hydrocarbon (m.p. 171.5-172.4° C) by treating the *iso* thiocyanoretene with a tertiary amine.

This hydrocarbon gave on heating with alcoholic hydrochloric acid the trimeric hydrocarbon mentioned above, and it added bromine instantaneously to give a dibromoretene. The corresponding iodine derivative could not be made under similar conditions.

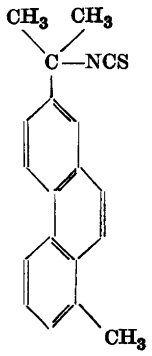
Hydrogenation in presence of a palladium catalyst at room temperature and ordinary pressure converted it quantitatively to retene.

From these facts we conclude that the new hydrocarbon is 1-methyl-7-isopropenyl-retene (Formula II). III is then a possible formulation of the trimeric hydrocarbon.

These results are not in harmony with the results of Talvitie and Laakso [4] who claim that 1-methyl-7-isopropenyl-retene is formed by dehydrogenation of retene at 500° C in the presence of a catalyst, because Talvitie and Laakso's compound melts at 163—164° C and adds iodine very easily to form a diiodide. The dibromide made from Talvitie and Laakso's hydrocarbon melts at 116—117° C [5] while our dibromide melts at 123—125° C.

The retenyl thiourethane could be converted into the corresponding retenylamine by treatment with alcoholic potassium hydroxide, but the yield was poor and large amounts of the trimeric hydrocarbon were formed. We found it impossible to acetylate the amine, and no hydroxyretene was formed by treatment with sodium nitrite in acid solution. In both cases the trimeric retene derivative was formed.

This instability of the retenyl amine and the corresponding hydroxyretene indicates that the substituents are attached to the 7 [1]-carbon atom.



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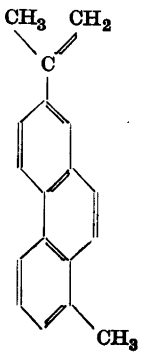

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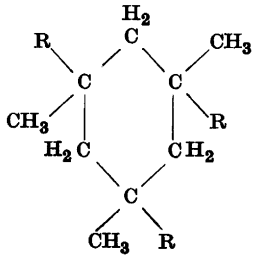
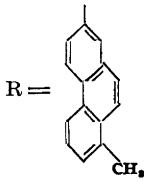


III



All attempts to introduce thiocyanogen in the nucleus of retene and phenanthrene failed.

EXPERIMENTAL

7^1-*iso*-Thiocyano-1-methyl-7-isopropylphenanthrene

To 250 ml of dry benzene solution of free thiocyanogen prepared from 120 g of lead thiocyanate and a little less than the calculated amount of bromine [6] was added a solution of 24 g of retene in 100 ml of dry benzene.

The mixture was illuminated from a mercury lamp for 12 hours at 38—40° C. The precipitated polymeric thiocyanogen was then filtered off and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in 300 ml of boiling ethanol, and the solution was treated three times with 1 g of carbon. The resulting colourless solution was then cooled to 0—5° C and allowed to stand at this temperature for 1 hour.

The white crystalline precipitate of *iso* thiocyanoretene was filtered off and dried in the air. Yield 20 g (67 % of the theoretical). M. p. 107.5—108° C (corr.).

$C_{19}H_{17}NS$ (291.2)	Calc.	C 78.5	H 5.91	N 4.82	S 11.0
	Found	» 78.8	» 5.86	» 4.80	» 10.99

N-Retenyl-(7^1)-thiocarbamyl-morpholine

10 g of *iso*-thiocyanoretene was dissolved in 100 ml of dry acetone and 5 g of morpholine was added. After standing at room temperature for a few minutes a white crystalline precipitate was formed.

The compound which is sparingly soluble in most organic solvents was purified by washing with boiling acetone. Yield 9.8 g (76 % of the theoretical). M. p. 163°—164°, 5 C (corr.).

$C_{23}H_{26}ON_2S$ (378.3)	Calc.	C 73.0	H 6.96	N 7.41	S 8.46
	Found	» 72.8	» 6.92	» 7.43	» 8.40

4-Retenyl-(7^1)-thiosemicarbazide

1 g of *iso*-thiocyanoretene was refluxed with a solution of 1 g of hydrazine-hydrate in 8 ml of ethanol for a few minutes. The retenylthiosemicarbazide crystallized from the boiling solution as white needles, which were almost insoluble in most organic solvents. Yield 0.9 g (81 % of the theoretical). M. p. 188—89° C (corr.)

$C_{19}H_{21}N_3S$ (323.2)	Calc.	C	71.0	H	6.23	N	12.91	S	9.72
	Found	»	71.2	»	6.56	»	12.80	»	9.69

N-Retenyl-(7^1)-thiourethane

3 g of *iso*-thiocyanoretene was dissolved in a solution of 1.5 g of sodium in 50 ml of abs. ethanol by gentle heating for a few minutes. The thiourethane separated as white crystals from the hot solution. Recrystallization from acetone. Yield 3.2 g (92 % of the theoretical). M. p. 177—78° C (corr.).

$C_{21}H_{23}ONS$ (337.2)	Calc.	C	74.8	H	7.41	N	4.16	S	9.50
	Found	»	74.5	»	7.19	»	4.18	»	9.47

7^1-Retenylamine

10 g of retenylurethane was refluxed with a solution of 5 g potassium hydroxide in 200 ml of 90 % ethanol for 24 hours. The solution was then diluted with 600 ml of water and extracted four times with 50 ml of ether. By addition of alcoholic hydrochloric acid the retenylamine hydrochloride was precipitated. Recrystallization from water. Yield 1.8 g (19 % of the theoretical). M. p. 277—80° C (corr.).

$C_{18}H_{20}NCl$ (285.7)	Calc.	N	4.89
	Found	»	4.88

0.1 g of the hydrochloride was dissolved in boiling water and the solution was made alkaline with sodium hydroxide. The retenylamine separated as white crystals. Recrystallization from ethanol. Yield 0.07 g (81 % of the theoretical). M. p. 120—121° C (corr.).

$C_{18}H_{19}N$ (249.2)	Calc.	C	87.1	H	7.63	N	5.62
	Found	»	86.6	»	7.55	»	5.61

"Trimeric retene" $(C_{18}H_{16})_3$

2 g of *iso*-thiocyanoretene was dissolved in 15 ml of boiling ethanol and 1.5 ml of conc. hydrochloric acid was added. The mixture was refluxed for five hours. A white crystalline precipitate was formed during the refluxing. Yield 1.3 g (81 % of the theoretical). M. p. 244—45° C (corr.).

$(C_{18}H_{16})_3$	Calc.	C	93.1	H	6.89	M. wt.	696	
	Found	»	93.0	»	6.98	» »	(acc. to Rast)	662

1-Methyl-7-isopropenylphenanthrene

10 g of *iso*-thiocyanoretene was refluxed with 20 ml of dimethylaniline for 3 hours. The reaction mixture was poured into dilute hydrochloric acid and the precipitate was dissolved in ether.

The ethereal solution was dried with calcium chloride, and after filtration evaporated to dryness. The residue was recrystallized three times from ethanol. Yield 3.5 g (44 % of the theoretical; a large amount of the trimeric hydrocarbon was formed). M. p. 171.5—172.4° C (corr.).

$C_{18}H_{16}$ (232.2)	Calc.	C	93.5	H	6.92
	Found	»	93.13	»	7.03

1-Methyl-7[1], 7[2]-dibromo-7-isopropylphenanthrene

0.23 g of 1-methyl-7-isopropenylphenanthrene was dissolved in 10 ml of dry ether at 0° C. To this solution was added drop by drop a solution of 0.16 g of bromine in 5 ml of ether. The mixture was then evaporated to dryness *in vacuo*. Yield 0.3 g (72 % of the theoretical). M. p. (after recrystallization from ether) 123—125° C (corr.). The compound is unstable, and hydrobromic acid is easily liberated.

$C_{18}H_{16}Br_2$ (392.0)	Calc.	C	55.0	H	4.14	Br	40.8
	Found	»	55.7	»	4.11	»	39.0

Hydrogenation of 1-methyl-7-isopropenylphenanthrene

0.1 g of the dehydroretene was dissolved in 50 ml of dry ether and a suspension of 50 mg palladium catalyst was added. Hydrogen was bubbled through the solution for four hours at room temperature. After filtration and evaporation to dryness the residue was recrystallized from ethanol. Yield 0.09 g. M. p. 97—98° C (corr.). The melting point was not depressed when the product was mixed with retene (melting point 97—98° C).

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A Thermostat for Temperatures between 0 and — 50° C

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In order to continue our investigations of the cuprous chloride — acetylene system [1] we required a thermostat for the temperature range 0 to — 50°C. Further, because each experiment might be expected to extend over several days, it was essential that the thermostat should be accurate and reliable in operation but at the same time economical to run. Although the new equipment is a development of earlier designs, a detailed description is probably justified because of its advantages.

The thermostat is based on a design by Simon [2,3], later improved by Fehér [4] and by Bell and Thomas [5], in which the cooling effect was obtained by the evaporation of sulphur dioxide, ammonia or ether under controlled pressure, the gases being sucked off by means of a water-jet pump. The new model operates with Freon-114 in a closed system, and is more economical than its predecessors and does not present the same health hazard.

The apparatus consists of a double-walled vessel, a manostat and a trap.

The double-walled vessel (Fig. 1) contains methanol as a bath liquid in its central part and freon in the annular space B between the walls. It is of welded stainless steel 1.5 mm thick and has a ground joint at the top for connection to the manostat. Alternatively, a steel lid with a lead gasket can be screwed to the vessel, thus making it possible to store freon in it at room temperature, when the apparatus is not in use. This feature and better heat conduction are improvements on the earlier designs in glass.

The steel vessel is insulated by a Dewar flask in a wooden box filled with saw-dust. The top is of wood and wallboard with cork over the apparatus in the bath. The methanol is stirred mecanically by a propeller.

The manostat (Fig. 2) is essentially a pressure-regulated valve, which keeps the pressure over the boiling liquid at a given value and hence the tem-

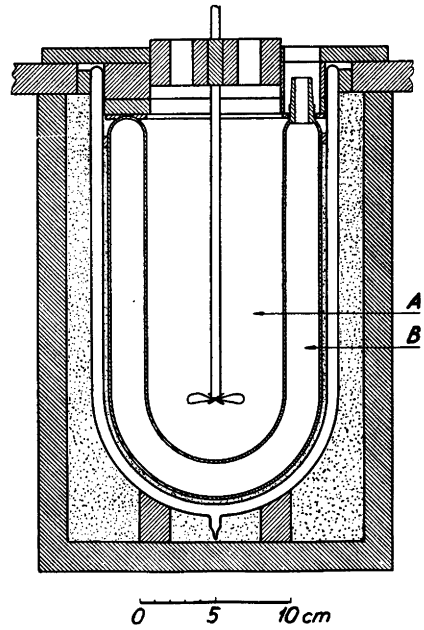

Fig. 1. A double-walled vessel with insulation.

perature in the thermostat bath at the corresponding constant temperature. The apparatus is developed from a design by Fehér [4], but has the additional advantage that it is independent of the room temperature. It can be described as a U-shaped mercury manometer with a sintered glass filter F (finest porosity from Le Pyrex, France) in one of its legs. It is connected to the double-walled vessel at A, and the filter communicates with the trap and its vacuum vessel at B. When the mercury surface M reaches F the flow of gas is interrupted

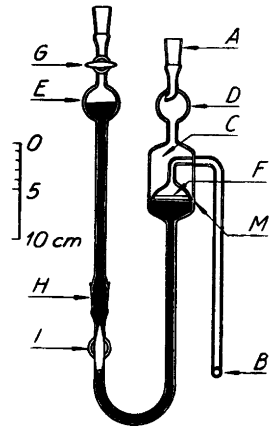

Fig. 2. The manostat.

until the pressure increases, the mercury level drops and the valve opens again. The mercury of course cannot pass through the filter.

The bulb C can hold all the mercury, and to prevent splashing there is a trap D over it. The other leg ends in the bulb E, which can be evacuated through the valve G. The leg is made in two pieces connected by a ground joint H and the length of the leg is determined by the temperature required. By means of the valve I it is possible to turn off the manostat. A vessel of 10 l capacity connected to the tube between the bath vessel and the manostat prevents rapid oscillations of the mercury level.

At first we found that, after some days, the mercury tended to clog the filter, but when a filter was carefully selected from a batch by testing the permeability to gases and resistance to mercury at 1 atmos. pressure for several hours, and precautions were taken to ensure that the mercury was clean, no clogging occurred even after months of continuous use. Our experience has shown that the filter should be capable of handling 2—3 times the calculated rate of flow of freon.

Parallel to the manostat there is a connection via a valve between the boiling freon and the trap. Normally this valve is closed, but it can be used to regulate the temperature by hand.

A glass tube leads from the manostat to the bottom of the trap, the upper part of which communicates with a vessel of 10 l capacity.

The trap holds 2 l and is cooled by solid carbon dioxide and acetone in an insulated box, which is removable for refilling. Fig. 3 shows the arrangement. The whole system can be evacuated through a valve on the vacuum vessel.

The thermostat is operated with Freon-114, $CClF_2 \cdot CClF_2$, which boils at 3.8° C/460 mm and has a vapour pressure of 1.8 atm. absolute at 20° C.

Manipulation is very simple. Initially, the trap and the double-walled vessel are cooled by dry ice, and the vessel filled with freon and connected to the manostat. The temperature is adjusted roughly, the system evacuated and the manostat released. In less than half an hour the temperature is stable.

After 8—12 hours the trap is one third filled with freon. This is forced back through the direct connection to the double-walled vessel by air pressure in the trap. The temperature falls by the colder freon from the trap and is adjusted by means of a heating coil and the system evacuated.

To turn off, all the freon is collected in the steel vessel, which is then closed.

Transient fluctuations in the temperature of the bath have not been observed; if any they must be less than 0.01° C.

A gradual temperature rise of about 0.08 °C in 12 hours at — 20 °C is due to the changing liquid level in the steel vessel and possibly to impurities in the freon. This drift can be diminished by draining the trap frequently.

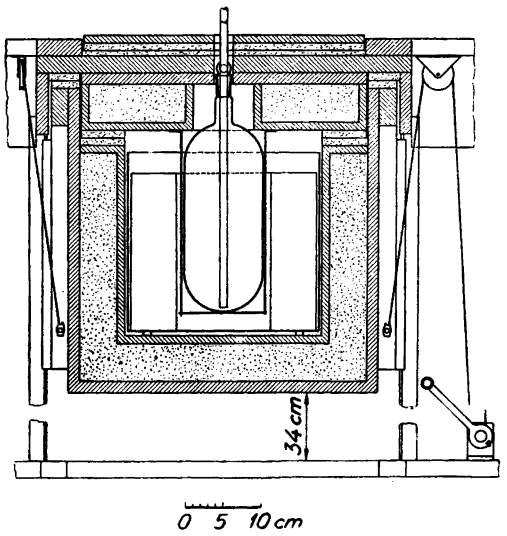

Fig. 3. The trap and insulation box.

The consumption of dry ice is about 5 kg per day, and freon must be added every 2 or 3 weeks.

After several months of continuous use we find that the apparatus is reliable and requires little servicing.

SUMMARY

A description is given of a thermostat for temperatures between 0 and — 50 °C, operating continously with freon-114 in a closed system. Accuracy ± 0.04 °C.

We wish to thank Professor Arne Ölander for his interest, and *Statens Tekniska Forskningsråd* for financial support.

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Determination of 4-Chloro-2-methylphenoxyacetic Acid in a Multicomponent System by Isotope Dilution Analysis

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In this journal Sjøberg [1] described a method for determination of 4-chloro-2-methylphenoxyacetic acid (4K-2M) using infrared spectrometry. Grabe [2] has elaborated a similar method based upon ultraviolet spectrometry.

The advantages of isotope dilution are that no impurities will disturb determination and the procedure is quite simple. The accuracy is about $\pm$ 1.5 %, *i. e.* about the same as that attained by the infrared spectrophotometric method.

Isotope dilution technique is generally known [3]. Consider a crude sample which is to be analyzed for one of its constituents X. To the sample is added a known quantity of radioactive X, with a known specific activity (= activity per unit weight). From the mixture some pure X (or X of a known purity) should in one way or the other be isolated and the specific activity of this is determined. The ratio of the final and initial specific activities depends on how much the radioactive X has been diluted by the amount of inactive X present in the original sample, and from the measured quantities this amount can be calculated.

Using this method for this investigation the principle becomes:

Radioactive 4K-2M is synthesized from radioactive chlorine (Cl^{36}). A solution of this active 4K-2M is prepared with a content of y mg of 4K-2M per mg solution. The specific activity of the solution we call x (counts per minute per mg solution). Suppose a mg of active solution is mixed with a sample containing b mg of (inactive) 4K-2M. Pure 4K-2M isolated from the mixture will give the specific activity:

$$c = \frac{a \cdot x}{b + a \cdot y} \text{ counts per minute per mg}$$

Carrying out the same experiment with corresponding A and B mg and calling the final specific activity C, we have:

$$C = \frac{A \cdot x}{B + A \cdot y}, \text{ and by division}$$

$$r = \frac{C}{c} = \frac{A}{a} \cdot \frac{b + ay}{B + Ay} \quad \text{or}$$

$$B = \frac{1}{r}\frac{A}{a} \cdot b + A \cdot y \cdot \frac{1 - r}{r}$$

A standard sample of pure 4K-2M is made from a known amount b mg of 4K-2M to which is added a mg of active solution.

In the above expression B is the only unknown quantity. Starting with a sample containing an unknown amount B mg of 4K-2M, adding A mg of active solution, isolating a final sample of pure 4K-2M and measuring $r =$ the ratio of specific activity of the final sample and of the standard sample, the formula gives the value of B.

Instead of isolating 4K-2M a derivative of the compound may be isolated. If the standard sample is the same derivative the formula is still valid.

Is it only possible to isolate 4K-2M (eventually a derivative) with a known purity P %, the expression which determines B will be (with an approximation giving no practical difference in B):

$$B = \left(\frac{1}{r}\frac{A}{a} \cdot b + A \cdot y \cdot \frac{1 - r}{r}\right) \frac{P}{100}$$

Here it should be emphasized that a high purity of the radioactive 4K-2M is not necessary. Contamination of the radioactive 4K-2M with other radioactive substances may give rise to errors, but this source of error is eliminated by preparing the active solution with an acceptably high content of these components in inactive form. When isolating pure 4K-2M derivative by the procedure of the analysis, the radioactive contaminants are removed together with inactive species of the same compounds.

EXPERIMENTAL

The investigation was carried out with mixtures containing the same constituents as in Sjøberg's nvestigation, that is in addition to 4K-2M: 6-chloro-2-methylphenoxyacetic acid (6K-2M), 4,6-dichloro-2-methylphenoxyacetic acid (4,6K-2M) and 2-methylphenoxyacetic acid (2M).

Preliminary experiments showed that isolation of 4K-2M as an acid of known purity from a mixture of the mentioned constituents could not be done simply.

Experimenting with different derivatives it was found that the anilide of 4K-2M had practically ideal properties for the purpose. The anilide is easy to prepare, easy to gain in a nearly pure state by recrystallization and the purity can be determined with good accuracy by the melting point.

Melting points are determined by a capillary method in a Hershberg apparatus [4]. The tube is made from conventional glass capillary with a diameter about 1—1.5 mm. The middle of this capillary is drawn into an even narrower capillary about 20—30 mm long and about 0.1—0.2 mm diameter. The middle of the narrowed portion of the capillary is then divided in a flame so that two tubes are produced from one capillary. The sample is placed in the narrow end of the tube. The temperature at which a meniscus forms in the narrow end is taken as the melting point. Additional temperature values may be observed where „sintering" occurs and where *all* the substance melts. The bath is heated at a rate of about 0.1° C per minute in the vicinity of the expected temperatures. The difference in melting points of two samples is determined by placing the two tubes in the Hershberg-apparatus at the same time; the two tubes being made from the same capillary. The difference in melting points (meniscus points) is determined with an accuracy of ± 0.1° C.

Limits given for melting points refer to the sintering and meniscus point.

Radioactivity was measured with a Madsen-tube (bellshaped end window-counter, window thickness = 3 mm/cm^2, back ground = about 20 counts per minute [5]) and a Brüel and Kjær electronic counter 6501.

About 50 mg of the fine crystalline sample was placed in an aluminium dish and weighed. The measured number of counts per minute is corrected for background and self-absorption. Application of the sample in a uniform layer in the aluminium dish is done by suspending the powder in methanol on the dish and smoothing the sample with a nickel-spatula during the evaporation of the methanol. Finally the sample is dried under an infra-red lamp and reweighed.

The accuracy of determining activity is limited by the counting error. The error of the total count depends on the counting time. When counting samples of the activity as used in these experiments (500—1 000 counts per minute) for about ½—1 hour the standard deviation of the number of counts will be about 0.7 %, *i. e.* the ratio r (of the two samples specific activities) is determined with a standard deviation of about 1 %. In comparison with this error, the errors arising from corrections for background and selfabsorption, weighing of the sample and application on the aluminium dish are small and may be ignored.

MATERIALS

The phenoxyacetic acids were prepared by methods similar to Sjøberg's. Another way used to get pure products was purifiing the anilide followed by saponification.

Table 1 gives the melting points and solubilities of the acids used.

The anilides were prepared by refluxing the acid in question with 2—3 times as much aniline (b. p. 184—185) one hour, precipitating with an excess of 4 N HCl, filtering and

Table 1. Properties of phenoxyacetic acids.

Acid	m. p. C°	Solubilities at 25° C mg per 100 ml of solution		
		water	carbon tetrachloride	benzene
4K—2M	119.6—119.8	75	600	3 100
2M	155.1—155.3	60	50 [1]	
6K—2M	108.9—109.3	220		
4,6K—2M	187.6—188.0	8	50	200

[1] From Sjøberg's investigation.

Table 2. Melting points and analyses of anilides of phenoxyacetic acids.

Anilide of	m. p. C°	Microanalyses (by F. Limborg)							
		C %		H %		Cl %		N %	
		calc.	found	calc.	found	calc.	found	calc.	found
4K—2M	129.8—130.0 [1] 129.5—129.7 [2]	65.4	65.7	5.12	4.9	12.9	12.9	5.08	5.3
2M	108.2—108.4	74.7	74.3	6.27	5.8	0		5.81	5.7
6K—2M	78.0— 78.3	65.4	65.5	5.12	5.0	12.9	12.9	5.08	5.0
4,6K—2M	100.8—101.1	58.1	58.1	4.23	4.2	22.9	22.9	4.52	4.4

[1] Recrystallized from methanol.
[2] Recrystallized from 61 % ethyl alcohol.

Table 3. Solubilities of anilides of phenoxyacetic acids.

Anilide of	Solubility at 25° C mg per 100 ml of solution	
	abs. ethyl alcohol	61 % ethyl alcohol
4K—2M	1 100	150
2M	2 600	700
6K—2M	10 000	1 500
4,6K—2M	3 300	300

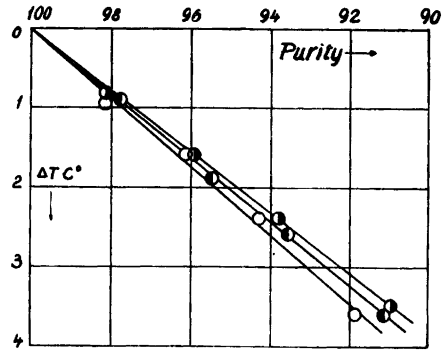

Fig. 1. Melting point depression of 4K-2M-anilide.

◑ *4,6K-2M-anilide*
◐ *6K-2M-anilide*
○ *2M-anilide*

Table 4. Purity determination from melting points.

Deviation of m. p. ΔT C°	0.1	0.2	0.3	0.4	0.5	0.6
Purity	99.8	99.5	99.3	99.0	98.8	98.5

washing with water and recrystallization from abs. ethyl alcohol or from 61 % alcohol (= 2 vol. alc. + 1 vol. water).

Tables 2 and 3 gives analytic data, melting points and solubilities of the anilides.

Radioactive 4K-2M was prepared by chlorination of 2-methylphenoxyacetic acid with radioactive chlorine *. (Cl^{36}, half life = $2 \cdot 10^6$ years, β-emitter, 0.66 Mev.) The starting material was about 10μ-Curie NaCl as a dilute aqueous solution. The chloride was precipitated as AgCl and dried. From AgCl the chloride was transformed to elementary chlorine by oxidation with potassium dichromate in sulfuric acid after Baubigny and Chavanne [6], with a yield of about 95 %. 1.03 g of methylphenoxyacetic acid dissolved in 10 g of acetic acid at 100° C was chlorinated by the chlorine produced from 1.947 g of active AgCl. The chlorinated solution was diluted 15 times with water and after heating to clear solution, crystallized when cool. Crystals of active 4K-2M were separated from the mother liquor and dried. Yield: 0.964 g, m. p. 118.5—119.0, estimated purity: 95 %.

The HCl evolved by the process together with other chlorine-containing fractions were transformed back to AgCl. Yield for consumed chlorine was about 80 %.

Preparation of radioactive solution: 420 mg of active 4K-2M, 198 mg of 4,6K-2M and 221 mg of 6K-2M were dissolved in 20 ml of ethyl alcohol and 6 ml of 1 *N* NaOH were added. Water was added to total weight 40.0 g. The content of 4K-2M is about 1 % that is $y \cong 0.010$ mg of 4K-2M per mg solution. Specific activity (x) about 4 counts per minute per mg solution when counting with the afore-mentioned apparatus.

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* The Cl^{36} samples used were from the U. S. Atomic Energy Commission, Oak Ridge.

Purity determination of 4K-2M-anilide

Mixtures of the anilide of 4K-2M and an anilide of other phenoxyacetic acids were prepared by dissolving these in acetone and evaporating to dryness. Fig. 1 shows the depression of the melting point (meniscus point) of 4K-2M-anilide by different contents of anilides of the other acids. By using the curves for purities above 99 % the different curves give practically the same corrections. Table 4 gives the purity at different melting point-differences (by extrapolation).

ANALYTICAL PROCEDURE

A quantity of the product to be analyzed, which is estimated to contain about 200 mg of 4K-2M is weighed. 1 ml of active solution is added and accuratly weighed (A mg). After eventually dissolving in NaOH the phenoxyacetic acids are precipitated by 4 N HCl and isolated by filtering or decanting.

1.5 ml of aniline is added and in a test-tube provided with a "cold finger" is refluxed for an hour. Some waterdrops from the wet acids will condense on the "cold finger" and may be removed with a piece of filterpaper. After cooling add 10 ml of 4 N HCl, boil again a moment and cool off. The anilides are washed with water, the supernatant being filtered to reduce loss. The wet crude anilides are recrystallized first from 5 ml of abs. ethyl alcohol and then two or more times from 61 % alcohol until the difference in melting point from that of the standard sample is less than 0.5° C that is to say a purity above 99 %. For technical products usually two or three recrystallizations from 61 % alc. are enough. After the last recrystallization the product is washed with about 1 ml of methanol and dried under an infra-red lamp. The yield of 4K-2M-anilide is about 100—150 mg. The specific activity as a ratio of the standard sample (prepared in the same way, but from a known amount (b mg) of pure 4K-2M) is measured ($= r$). From the melting point the purity (R %) is determined and the amount (B mg) of 4K-2M in the original sample is calculated.

RESULTS

Following samples were analyzed:

Table 5.

Sample	Composition							
	4K-2M		2M		6K-2M		2M-4, 6K	
	mg	%	mg	%	mg	%	mg	%
A	400.7	66.7	40.2	6.7	119.5	19.9	39.8	6.7
B	292.4	59.5	40.2	8.2	119.5	24.3	39.8	8.1
C	256.2	72.0	20.1	5.6	59.8	16.8	19.9	5.6

In the case of sample A, several recrystallizations were performed and between each recrystallization from 61 % alcohol, the melting point and activity were determined.

Table 6. Analyses of known mixtures (see Table 5).

Sample	Deviation of m. p. from standard ΔT C°	Purity R %	Active solution added A (a) mg	Specific activity in relation to standard r	Amount of 4K − 2M		Deviation %
					found B mg	calc. mg	
Standard	0	100.0	1855.8	1.000		395.0	
A	1.1	97.5	1876.3	0.973	401	400.7	+ 0.1
A	0.5	98.8	1876.3	0.991	398	400.7	− 0.7
A	0.3	99.3	1876.3	0.997	399	400.7	− 0.4
A	0.2	99.5	1876.3	0.999	398	400.7	− 0.7
B	0.5	98.8	1838.0	1.296	294	292.4	+ 0.6
C	0.3	99.3	924.4	0.771	256	256.2	− 0.1

DISCUSSION

By estimation of the accuracy of the method it is obvious that all other uncertainties may be ignored in comparison with the uncertainty of r. The quantity y is rather inaccurratly determined but the term with y only represents about a per cent or less of the total value.

The purity R may give rise to errors but if the purity determined by melting point is above 99 % it is not probable that impurities even though unknown will lead to inaccurate results.

SUMMARY

4-Chloro-2-methylphenoxyacetic acid has been determined in mixtures with other phenoxyacetic acids by an isotope dilution method.

The standard deviation of the result is calculated to be about 1 %, *i. e.* the real value is with a 90 % probability between the determined value $\pm$ 1.64 %.

The author gratefully acknowledges the opportunity of carrying out the radioactivity measurements at the Zoophysiological Laboratory of the University of Copenhagen.

Dr. A. Hobgen kindly revised the English text.

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Dependence of Nitrate Reduction in Green Plants on Reducing Substances

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The decisive role of reducing substances (ascorbic acid, reductone, and SH-compounds) in the reduction of nitrate and nitrite in green plants has been ascertained during some years in this laboratory by growth experiments with pea seedlings and wheat embryos isolated from cotyledons and endosperm [1]. Similarly, it has been noted that a cotyledonless pea, growing in a nutrient solution without combined nitrogen but inoculated with an effective strain of pea *Rhizobium*, grows only if reducing substances are added to the nutrient solution although white nodules are formed without any addition [2]. On the other hand, in a nutrient solution containing ammonium nitrogen, the growth is good even without reducing substances. Used in greater amounts they will even injure the growth [1].

A quantity of 100–120 mg ascorbic acid per plant given in three doses at intervals of about a week produced in best cases with cotyledonless pea in nitrate nutrient solution a growth almost comparable with that of normal pea. *Cotyledons were thus successfully replaced by ascorbic acid.* On the basis of the results it seems that the most important function of the cotyledons and the endosperm is to produce reducing substances to the plant at the early stage of its growth. The action of the reducing substances affects probably primarily the iron metabolism [2].

Nitrate reduction in the cotyledonless pea plants is presented by data in Table 1.

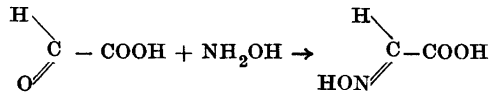
It appears from the results that when a cotyledonless pea grows in nitrate nutrient solution without ascorbic acid, nitrate-N accumulates in the plant in very great amounts, in extreme cases up to 20 % of total N. The growth of plants is then nil or very poor. In parallel experiments, the addition of ascorbic acid, which produces a vigorous growth, lowers the nitrate content of the plants very much, to less than 1 % of total N. The percentage of nitrite nitrogen also drops sharply by the addition of ascorbic acid, thus nitrite, too, is accumulated in abundance though less by far than nitrate. It is interesting to note, that when a cotyledonless pea seedling grows in the dark in a nutrient solution containing cane sugar and nitrate (a modified method of

Table 1. Cotyledonless pea (Torsdag) in sterile culture system. Cotyledons removed 5 days after moistening the seeds. One seedling in 1 litre suction flask. Mineral nutrient solution with 243 mg $NaNO_3$ (40 mg N) and 1 mg Fe as $FeCl_3$ per litre. Sterile seedling transferred to suction flask 21. XI. 1949. Experiment interrupted 15. XII. 1949, 13. I. 1950 and 3. II. 1950. Ascorbic acid (Aa) added in three doses, 50 mg each, to each flask 21. XI, 10. XII, and 20. XII. Artificial light.

No.	N-source and additions	Date of harvest	Fresh weight g	Total N, mg	N in plant extract, mg	NO_3—N γ	NO_3—N % of total N	NO_2—N γ	NO_2—N % of total N	NO_2—N % of NO_3—N
1	40 mg NO_3—N	15. XII	0.8	3.2	—	510	15.9	3.7	0.12	0.7
2	—„—	15. XII	0.3	1.7	—	200	11.8	3.5	0.20	1.8
3	—„—	13. I	0.4	1.8	0.9	340	18.8	2.6	0.14	0.8
4	—„—	13. I	1.7	5.1	0.8	250	5.0	2.8	0.06	1.1
5	—„—	13. I	2.1	6.8	1.4	225	3.3	4.6	0.07	2.0
6	40 mg NO_3—N + 150 mg Aa	15. XII	3.0	8.8	—	70	0.8	10.0	0.12	14.3
7	—„—	13. I	10.7	44.1	6.7	130	0.3	4.2	0.01	3.2
8	—„—	13. I	13.8	42.1	8.4	160	0.4	4.2	0.01	2.6
9	—„—	13. I	4.0	15.6	4.7	70	0.4	4.8	0.03	6.9
10	—„—	3. II	9.2	41.5	10.5	225	0.5	9.0	0.02	4.0
11	—„—	3. II	6.1	26.8	5.3	170	0.6	5.6	0.02	3.3

Bonner [3]), nitrate and nitrite are not accumulated to any noteworthy extent, although even then an addition of ascorbic acid promotes the growth. This implies that the mode of nitrate reduction may be different in the light (in green parts of the plant) and in the dark (in roots), as Burström [4] has suggested*. However, it must also be considered that the seedlings utilize nitrate comparatively little in this system, inspite of ascorbic acid addition.

* The hypothesis is attractive that the reduction of nitrate in the green parts of plants proceeds partly only as far as, *e.g.* to hydroxylamine, and this then reacts with compounds containing the CO-group, *e.g.*, glyoxylic acid, produced by the CO_2-assimilation. The reaction



is very intense according to the determinations made in this laboratory, and besides, the enzyme system which oxidizes glycolic acid to glyoxylic acid is present according to Tolbert and Burris (*J. Biol. Chem.* **186** (1950) 791) in the green parts of plants but not in roots. It is activated by light in intact etiolated plants. The said authors suggest that glycolic acid and the enzymatic system for its oxidation may be concerned with photosynthesis. According to Benson and Calvin (*J. Exp. Bot.* **1** (1950) 63) glycolic acid is one of the earliest products to contain C^{14} after a short exposure of plants to $C^{14}O_2$.

It is seen from Table 2 that the cotyledonless peas did not grow at all with nitrite-N without ascorbic acid while in most cases and addition of ascorbic acid produced a fairly good growth.

Table 2. Parallel experiments with those in Table 1, but $NaNO_2$ as the source of nitrogen.

No.	N-source and additions	Date of harvest	Fresh weight g	Total N, mg	N in plant extract, mg	NO_3—N γ	NO_3—N % of total N	NO_2—N γ	NO_2—N % of total N	NO_2—N % of NO_3—N
1	40 mg NO_2—N	15. XII	0.40	3.1	2.1	170	5.49	7.6	0.25	4.5
2	—„—	3. II	0.44	5.2	2.9	70	1.35	4.2	0.08	6.0
3	—„—	3. II	0.08	1.9	0.6	45	2.37	7.2	0.37	16.0
4	—„—	3. II	0.08	3.6	1.9	50	1.38	4.6	0.13	9.2
5	—„—	3. II	0.07	2.8	—	48	1.71	5.6	0.20	11.7
6	40 mg NO_2—N + 150 mg Aa	3. II	11.36	38.9	10.7	130	0.33	7.7	0.02	5.9
7	—„—	3. II	13.61	41.9	13.2	155	0.37	10.0	0.02	6.5
8	—„—	3. II	1.85	10.2	2.5	95	0.93	8.5	0.08	9.0
9	—„—	3. II	5.30	22.8	5.2	160	0.70	4.9	0.02	3.1

The recent finding of Hewitt *et al.*[5] of the notable decrease in the ascorbic acid content of plants caused by the lack of molybden is very interesting and is apt to throw more light on the action of molybden. In our experiments the nutrient solution has contained molybden in so great amounts that a further addition has caused no increase in the growth. To make certain, it has been a rule during the last two years to add to the nutrient solution a mixture of trace elements containing also molybden.

The effect of the addition of reducing substances has been examined not only with plants whose cotyledons or endosperm were removed at an early stage of germination but also with normal plants. When pea plants, whose cotyledons were not removed, were grown in sterile nitrate nutrient solution with and without ascorbic acid, it was noted that the plants administered with ascorbic acid contained noticeably less nitrate nitrogen than those grown without ascorbic acid. In a previous paper[6] we have recorded results of experiments, in which the quantity of NO_3-N in the nutrient solution has been comparatively low, *viz.*, 15, 30 and 60 mg per plant.

When the nutrient solution contains plenty of nitrate nitrogen (150—300 mg N per plant per litre nutrient solution) an addition of ascorbic acid also causes an increase in the crop. When growing without ascorbic acid the peas then regularly develop chlorosis. The plants supplied with ascorbic acid soon overcame the slight chlorosis that first developed and the growth continued normally. Results of these experiments are given in Table 3.

Table 3. Normal pea plant (Torsdag) in nitrate nutrient solution. Sterile culture system, 1 litre suction flasks. Experiment started 5. V. 1950. 1 mg Fe as $FeCl_3$ per litre. Ascorbic acid (Aa) given in 50 mg doses May 5, 12, 28, and June 9. Date of harvest appears from the table.

No.	N-source and additions	Date of harvest	Fresh weight g	Total N, mg	N in plant extract, mg	NO_3—N γ	NO_3—N % of total N	NO_3—N γ	NO_3—N % of total N	NO_3—N % of NO_3—N
3	150 mg NO_3—N		10.7	31.0	7.6	1700	5.5	15.0	0.05	0.9
4	150 mg NO_3—N + 200 mg Aa	June	12.7	38.8	10.7	1400	3.6	9.8	0.03	0.7
5	300 mg NO_3—N	1—3	8.3	36.3	13.5	1900	5.2	23.0	0.06	1.2
6	300 mg NO_3—N + 200 mg Aa		13.4	44.0	13.3	1500	3.4	9.5	0.02	0.6
9	150 mg NO_3—N		20.9	76.5	14.8	5190	6.8	57.7	0.08	1.1
10	150 mg NO_3—N + 200 mg Aa	June	35.7	127.6	32.8	4880	3.8	20.7	0.02	0.4
11	300 mg NO_3—N	12—16	8.8	36.6	6.4	5290	14.4	12.9	0.04	0.2
12	300 mg NO_3—N + 200 mg Aa		26.8	102.7	26.5	9000	8.8	—	—	—
15	150 mg NO_3—N		19.6	87.3	18.4	5990	6.9	38.8	0.04	0.6
16	150 mg NO_3—N + 200 mg Aa	June	32.9	142.1	33.5	1395	1.0	27.0	0.02	1.9
17	300 mg NO_3—N	26—27	15.9	67.3	14.5	7620	11.3	21.5	0.03	0.3
18	300 mg NO_3—N + 200 mg Aa		26.5	98.4	27.8	4900	5.0	20.0	0.02	0.4

A similar effect on the nitrate content of plants as produced with an addition of ascorbic acid is also brought about with an addition of sodium sulphide to the nutrient solution. In open pot cultures with either quartz sand or soil as a root support, the nitrate content of plants is also often lowered by the effect of sulphide.

Our idea of the function of the reducing substances in the plants is that they lower the redox potential and effect in this way on the uptake and assimilation of iron. As a result of the disturbance in the iron metabolism the reduction of nitrate is checked or weakened. Chlorosis, which appears in pea plants in our nutrient solution with plenty of nitrate but no reducing substances, is an indication of the disturbance in the iron metabolism.

SUMMARY

The addition of reducing substances to the root support promotes nitrate reduction in normal pea plants. In sterile cultures the effect is strong and distinct at least in the experimental conditions used. In open pot cultures the effect is also often to be found. The primary influence, however, affects the uptake and metabolism of iron.

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On the Mechanism of the Intestinal Fat Absorption. II

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We have recently[1] investigated the composition of lymph fat collected from the ductus thoracicus of the cat after feeding C^{14} labelled fat. The recoveries of this fat in the lymph were, however, low, *viz.* 4.3—12.8 per cent probably because the animals were anesthetized throughout the entire period of lymph collection. In the present study we have used rats having the intestinal lymph duct cannulated by the Bollman *et al.* technique [2] in order to get better recoveries and more reliable results.

Table 1 shows the types of fats given by stomach tube to adult male rats under a light ether anestesia at least 18 hours after the operation. Some of the animals were used two or more times. In these cases the animals were fasted 12 hours before the fat administration. The lymph was collected in two portions 0—10 and 10—24 hours after the feeding, extracted with ethanol: ether 3 : 1 and then with light petroleum. The total fat from the 0—10 hours samples were separated on columns of magnesium oxide, the neutral fat was eluated with acetone, the phospholipids with metanol. The phospholipids thus eluated were the choline containing phospholipids which constituted 90—100 per cent of the lymph phospholipids [3]. The amounts of non-choline containing phospholipids obtained by dissolving the column with hydrochloric acid were too small to be accurately assayed, and hence the values given here only refer to the fatty acids of the choline containing phospholipids. The total fat from the 10—24 hours portions and the two fractions from the first portions were saponified and the activity of the fatty acids determined after wet combustion [3].

The recoveries in the lymph of the fat fed varied widely. This is probably due to the fact that the conditions of the animals after the operation were different. In some cases the fat rapidly passed the intestine and appeared in the faeces in larger quantities than is found in the normal animal [4]. Diarrhea occurred in some animals especially after feeding hydrolysed corn oil. The

complete quantitative evaluation of the significance under normal conditions of the lymphatic pathway in fat absorption can not be made in these experiments as accessory intestinal lymphatics are ligated and no data are available regarding how much of the small intestine are drained by the main lymphatic which was cannulated.

In all three groups of experiments, however, the same maximum recoveries were found after 24 hours, *i. e.* 80.9, 85.0 and 87.5 per cent of the activity given. These results indicate that under good conditions most of the absorbed fat is transported via the lymphatic channels to the systemic circulation whether fed as glycerides or free fatty acids. These findings do not accord with the partition hypothesis of Frazer [5, 6]. They are, however, in agreement with earlier work of Munk [6] and Ivy [7] and recent work of Tidwell [8] with chylomicroncounting and of Bloom *et al.*[9] using the same technic as we used but without investigating the composition of the lymph fat and of Bollman *et al.*[10] investigating the intestinal lymph of unanesthetized dogs.

The proportions of neutral fat and phospholipids in the lymph were in all three cases about the same. Ninety per cent of the fatty acids were present in the neutral fat and the remaining 10 per cent in phospholipids. The neutral fat consisted chiefly of triglycerides; cholesterol and cholesterol esters representing only a minor part of this fraction. No free fatty acids or soaps appeared in the lymph [3].

The specific activity of the neutral fat fatty acids were in all three types of experiments about 80—90 per cent of the activity of the fatty acid mixture fed when assayed after hydrolysis. This finding evidence a very active synthesis of glycerides in the intestinal mucosa from free fatty acids fed in A and in B as indicated by work of Munk [6], Sinclair [11] and Ivy [7] and in contradistinction to Frazer [5].

The specific activities of the phospholipid fatty acids also showed about the same values in the three types of experiments. This finding is somewhat different from the results in our experiments on cats [1] and indicates that the glycerides might be completely hydrolysed in the intestinal lumen of the rat and then resynthesized in the intestinal wall in agreement with the theories of Verzar [12]. If the hydrolysis was only partial in the intestinal lumen as supposed by Frazer the highly active free fatty acids given in A in a small amount together with inactive corn oil, were only partly diluted by the free fatty acids liberated on partial hydrolysis of the glycerides. The free fatty acid mixture available for glyceride synthesis should then have a specific activity higher than it would if the hydrolysis were complete as in C. In B the specific activity of the eventual free fatty acids is independent of the degree of hydrolysis. As other work in this laboratory [5] indicates that the synthesis of glycerides in

Table 1. The composition of rat intestinal lymph fat. Activity % = specific activity in % of specific activity of administered fatty acids.

Number of rats	Form of fat administered	Lymph in ml 0—10 h	Total fat in lymph mg 0—10 h	Total cholesterol in lymph mg 0—10 h	Per cent of administered activity recovered in lymph		Fatty acids recovered in lymph as: 0—10 h			
							Phospholipid fatty acids		Neutral fat fatty acids	
					0—10 h	0—24 h	Weight %	Activity %	Weight %	Activity %
5	0.5 ml corn oil + 2.5 mg active palmitic acid —1—C^{14}* A	10.4 (16—7)	351 (482—230)	6.5 (9.9—3.2)	52.6 (78.8—35.8)	57.0 (80.9—38.4)	10.9 (14.0—8.3)	50.3 (55.5—43.6)	89.1 (91.7—86.0)	88.0 (99.1—78.8)
6	0.5 ml corn oil transesterified with 2.5 mg active palmitic acid —1—C^{14}* B	13 (16.5—8.5)	366 (644—196)	7.5 (12.3—4.9)	49.3 (83.4—29.1)	61.7 (85.0—30.2)	11.3 (12.8—9.4)	40.2 (49.7—29.4)	88.7 (90.6—29.4)	79.4 (95.7—64.0)
5	0.5 ml hydrolysed corn oil + 2.5 mg active palmitic acid —1—C^{14}* C	14.4 (18—12)	260 (426—124)	6.4 (10.6—4.1)	39.5 (72.0—14.5)	62.3 (87.5—45.6)	11.3 (13.3—9.4)	37.2 (50.6—26.6)	88.7 (90.6—86.7)	85.1 (96.8—78.8)

* Specific activity about $2 \cdot 10^5$ counts per minute when assayed as $BaCO_3$.

the intestinal wall is in some way related to the phospholipids of the intestinal mucosa, it seems probable that a partial hydrolysis in experiment A would have been reflected in the specific activity of the phospholipids given off from the intestinal wall into the lymph.

The results of this investigation show that in the rat under the conditions of these experiments about 3 per cent of the active palmitic acid fed is transported in the lymph to the blood as phospholipid fatty acids. Thus the intestinal wall supplies a quantitatively important part of the blood phospholipids during fat absorption in the rat as has previously been indicated to be the case in the cat [1].

SUMMARY

The mechanism of the intestinal fat absorption has been studied with C^{14} labeled fat in rats with the intestinal lymph duct cannulated.

It has been found that:

1. Absorbed fat is mainly transported via lymphatic channels to the systemic circulation wether fed as glycerides or as free fatty acids.

2. Free fatty acids administered alone or together with glycerides appear in the lymph in glycerides and phospholipids. No free fatty acids or soaps appear in the lymph.

3. The intestinal wall supplies a quantitatively important part of phospholipids to the blood during fat absorption.

4. A complete hydrolysis of the fat in the intestinal lumen might occur in the rat.

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A Kinetic Study of the Reactions of Alkyl Esters of Arylsulphonic Acids with Water, Alcohols, Hydroxyl Ion, and Ethoxyl Ion

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Chemically, the esters of sulphonic acids differ greatly from those of carboxylic acids. The latter are stable when dissolved in the corresponding alcohol, and their hydrolysis in pure water is, in general, very slow, whereas the former react rather easily with water and also with alcohol,

$$RSO_2 \cdot OR' + H_2O \rightarrow RSO_2 \cdot OH + R'OH \quad (A)$$
$$RSO_2 \cdot OR' + R''OH \rightarrow RSO_2 \cdot OH + R'OR'' \quad (B)$$

These two reactions have been kinetically studied by several early workers [1,2], but, with one exception, at one temperature only. The present paper presents the results of a series of experiments carried out with some aromatic sulphonic esters at different temperatures.

EXPERIMENTAL

Chemicals. The esters were prepared by the method of Krafft and Roos [3] from the corresponding sulphonyl chlorides and alcohols, and purified by distillation at reduced pressure in an all-glass apparatus. The physical characteristics of the esters were:

Ethyl ester of benzenesulphonic acid, b. p. 152–152.5°/12 mm,
$d_{15°}$ 1.2213, $d_{20°}$ 1.2167, $d_{25°}$ 1.2124, $d_{40°}$ 1.2083.

Methyl ester of benzenesulphonic acid, b. p. 143°/10 mm, $d_{17°}$ 1.2730.

Ethyl ester of *p*-chlorobenzenesulphonic acid, b. p. 162°/12 mm, m. p. 23.5°.

Ethyl ester of *p*-toluenesulphonic acid, b. p. 176°/16 mm, m. p. 32–33°.

These values are in good agreement with the most reliable values reported in literature. The purity of esters was also checked by complete alkaline hydrolysis.

The water used as solvent in the experiments was ordinary distilled water, freed from carbon dioxide by suction of CO_2-free air through the water for several hours. The

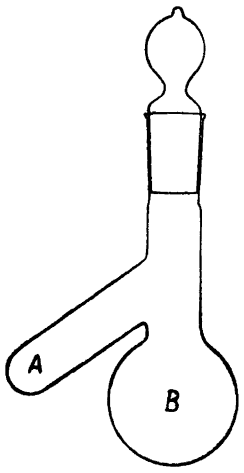


Fig. 1. Reaction vessel.

methanol, "Methanol acetonfrei", from Schering, was purified by the method of Lund and Bjerrum [4]. In the last distillation, however, instead of tribromobenzoic acid anhydrous copper sulphate was used. To get pure water-free ethanol, usual commercial 95 per cent ethanol was first dried by refluxing with calcium oxide, distilled, and treated by the method of Lund and Bjerrum. Acetone was Schering's "Aceton, reinst" which was purified by drying over potassium carbonate and subsequent slow distillation through an efficient column. It had a constant boiling point within 0.1°, and it did not give any reaction with potassium permanganate.

Benzenesulphonic acid used in some experiments was a specimen of Schering.

Method. The most kinetic experiments were carried out in glass-stoppered reaction vessels with two compartments *A* and *B* (Fig. 1). The vessel was weighed, and a few drops of ester were introduced into *A*; then it was weighed again. The difference between the two weighings gives the weight of the ester. A calculated amount of solvent was then measured by means of a pipette and a micro burette into the bulb *B*. The vessel was closed with the stop-cock and kept in the thermostat until the reactants had acquired its temperature. Then the reaction was started by mixing the reactants by vigorous shaking, the time being recorded. After a few seconds all of the ester had always dissolved. After suitable time intervals reaction vessels were taken from the thermostat and quickly cooled by putting in a mixture of ice and water; the acid formed was titrated, cresol red being used as indicator. At temperatures in the neighbourhood of and over the boiling point of the solvent the reactions were carried out in sealed ampoules of about 12 ml capacity.

In experiments where NaOH was used as catalyst, a measured amount of the NaOH-solution was placed in *B* and the reaction started as given above; in this case the reaction was stopped by an excess of 0.02 *N* hydrochloric acid and titrated back with 0.02 *N* baryta solution. The reactions between ethyl benzenesulphonate and ethoxyl ion were carried out in the same reaction vessels; 5 ml of 0.1 *N* solution of the ester in absolute ethanol were placed in *A*, and in *B* 5 ml of 0.1 *N* sodium ethylate solution (prepared by dissolving sodium in absolute ethanol), thermostated, and mixed by shaking. The reaction was stopped by an excess of 0.02 *N* hydrochloric acid and titrated back with baryta solution, using cresol red as indicator.

The thermostat was electrically controlled and its temperature could be kept constant within 0.02°. The thermometers were checked against standard thermometers.

The formation of ether in reaction B is clearly observed by smell when a concentrated ethanol solution of ethyl benzenesulphonate is heated in an ampoule at 50° for some hours and the ampoule is broken. No decomposition was observed when a solution of ethyl benzenesulphonate in dry acetone was boiled on a water bath for several hours under a reflux condenser.

Rate calculations. The solubility of sulphonic esters in water is very poor; however, in the experiments in pure water 0.01 *N* solutions could be used. In the other solvents the ester was 0.05 *N*. As water or alcohol is in such great excess that its concentration hardly changes during the course of the reaction, for reactions (A) and (B) the rate equation

$$dx/dt = k_1\ (a-x),\ \text{or}\ k_1 = 1/t \cdot \ln\ [a/(a-x)],$$

where $(a-x)$ is the concentration of the ester at time t, could be used. If p is the percentage change at time t, for calculation of the first order velocity constant, the equation may be written in the very convenient form

$$k_1 = \frac{2.303}{t} \log \frac{100}{100-p}$$

This formula gave a good constancy for k_1, as is exemplified by the following two typical runs:

Solvolysis of ethyl benzenesulphonate in pure water, a = 0.01 mole/liter, 60.05°.

t mins.	7	12	16	20	37	40	50	60
Percentage change	17.3	27.6	35.7	42.7	65.4	68.1	76.5	80.8
$10^4 k_1$ sec.$^{-1}$	4.55	4.50	4.61	4.64	4.77	4.77	4.82	4.58

Solvolysis of ethyl p-chlorobenzenesulphonate in abs. ethanol, a = 0.05 mole/liter, 75.00°

t mins.	20	36	50	72	85	95	112	122	130
Percentage change	12.1	21.0	27.8	36.8	42.7	45.6	51.2	53.9	56.4
$10^4 k_1$ sec.$^{-1}$	1.07	1.10	1.09	1.06	1.10	1.07	1.07	1.06	1.07

In the other runs the constancy of k_1 was of about the same order. To save space the runs are not given in detail.

In the presence of sodium hydroxide the reaction is

$$RSO_2OR' + OH^- \rightarrow RSO_2O^- + R'OH \qquad (C)$$

but we are also concerned with the reaction (A). Thus the over-all rate of reaction is given by the expression

$$dx/dt = k_1\ (a-x) + k_2\ (a-x)\ (b-x)$$

where $(a-x)$ is the concentration of the ester, and $(b-x)$ that of hydroxyl ions at time t, k_1 the first order rate constant for (A), and k_2 the bimolecular constant for (C). However, the reaction (C) is so much faster than (A) that if a relatively great concentration of the alkali is used, the latter reaction may be neglected. To facilitate calculations,

the experiments were conducted with equimolecular concentrations of ester and hydroxyl ion, and k_2 computed from the usual expression

$$k_2 = \frac{1}{t}\left[\frac{1}{a-x} - \frac{1}{a}\right], \text{ or } k_2 = \frac{100}{at}\left[\frac{1}{100-p} - \frac{1}{100}\right].$$

E. g. in water at 50° C, with the initial concentration $a = 0.01$ mole/liter, was obtained:

t mins.	4	7	9	11	13	16	19
Percentage change	15	23.6	27.8	32.3	35.7	42.4	45.1
$10^2 k_2$ l mole^{-1} sec.$^{-1}$	7.29	7.39	7.15	7.24	7.16	7.69	7.21

The mean of the series is $k_2 = 0.0730$. At this temperature k_1 is only 0.000482. However, to avoid a significant decrease of the ester concentration due to reaction (A), the runs were taken only to about 50 per cent change.

In similar manner, for evaluation of the velocity constant of the reaction between ethyl benzenesulphonate and sodium alcoholate in absolute ethanol:

$$C_6H_5SO_2OC_2H_5 + C_2H_5O^- \rightarrow C_6H_5SO_2O^- + (C_2H_5)_2O \qquad (D)$$

the reaction (B) can be omitted and the simple bimolecular formula used, as show the following values obtained at 40° C with equal initial concentrations of both rectants:

t mins.	30	45	60	75	90	110	120	170
Percentage change	15.2	21.2	27.0	31.4	35.2	39.2	41.2	50.4
10^3 k_2 l mole^{-1} sec.$^{-1}$	1.99	2.00	2.05	2.04	2.01	1.94	1.94	2.00

The mean is $k_2 = 2.00 \cdot 10^{-3}$. For the reaction (B) is at this temperature $k_1 = 1.40 \cdot 10^{-6}$. Morgan and Cretcher [5], in their investigation on the reaction, also used this simplified method for the evaluation of k_2.

The rate constants were determined at four or five temperatures and are means of 6 to 10 determinations representing 10 to 60 percentage change. From the k-values, corresponding different temperatures, the parameters A and E of the Arrhenius equation

$$k = Ae^{-E/RT}$$

were calculated using the method of the least squares. The Arrhenius equation was always obeyed within the experimental error. The results are summarized in Tables 1—3.

Table I. Solvolysis of sulphonic esters.

Ester	Solvent	Temp. °C	$10^5 k_1$ sec^{-1}.	*E* cal	$\log_{10} A$
$C_6H_5SO_2OEt$	Pure water	25.0	1.03	22 030	11.13
		30.0	1.60		
		40.0	5.77		
		50.0	17.4		
		60.0	47.3		
$C_6H_5SO_2OEt$	Abs. methanol	40.0	0.263	22 090	9.84
		50.0	0.800		
		60.0	2.29		
		74.9	9.62		
		80.0	14.2		
$C_6H_5SO_2OEt$	Abs. ethanol	40.0	0.139	22 000	9.50
		50.2	0.421		
		60.0	1.22		
		74.9	4.82		
$C_6H_5SO_2OEt$	0.01 *N* $C_6H_5SO_2OH$ in water	40.0	6.41	22 080	11.22
		50.0	18.6		
		60.0	52.9		
		75.0	226		
$C_6H_5SO_2OEt$	0.05 *N* KCl	40.0	6.13	22 130	11.24
		50.0	19.3		
		60.0	52.9		
		64.8	83.4		
$C_6H_5SO_2OEt$	Acetone-water containing 56 weight per cent acetone	40.0	0.291	21 730	9.64
		50.0	0.868		
		60.0	2.42		
		75.0	9.74		
$C_6H_5SO_2OMe$	Pure water	25.0	1.15	21 030	10.48
		40.0	6.45		
		50.1	18.5		
		60.0	48.4		
p-$CH_3.C_6H_4SO_2OEt$	Abs. ethanol	40.0	0.0875	22 320	9.52
		50.0	0.265		
		64.8	1.25		
		75.0	3.17		
p-$CH_3.C_6H_4SO_2OEt$	56 per cent acetone	40.0	0.177	21 860	9.58
		50.0	0.530		
		64.8	2.39		
		75.0	6.11		

Table 1 continued.

p-Cl.$C_6H_4SO_2OEt$	Abs. ethanol	40.0	0.300	22 670	10.31
		50.0	0.919		
		60.0	2.83		
		75.0	11.5		
p-Cl.$C_6H_4SO_2OEt$	56 per cent acetone	40.0	0.749	21 250	9.71
		50.0	2.16		
		60.0	6.05		
		75.0	23.0		

Table. 2. The reaction $C_6H_5SO_2OEt + NaOH \rightarrow C_6H_5SO_2ONa + EtOH$ in water.

Temp. °C	$10^3 k_2$ l mole^{-1}sec.$^{-1}$	E cal	$\log_{10} A$
40.0	9.20	21 120	12.70
50.2	25.2		
60.0	74.1		
75.0	273.0		

Table 3. The reaction $C_6H_5SO_2OEt + EtONa \rightarrow C_6H_5SO_2ONa + EtOEt$ in absolute ethanol

Temp. °C	$10^3 k_2$ l mole^{-1} sec.$^{-1}$	E cal	$\log_{10} A$
25.0	0.372	20 880	11.87
40.0	2.04		
49.9	5.78		
60.1	15.2		

DISCUSSION

General. From Table 1 we see that the specific rate of the solvolytic reaction of ethyl benzenesulphonate with water is about 20 times as great as that of the reaction with methanol and about 40 times as great as that of the reaction with ethanol. The solvolysis of methyl ester is a little faster than that of ethyl ester. Chlorine as substituent in the aromatic nucleus has an accelerating influence, while methyl group reduces the reaction velocity, as already demonstrated in Demeny's study of the effect of a substituent upon the rate at which an alkyl sulphonate hydrolyzes [1k]. The independence of k_1 on the acid formed in the reaction and the experiments carried out in the presence of added benzenesulphonic acid show that the hydrolysis is not catalyzed by acids. This is consistent with previous observations [1e,g,6]. However, in the

presence of added sulphonic acid k_1 is a little greater than in pure water. This result and the observation of McCleary and Hammett [6] that in 60 per cent dioxane-water k_1 slightly increases during the reaction is easily explained by the dependence of activity coefficients on the concentration.

One conspicuous point is that the activation energies of the solvolytic reactions of ethyl benzenesulphonate with water, methanol, and ethanol are practically equal, about 22 000 cal, and that the differencies in the k-values are solely due to differencies in the parameter A of the Arrhenius equation. For the (bimolecular) reactions between ethyl benzenesulphonate and hydroxyl ion (in water) or ethoxyl ion (in absolute ethanol) the activation energy is only about 1 000 calories lower (Tables 2 and 3). For the latter reaction Morgan and Cretcher [5] found that $E = 21\ 750$ cal and $\log A = 12.56$, which values are somewhat greater than those of the Table 3. Morgan and Cretcher estimated k_2 at two temperatures only.

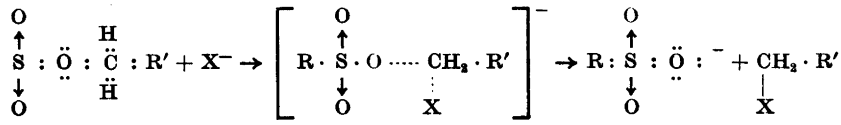
Concerning the parameters of the Arrhenius equation, the alkaline hydrolysis of ethyl benzenesulphonate differs greatly from that of ethyl benzoate. As is generally the case for the alkaline hydrolysis of carboxylic esters, the activation energy and frequency factor have markedly low values for the latter reaction; in water $E = 12\ 700$ cal and $\log A = 7.76$ (unpublished results). For the alkaline hydrolysis of ethyl benzenesulphonate in water $E = 21\ 100$ cal and $\log A = 12.70$ or $A = 5 \times 10^{12}$. This value of A is somewhat greater than the theoretical collision frequency $Z \approx 3 \times 10^{11}$, so that in the expression $A = PZ$ the probability factor P is about 17. For the reaction between ethoxyl ion and ethyl benzenesulphonate $E = 20\ 880$ cal and $\log PZ = 11.87$ or $P \approx 2$. For the alkaline hydrolysis of ethyl benzoate in water we obtain from $\log PZ = 7.76$ the value $P \approx 2.\ 10^{-4}$.

Acetone in the solvent reduces the reaction velocity very much. This effect is entirely due to a decrease of the frequency factor, the activation energy remaining practically constant or rather decreasing somewhat.

The mechanism of the reaction. The values of E and A indicate that the mechanism of the hydrolysis of sulphonic esters differs greatly from that of carboxylic esters. The hydrolysis carried out in water enriched in ^{18}O has shown that in the latter case the cleavage is in the C—OR link [7]. Sulphonic esters, again, hydrolyse, as suggested even as early as 1912 by Ferns and Lapworth [8], and as the experiments [9] with ^{18}O show, with O—C and not S—O bond-fission. Hinshelwood, Laidler, and Timm [10] have shown, in a theoretical treatment, that one 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, *i. e.*, to stretch it from its normal length to the length in the transition state. In alkaline hydrolysis

of carboxylic esters the repulsion is least at the carbonyl carbon, and therefore the hydroxyl ion attacks this atom; the over-all reaction is of the type where the repulsion energy governs the situation [10]. Since the repulsion is weak, the activation energy is small.

In the sulphonic acids two oxygen atoms are bound to sulphur with semipolar links, the two lone electron pairs of the sulphur atom being shared with oxygen. Thus the sulphur atom carries a positive charge. However, since no double bond exists and consequently the electron displacements around the central atom are impossible, the hydroxyl ion can not be attached to the sulphur and the reaction takes place by another mechanism. The positive charge of the sulphur atom causes electrons in the chain to be drawn towards the sulphur: S ← O ← C, which renders the carbon atom of the alkyl group weakly positive. Therefore, the hydroxyl ion adds at this atom. Because, however, the attraction between the negative ion and the carbon atom is small, *i. e.*, the repulsion large, the activation energy is large. Reactions (C) and (D) may accordingly be represented *cf.*[11,9,12]



where X^- is OH^- or $C_2H_5O^-$ (or any nucleophilic reagent). Thus here the ethereal oxygen with the bond electrons follows the acid side and this radical comes off as an ion. The experiment carried out in the presence of KCl shows that the reaction with Cl^- is extremely slow *cf.*[6].

If the standard state is taken as corresponding to a concentration of 1 mole per liter, we obtain by the formula

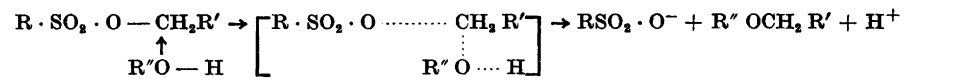
$$A = e\frac{kT}{h}e^{\Delta S^{\neq}/R}$$

for the reaction (C) at 300° K from $A = 5.0 \times 10^{12}$ for the standard entropy of activation $\Delta S^{\neq} = -2.44$ cal deg.$^{-1}$ mole^{-1}, whereas the value $A = 2.8 \times 10^{11}$, which equals the theoretical collision frequency Z, gives $\Delta S^{\neq} = -8.2$ cal deg.$^{-1}$ mole $^{-1}$. For the reaction (D) we obtain from the experimental result $A = 7.4 \times 10^{11}$ $\Delta S^{\neq} = -6.6$ cal deg.$^{-1}$ mole $^{-1}$. These values of $\Delta S^{\neq}$ indicate that the transition state in reactions (C) and (D) has a loose structure [13], the bond between the ethereal oxygen and the alkyl carbon being largely stretched. For the alkaline hydrolysis of ethyl benzoate in water

the entropy of activation has the very negative value $\Delta S^{\neq} = -24.9$ cal. deg.$^{-1}$ mole^{-1}, which is most easily understood in terms of the electromeric displacement and of the change in solvation in the formation of the transition state.

As stated above, the usual mode of alkaline hydrolysis of carboxylic esters involves a bimolecular attack of carbonyl carbon by hydroxyl ion and acyl-oxygen fission. However, recent work by Kenyon, Balfe, and their collaborators [14] shows that in some cases in alkaline hydrolysis of carboxylic esters the mechanism which involves alkyl-oxygen fission, as in the hydrolysis of sulphonic esters, is operating.

For the solvolysis a corresponding mechanism can be presented:



R'' is a hydrogen atom or an alkyl group. Here it is the uncharged $R''OH$ which attacks the carbon atom of the CH_2R' group, and since the electrostatic attraction between this carbon atom and the dipole is less than between this carbon and a negative ion, the experimental result that for the solvolysis the activation energy is somewhat greater than for the reactions (C) and (D), is readily understandable. It is also natural that, on the one hand, the activation energy is nearly the same for (C) and (D), and the near-constancy of E for the solvolytic reactions on the other hand. In a solvolytic reaction there is a considerable re-arrangement of energy among the various degrees of freedom to accompany the formation of the activated complex, *i. e.*, a small probability of formation of the activated complex, and, therefore, the entropy of activation is appreciably negative, the more the more complicated is the reagent molecule. Thus, the standard state being a concentration of 1 mole per liter, we obtain from the bimolecularly calculated log A values for the solvolysis of ethyl benzenesulphonate with water, methanol, or ethanol for $\Delta S^{\neq}$ the values — 17.6, — 22.8 or 23.6 cal deg.$^{-1}$ mole^{-1}.

Acetone reduces remarkably the rate of the reaction, as is seen from the following values concerning the solvolysis of ethyl benzenesulphonate with water:

water $(k_2)_{50}^{\circ} = 29.0 \cdot 10^{-7}$	$E = 22\,030$ cal	log A =	9.68
56 per cent acetone $3.89 \cdot 10^{-7}$	21 730		8.29

Here k_2 is the bimolecular rate constant of reaction (A), calculated from the Arrhenius equation and log A also refers to the bimolecular reaction. The retarding effect is wholly in the A term, with E remaining unchanged or

ratl er decreasing a little. The decrease of A, or a more negative value of the entropy of activation, is probably explained in terms of the solvation entropy[13]. The dipole moment of acetone (2.72 D) is much larger than that of water (1.84 D), and thus the acetone dipoles compete successfully with water dipoles in the solvation of reagents and activated complex, and this might make the entropy of activation more negative, since it is possible that it renders the rearrangement of the energy in the formation of transition state more complex. The eventual small decrease of E might be explained by an inrease of attraction between R″OH and CH_2R' as the dielectric constant of the medium decreases.

A comparison of reactions of substituted ethyl benzenesulphonates with ethanol and water (56 per cent acetone) is given in Table 4.

Table 4. The substituent influences.

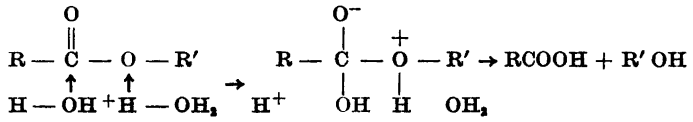
	Ethanol			56 per cent acetone		
	$10^7\ (k_2)_{50}°$	E cal	$\log_{10}A$	$10^7(k_2)_{50}°$	E cal	$\log_{10}A$
p-CH_3	0.980	22 320	8.09	2.38	21 860	8.24
H	1.58	22 000	8.07	3.89	21 730	8.29
p-Cl	3.43	22 670	8.87	9.76	21 250	8.36

Because the benzene ring which carries the substituents is remote from the seat of reaction, the transmission of the substituent influences must be considerably damped. A substituent which attracts electrons (chlorine) raises, a substituent which repels electrons (methyl group) lowers the velocity of reaction. In acetone water the changes in velocity are principally accounted for by change in the activation energy. In the reaction with ethanol the changes in E and A are irregular.

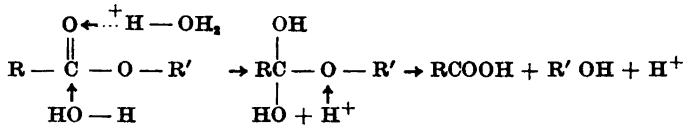
From the alcohol side the substituent influence is, because of the close proximity of the seat of reaction, more powerful, as a comparison of water hydrolysis of methyl and ethyl benzenesulphonates shows. It is true that the rate constants do not differ much, but for the methyl ester E is 1 000 cal lower and A considerably smaller than for the ethyl ester, their changes thus operating in opposite directions. Substitution of a hydrogen atom in CH_3 with CH_3, which repels electrons, makes the C-atom of the former less positive and thus the repulsion of an approaching water molecule larger; accordingly, the activation energy will be increased. On the other hand, the increase of the activation energy causes the dotted bonds in the transition state to be more stretched, whence the entropy of activation in case of the ethyl ester is less negative, *i. e.*, A is larger.

Now the question arises whether in the reactions considered the most important step involves the overcoming of the repulsion of the approaching reagent molecule, or whether the stretching of the bond SO—C, which is to be disrupted, is the controlling step, or, perhaps, whether the reaction proceeds by a compromise between these two mechanisms. Several points discussed above seem to denote that the reaction is of the type in which probably both repulsion energy and bond-stretching energy are of importance. To these facts may be added the small difference in activation energies between reactions (C, D) and (A, B). More general conclusions must, however, be postponed to a further investigation, which is in progress.

A reaction catalyzed by hydrogen ion would take place with another mechanism than that described above for the uncatalyzed solvolysis. A mechanism similar to that in the acid hydrolysis of carboxylic esters would here, too, be the only possible. According to the Lowry mechanism [15], the proton combines with the ethereal oxygen, and the second step involves attachment of hydroxyl ion from water to carbonyl carbon:



However, since the mesomerism of the ester grouping renders carbonyl oxygen fractionally negative and ethereal oxygen fractionally positive, it has also been suggested [16] that the proton is more likely to be attached to the former,and then hydroxyl ion from water is attached to the now very positive carbonyl carbon:



Because, owing to the lack of double bond in a sulphonic ester molecule, the electronic displacements necessary to either of these processes are impossible, the corresponding reaction does not occur, and consequently the hydrogen ion has no catalytic effect on the decomposition of sulphonic esters.

SUMMARY

The reactions of several alkyl benzenesulphonates with water, alcohols, hydroxyl ion, and ethoxyl ion were kinetically investigated. The results and the mechanisms of reactions which are definitely different from that of the hydrolysis of carboxylic esters are discussed.

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The Alcoholysis of Some Aromatic Sulphonyl Chlorides

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The work of Tommila and (Miss) Lindholm [1] has shown that for the solvolysis of ethyl benzenesulphonate the activation energies are within experimental error constant and that the differences in reaction velocities are entirely due to different frequency factors. The present paper is an attempt to elucidate the alcoholysis of sulphonyl clorides. The hydrolysis of sulphonyl chlorides has been investigated by Berger and Olivier [2], and Hedlund [3].

On account of the practical importance of the alkyl esters of aromatic sulphonic acids as alkylating agents and because of the difficulties in the other preparation methods, the reactions between the different sulphonyl chlorides and alcohols are, concerning the sort and quantities of the products, cleared up fairly extensively. The reaction

$$\begin{array}{l} \mathrm{O} \\ \uparrow \\ \mathrm{RS} - \mathrm{Cl} \\ \downarrow \\ \mathrm{O} \end{array} + \mathrm{R'OH} \rightarrow \begin{array}{l} \mathrm{O} \\ \uparrow \\ \mathrm{RS} - \mathrm{OR'} \\ \downarrow \\ \mathrm{O} \end{array} + \mathrm{HCl} \qquad \text{(A)}$$

is rather slow at and below room temperature [4], but the alcoholysis of the ester formed,

$$\mathrm{RSO_3R'} + \mathrm{R'OH} \rightarrow \mathrm{RSO_3H} + \mathrm{R'OR'} \qquad \text{(B)}$$

obtains first at high temperatures a velocity [1] to disturb the kinetic investigation of the first reaction.

Probably only Goubau [5] has kinetically investigated the alcoholysis of sulphonyl chlorides.

EXPERIMENTAL

Materials. — Benzenesulphonyl chloride: A product prepared according to *Organic Syntheses*, Part XV, was distilled twice in an all-glas apparatus at reduced pressure; b. p. 105°/6.5 mm.

p-Chlorobenzenesulphonyl chloride, prepared according to Ullmann und Korselt [6] and crystallised several times from light petroleum (b. p. 40—50°), had after some days' staying in a vacuum desiccator with solid KOH m. p. 53.8—53.9°.

p-Toluenesulphonyl chloride: Schering's specimen "reinst für wissenschaftliche Zwecke", was crystallised twice from light petroleum and had after some days' staying in a vacuum desiccator m. p. 69.1°.

The alcohols were commercial products, purified by the method of Lund and Bjerrum as described before [1].

Acetone: A commercial product of high purity was dried over potassium carbonate and then slowly distilled through a Widmer column.

Rate measurement. — The proceeding of the reaction (A) was followed by measuring after appropriate time intervals the amount of HCl formed. The experiments were conducted in reaction vessels described before [1]. About 100 mg (or 200 mg) of sulphonyl chloride were accurately weighed into the side tube and by means of a burette a calculated amount of alcohol (between 6 and 30 ml) was measured into the bulb, so that the initial concentration of the chloride was 0.05 mole/liter (in some series 0.1 or 0.2 mole/l). The reaction vessels were kept in the thermostat until the reactants had acquired its temperature (at 0° about 30 mins., at 15° and 25°, to avoid the attack of alcohol vapour upon the chloride, only 5 mins.) and the reaction was started by shaking the tube. The chloride always dissolved at once.

Of the analytical methods of which several were proved, the best results were obtained by a slight modification of the procedure described by Goubau [5]. The reaction was stopped by sudden pouring of the reaction mixture into 50 ml of acetone which was precooled to 0° C and the reaction vessel rinsed with 20 ml of 0° acetone. Then the acid was quickly titrated with $N/50$ baryta solution, cresol red as indicator. All titrations were carried to a standard tint. The acetone always required a few tenths of ml of $N/50$ alkali, which were taken into account in the calculations. The precence of a large excess of acetone and the low temperature make the reaction with alcohol and water so slow that titration is possible. However, it is necessary to operate very quickly and the titration is to be stopped at once when the standard tint is reached, owing to the extremely rapid decomposition caused by an excess of alkali.

The reaction velocities were determined at 25°, 15° and 0°. For 25° and 15° the thermostat could be regulated to within 0.01°. Experiments at 0° were carried out in a well-isolated vessel which contained crushed ice and water. All thermometers were checked against standard thermometers.

Calculation of rate constants. — The course of the reaction is expressed by the usual first order formula $dx/dt = k_1 (a-x)$; this may be exemplified by the following typical series, obtained for benzenesulphonyl chloride in absolute ethanol at 25° C, the initial concentration being 0.05 mol/liter:

Time mins.	75	129	135	195	220	318	420	669	947	1470
Percentage change	9.2	13.7	15.8	20.6	23.8	32.3	39.6	55.5	68.2	85.3
$10^5 \cdot k_1$ sec.$^{-1}$	2.13	1.89	2.12	1.97	2.06	2.05	2.00	2.01	2.02	2.17

In every case at least six, but usually more measurements were made at each temperature.

RESULTS AND DISCUSSION

Rate constant and concentration. — Goubau [5] found that the first order formula does not give constant values for k, but the velocity constant increases with the time. He therefore concluded that the hydrogen ion formed catalytically accelarates the reaction, and, in fact, he got higher values for k when HCl was added to the reaction mixture. Hedlund, on the contrary, came to the result that HCl somewhat *decreases* the rate of *hydrolysis* of sulphonyl chlorides, but the influence is of the same order of magnitude as that of neutral salts. To reinvestigate any influence of hydrogen ion upon the alcoholysis we carried out experiments with benzenesulphonyl chloride in absolute ethanol using different concentrations. Our results are given in Table 1.

Table 1. The alcoholysis of benzenesulphonyl chloride in absolute ethanol at 25°, initial concentration a. (The values of k are means of about eight observations.)

a mole/liter	0.01	0.05	0.1	0.2
$10^5\, k_1$ sec.$^{-1}$	2.028	2.045	2.137	2.172

We see that k_1 increases a little with the initial concentration of the sulphonyl chloride, but the effect is a very feeble one. In the measuring series carried out with the same initial concentration it can be neglected, as shown by the example given above. On the ground of these results there is no evidence of hydrogen-ion catalysis in the alcoholysis of sulphonyl chlorides. The feeble increase of k_1 with the initial concentration can be explained by relating the rate constant to that in very dilute solution by using activity coefficients.

Influence of light. — Two series of experiments were carried out with *p*-chlorobenzenesulphonyl chloride in ethanol at 25.0° C, the one series in bright sun light, the other in dark. The values for k_1 were $2.40 \cdot 10^{-5}$ in the sun light and $2.38 \cdot 10^{-5}$ in dark; thus no influence of light was noted.

Rate constants and parameters of the Arrhenius equation. — The first order rate constants k_1, as mean values of the measuring series, are given in Table 2. The Table also includes the values of activation energy E and frequency factor A, according to the simple equation of Arrhenius,

$$k = Ae^{-E/RT}$$

calculated from the k_1-values at different temperatures by the method of least squares. The plot of experimental values of log k_1 against $1/T$ showed that the Arrhenius equation was followed within the experimental error in every case.

Table 2. The values of k_1, E and log A. In all cases the initial concentration of the sulphonyl chloride a = 0.05 mole/liter.

	Temp., °C	$10^6 \cdot k_1$ sec.$^{-1}$	E cal	$\log_{10}A$
$C_6H_5SO_2Cl$, methanol	0.0	7.79	16 670	8.23
	15.0	37.5		
	25.0	103		
$C_6H_5SO_2Cl$, ethanol	0.0	1.47	16 980	7.76
	15.0	7.26		
	25.0	20.5		
$C_6H_5SO_2Cl$, *n*-propanol	0.0	0.888	17 090	7.63
	15.0	4.60		
	25.0	12.5		
p-Cl · $C_6H_4SO_2Cl$, ethanol	0.0	1.58	17 530	8.23
	15.0	8.49		
	25.0	23.7		
p-CH_3 · $C_6H_4SO_2Cl$, ethanol	0.0	1.27	17 770	8.32
	15.0	6.85		
	25.0	19.8		

Mechanism of the reaction. — It is seen from Table 2 that chlorine as a substituent in the aromatic nucleus somewhat enhances and the methyl group somewhat reduces the reaction velocity. However, the activation energy E is clearly higher for the *p*-CH_3 *and* *p*-Cl compounds than for the unsubstituted chloride. The introduction of an electron-attracting substituent *e. g.* chlorine into the sulphonyl chloride molecule will (*a*) decrease the electron density at the S-atom, and (*b*) strengthen S-atom's hold on the S—Cl bond electrons, or decrease the ionic character of this bond. These two effects act in opposite directions. The first effect, because of lowering of the repulsion of an approaching alcohol molecule, tends to decrease the activation energy, whereas the second one tends to increase it. An electron-repelling substituent (*e. g.* methyl group) acts in opposite direction. The above-mentioned experimental results show that in the alcoholysis of *p*-chloro benzenesulphonyl chloride the effect of the substituent on E is governed mainly by the strength of the S—Cl bond, whereas in the alcoholysis of *p*-toluenesulphonyl chloride the repulsion energy is the most important factor. This indicates that in the alcoholysis of unsubstituted benzenesulphonyl chloride the S—Cl bond strength and the repulsion energy contribute nearly equally to the activation energy.

In both the cases the substituent also causes an increase of the parameter A of the Arrhenius equation, and therefore the reaction velocity, despite the increase of activation energy, is either only a little decreased (*p*-toluenesulphonyl chloride) or is increased (*p*-chlorobenzenesulphonyl chloride). This increase of A denotes, according to the theory of absolute reaction rates, that the entropy of activation is less negative than in the alcoholysis of the unsubstituted compound.

As to the dependence of the reaction velocity on the alcohol, the table shows that the rate of the alcoholysis in the temperature range investigated changes in the order: methanol > ethanol > propanol. This is partly due to changes in E, partly to changes in A, both influencing in the same direction. As usual in bimolecular reactions, the frequency factors are smaller or the entropies of activation more negative for reactions involving more complex molecules having a large number of degrees of freedom [7]. The differences in the activation energies are probably due to different repulsion energies between the reactants.

A comparison of our results with the work of Hedlund [3] concerning the hydrolysis of sulphonyl chlorides, from which some data are taken into Table 3, shows that the hydrolysis in pure water is much faster than the alcoholysis in pure alcohols.

Table 3. Hydrolysis of sulphonyl chlorides (Hedlund), 25° C.

RSO_2Cl	Water			42 per cent acetone		
	k_1 sec.$^{-1}$	E	log A	k_1 sec.$^{-1}$	E	log A
p-$CH_3 \cdot C_6H_4$–	0.00385	17 690	12.35	0.000233	14 270	8.62
C_6H_5–	0.00307	17 420	12.07	0.000303	14 430	8.85
p-$O_2N \cdot C_6H_4$–	0.00272	18 060	12.46	0.00174	12 870	8.46
m-$O_2N \cdot C_6H_4$–	0.00144	18 810	12.74	0.000717	13 780	8.83

Methyl substitution increases, whereas the electron-attracting nitro group decreases the rate of hydrolysis in pure water, but in 42 per cent acetone the effect is reversed. Accordingly, in pure water, the substituent influences are contrary to those in the alcoholysis in pure alcohols. Concerning the activation energies, however, the effects act in same direction.

For the hydrolysis in water the activation energy is probably a little higher than for the alcoholysis in pure alcohols, but the frequency factors are much higher. Accordingly, the entropies of activation are much more negative for

the alcoholysis than for the hydrolysis. This is in harmony with general experience. In the first case one of the reactants is more complex, and in such cases there is a considerable rearrangement of energy among the degrees of freedom, which causes the formation of the activated complex to be followed by a large decrease in the entropy and a decrease in the reaction rate.

The mechanism proposed above is the S_N2 mechanism of Ingold and Hughes [8]. However, because an electron-repelling substituent (methyl group) increases and an electron-attracting substituent (nitro group, halogen) decreases the velocity of reaction in water [3], it is possible that the hydrolysis in water proceeds, at least partly, by a S_N1 mechanism, or is a two-stage reaction

$$RSO_2Cl_s \rightarrow RSO_2^+{}_s + Cl_s^- \qquad \text{slow}$$

$$RSO_2^+{}_s + HOH \rightarrow RSO_3H_s + H_s^+ \qquad \text{fast}$$

The suffix s means that the component is solvated. It is reasonable to assume that water has a more powerful affinity for chloride ion than alcohol and so facilitates ionisation of the sulphonyl chloride.

SUMMARY

The alcoholysis of some sulphonyl chlorides has been kinetically investigated. The reaction mechanism is discussed.

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Action of Strong Acids on Acetylated Glycosides

IX.* Transglycosidation of 6-Glucose β-Maltoside

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When starch is hydrolyzed by "malt amylase", the parts of the molecule which contain the 1, 6-linkages are attacked much more slowly than the remainder of the molecule and give rise to the so-called *limit dextrins*, which are polysaccharides containing these 1,6-linkages [1]. The simplest saccharide of this type, *iso*-maltose, has recently been isolated in a crystalline state from amylopectin hydrolysate [2]. A *limit dextrin* with three glucose units has been isolated by Örtenblad and Myrbäck [3], and Myrbäck and Ahlborg [4] have shown that this trisaccharide contains one maltose and one *iso*-maltose bond. There are accordingly two possible structures for this substance, namely 6-glucose α-maltoside and 4-glucose α-*iso*-maltoside, the former of which could conceivably be prepared by a method analogous to the synthesis of *iso*-maltose [5]. In the present paper the synthesis of 6-glucose α-maltoside by such a method is described.

β-6-Glucose β-maltoside hendecaacetate was prepared by the condensation of maltose bromide heptaacetate and β-1,2,3,4-glucose tetraacetate. In order to transform the β-glycosidic linkage into the α-form, the product was treated with titanium tetrachloride in chloroform. In this process the acetoxyl group at carbon atom 1 was simultaneously replaced by chlorine, but by subsequent treatment with mercuric acetate in acetic acid, this substitution was reversed. The β-6-glucose α-maltoside hendecaacetate was obtained as an amorphous powder, the specific rotation of which varied between + 85° and + 97° for different preparations. From the values for the specific rotation of β-6-glucose β-maltoside hendecaacetate (+ 42°), β-gentiobiose octaacetate (— 5°) and β-*iso*-maltose octaacetate (+ 98°), the specific rotation of pure β-6-glucose

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α-maltoside hendecaacetate may be estimated to be about + 114°. A similar calculation but based on the specific rotations of β-maltotriose hendecaacetate (+ 86°) [6], β-*iso*-maltose octaacetate and β-maltose octaacetate (+ 63°), gives the value + 111°. The low rotation exhibited by the amorphous β-6-glucose α-maltoside hendecaacetate indicated a low state of purity. The substance was deacetylated and the free trisaccharide obtained as a slightly yellow, amorphous powder which had a specific rotation of + 128°. By paper partition chromatography it was demonstrated that the sample contained not only trisaccharide but also di- and monosaccharides *, but from the intensity of the spots it was evident that the trisaccharide was the chief constituent. The trisaccharide fraction itself almost certainly contained a small amount of unchanged 6-glucose β-maltoside and possibly also altrose derivatives as by-products of the transglycosidation reaction (Compare Part VIII [7]). From analogy with the *iso*-maltose synthesis, however, one may assume that at least 50 % of the amorphous product consists of the desired substance, 6-glucose α-maltoside.

EXPERIMENTAL

β-6-Glucose β-maltoside hendecaacetate

A mixture of β-1,2,3,4-glucose tetraacetate (12 g), freshly prepared silver oxide (11 g), drierite (30 g) and absolute chloroform (60 ml) was shaken mechanically in a brown bottle for one hour. A solution of iodine (225 mg) and amorphous maltose bromide heptaacetate (21 g) in absolute chloroform (60 ml) was added in ten portions during the next hour, and the shaking continued overnight. The mixture was then filtered through a layer of Celite and the filter well washed with chloroform. The combined chloroform solutions were washed with a small amount of sodium thiosulfate solution and with water, dried over calcium chloride and concentrated under reduced pressure. The residue was dissolved in boiling ethanol (450 ml) and on cooling almost pure β-6-glucose β-maltoside hendecaacetate (12,8 g), melting at 229—230° **, crystallized out as fine needles. One recrystallization raised the melting point to 233—234°; further recrystallizations did not change this value. The substance showed $[\alpha]_D^{20}$ + 42° (chloroform, $c = 2$).

$C_{40}H_{54}O_{27}$ (966.8)	Calc.	C	49.7	H	5.63
	Found	»	49.8	»	5.66

* We are indebted to Fil. lic. Gunnar Neumüller, Biokemiska Institutet, Stockholms Högskola, for carrying out the chromatographic analysis.

** All melting points uncorrected.

β-6-Glucose α-maltoside hendecaacetate

β-6-Glucose β-maltoside hendecaacetate (5 g) was dissolved in absolute chloroform (70 ml) and titanium tetrachloride (6 g) in chloroform (50 ml) was added, whereupon a yellow precipitate was formed. The mixture was refluxed on a glycerol bath, kept at about 70°, for five hours and then after cooling poured into ice water (500 ml). The yellow precipitate dissolved and the almost colourless chloroform layer was separated, washed with ice water (4 × 400 ml), dried over calcium chloride and concentrated under reduced pressure. The residual sirup was dissolved in a solution of mercuric acetate (4 g) in acetic acid (40 ml). After two hours the solution was poured into water (500 ml) and extracted with chloroform (2 × 50 ml). The chloroform solution was washed with sodium hydrogen carbonate solution and water, dried over calcium chloride and concentrated under reduced pressure. The residual sirup was dissolved in boiling ethanol (300 ml) and on cooling unchanged starting material (3.0 g) of m.p. 231—232° precipitated. The mother liquors were concentrated to 5 ml and a further amount of the starting material (0.1 g) was recovered. When the remaining ethanolic solution was poured into water an almost colourless, amorphous powder (1.1 g) precipitated which resisted all attempts to obtain it in a crystalline state. After treatment with acetic anhydride in pyridine, a further amount of rather impure starting material (0.3 g, m.p. 223—224°, $[\alpha]_D^{20}$ + 46°) could be recovered, together with an amorphous powder (0.5 g) which had the specific rotation of + 97°, the chief constituent of which should be β-6-glucose α-maltoside hendecaacetate.

6-Glucose α-maltoside

The amorphous β-6-glucose α-maltoside hendecaacetate described above (1.0 g) was dissolved in absolute methanol (30 ml), the solution chilled to 0° and 0.5 N sodium methylate in methanol (1.5 ml) added. The solution was kept at 0° for 24 hours and was then diluted with water (100 ml). The solution was freed from ionic material by passing through Amberlite resins IR-120 and IR-4B and was then concentrated to a sirup under reduced pressure. The residual water was removed by repeated stirring with absolute ethanol and evaporating to dryness at room temperature in a vacuum desiccator containing calcium chloride. All attempts to crystallize the amorphous product have failed.

Yield 0.45 g. $[\alpha]_D^{20}$ + 128° (water, c = 2).

$C_{18}H_{32}O_{16}$ (504.4)	Calc.	C	42.9	H	6.40
	Found	»	42.0	»	6.45

SUMMARY

A potential *limit dextrin*, 6-glucose α-maltoside, has been prepared by transglycosidation of the corresponding β-glycoside. It could not be obtained in a state of purity.

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Short Communications

The Solubility of Calcium Oxalate Monohydrate in Ammonium Oxalate Solutions at 25° C

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In the course of an investigation on the separation of traces of calcium by precipitation with strontium oxalate as a collector, it was soon found that one of the chief sources of error was the unexpectedly high blank obtained from the ammonium oxalate solution. The solubility of calcium oxalate in ammonium oxalate solutions does not seem to have been determined. Calculation of the solubility from the solubility product may give erroneous results on account of complex formation, this being the case with regard to magnesium oxalate. A determination of the solubility seems therefore to be warranted.

Experimental. Calcium oxalate monohydrate was prepared in two different ways: Preparation A was precipitated in an excess of calcium, Preparation B in an excess of ammonium oxalate. The precipitation was performed at room temperature and the resulting very finegrained precipitate recrystallized from slightly acidic solution by heating for 24 h at 100° C (Kolthoff and Sandell [1], Mc Comas and Rieman [2]). The preparations were kept under water. No significant difference in the solubilities of these two preparations in either water or ammonium oxalate solutions was obtained.

About 0.25 g of the preparation was added to 250 ml of solution and stirred for 1–2 h at 25.0 ± 0.1° C. Preliminary experiments showed, in agreement with Mc Comas and Rieman's [2] statement as to the dissolution velocity of calcium oxalate in water, that the solution equilibrium is rapidly established and no change is obtained after 1 hour. The solution was sucked off through a porcelain filter stick; the first portion of the filtrate was rejected. 200 ml of the solution were evaporated to dryness in a platinum dish and the ammonium oxalate removed by heating. The residue was dissolved in dilute hydrochloric acid, evaporated to dryness and again dissolved in 10.0 ml 0.3 *M* hydrochloric acid. When determining the solubility in water, only 100 ml of the filtrate were taken for analysis and the final volume was 25.0 ml.

The calcium concentration of the solutions was determined by flame spectrography of the 4227 Å line according to Lundegårdh [3]; the sensitivity of the modification used was such that a density of 0.1 on the photographic plate was obtained from a solution, $2 \cdot 10^{-5}$ *M* in calcium. No blank was obtained from the reagents which had been specially purified.

Discussion of the results. The results are given in Table 1. The solubility in water is the mean value of 6 determinations with a standard deviation of ± 0.4 %. Every value for the solubility in ammonium oxalate solutions is the mean of 4 determinations with a standard deviation of ± 4.3 %.

The solubility in water ($4.90 \cdot 10^{-5}$ *M*) is in good agreement with the earlier results of Kohlrausch [4] ($4.85 \cdot 10^{-5}$ *M*) and Pedersen [5] ($4.84 \cdot 10^{-5}$ *M*). From this solubility the thermodynamic solubility

Table 1. The solubility at 25° C of calcium oxalate monohydrate in ammonium oxalate solutions and in water.

Ammonium oxalate, molarity	Ionic strength	Square of mean activity coefficient	Solubility of $CaC_2O_4 \cdot H_2O$, molarity $\times 10^6$ calculated	found
0	$1.96 \cdot 10^{-4}$	0.88	—	49.0
0.001	0.003	0.62	3.4	5.9
0.003	0.009	0.46	1.5	4.2
0.01	0.03	0.28	0.76	3.8
0.03	0.09	0.16	0.44	4.3
0.1	0.3	0.060	0.35	5.3
0.3	0.9	0.027	0.26	8.8

product was calculated to be $2.12 \cdot 10^{-9}$, using the mean activity coefficient calculated from the formula

$$-\log \gamma = 0.5\, z^2 \sqrt{\mu}/(1 + \sqrt{\mu})$$

The activity coefficients used for calculating the solubility in the oxalate solutions having an ionic strength of 0.003—0.09 were interpolated from tables published by Kielland [6]; for an ionic strength of 0.3—0.9, they have been calculated from Mc Comas and Rieman's [2] values on the solubility of calcium oxalate monohydrate in sodium chloride solutions.

The considerable differences between observed and calculated values indicate complex formation. Minimum solubility occurs at an oxalate concentration of about 0.01 *M* but the solubility differences in the concentration range 0.001—0.1 *M* ammonium oxalate are not large; 5 micromoles per litre, *i. e.* 0.2 mg Ca per litre, may be accepted as an approximate mean.

The author wishes to thank Miss Ulla Friberg for valuable help with the experimental work.

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Tetragonal Tungsten Bronzes of Degenerated Perovskite Type

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The existence of isomorphous tetragonal sodium and lithium tungsten bronzes, Me_xWO_3, of previously unknown structural type was reported in a recent paper from this Institute [1]. This note will present an account of the determination of the crystal structure of these compounds.

The investigation was based on powder and single crystal photographs of the sodium compound, the preparation examined having the composition $Na_{0.10}WO_3$. The radiation used was Cu-*K*. The lattice constants of the tetragonal unit cell, as obtained from the powder photographs are (referred to the wave-length of Cu-*Ka* radiation equal to 1.5418 Å):

$$a = 5.248 \text{ Å}, \quad c = 3.895 \text{ Å}, \quad V = 107.3 \text{ Å}^3$$

The observed density of 7.25 corresponds to a cell content of 2 formula units of $Na_{0.10}WO_3$.

The Laue symmetry was found from Weissenberg photographs to be *4/mmm*. The only systematic absences are *hk*0 reflections with $h + k$ odd, which unequivocally suggests *P4/nmm* as the most probable space-group.

A striking feature of the diffraction pattern is the decisive significance of the *l* index for the intensities of the reflections. In rough outline the following regularities could be observed:

for reflections *hkl* ($h + k$ even): *hk*0 (strong), *hk*1 (strong), *hk*2 (medium), *hk*3 (weak), *hk*4 (not visible), *hk*5 (weak, only β reflections observed);

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A striking feature of the diffraction pattern is the decisive significance of the *l* index for the intensities of the reflections. In rough outline the following regularities could be observed:

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for reflections *hkl* ($h + k$ odd): *hk*0 (absent), *hk*1 (medium), *hk*2 (medium), *hk*3 (medium), *hk*4 (medium).

Table 1. Observed and calculated intensities in Weissenberg photographs of $Na_{0.10}WO_3$. Rotation axis [110]. Cu-K radiation.

Zero layer line:

110^{vst}_{79} 220^{vst}_{23} 330^{m}_{10} 440^{m}_{8} $550(\beta)^{w}_{1.2}$

001^{vst}_{76} 111^{vst}_{38} 221^{st}_{15} 331^{m}_{7} 441^{m}_{7} $551(\beta)^{w}_{2}$

002^{st}_{12} 112^{m}_{10} 222^{m}_{5} 332^{m}_{3} 442^{m}_{5}

$003^{w}_{1.2}$ $113^{w}_{1.0}$ $223^{w}_{.8}$ $333^{w}_{1.0}$ $443(\beta)^{-}_{.1}$

$004^{-}_{.02}$ $114^{-}_{.02}$ $224^{-}_{.02}$ $334(\beta)^{-}_{.005}$

$005(\beta)^{vw}_{.2}$ $115(\beta)^{vw}_{.2}$ $225(\beta)^{vw}_{.4}$

First layer line:

010^{-}_{0} 120^{-}_{0} 230^{-}_{0} 340^{-}_{0} 450^{-}_{0}

011^{st}_{11} 121^{m}_{4} $231^{w}_{1.9}$ $341^{w}_{1.1}$ 451^{m}_{3}

012^{st}_{12} 122^{m}_{7} 232^{m}_{4} 342^{m}_{4}

013^{m}_{8} 123^{m}_{7} 233^{m}_{6} 343^{st}_{12}

014^{m}_{7} 124^{m}_{8} 234^{st}_{15}

Second layer line:

020^{vst}_{60} 130^{vst}_{20} 240^{m}_{8} 350^{m}_{9}

021^{vst}_{33} 131^{st}_{14} 241^{m}_{6} 351^{m}_{8}

022^{m}_{8} 132^{m}_{5} 242^{w}_{3} 352^{m}_{7}

$023^{-}_{1.1}$ $133^{-}_{.8}$ $243^{-}_{1.2}$

$024^{-}_{.02}$ $134^{-}_{.02}$

Third layer line:

030^{-}_{0} 140^{-}_{0} 250^{-}_{0}

031^{m}_{3} $141^{w}_{1.5}$ $251^{w}_{1.3}$

032^{m}_{6} 142^{m}_{4} 252^{m}_{5}

033^{m}_{7} 143^{m}_{8} 253^{st}_{23}

034^{m}_{11}

Fourth layer line:

040^{st}_{15} 150^{m}_{8} 260^{st}_{13}

041^{m}_{11} 151^{m}_{7} 261^{st}_{13}

042^{m}_{4} 152^{m}_{4}

$043^{vw}_{1.0}$ 153^{w}_{2}

$044^{-}_{.1}$

vst = very strong, st = strong, m = medium, w = weak, vw = very weak

A close examination furthermore revealed that the structure amplitudes $|F(hkl)|$ of the reflections of each of these two series are, within the error of estimation, independent of the values of h and k, but varying with l only. These regularities can only be accounted for if the two tungsten atoms of the unit cell occupy a twofold point position $2(c): 0\,\frac{1}{2}\bar{z}$, $\frac{1}{2}0z$, with $\bar{z}$ equal to 0.065 ± 0.01 or 0.435 ± 0.01. The latter value of the parameter will be arbitrarily adopted for the following discussion. Table 1 gives a comparison between the observed intensities and those calculated on the basis of this arrangement of the tungsten atoms.

It is not possible to find the positions of the oxygen and sodium atoms from

the X-ray data as the scattering power of these atoms is too low in comparison with that of the tungsten atoms. The arrangement of the latter, however, is very similar to that derived by Hägg [2] for the cubic sodium tungsten bronze of perovskite type. This fact suggests that the two structures are closely related, and leads to the following atomic configuration of the tetragonal bronze, which corresponds throughout to plausible interatomic distances:

Cell content: $2\ Na_xWO_3$
Space-group: $D_{4h}^7 - P4/nmm$
2 W in 2(*c*): $0\frac{1}{2}z$, $\frac{1}{2}0\bar{z}$ $z = 0.435$
$2x$Na in 2(*a*): 000, $\frac{1}{2}\frac{1}{2}0$
2 O in 2(*c*): $0\frac{1}{2}z$, $\frac{1}{2}0\bar{z}$ $z \approx 0.935$
4 O in 4(*e*): $\frac{1}{4}\frac{1}{4}\frac{1}{2}$, $\frac{3}{4}\frac{3}{4}\frac{1}{2}$, $\frac{1}{4}\frac{3}{4}\frac{1}{2}$, $\frac{3}{4}\frac{1}{4}\frac{1}{2}$

The structure may be described as built up of deformed WO_6 octahedra, joined by sharing corners. The alkali metal atoms are statistically distributed and occupy approximately 10 per cent of the major interstices of the lattice. (It must be emphasized that this scheme presupposes the unit cell dimensions and symmetry of the actual structure to be identical with those of the tungsten lattice.)

The tungsten atoms form puckered networks extending parallel to the *ab* plane, by being a little displaced alternately + 0.25Å and − 0.25 Å in the direction of the *c* axis. In this respect which constitutes the difference from a lattice of perovskite type, the structure is reminiscent of the tungsten trioxide structure of deformed ReO_3-type, the metal atoms of which show similar displacements parallel to two of the axes of the cubic substructure cell (± 0.23 Å and ± 0.24 Å respectively)[3]. It is noteworthy that another tetragonal sodium tungsten bronze, $Na_xWO_3 (x \approx 0.3)$, of complicated structure also contains puckered networks of tungsten atoms [4].

These structural relationships evidently demonstrate the character of the bronzes of degenerated perovskite type to be a distinct intermediate state in the transitions with decreasing alkali metal content from the cubic sodium [2] and lithium [5] bronzes of perovskite type to tungsten trioxide.

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5. Magnéli, A., and Nilsson, R. *Acta Chem. Scand.* **4** (1950) 398.

Received May 5, 1951.

Estimation of Xylocaine * by Nitration

BERTIL ÖRTENBLAD

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Comprehensive experiments have shown that Xylocaine (α-diethylamino-2,6-acetoxylidide) can be nitrated in a simple manner with analytical reproducibility. However the nitration conditions must be rigidly controlled since the course of the reaction is easily affected by variations in the reagent and the temperature employed. Nitration at 80° C with one part of conc. nitric acid and five parts of acetic acid seems to be most suitable. Hydrogenation of the nitration product is subject to similar limitations. The method would appear to be specially suitable for estimation of Xylocaine in small amounts, in dilute solutions and possibly also in biological material. The procedure and applicability of the method are being further investigated at present.

* Regd. trade mark.

the X-ray data as the scattering power of these atoms is too low in comparison with that of the tungsten atoms. The arrangement of the latter, however, is very similar to that derived by Hägg [2] for the cubic sodium tungsten bronze of perovskite type. This fact suggests that the two structures are closely related, and leads to the following atomic configuration of the tetragonal bronze, which corresponds throughout to plausible interatomic distances:

Cell content: $2\ Na_xWO_3$
Space-group: $D_{4h}^7 - P4/nmm$
2 W in 2(*c*): $0\frac{1}{2}z$, $\frac{1}{2}0\bar{z}$ $z = 0.435$
$2x$Na in 2(*a*): 000, $\frac{1}{2}\frac{1}{2}0$
2 O in 2(*c*): $0\frac{1}{2}z$, $\frac{1}{2}0\bar{z}$ $z \approx 0.935$
4 O in 4(*e*): $\frac{1}{4}\frac{1}{4}\frac{1}{2}$, $\frac{3}{4}\frac{3}{4}\frac{1}{2}$, $\frac{1}{4}\frac{3}{4}\frac{1}{2}$, $\frac{3}{4}\frac{1}{4}\frac{1}{2}$

The structure may be described as built up of deformed WO_6 octahedra, joined by sharing corners. The alkali metal atoms are statistically distributed and occupy approximately 10 per cent of the major interstices of the lattice. (It must be emphasized that this scheme presupposes the unit cell dimensions and symmetry of the actual structure to be identical with those of the tungsten lattice.)

The tungsten atoms form puckered networks extending parallel to the *ab* plane, by being a little displaced alternately + 0.25Å and − 0.25 Å in the direction of the *c* axis. In this respect which constitutes the difference from a lattice of perovskite type, the structure is reminiscent of the tungsten trioxide structure of deformed ReO_3-type, the metal atoms of which show similar displacements parallel to two of the axes of the cubic substructure cell (± 0.23 Å and ± 0.24 Å respectively)[3]. It is noteworthy that another tetragonal sodium tungsten bronze, $Na_xWO_3 (x \approx 0.3)$, of complicated structure also contains puckered networks of tungsten atoms [4].

These structural relationships evidently demonstrate the character of the bronzes of degenerated perovskite type to be a distinct intermediate state in the transitions with decreasing alkali metal content from the cubic sodium [2] and lithium [5] bronzes of perovskite type to tungsten trioxide.

The author is indebted to Miss Birgitta Blomberg for her valuable assistance. The work has been supported by a grant from the Swedish Natural Science Research Council, whose assistance is gratefully acknowledged.

1. Magnéli, A., and Blomberg, Birgitta *Acta Chem. Scand.* In print.
2. Hägg, G. *Z. physik. Chem.* (B) **29** (1935) 192.
3. Braekken, H. *Z. Krist.* **78** (1931) 484.
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Received December 28, 1950.

On the Kinetics of Formation of Precipitates of Sparingly Soluble Salts *

J. A. CHRISTIANSEN and ARNE E. NIELSEN

Institute of Physical Chemistry, University of Copenhagen, Copenhagen, Denmark

To get information regarding the mechanism of precipitation we have made preliminary experiments on the systems bariumchloride + sulphuric acid and silver nitrate + potassium chromate.

For the more concentrated solutions (0.01 molar) we used a flow method according to Roughton's principle [1]. Three glass tubes, two for inflow of the two component solutions and one for outflow of the mixture were cemented into a "crosshead" from perspex, containing the mixing chamber and adjacent channels for inflow and outflow. The outflow tubing had a bore of about 2 mm.

In the outflow tube the place and thus the time of occurrence of visible precipitation could be observed. In that way precipitations occuring in times after the mixing from 10^{-3} seconds to about 10^{-1} seconds could be observed. By stopping the flow and noting the time of appearance of visible precipitation observations could be taken in times from about 1 to about 10 seconds, while longer times than that could be observed simply by mixing of the two solutions in a beaker.

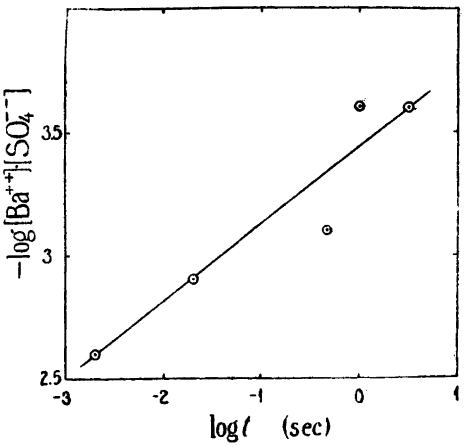

Fig. 1. The diagram represents the relation between the product of the concentration of barium ions and sulphate ions and the time of visible precipitation. Dekadic logarithms, time in seconds, concentration in moles/liter.

The scale of times thus extends over an interval of 4 to 6 powers of ten.

The times for visible precipitation could only be determined to within a factor of

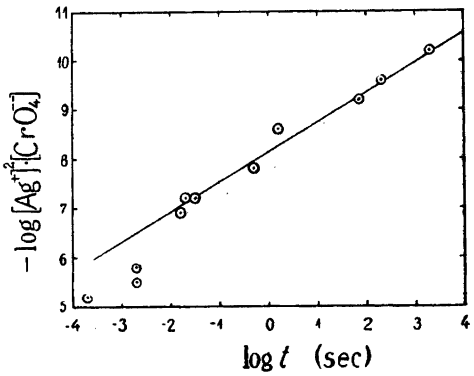

Fig. 2. The diagram represents the relation between the product of the concentration of silver-ions (squared) and of chromate ions and the time of visible precipitation.

* The content of the following three notes was given in a lecture to a joint meeting of Kemiska Föreningen, Lund, Sweden and Kemisk Forening, København, held in Copenhagen May 19. 1951.

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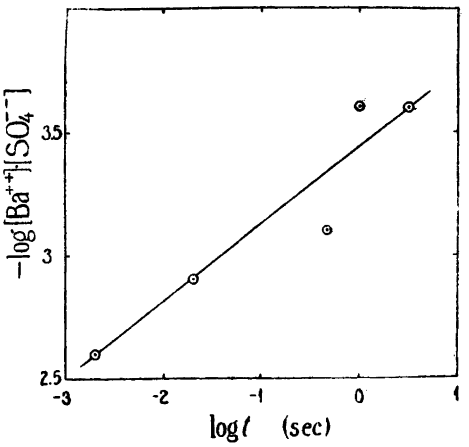

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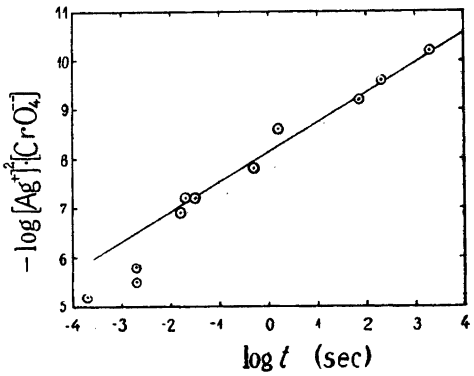

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about 2. We take them to mean the times of appearance of a certain constant and not quite small fraction of the maximum density of the precipitate.

The experiments were difficult to perform when the concentrations of the component solutions were not practically equivalent, and our results are therefore valid only for this case. The temperature was about 20° C.

The results are plotted in Figs. 1 and 2 in logarithmic diagrams. It is obvious that notwithstanding the inaccuracy of the single experiment the results can be fairly well expressed by relations of the form.

$$t \cdot c_0^{p-1} = k$$

where t is the time of precipitation, c_0 the original molar concentration taken as the i'th root of the ionic product, if one molecule dissociates into i ions, p a constant and k another constant.

For bariumsulphate we find

$$t \cdot c_0^7 = 10^{-11.8}$$

and for silverchromate

$$t \cdot c_0^5 = 10^{-13.5}$$

We remark that for bariumsulphate an exponent 6 would give about as good an agreement with the experiments as 7, while for silverchromate neither the exponents 4 nor 6 could very well agree with the experiments.

In a paper on precipitation of calciumfluoride A. Tovborg Jensen [2] has found a similar relationship between what he calls the incubation time and the concentration but experimentally his incubation times are defined as the times of appearance of visible precipitation just as ours. His time range extends only over about 2 powers of ten but he has been able to show that his times are reproducible inside a factor which is less than 1.5. Tovborg Jensens experiments from his table 3 p. 102 can be represented either by the expression

$$\left.\begin{matrix} tc_0^7 = 10^{-14.31} \\ \text{or by} \\ tc_0^8 = 10^{-16.81} \end{matrix}\right\} \text{ 18° C; time in seconds}$$

If the precipitation time is assumed to mean the time t_a when the reaction has taken place to a constant fraction a of the maximum extent it can be shown that if the reaction velocity s is of the form

$$s = k_p \cdot c^p$$

where c is the instantaneous concentration, integration yields the expression

$$t_a \; c_0^{p-1} = k_a$$

We therefore conclude that the velocity of precipitation is proportional to the 7th or 8th power of the instantaneous concentration in the case of bariumsulphate, to the 8th or 9th power in the case of calciumfluoride and to the 6th power in the case of silverchromate.

1. Roughton, F. J. W. *e. g. Proc. Roy. Soc. (London)* **A 155** (1936) 258.
2. Tovborg Jensen, A. *Z. physik. Chem.* **A 180** (1937) 93.

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Kinetic Determination of the Size of Crystal Germs

J. A. CHRISTIANSEN and ARNE E. NIELSEN

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According to well known assumptions the formation of visible crystals in a supersaturated solution takes place in at least two steps *viz.* the formation of in-

about 2. We take them to mean the times of appearance of a certain constant and not quite small fraction of the maximum density of the precipitate.

The experiments were difficult to perform when the concentrations of the component solutions were not practically equivalent, and our results are therefore valid only for this case. The temperature was about 20° C.

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According to well known assumptions the formation of visible crystals in a supersaturated solution takes place in at least two steps *viz.* the formation of in-

visible "germs" and their growth to visible dimensions. Now it follows from the theory of Smoluchowsky [1] which has lately been used and extended by La Mer [2] that the velocity of growth of germs in a supersaturated solution is proportional to the instantaneous concentration in a low power. In the above note it has been shown that the velocities of precipitation of some slightly soluble salts in sharp contrast to this are proportional to the concentrations in powers from 6 to 9. Therefore the rate-determining step in the experiments described in the note cannot be the growth but must be the formation of crystal germs.

Elementary kinetical considerations show that the power in which the instantaneous concentrations appear in the velocity expression is equal to the number of single particles (ions) in a germ, and it is therefore possible to determine this number by kinetic experiments.

However, the experiments are at the present stage not in all cases sufficiently accurate to decide with certainty which of two consecutive whole powers is the right one.

Stoichiometric arguments can however be used for the decision, if we assume that a germ is always electroneutral.

That this assumption is probably correct can be seen from the more detailed discussion of the mechanism of the formation of germs given in the note below. It is evident from this that it is an essential property of the germ that it has a low probability of loosing a particle. Now from electrostatics it follows immediately that a charged complex particle containing several single particles has a much greater probability of loosing an ion than a neutral one and our assumption is therefore probably correct.

If the stoichiometric composition of the precipitate shows that it contains i ions, the number of particles in the germ must thus be an integer times i.

For bariumsulphate we had for the exponent p the choice between 7 and 8. When we assume the germ to be electroneutral, we must choose $8 = 4 \cdot 2$, which means that the germs contain 4 bariumions and 4 sulphate ions.

For silverchromate we found the number $6 = 2 \cdot 3$ meaning that a germ of this substance contains 4 silverions and 2 chromateions.

For calciumfluoride we calculated from Tovborg Jensens experiments [3] the number 8 or 9. The condition of neutrality decides for $9 = 3 \cdot 3$ which means that germs of this substance contain 3 calciumions and 6 fluorions.

It is thus seen that by an experimental investigation of the kinetics of precipitation combined with stoichiometric considerations we can determine the number of single particles in a crystal germ or as we may say the size of the germ.

That some sort of "germs" are necessary to initiate the crystallization of a supersaturated solution is practically an empirical fact, but they have always been assumed to contain many single particles. To quote an example La Mer [4] who follows Frenkel [5] speaks of embryos, complex particles which contain hundreds of single particles and nuclei which are still greater.

In contrast to this we have found that the germs contain only rather few particles and it is to avoid confusion with Frenkel's and La Mer's nomenclature that we have chosen the name germ.

1. Smolychowski, M. *Z. physik. Chem.* **92** (1918) 129.
2. La Mer, V. K., and Dinegar, R. H. *J. Am. Chem. Soc.* **72** (1950) 4847.
3. Tovborg Jensen, A. See the preceding note.
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5. Frenkel, J. *Kinetic theory of liquids.* Oxford (1945) chapter 7.

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On the Mechanism of Precipitation of Sparingly Soluble Salts, Preliminary note

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The kinetic laws which seem to govern the formation of crystal germs in supersaturated solutions of different sparingly soluble salts leads to the assumption of a mechanism which can be described as follows:

Let us take as an example the precipitation of silverchromate. We shall denote by A a silver ion and by B a chromate ion.

By mere chance it may happen that two such ions form what we shall call a cluster of the second order, this meaning that the two particles are in the same volume-element of molecular dimensions ($\sim 10^{-27}$ liter). This may be illustrated by means of the reaction equation

$$A + B \rightleftharpoons X_2$$

where X_2 is the cluster of the second order.

Now such a cluster may capture a third particle according to the equation

$$A + X_2 \rightleftharpoons X_3$$

and so on until at last by the capture of a sixth particle a germ (Q_6) of the sixth order is formed according to the equation

$$A + X_5 \rightarrow Q_6$$

To understand the experimentally determined kinetics we must assume that a "germ" is different from a "cluster" in so far as it is much more probable, that it will capture a particle than it will loose one. The whole sequence of reactions thus becomes

$$\begin{aligned} A + B &\rightleftharpoons X_2 && (\pm 1)\\ A + X_2 &\rightleftharpoons X_3 && (\pm 2)\\ A + X_3 &\rightleftharpoons X_4 && (\pm 3)\\ B + X_4 &\rightleftharpoons X_5 && (\pm 4)\\ A + X_5 &\rightarrow Q_6 && (\pm 5) \end{aligned}$$

As now the clusters must be assumed to be very short living we may calculate the velocity s of the formation of germs by using the conditions of stationarity of the concentrations of the clusters. When b is the instantaneous concentration of B-particles, w_1 the probability per unit time for a B-particle to react according to the scheme $(+1)$, w_{-1} the probability (p.u.t.) for X_2 to react according to the scheme (-1), w_2 the probability (p.u.t.) for X_2 to react according to $(+2)$ a.s.o. we get [1]

$$\begin{aligned} b/s = {} & 1/w_1 + w_{-1}/w_1w_2 + w_{-1}w_{-2}/w_1w_2w_3 \\ & + w_{-1}w_{-2}w_{-3}/w_1w_2w_3w_4 \\ & + w_{-1}w_{-2}w_{-3}w_{-4}/w_1w_2w_3w_4w_5 \end{aligned}$$

From elementary considerations it turns out that $bw_1 = x_2^0w_{-1}$; $bw_1w_2 = x_3^0w_{-1}w_{-2}$ and so on, where x_i^0 means the (virtual) thermodynamical equilibrium concentration of X_i (not the actual steady state concentration of X_i). We may therefore write $1/s$ in the form

$$\begin{aligned} 1/s = {} & 1/bw_1 + 1/x_2^0w_2 + 1/x_3^0w_3 + 1/x_4^0w_4 + \\ & + 1/x_5^0w_5 \end{aligned}$$

A rough estimate taking into account the effect of electrostatic attractions shows that the denominators of the members in the sum decrease from the left to the right by large factors (100 — 1 000) when the concentrations of A and B are not too large. We may therefore neglect all the

members except the last one and we get thus

$$s = x_5^0 w_5$$

as the velocity of germ-formation.

In this calculation we have neglected reactions between clusters. The reason is that the concentration of clusters even of low orders (*e. g.* two) is small as compared to the concentration of single particles. Furthermore we have neglected the possibility that the germ $^*Q_6$ may loose a particle, or rather we have assumed that a sixth member in the sum derived from the fifth by multiplication with w_{-5}/w_6 is small against the fifth. This again means that we have assumed that a germ has a much greater probability of capturing a particle than of loosing one. In contrast to this we estimated above that a cluster has a much greater probability for loss than for capture. As now the assumption of this contrast is necessary to explain the kinetics we arrive at the conclusion, that a "germ" is qualitatively different from a "cluster". It is as if by capturing its last particle the germ falls into a potential well, which it only with difficulty can leave. By closer consideration of the circumstances one gets the impression that electrostatic forces are not sufficient to explain the formation of germs, but that other not so evident forces must come into play.

The further fate of the germs, when once they have been "born" is of course to grow by capturing new particles, until eventually the ionproduct of the solution has reached the saturation value.

Returning finally to the velocity expression it is seen that the product $x_5^0 w_5$ must be of the form $k_6\ a^4b^2$ or if we define c as the third root of the ionproduct a^2b:

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It is this formula which by integration yields an expression of the form

$$t_a\ c_0^5 = k_a$$

which was discussed in a previous note.

It must be added that both the experiments and the calculations hitherto applied are very crude, so crude that even changes in the orders of the different germs are not quite excluded, but such changes would not affect the general results which are that the size (order) of crystal germs can be determined by kinetical experiments and that the germs are far less than hitherto has been assumed.

Experimental and theoretical work on the problem is being continued.

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A Preliminary Report on the Synthesis of Taurine and Cystamine labelled with Radioactive Sulfur

LORENTZ ELDJARN*

Biochemical Department, Medical Nobel Institute, Stockholm, Sweden

Taurine, free and in combination with the cholic acids, has been demonstrated to exert a series of physiological, pathological and pharmacological actions. In order to investigate its metabolism and some of the above functions by means of isotopic methods, a synthesis for the labelling of this compound with radioactive sulfur has been worked out. The labelled taurine will then be combined with the cholic acids by chemical or biological synthesis.

The formation of taurine from L(+)-cysteine through L(−)-cysteic acid has been demonstrated to take place in the animal organism [1, 2]. Certain findings,

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such as the lack of cysteic acid decarboxylase in some animals known to produce taurine, indicates, however, that taurine might be formed in another manner. Its possible formation from 1-amino-2-mercapto ethane will be investigated by means of this compound labelled with radioactive sulfur.

The syntheses of taurine described in the literature have been critically studied and found unsuitable for its labelling with radioactive sulfur [3-12]. We therefore have worked out the following synthesis, which has been found suitable for semi-micro work and which gives an overall yield of approximately 50 per cent from the sulfur of thiocyanate.

The conversion of labelled sulfate to thiocyanate may be performed according to Wood (1947) [13]. The thiocyanate is reacted with 1-benzoylamino-2-brom ethane. The latter is prepared by benzoylating the hydrobromide of 1-amino-2-brom ethane in dry pyridine. The reaction

$$SCN^- + C_6H_5CONHCH_2CH_2Br = Br^- + \\ + C_6H_5CONHCH_2CH_2SCN$$

proceeds smoothly in 100 per cent ethanol at 50 degrees C with an average yield of 90 per cent. The resultant 1-benzoylamino-2-thiocyanate of ethane melts after recrystallisation from aqueous ethanol at 80° C (uncorr.) and forms small, white crystalline needles.

On hydrolysis of the 1-benzoylamino-2-thiocyanate of ethane with 2 eqv. of potassium hydroxyde and the simultaneous oxidation of the mercaptan formed to the disulfide by bubbling air through the alcoholic solution, the dibenzoyl-cystamine of m.p. 132.5° C (uncorr.) is formed in almost quantitative yield. This is hydrolyzed to cystamine by boiling with 22 per cent hydrochloric acid, and can be either reduced to the hydrochloride of 1-amino-2-mercapto ethane, or oxidized according to Schöberl [14] to taurine in about 60 per cent yield. The taurine separates on the addition of ethanol to the aqueous solution as typical crystalline needles of m. p. 228—230° C.

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The Structure of Acetaldehyde-ammonia, and a Note on its Anhydrous Form

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Universitetets Kjemiske Institutt, Blindern—Oslo, Norway

Acetaldehyde-ammonia (m. p. 94—96° C) crystallizes in the rhombohedral space group $D_{3d}^5 - R\bar{3}m$. [1, 2]. The hexagonal lattice constants as determined by the author are: $a = 11.29$ Å, $c = 15.86$ Å. There are 18 units of $CH_3 \cdot CHO \cdot NH_3$ in the hexagonal unit cell. The 18-fold position of this space group is a special one, involving a symmetry plane, whereas the 6-fold position requires the point symmetry $C_{3v}-3m$.

According to an X-ray investigation carried out by Moerman [2] the solid com-

such as the lack of cysteic acid decarboxylase in some animals known to produce taurine, indicates, however, that taurine might be formed in another manner. Its possible formation from 1-amino-2-mercapto ethane will be investigated by means of this compound labelled with radioactive sulfur.

The syntheses of taurine described in the literature have been critically studied and found unsuitable for its labelling with radioactive sulfur [3-12]. We therefore have worked out the following synthesis, which has been found suitable for semi-micro work and which gives an overall yield of approximately 50 per cent from the sulfur of thiocyanate.

The conversion of labelled sulfate to thiocyanate may be performed according to Wood (1947) [13]. The thiocyanate is reacted with 1-benzoylamino-2-brom ethane. The latter is prepared by benzoylating the hydrobromide of 1-amino-2-brom ethane in dry pyridine. The reaction

$$SCN^- + C_6H_5CONHCH_2CH_2Br = Br^- + \\ + C_6H_5CONHCH_2CH_2SCN$$

proceeds smoothly in 100 per cent ethanol at 50 degrees C with an average yield of 90 per cent. The resultant 1-benzoylamino-2-thiocyanate of ethane melts after recrystallisation from aqueous ethanol at 80° C (uncorr.) and forms small, white crystalline needles.

On hydrolysis of the 1-benzoylamino-2-thiocyanate of ethane with 2 eqv. of potassium hydroxyde and the simultaneous oxidation of the mercaptan formed to the disulfide by bubbling air through the alcoholic solution, the dibenzoyl-cystamine of m.p. 132.5° C (uncorr.) is formed in almost quantitative yield. This is hydrolyzed to cystamine by boiling with 22 per cent hydrochloric acid, and can be either reduced to the hydrochloride of 1-amino-2-mercapto ethane, or oxidized according to Schöberl [14] to taurine in about 60 per cent yield. The taurine separates on the addition of ethanol to the aqueous solution as typical crystalline needles of m. p. 228—230° C.

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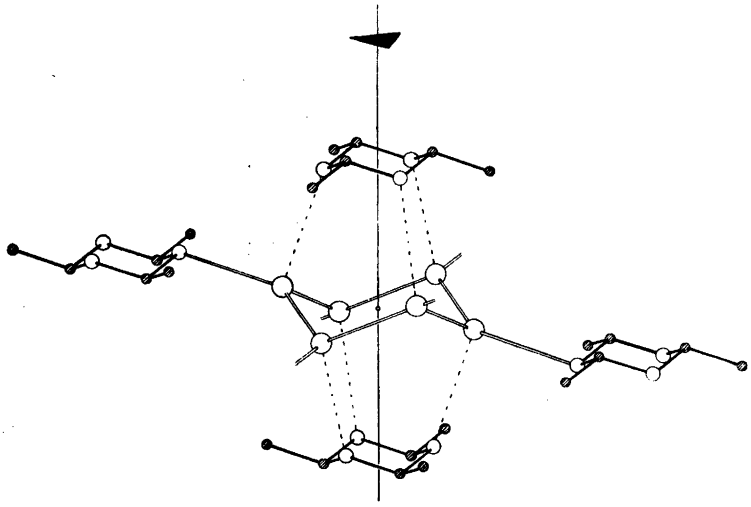
According to an X-ray investigation carried out by Moerman [2] the solid com-



O N C

Fig. 1.

pound is a trihydrate of 2,4,6-trimethyl-hexahydro-1,3,5-triazine, in correspondence with the view of Delépine [3]. However, the intensity material employed by Moerman was rather poor, and a re-investigation has therefore been carried out.

As a first trial a structure was considered based on the following principles: The main part is a hydrogenated triazine ring in the "chair" form with tetrahedral valency angles throughout as in an ideal *cyclo*hexane ring. One methyl group is bonded to each carbon atom of the ring in $\varkappa$ position. The C—N and C—C bond lengths are assumed to be 1.47 Å and 1.54 Å, respectively.

This molecule is placed with the center of the ring in the 6-fold position, the coordinates of which are (0, 0, z). All carbon and nitrogen atoms have then to lie in symmetry planes intersecting at 60°. The oxygen atoms of the water molecules must also be placed in these symmetry planes.

Consideration of ($hk0$) reflexions gives the distance of the oxygen atoms from the threefold axis, whereas the z parameters are determined by means of the ($0kl$) reflexions. A satisfactory correspondence between observed and calculated intensities was obtained with the following relationship between hydrogenated triazine rings and the water molecules: Between each nitrogen atom of the ring and one oxygen atom a hydrogen bond is established, having a direction corresponding to a $\varkappa$ bond in *cyclo*hexane and a length of 2.91 Å. The center of the ring has a z parameter equal to 0.26. As a result of this another hydrogen bond is established between nitrogen and oxygen atoms, hav-

ing a length of 3.18 Å and making an angle of about 20° with the threefold axis. Further, six oxygen atoms are linked together by means of hydrogen bonds, all having the length of 2.71 Å. An oxygen ring in the "chair" form of the *cyclo*hexane type is thus formed. The center of this ring coincides with a symmetry center of the lattice.

In Moerman's structure the methyl groups are situated nearly in ε position to the carbon atoms of the ring. Further, his parameters lead to a covalent C—C distance of 2.08 Å and a van der Waals' distance between two methyl groups of 2.67 Å. The hydrogen bond lengths between nitrogen and oxygen atoms are 2.67 Å and between two oxygen atoms 3.36 Å.

All these values differ greatly from those obtained in the present investigation. Here, all intensities of reflexions obtainable with Cu-K radiation were estimated and a Fourier section in the plane $y = -x$ was carried through. Only small deviations from the ideal structure pictured above were observed. A complete account of the analysis will be given shortly.

Fig. 1 gives a picture of the structure. The central ring is that formed by six oxygen atoms. Of neighbouring hydrogenated triazine rings two have their centers on the threefold axis, and six are arranged trigonally around the axis. Of these last rings only two are drawn. Covalent bonds are represented by heavy lines, hydrogen bonds of normal length by double lines and the longer hydrogen bonds by broken lines. Hydrogen atoms are altogether omitted from the Figure.

If acetaldehyde-ammonia is left for some hours in a vacuum desiccator containing sulfuric acid or even calcium chloride, it loses water and small apparently cubic crystals are obtained. Delépine analyzed this compound and found it to have the composition $(C_2H_5N)_x$.

Examinations carried out by means of a polarizing microscope and X-rays showed that the crystals are really cubic with a lattice constant $a = 14.56$ Å. Some freshly prepared crystals were observed to have very nearly the same density as quinoline ($d = 1.093$). The number of molecules of the form $C_6H_{15}N_3$ is therefore equal to *sixteen*.

Systematic absences of reflexions lead unambigously to the space group T_h^6-Pa3. In this space group the general position is a 24-fold one, but there is an 8-fold special position involving a threefold axis.

It is therefore possible to place two substituted triazine rings with their centers in two eightfold positions. This conclusion strongly supports the view of Delépine that the triazine rings of the acetaldehyde-ammonia structure are left intact when the water molecules are removed.

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On the Mode of Action of Peptides as Growth Factors for *Leuconostoc mesenteroides*

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In a previous paper [1] we recorded results showing that *Leuconostoc mesenteroides* P-60, which requires glycine for an amino acid, is also able to use glycine peptides instead of glycine. In connection with the present study we were particularly interested in ascertaining whether the action of peptides was due to the cleavage of glycine from them caused by bacterial cells, hence, the growth-affecting factor being always free glycine, or whether bacteria may utilize glycine peptides without prior hydrolysis. The former explanation strikes at first more natural, but some observations reported in the literature seem to favour the latter one. Particular reference should be made in this connection to the findings of Simmonds and Fruton [2] of the exceptional activity of proline peptides which is greater than that of the equimolar free proline. In our investigations, however, no glycine peptide exceeded in activity the free glycine.

To elucidate the problem, we examined the hydrolysis of some glycine peptides by the action of *Leuconostoc mesenteroides* P-60. The results are reported here.

EXPERIMENTAL

The bacterial mass was grown in a nutrient solution containing 1 % glucose, 1 % sodium citrate, 0.5 % Bacto-tryptone, and 1.5 % Bacto-yeast extract. A stab culture of the strain was first inoculated into 10 – 20 ml of the above nutrient solution and allowed to grow for about 1 day at 37° C. Then it was further inoculated into 500 ml of the same nutrient solution and kept again for 1 day at 37° C. The bacterial mass was then aseptically centrifuged, washed once and suspended in 15 – 20 ml of 0.9 % NaCl-solution. Dry weight of bacteria was determined from the suspension after standing overnight at 103° C.

The examination included the following peptides: DL-alanylglycine (Schuchardt), DL-leucylglycine (University of California), glycylglycine (the same), glycyl-L-tyrosine

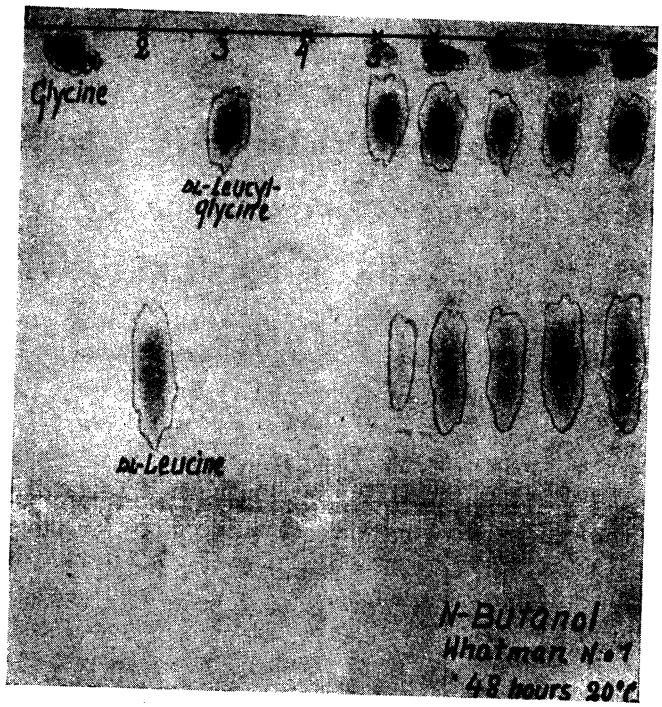

Fig. 1. Photograph of one-dimensional paperchromatogram of the hydrolysis of DL*-leucyl-glycine with bacteria.*

1) Glycine alone
2) DL*-Leucine alone*
3) DL*-Leucylglycine alone*
4) Bacteria alone after 45 h
5—9) DL*-Leucylglycine with bacteria*
5) after 3 h
6) » 9 »
7) » 21 »
8) » 32 »
9) » 45 »

methylesterhydrochloride (Sir Ian Heilbron) and leucyl-DL-phenylalanine methylesterhydrochloride (the same). The peptide concentration of the three first mentioned ones was 10 mg/ml and that of the two last mentioned 15 mg/ml. The concentration of the respective amino acids was: glycine, alanine, and leucine 5 mg/ml, tyrosine and phenylalanine 10 mg/ml.

The amino acid and peptide solutions, 4 ml each, were sterilized in an autoclave at 112° C in glass ampoules for 5 min. After this 1 ml of bacterial suspension, corresponding to 15—18 mg dry bacteria, was pipetted into each ampoule. Respectively, 1 ml of distilled

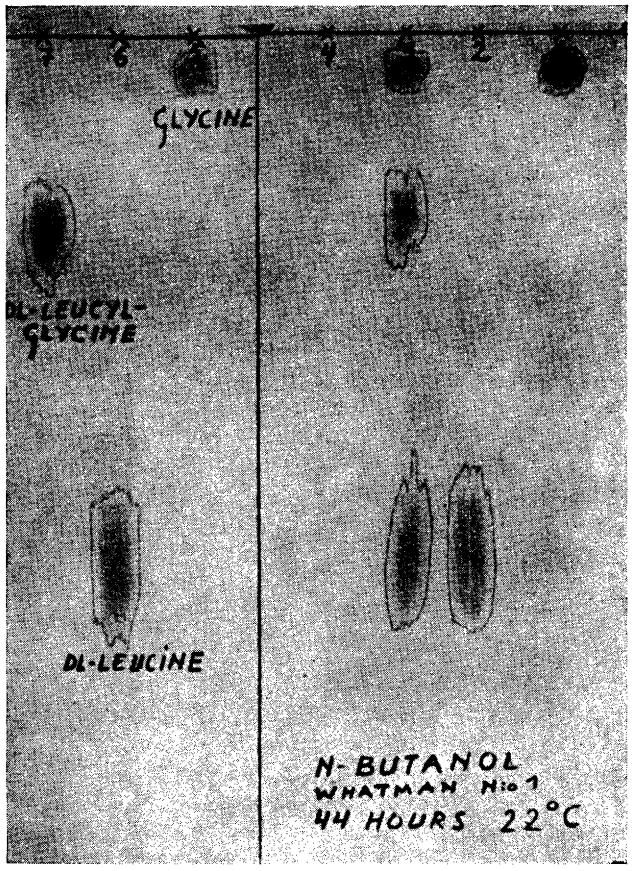

Fig. 2. Photograph of one-dimensional paperchromatogram of the hydrolysis of DL-*leucylglycine with bacteria after 24 hours.*

1) Glycine with bacteria
2) DL-*Leucine with bacteria*
3) DL-*Leucylglycine with bacteria*
4) Bacteria alone
5) Glycine alone
6) DL-*Leucine alone*
7) DL-*Leucylglycine alone*

water was pipetted into the control solutions. The tubes were again sealed and warmed on a water-bath to 37° C.

After hydrolysis the bacterial mass was centrifuged and amino acids and peptides were determined in the clear solution by means of one-dimensional paperchromatography employing the technique introduced by Miettinen and Virtanen [3]. In some of the experi-

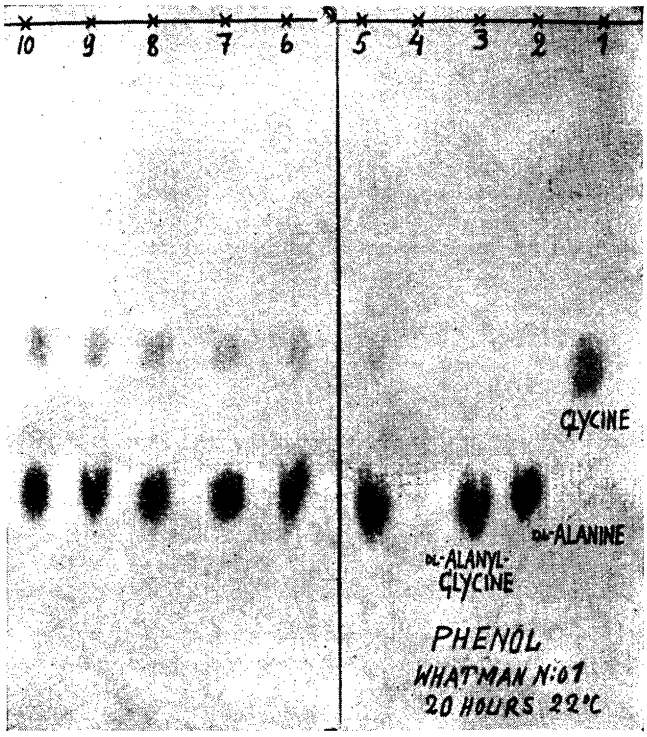

Fig. 3. Photograph of one-dimensional paperchromatogram of the hydrolysis of DL-*alanylglycine with bacteria.*

1) Glycine alone
2) DL-*Alanine alone*
3) DL-*Alanylglycine alone*
4) Bacteria alone after 24 h
5—10) DL-*Alanylglycine with bacteria*
5) after 3 hours
6) » 6 »
7) » 9 »
8) » 12 »
9) » 18 »
10) » 24 »

ment series, besides, 1 % ninhydrin solution (1 : 1) was used for following the hydrolysis of peptides according to Abderhalden [4]. The intensity of the red-blue colour, obtained with ninhydrin in 2 h at 21° C in experiments with glycyl-L-tyrosine and glycyl-DL-phenylalanine, was determined with a Klett-Summerson photoelectric colorimeter using filter S 52.

In order to find out how the leucine component of leucylglycine acts in the growth experiments of *Leuconostoc mesenteroides* an experiment was carried out, using the same

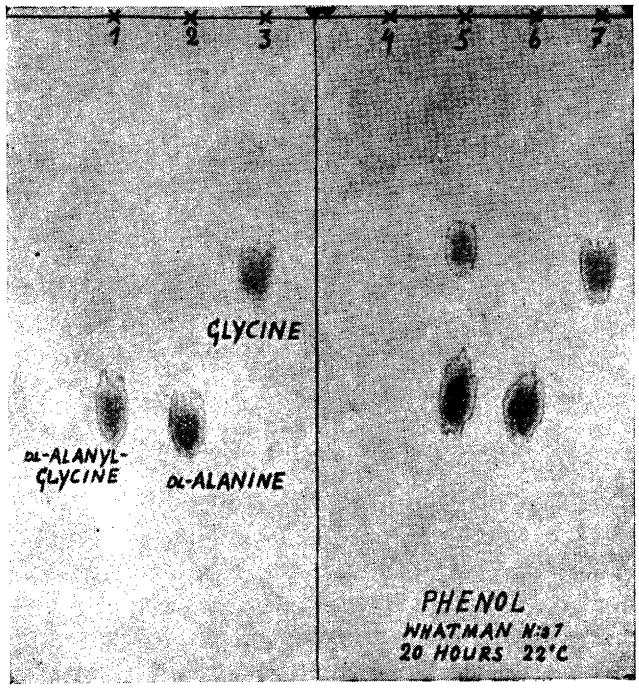

Fig. 4. Photograph of one-dimensional paperchromatogram of the hydrolysis of DL-*alanyl-glycine with bacteria after 24 hours.*

1) Alanylglycine alone
2) DL-*Alanine alone*
3) Glycine alone
4) Bacteria alone
5) DL-*Alanylglycine with bacteria*
6) DL-*Alanine with bacteria*
7) Glycine with bacteria

method as earlier [1], in a leucine-free basal medium, prepared according to Henderson and Snell [5].

RESULTS

The results of our hydrolysis experiments are presented in Figs. 1—5 and in Table 1. The photographs were taken from the paperchromatograms, which were obtained from DL-leucylglycine, DL-alanylglycine and glycylglycine before and after the hydrolysis with bacterial mass and from the respective amino acids using one-dimensional run in butanol or in phenol. As appears from the figures, all these peptides are hydrolyzed by the bacterial mass. The longer

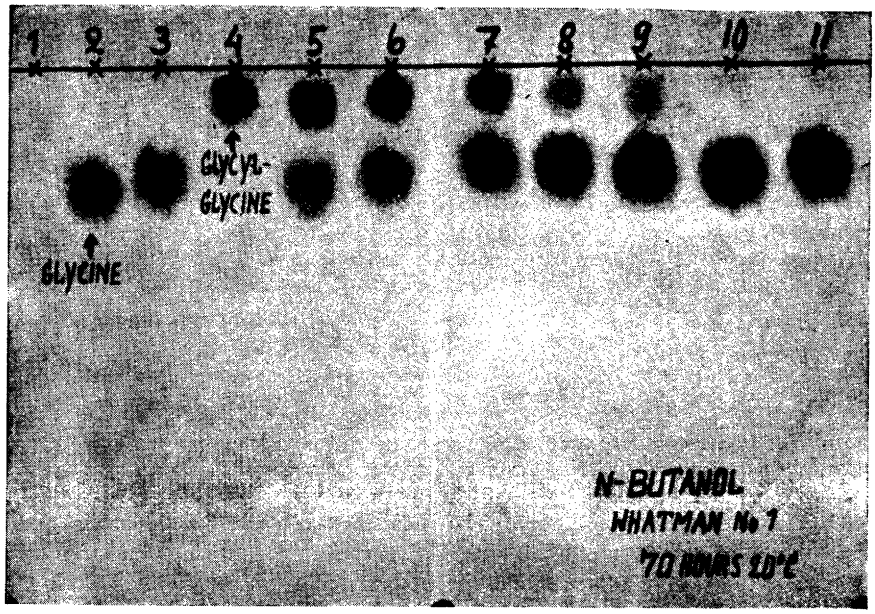

Fig. 5. Photograph of one-dimensional paperchromatogram of the hydrolysis of glycylglycine with bacteria.

1) Bacteria alone after 24 h
2) Glycine alone
3) Glycine with bacteria
4) Glycylglycine alone
5—11) Glycylglycine with bacteria
5) after 1 ½ h
6) » 3 »
7) » 6 »
8) » 12 »
9) » 19 »
10—11) » 24 »

the time of hydrolysis, the higher the hydrolysis degree. DL-leucylglycine and DL-alanylglycine are slightly hydrolyzed already within 3 hours and glycylglycine within 1 ½ h. Within 24 hours the hydrolysis of glycylglycine was quantitative.

Bacterial mass alone did not produce compounds detectable in paper chromatograms.

Hydrolyzation of DL-leucylglycine and DL-alanylglycine was also noted by colour reaction with 1 % ninhydrin solution at room temperature. The inten-

Table 1. Hydrolysis of glycyl-L-tyrosine methylesterhydrochloride and glycyl-DL-phenylalanine methylesterhydrochloride with bacteria after 24 hours at 37° C. 1 % ninhydrin solution added to the solution under examination in ratio 1 : 1 according to R. Abderhalden.

	Optical density		Optical density
Glycine	0.346	Glycine	0.336
Tyrosine	0.028	Phenylalanine	over 1.800
Glycyl-L-tyrosine methylester-hydrochloride	0.210	Glycyl-DL-phenylalanine methylesterhydrochloride	0.190
Bacteria alone	0.028	Bacteria alone	0.036
Glycyltyrosine methylesterhydrochloride with bacteria	0.432	Glycyl-DL-phenylalanine methylesterhydrochloride with bacteria	0.334

Both experiments indicate that hydrolysis has taken place. Provided that the results are quantitatively comparable, the hydrolysis of glycyl-L-tyrosine methylesterhydrochloride is almost complete, whereas that of glycyl-DL-phenylalanine methylester is insignificant. Phenylalanine gives with ninhydrin so intense a colour that a hydrolysis of phenylalanine, proceeded to a high extent, raises the colour intensity to manyfold. However, it must be taken into account that tyrosine and phenylalanine have been free in the controls, whereas they are liberated as methylesters in the hydrolysis. This affects considerably the colour intensity.

sities of colour were then consistent with the results obtained by paperchromatography.

Since hydrolysis of glycyl-L-tyrosine methylester and glycyl-DL-phenylalanine methylester could not be proved for certain by paperchromatography, 1 % ninhydrin was used for the purpose (Table 1). Glycyl-L-tyrosine methylester gave with the bacterial mass in 24 h a distinctly more intense red-blue colour than peptide alone. The greater intensity must be due to the partial hydrolysis of peptide since glycine produces a comparatively strong colour reaction. Likewise, glycyl-DL-phenylalanine gave with bacterial mass in 24 h a more intense red-blue colour than peptide alone. Hydrolysis must therefore have taken place. Phenylalanine gave a very intense colour, whereas tyrosine gave practically none at all.

In comparing the results of the hydrolysis experiments with those obtained in the growth experiments with peptides [1], it can be ascertained that the active normal peptides, which are composed of amino acids, also are regularly hydrolyzed. This suggests that glycine, set free on the cleavage of peptides, is responsible for the action of glycine peptides. This concept is also supported by the observation made in this work that leucylglycine is approximately as active both as glycine and leucine.

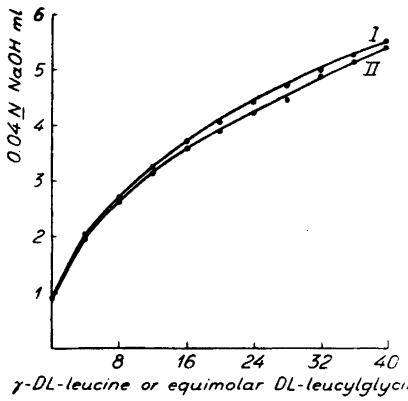

Fig. 6. DL-*leucine activity of* DL-*leucylglycine in growth experiment with Leuconostoc mesenteroides P-60. (Growth 72 h at 37° C, basal medium according to Henderson & Snell.)*

I = DL-*leucine 100 % activity.*
II = DL-*leucylglycine 97 % activity.*

On the contrary, benzoylglycine, which is glycine-active in the growth experiments with *L. mesenteroides*, was not hydrolyzed, at least not in detectable amounts. It would therefore seem likely that benzoylglycine as such would replace glycine. Its non-amino acid component (benzoic acid) would thus change the mode of action of peptide. It is possible that benzoylglycine joins the enzyme on the surface of the bacterial cells and is not hydrolyzed until in the cell during the metabolism. This concept is, however, quite hypothetical.

There is still reason to emphasize the fact that our hydrolysis experiments were carried out with greater amounts of bacteria than our growth experiments. Thus the hydrolysis velocity is not directly comparable with the glycine activity of peptides in the growth experiments. With such amounts of bacteria, which come into question in the growth experiments, hydrolysis of peptides is not detectable by the technique employed by us. Therefore our experiments do not suffice to solve convincingly the problem whether the hydrolysis of peptides is so rapid that the liberation of glycine from normal peptides would explain the mode of action of active glycine-peptides in the growth experiments with *Leuconostoc mesenteroides*. The results show at any rate for certain that this organism hydrolyzes the normal glycyl and leucine peptides which can replace the said amino acids in the growth experiments.

SUMMARY

Leuconostoc mesenteroides P-60 hydrolyzes leucylglycine, alanylglycine and glycylglycine, which replace glycine in growth experiments with this organism. Similarly, hydrolysis has been noted with glycyl-L-tyrosine methylester and

glycyl-DL-phenylalanine methylester, which also show activity with the same bacteria. The results suggest that the activity of glycine peptides depends on the hydrolysis of these peptides to free amino acids. DL-Leucylglycine replaces as well leucine as glycine in the growth experiments with *L. mesenteroides*, which is to be expected if the amino acids set free by hydrolysis are the active factors in the growth experiments.

Benzoylglycine is also glycine-active in the growth experiments with *L. mesenteroides*. Its hydrolyzation could not be noted with this organism.

The authors wish to express their thanks to Sir Ian Heilbron and Dr. A. H. Cook, Imperial College of Science and Technology, London, England, for the samples of peptide esters, and to Prof. Dr. M. S. Dunn, University of California, U.S.A., for the samples of peptides.

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The Addition of Hydrogen Sulphide to Some Unsaturated Aliphatic Nitriles and Carboxylic Acids

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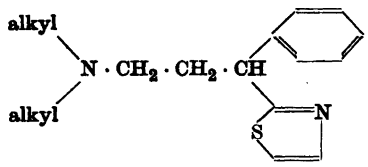
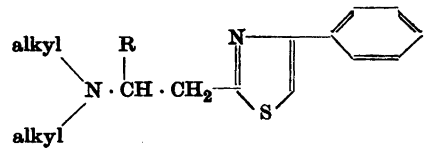
In a previous communication [1] the syntheses of a number of aminoalkyl derivatives of 2-benzylthiazole, of the type I, were described. In connection with this work it was desired to prepare a series of 2-aminoalkyl-4-phenylthiazoles, II.



I



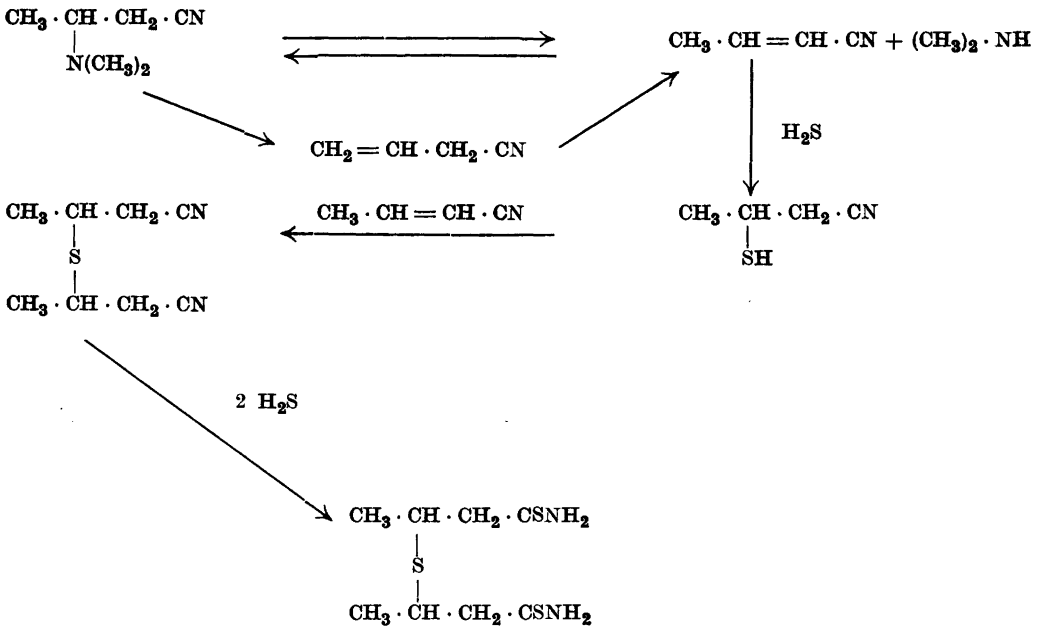
R = H or CH_3

II

As starting materials for these syntheses β-dialkylaminosubstituted thiobutyramides and thiopropionamides were required, and attempts were made initially to prepare β-dimethylaminothiobutyramide by the addition of hydrogen sulphide to β-dimethylaminobutyronitrile in ethanolic solution at 70° using sodium ethoxide as a catalyst. A crystalline product melting at 169—170° was obtained, but this, however, did not have the properties of the expected thioamide. It was insoluble in acids, and analyses showed that it had the composition $C_8H_{16}N_2S_3$. Two thioamide groups were present as one mole of the substance reacted with two moles of ω-bromoacetophenone or ω,p-dibromoacetophenone to give thiazoles of the compositions $C_{24}H_{24}N_2S_3$ and $C_{24}H_{22}Br_2N_2S_3$ respectively. The former thiazole was oxidised by treatment with hydrogen peroxide in acetic acid to a compound having the composition $C_{24}H_{24}N_2OS_3$, and it was thus probable, that the third sulphur atom

in the molecule was present as a sulphide group. The thioamide yielded, on hydrolysis with concentrated hydrochloric acid, an acid having equivalent weight 103, and melting at 84—84.5°. These constants agree with those recorded for the higher melting form of β,β'-thiodibutyric acid, prepared by Lovén and Johansson[2] and later shown by Johansson[3] to be the racemic form of the acid. The product obtained from the hydrolysis of the thioamide gave no melting point depression with an authentic sample of acid synthesised by the method of Lovén and Johansson[2], and the corresponding sulphones obtained from the thio acids by oxidation with potassium permanganate or bromine were also identical. It was thus evident that the reaction between β-dimethylaminobutyronitrile and hydrogen sulphide yielded *dl*-β,β'-thiodi-(thiobutyramide), if it be assumed that no inversion occurred during the hydrolysis.

The formation of this compound can be accounted for by the following reaction sequence



The first step is assumed to be the decomposition of β-dimethylaminobutyronitrile into dimethylamine and crotononitrile or possibly allyl cyanide, a type of reaction which has been reported[4,5] to take place with other β-dialkylaminonitriles on heating. This reaction is probably reversible but proceeds

largely to completion owing to the removal of the crotononitrile by reaction with hydrogen sulphide. If allyl cyanide is the primary decomposition product, it would be expected to isomerise into crotononitrile almost instantaneously under conditions employed.

The second decomposition product, dimethylamine, acts as a catalyst for the addition of the hydrogen sulphide to crotononitrile, for the addition of β-mercaptobutyronitrile to another mole of crotononitrile, and for the formation of the thioamide. It is of course not impossible that the reactions occur in a different order. The sodium ethoxide added initially as a catalyst is of no importance, as the same reaction product is obtained in its absence.

In order to investigate the unexpected formation of β,β′-thiodi-(thiobutyramide) somewhat more closely, some other β-dialkylaminosubstituted or unsaturated nitriles were treated with hydrogen sulphide under similar conditions. β-Diethylaminobutyronitrile, allyl cyanide, and crotononitrile all yielded β,β′-thiodi-(thiobutyramide). If a very large excess (10 moles) of diethylamine, or better triethylamine, was used, excellent yields were obtained. It should be noted that acetyl and benzoyl derivatives of β-amino- and β-methylaminobutyronitrile are quite stable under the same conditions and give the expected β-acetylamino- and β-benzoylaminothiobutyramides [6]. The fact that allyl cyanide and crotononitrile give the same product is due to the rapid base catalysed isomerisation of the former compound. This isomerisation has been thorougly studied [4,7–10], the resonance between the double bond and the cyanide group being the cause of the stability of crotononitrile. When sodium ethoxide alone was used as catalyst, allyl cyanide was isomerised to crotononitrile, and no reaction with hydrogen sulphide could be detected.

β-Dialkylaminosubstituted propionitriles seem to behave in an analogous manner to the butyronitriles. β-Diethylaminopropionitrile or acrylonitrile yielded a compound of m.p. 94—95° and having the composition $C_6H_{12}N_2S_3$, which was shown to be β,β′-thiodi-(thiopropionamide). Hydrolysis of this product gave an acid of m.p. 130—131° and equivalent weight 89, which proved to be identical with an authentic sample of β,β′-thiodipropionic acid.

When sodium ethoxide was used as catalyst, acrylonitrile added on ethanol forming β-ethoxypropionitrile, and no reaction with hydrogen sulphide could be detected. It is of interest to note, that in this case, the sodium ethoxide failed to catalyse the addition of hydrogen sulphide to the cyanide group, though it is usually considered to be superior to amines in this respect [11].

A related reaction has been reported by Gershbein and Hurd [12], who obtained β,β′-thiodipropionitrile from acrylonitrile and hydrogen sulphide using sodium methoxide or "Triton B" (trimethylbenzylammonium hydroxide) as catalyst by carrying out the reaction in the absence of solvent at 70° at

atmospheric pressure. The same compound was obtained by Lieser and Kemmner [13] using acetone as solvent, and sodium sulphide as catalyst at room temperature. The formation of β,β'-thiodipropionitrile under these conditions, which are somewhat milder than those employed in the present investigation, is, however, evidence for the validity of the reaction sequence outlined above.

On the other hand, when γ-piperidinobutyronitrile was treated with hydrogen sulphide under the usual conditions, γ-piperidinothiobutyramide was smoothly formed.

In the hope of finding an easy route to β,β'-thiodibutyric acid, crotonic acid was treated with hydrogen sulphide in ethanolic solution in the presence of diethylamine. However, the only product was β-mercaptobutyric acid identified by conversion into β-benzylsulphonylbutyric acid. Vinylacetic acid, which is easily isomerised by bases to crotonic acid [8,14] gave the same product.

Acrylic acid when treated in this manner yielded β-mercaptopropionic acid together with β,β'-dithiodipropionic acid, apparently formed by oxidation of the mercapto acid.

A similar synthesis of a mercapto acid has recently been reported by Földi and Kollonitsch [15], who prepared β-mercapto-α-carboxy*iso*valeric acid by the addition of hydrogen sulphide to *iso*propylidenemalonic acid in ethanol solution and with triethylamine as catalyst.

EXPERIMENTAL

β-Dimethylaminobutyronitrile and hydrogen sulphide

β,β'-Thiodi-(thiobutyramide). β-Dimethylaminobutyronitrile (20 g, prepared according to Bruylants [4]) was dissolved in absolute ethanol (200 ml) in which sodium (0.5 g) had been dissolved. The mixture was saturated with hydrogen sulphide at − 20°, and then kept at 70° in a sealed glass bottle for sixteen hours. After cooling, the solvent was evaporated at reduced pressure, and the solid residue washed thoroughly with water and collected. The crude product (12.4 g) which melted at 149—153° was recrystallised three times from ethanol, giving white prisms, m. p. 169—170° (dec.).

$C_8H_{16}N_2S_3$ (236.4)	Calc.	C 40.7	H 6.82	N 11.9	S 40.7
	Found	» 41.0	» 6.84	» 11.7	» 40.9

In another experiment under the same conditions but with the omission of sodium, 4.1 g of crude product was obtained from 5.6 g of β-dimethylaminobutyronitrile. The purified thioamide was identical with the thioamide obtained above.

Hydrolysis of β,β'-thiodi-(thiobutyramide) to dl-*β,β'-thiodibutyric acid.* The thioamide (1.0 g) was refluxed with concentrated hydrochloric acid (10 ml) until hydrogen sulphide

was no longer evolved (2 hours). The solution was extracted with ether (5 × 20 ml), the ether extract dried, and the solvent evaporated. The crystalline residue (0.9 g) was recrystallised twice from benzene — light petroleum, from which it separated in white plates, m. p. 84—84.5°, undepressed by an authentic specimen of *dl-β,β'*-thiodibutyric acid prepared by the method of Lovén and Johansson [8]. Titration of the product with 0.1 *N* sodium hydroxide gave an equivalent weight of 103.5 (calc. for *β,β'*-thiodibutyric acid 103.1).

Oxidation of dl-*β,β'-thiodibutyric acid to* dl-*β,β'-sulphonyldibutyric acid.* The thio acid (100 mg) was dissolved in water (1 ml) and a slight excess of bromine was added. After a few minutes the sulphone began to separate as white plates (86 mg). After recrystallisation from acetone — light petroleum the compound had m.p. 170—171° undepressed by an authentic sample of *β,β'*-sulphonyldibutyric acid prepared by the method of Lovén and Johansson [8] by the oxidation of the thio acid with potassium permanganate. Equivalent weight: found 118.8, calcd. 119.1.

*β,β'-Di-[4-(*p*-bromophenyl)-thiazolyl-2]-diisopropyl sulphide.* In order to show the presence of two thioamide groups, *β,β'*-thiodi-(thiobutyramide) (2.0 g, 8.5 millimoles) and *ω,p*-dibromoacetophenone (4.85 g, 17.5 millimoles) were refluxed in ethanol (15 ml) for half an hour. The precipitate which formed on cooling was collected, suspended in dilute ammonia, and the mixture shaken for an hour at room temperature. The solid (4.65 g) was collected and recrystallised twice from ethanol-acetone (4 : 1) forming white leaflets, m.p. 132—133°.

$C_{24}H_{22}Br_2N_2S_3$ (594.5)	Calc.	C	48.5	H	3.73	S	16.2
	Found	»	47.9	»	3.64	»	16.3

β,β'-Di-(4-phenylthiazolyl-2)-diisopropyl sulphide was prepared from the thioamide (2.5 g) and *ω*-bromoacetophenone (4.65 g) in the same manner as above. The crude product (4.0 g) was crystallised twice from light petroleum and then melted at 66—67°.

$C_{24}H_{24}N_2S_3$ (436.6)	Calc.	C	66.0	H	5.54	S	22.0
	Found	»	66.3	»	5.52	»	21.8

β,β'-Di-(4-phenylthiazolyl-2)-diisopropyl sulphoxide. The thiazole compound (250 mg) obtained above was dissolved in glacial acetic acid (5 ml), and 30 % hydrogen peroxide (0.3 ml) was added. The solution was kept at room temperature for a week and was then poured into excess of water. The precipitated oil solidified after a few days, giving white crystals (210 mg), melting at 89.5—90° after recrystallisation twice from acetone — light petroleum.

$C_{24}H_{24}N_2OS_3$ (452.6)	Calc.	C	63.7	H	5.35	N	6.19
	Found	»	63.9	»	5.58	»	6.13

γ,γ'-Thiodi-(thiobutyramide). As a comparison with the *β,β'*-compound, this thioamide was prepared from *γ,γ'*-thiodibutyronitrile [16] (8.0 g) and diethylamine (70 g) in ethanol (100 ml) under the usual conditions. The crude thioamide (7.4 g) was recrystallised twice from methanol and then melted at 88—90°.

$C_8H_{16}N_2S_3$ (236.4)	Calc.	N	11.9	S	40.7		
	Found	»	11.6	»	40.0		

γ,γ'-Di-(4-phenylthiazolyl-2)-dipropyl sulphide. For further characterization, γ,γ'-thiodi-(thiobutyramide) (0.6 g) was converted to the thiazole by refluxing it in ethanol (6 ml) with ω-bromoacetophenone (1.1 g) for one hour. Working up in the usual way gave a semi-solid product, which was dissolved in a boiling mixture of ethanol and light petroleum. On cooling, white crystals (0.5 g) were deposited. After one more recrystallisation the thiazole compound melted at 49—50°.

$C_{24}H_{24}N_2S_3$ (436.6)	Calc.	C	66.0	H	5.54
	Found	»	65.8	»	5.59

β-Diethylaminobutyronitrile and hydrogen sulphide

β-Diethylaminobutyronitrile. This compound, which was required as a starting material, has not been described in the literature. Bruylants [4] stated that attempts to add diethylamine to allyl cyanide in a manner analogous to the preparation of β-dimethylaminobutyronitrile, only resulted in the rearrangement of the allyl cyanide to crotononitrile. It has now been found, however, that if distillation of the crude product at atmospheric pressure was avoided, β-diethylaminobutyronitrile could be obtained in good yield (79 %) by this method. Allyl cyanide (20 g) and 33 % diethylamine (90 g) were mixed with cooling and set aside at room temperature for three days, during which time the mixture separated into two phases. The mixture was saturated with potassium carbonate, and the top layer separated and fractionated, yielding 33 g of product, b. p. 83—85°/11 mm, n_D^{20} 1.4388.

$C_8H_{16}N_2$ (140.2)	Calc.	N	20.0	Eq. wt.	140.2
	Found	»	19.9	»	139.5

(Mixed indicator: bromo-cresol green-methyl red)

β-Diethylaminobutyronitrile (5 g) when treated under the usual conditions with hydrogen sulphide in ethanol (50 ml) to which sodium (0.15 g) had been added yielded 1.55 g of crude product, which after purification was identical with β,β'-thiodi-(thiobutyramide). If, instead of sodium, diethylamine (26 g) was used as catalyst, a higher yield (3.65 g) was obtained.

Allyl cyanide and hydrogen sulphide

Allyl cyanide was prepared from allyl bromide [17], or from allyl alcohol by the excellent method of Breckpot [18], which was more convenient.

Allyl cyanide (5.0 g, 0.075 mole) was treated with hydrogen sulphide in ethanol (50 ml) in the usual way, various amounts of different catalysts being used. With diethylamine (55 g, 0.75 mole), 6.2 g of crude crystalline product was obtained: triethylamine (75.8 g, 0.75 mole) gave a still better yield (8.7 g). The products proved after purification to be identical with β,β'-thiodi-(thiobutyramide). In some experiments when only small amounts of amine (0.02 mole) were used viscous oils were obtained, from which no identifiable products could be isolated. If the amine was replaced by sodium (0.115 g, 0.005 mole) the allyl cyanide was isomerised to crotononitrile (3.4 g), b.p. 114—116°, n_D^{20} 1.4204, together with a tarry destillation residue.

Crotononitrile and hydrogen sulphide

Crotononitrile (mixture of *cis*- and *trans*-form) was prepared by shaking allyl cyanide with *N* sodium hydroxide for 12 hours, as described by Letch and Linstead [10]. Treatment of the nitrile (5.0 g) in ethanol (50 ml) with hydrogen sulphide in the presence of diethylamine (55 g) yielded 7.5 g of crude β,β′-thiodi-(thiobutyramide).

β-Diethylaminopropionitrile and hydrogen sulphide

β,β′-Thiodi-(thiopropionamide). β-Diethylaminopropionitrile [5] (10 g) and diethylamine (29 g), dissolved in ethanol (50 ml), were allowed to react with hydrogen sulphide under the usual conditions. The crystalline compound obtained (6.85 g) melted at 94.5—95.5° after three recrystallisations from acetone — light petroleum.

$C_6H_{12}N_2S_3$ (208.4)	Calc.	C	34.6	H	5.81	S	46.2
	Found	»	35.1	»	5.86	»	46.0

Hydrolysis of β,β′-thiodi-(thiopropionamide) to β,β′-thiodipropionic acid. β,β′-Thiodi-(thiopropionamide) (0.7 g) was refluxed with concentrated hydrochloric acid (10 ml) for two hours. The solution was extracted with ether (5 × 20 ml), and evaporation of the ether gave a crystalline residue (0.6 g), which, after recrystallisation from ethyl acetate-light petroleum, melted at 130—131°. Titration with alkali gave an equivalent weight of 89.3, in good agreement with the value of 89.1 for β,β′-thiodipropionic acid and no depression in m.p. was obtained on admixture with an authentic specimen of this acid.

Bromine oxidation of the acid yielded β,β′-sulphonyldipropionic acid; eq. wt. 105.4 (calc. 105.1). After recrystallisation from ethanol the compound melted at 223—225° in agreement with the values recorded by Holmberg [19] (220—222°) and by Larsson [20] (225°), but at variance with the value of 210° first reported by Lovén [21].

β,β′-Di-(4-phenylthiazolyl-2)-diethyl sulphide. This compound was prepared by refluxing β,β′-thiodi-(thiopropionamide) (0.4 g) and ω-bromoacetophenone (0.84 g) in ethanol (5 ml) for one hour. The crude thiazole (0.7 g), which was isolated as previous thiazoles, was recrystallised twice from ethanol and then melted at 68—69°.

$C_{22}H_{20}N_2S_3$ (408.6)	Calc.	C	64.7	H	4.94	N	6.86
	Found	»	64.5	»	4.89	»	6.99

Acrylonitrile and hydrogen sulphide

Acrylonitrile (6.1 g) when heated with diethylamine (42 g) in ethanol (50 ml) and hydrogen sulphide following the usual procedure, yielded 11 g of crude β,β′-thiodi-(thiopropionamide).

In another experiment, acrylonitrile (15 g) was treated with hydrogen sulphide in ethanol (75 ml) to which sodium (0.2 g) had been added. The product was an oil which boiled at 169—170° (11.2 g), apparently consisting of β-ethoxypropionitrile, formed by the addition of ethanol to the acrylonitrile [22].

γ-Piperidinobutyronitrile and hydrogen sulphide

γ-Piperidinothiobutyramide. γ-Piperidinobutyronitrile [5] (4.0 g) and diethylamine (19 g) were dissolved in ethanol (50 ml) and treated with hydrogen sulphide in the usual way. After removal of the solvent at reduced pressure, a dark viscous oil was obtained, which crystallised on trituration with ethanolic hydrogen chloride giving white crystals (3.4 g), which melted at 155—156° after recrystallisation twice from ethanol.

$C_9H_{18}N_2S \cdot HCl$ (222.8)	Calc.	C	48.5	H	8.59	N	12.6
	Found	»	47.9	»	8.44	»	12.6

Crotonic acid and hydrogen sulphide

A mixture of solid crotonic acid (10.0 g), diethylamine (87.5 g), and ethanol (100 ml) was saturated with hydrogen sulphide at — 20°, and heated at 70° overnight in a sealed vessel. The solvent was removed at reduced pressure, and the residue acidified with hydrochloric acid and extracted with ether (5 × 50 ml). After evaporation of the ether, the oily residue was distilled *in vacuo* giving 7.1 g of product, b.p. 70°/0.15 mm, n_D^{20} 1.4781. The compound was titrated with alkali and with iodine giving the equivalent weights 120.8 and 119.2, respectively (calc. for β-mercaptobutyric acid 120.1). Holmberg and Schjånberg [23] record n_D^{20} 1.4782 for β-mercaptobutyric acid.

The acid was identified by benzylating it following the directions of Holmberg and Schjånberg [23]. Bromine oxidation of the oily β-benzylthiobutyric acid in aqueous solution gave a product which was recrystallised twice from water, forming prisms of m. p. 130.5—131° undepressed on admixture with an authentic specimen of β-benzylsulphonylbutyric acid. Eq. wt.: found 242.2, calc. 242.2.

Vinylacetic acid and hydrogen sulphide

Vinylacetic acid [24] (10 g) yielded under the conditions of above experiment 5.4 g of β-mercaptobutyric acid, identified as the benzylsulphonyl derivative.

Acrylic acid and hydrogen sulphide

Acrylic acid (4.3 g) was treated with hydrogen sulphide in ethanol (50 ml) in the presence of diethylamine (44 g) in the usual manner. Distillation of the reaction product *in vacuo* gave an oil (2.3 g), b.p. 65°/0.2 mm. Titrations with alkali and iodine gave the equivalent weights 107.1 and 104.8, respectively (calc. for β-mercaptopropionic acid 106.1). The mercapto acid was dissolved in water and oxidised with iodine giving crystals of m.p. 154—155°, undepressed by an authentic specimen of β,β′-dithiodipropionic acid. Extraction of the distillation residue with ethyl acetate gave a further 0.9 g of the same material.

SUMMARY

Hydrogen sulphide reacts with β-diethylamino- and β-dimethylaminobutyronitrile in ethanol at 70° to form β,β′-thiodi-(thiobutyramide). The same product is obtained from allyl cyanide or crotononitrile in the presence of diethylamine or triethylamine.

Under similar conditions β-diethylaminopropionitrile and acrylonitrile give β,β′-thiodi-(thiopropionamide).

Crotonic acid and vinylacetic acid add hydrogen sulphide under the same conditions to give β-mercaptobutyric acid, while acrylic acid yields β-mercaptopropionic acid.

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Comparison between the Acetylcholinesterases of *Helix* Blood and Cobra Venom. I. The Hydrolysis of Acetylcholine and Its Inhibition by Various Compounds

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Much work has been carried out during recent years with mammalian esterases which split acetylcholine and other choline esters. The specificities of these enzymes are now fairly well known. One type is the acetylcholinesterase or aceto-cholinesterase [1] ("specific", "true" cholinesterase), present mainly in nervous tissues and in erythrocytes. The other type (cholinesterase, butyro-cholinesterase, "pseudo" or "non-specific" cholinesterase) occurs in the blood plasma of certain animals. These two types are best differentiated by using acetylcholine and butyrylcholine as substrates and by testing the activity-substrate concentration relationship; for details, see a recent review [2].

For the erythrocyte and nerve acetylcholinesterases no characteristics so far tested have been found to separate them from each other. In certain animals, however, there occur esterases which have most of the characteristics of an acetylcholinesterase but which differ from the latter in certain respects. The blood of *Helix pomatia*, for instance, has a high acetylcholine splitting power [3–6]. The esterase responsible for this shows the same specificity pattern and a similar activity-substrate concentration relationship as the erythrocyte and nerve esterase [7], but there are certain differences (discussed below) which separate it from other known acetylcholinesterases. A similar situation holds for the esterase of cobra venom. This has a high acetylcholine splitting activity [8]. The venoms from various species do not always show this property [9]. Non-choline esters are hydrolysed by the cobra venom [10–16], the esterase of which shows the same specificity pattern as other acetylcholinesterases and gives the activity-substrate concentration relationship typical of these [7].

The main purpose of the present study is to compare some properties of the esterases of *Helix pomatia* blood and cobra venom (*Naja naja*). The first part deals with the enzymic hydrolysis of acetylcholine and the inhibition of this reaction by certain "anticholinesterases". In a second paper [19], published as a second part of this study, the hydrolysis of various choline and non-choline esters will be discussed.

METHODS

Experimental

The esterase activity was measured with the Warburg manometric technique previously described in detail [6,17]. The enzyme activity is expressed in b_{30} values, *i. e.*, the amount of CO_2 in μl evolved during 30 minutes minus the corresponding value for non-enzymic hydrolysis. Measurements were made at 25° C. Substrates, enzymes, and inhibitors were dissolved in bicarbonate-Ringer's solution.

The edible snails, *Helix pomatia*, were collected in the early autumn. The blood was drawn from the circulus venosus and kept in the refrigerator. No change of activity was observed in 3 months.

A dried and crystallised preparation of cobra venom (*Naja naja*) was used. The venom was dissolved in bicarbonate-Ringer's solution. The solutions were stable when kept in the refrigerator for about 3 months. Fresh solutions, however, behaved differently from old ones. The solutions were made at least 4 hours before use, unless otherwise stated. The dried venoms from the Viperidae, *Crotalus horridus* and *Ancistrodon contortrix*, were used in a few preliminary experiments.

Acetylcholine chloride was used as substrate in all experiments. The inhibitors used were: choline chloride, methylene blue, physostigmine sulphate, prostigmine bromide, and tetraethyl pyrophosphate (TEPP) (pure), all dissolved in bicarbonate before use.

Graphical presentation of results

The results obtained in experiments with various inhibitor concentrations (I) and constant substrate concentration (S) have been analysed by the procedure worked out in a recent paper [6]. In the case of competitive inhibition and constant concentration of enzyme and substrate, a plot of the inhibition, expressed as v/v', against the concentration of inhibitor gives a straight line:

$$\frac{v}{v'} = 1 + [\mathrm{I}] \frac{K_S}{K_I ([\mathrm{S}] + K_S)}$$

The initial reaction velocities v and v' represent the enzyme activities in the absence of the inhibitor and in its presence respectively; both v and v' are expressed in b_{30} values. K_S and K_I are the dissociation constants of the enzyme-substrate and enzyme-inhibitor complexes respectively. This method has been applied recently in evaluating the action of choline [6] and other inhibitors [17, 18] on acetylcholine splitting enzymes.

The experimental results obtained by varying the substrate concentration keeping the inhibitor concentration constant have been recorded in activity-pS diagrams (pS is the negative logarithm of substrate concentration.)

RESULTS

Esterase activity

Helix blood. The blood of *Helix pomatia* is several times more active towards acetylcholine than the blood of vertebrates. Within certain limits there is a direct proportionality between blood concentration and rate of acetylcholine hydrolysis. This substate gives a bell-shaped activity-pS curve, characteristic of the acetylcholinesterases (Fig. 2). This relationship has been reported in detail in previous papers by the present author [5–7].

Snake venom. Zeller [9] showed that the venoms of the species of the Colubridae possess marked cholinesterase activity while the venoms of the species of the Viperidae show no such activity. The latter observation by Zeller was confirmed with the two viper venoms. These do not split acetylcholine even when the venom concentration is 100 times that used in the experiments with the cobra venom. It was also proved that the non-active venoms do not contain esterase inhibitors. The results are shown in Table 1.

Table 1. Comparison between the enzyme activities of three different snake venoms and mixtures of these.

Snake venom 0.1 mg of each per 2.00 ml reaction mixture	b_{30}
Naja naja	159.5
Crotalus horridus	0
Ancistrodon contortrix	0
N. naja + *C. horridus*	156.5
N. naja + *A. contortrix*	143.5

The observation has been made that the solutions of the cobra venom prepared immediately before use do not give the same activity as old solutions. A typical example is illustrated in Fig. 1. The difference between the hydrolysis curves for acetylcholine of a fresh solution and two old solutions (one week and four hours respectively) is distinct. With the fresh solution the hydrolysis rate increases during the reaction. No explanation for this induction period has been found; the enzyme may be absorbed on inert proteins and slowly go into solution.

The esterase activity of the cobra venom is comparatively very high. The b_{30} value obtained at optimum acetylcholine concentration and calculated

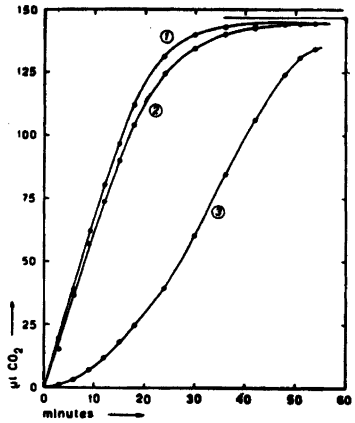

Fig. 1. Total hydrolysis of acetylcholine (3.30 × 10^{-3} M) by cobra venom dissolved in bicarbonate Ringer's solution. Venom concentration: 0.1 mg per 2.00 ml reaction mixture.

1. *One week old solution;*
2. *Four hours old solution;*
3. *Solution prepared immediately before filling of flask.*

for one mg dried venom is 1600—1800. This value is about the same as was found by Zeller [9]. When comparing this with the esterase activity of other material we have to remember that the natural venom contains about 70 % water, giving an activity for the native venom of 480—540. The approximate values for certain other highly active tissues under similar conditions are: horse plasma, 4; *Helix* blood, 6; the dart sac of *Helix*, 10; nucleus caudatus of human brain, 20; the electric organ of *Electrophorus*, 100. Snake venom is therefore the most active tissue known, particularly as venoms from other Colubridae species are still more active than that of the cobra [9]. The activities of the various solutions of cobra venom made at different times are not always the same, which might be explained by variations in the enzyme content of different crystals of the venom.

Direct proportionality between rate of acetylcholine hydrolysis and concentration of snake venom is obtained (Table 2) provided the enzyme concentration is not too high.

Most probably the cobra venom does not contain an ali-esterase. This will be discussed in a second paper [19].

Precipitation of the esterases by ammonium sulphate. Some preliminary experiments have been performed in precipitating the esterases with ammonium sulphate. Table 3 shows the results. In both cases the enzymes may be separated from non-active material by fractionation. The *Helix* blood enzyme

Table 2. Rate of enzymic hydrolysis of acetylcholine (1.10×10^{-2} M) as function of snake venom concentration.

mg cobra venom per 2.00 ml reaction mixture	b_{30}	b_{30}/0.1 mg
0.08	121.5	152
0.10	155	155
0.12	195	162.5
0.16	249	155.5
0.20	311.5	156
0.28	429	153.5
0.40	597.5	149.5

is precipitated maximally at about 60 % saturation, the snake venom esterase at a somewhat higher sulphate concentration (70 %). In both cases the enzymes can be absorbed completely onto infusorial earth from a solution of the sulphate precipitate. Bicarbonate solution brings about partial elution, but complete elution has so far proved impossible.

Esterase inhibition at various substrate concentrations

The inhibition of the enzymic hydrolysis of acetylcholine by choline chloride, methylene blue, physostigmine sulphate, prostigmine bromid, and TEPP is demonstrated in Fig. 2.

Table 3. Precipitation by ammonium sulphate. Helix blood diluted with distilled water of the same volume; the activity tested after dilution 1 to 10. Snake venom, 25 mg per 50 ml bicarbonate solution.

g ammonium sulphate per 10 ml enzyme solution	b_{30}			
	Helix blood		Cobra venom	
	Centrifugate	Precipitate	Centrifugate	Precipitate
—	193	—	294	—
2.00	190	3	291	no prec.
2.50	159	31	—	—
3.00	92	148	229	56.5
4.00	77	156	50	251
5.00	—	—	75	123

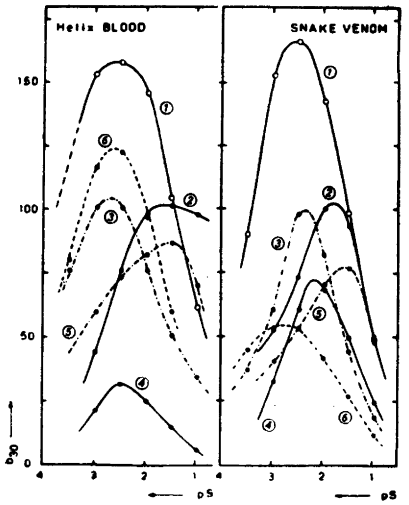

Fig. 2. Activity-pS curves for the enzymic hydrolysis of acetylcholine by Helix blood and snake venom respectively in the presence of certain inhibitors. Concentration of blood and venom per 2.00 ml reaction mixture: 25 µl blood and 0.1 mg venom respectively. The inhibitor concentration is the same in both series unless otherwise stated.

1. *Control (no inhibitor present);*
2. *Choline: 7.16 × 10^{-2} M;*
3. *Physostigmine: 3.08 × 10^{-9} M;*
4. *Prostigmine: 6.60 × 10^{-9} M, blood; 1.58 × 10^{-7} M, venom;*
5. *Methylene blue: 8.57 × 10^{-5} M, blood; 2.14 × 10^{-5} M, venom;*
6. *TEPP: 1.45 × 10^{-9} M, blood; 8.70 × 10^{-9} M, venom.*

Choline. In a recent paper [6] it has been shown that choline causes a shift to higher values of the optimum acetylcholine concentration for the esterases of erythrocytes and nerve tissues. This change of optimum substrate concentration is also observed for the snake venom esterase (Fig. 2, to the right). This shift of optimum is such that no inhibition is observed by 10^{-2} *M* choline when the acetylcholine concentration is higher that 3 x 10^{-2} *M*; at 3 x 10^{-3} *M* acetylcholine, however, the activity is inhibited 56 % by the same choline concentration.

A similar shift of optimum substrate concentration is observed with the *Helix* blood esterase. The action of choline, however, is such that at high acetylcholine concentration choline has a weak activating effect on the enzyme activity. An explanation for this may be that the blood contains a trace of a second cholinesterase (a butyro-cholinesterase) which is not inhibited by excess of acetylcholine.

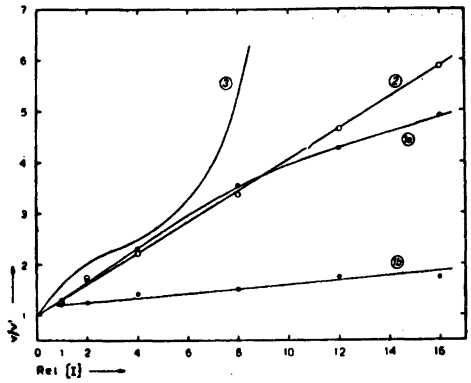

Fig. 3. Enzyme inhibition by choline chloride as function of inhibitor concentration. v = velocity in the absence, v' = in the presence of choline, expressed in μl CO_2 evolved in 30 minutes (b_{30}). Relative inhibitor concentration (Rel. [I]), 1 = 1.79 × 10^{-2} M choline. pI_{50} = negative logarithm of molar inhibitor concentration giving 50 % inhibition.

Enzyme	*Acetylcholine*	pI_{50}
1a. Helix blood	*3.30 × 10^{-3} M*	*1.27*
1b. » »	*3.30 × 10^{-2} M*	*(0.48)*
2. Cobra venom	*3.30 × 10^{-3} M*	*1.24*
3. Erythrocytes (human)	*1.10 × 10^{-3} M*	*1.47*

Methylene blue. This dye causes a shift of substrate optimum for both esterases (Fig. 2), as previously found for the erythrocyte esterase [17].

Physostigmine and prostigmine. Neither of these inhibitors shift the optimum acetylcholine concentration, except that at relatively high concentrations a slight change of substrate optimum is observed with physostigmine. This is in sharp contrast to the findings with the acetylcholinesterases of nerve tissues, erythrocytes, and electric tissue [6, 18].

TEPP. As expected, the inhibition by this irreversible inhibitor (inactivator) is independent of substrate concentration.

Esterase inhibition as a function of inhibitor concentration

The results of these experiments have been analysed by the graphical method described above. They are recorded in Figs. 3, 4, 6, and 7.

Choline. As described above, choline inhibits the *Helix* blood esterase in a way which differs from that for other acetylcholinesterases. At relatively low acetylcholine concentration (3 × 10^{-3} M) and a choline concentration

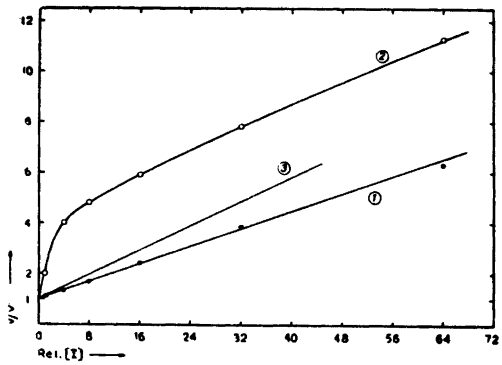

Fig. 4. Enzyme inhibition by methylene blue as function of inhibitor concentration. Acetylcholine concentration: 3.30 × 10^{-3} M. Rel. [I], 1 = 5.35 × 10^{-6} M. Description as in Fig.3.

Enzyme	pI_{50}
1. Helix blood	*4.19*
2. Cobra venom	*5.27*
3. Erythrocytes (human)	*4.36*

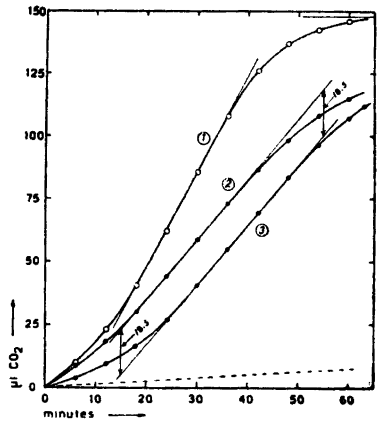

Fig. 5. The course of acetylcholine (3.30 × 10^{-3} M) hydrolysis by the action of cobra venom in the presence of prostigmine bromide (7.92 × 10^{-8} M). The parallelism between those parts of the inhibitory curves which represent equilibrium between enzyme, substrate, and inhibitor is illustrated. The dotted line refers to non-enzymic hydrolysis. Fresh solution of cobra venom.

1. *Control; b_{30} 110;*
2. *Prostigmine and acetylcholine simultaneously mixed with the enzyme; b_{30} 67.5;*
3. *Enzyme incubated 70 minutes with prostigmine before the addition of acetylcholine; b_{30} 67.5.*

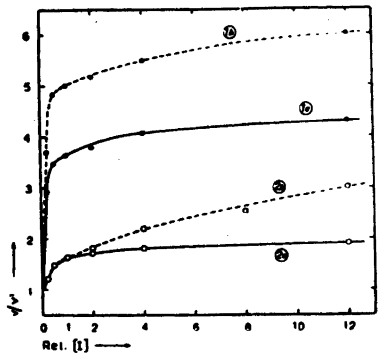

Fig. 6. Enzyme inhibition by physostigmine sulphate and prostigmine bromide as function of inhibitor concentration. Acetylcholine concentration: 3.30 × 10^{-3} M. Description as in Fig. 3.

Enzyme	*Inhibitor*	*Rel. [I]*
1a. Helix blood	*Physostigmine*	*7.70 × 10^{-10} M*
1b. » »	*Prostigmine*	*1.65 × 10^{-9} M*
2a. Cobra venom	*Physostigmine*	*7.70 × 10^{-10} M*
2b. » »	*Prostigmine*	*1.98 × 10^{-8} M*

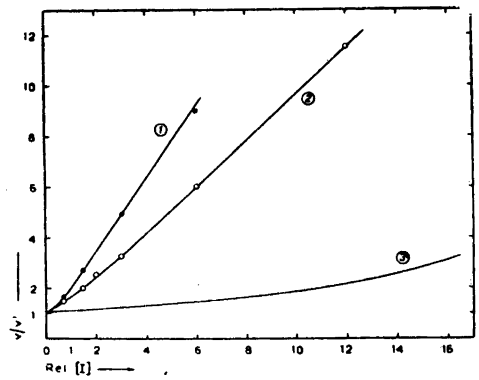

Fig. 7. Enzyme inhibition by TEPP as function of inhibitor concentration. Acetylcholine concentration: 3.30 × 10^{-3} M. Rel. [I], 1 = 2.9 × 10^{-9} M. Description as in Fig. 3. Enzyme solutions incubated 60 minutes with TEPP before mixed with the substrate.

Enzyme	*pI_{50}*
1. Helix blood	*8.54*
2. Cobra venom	*8.36*
3. Erythrocytes (human)	*7.49*

not higher than 0.1 M a straight line is obtained in the graphical method, shown in Fig. 3. With increasing choline concentration the degree of inhibition does not increase in a way expected for a true competitive inhibition. When the substrate concentration is relatively high ($> 3 \times 10^{-2}$ M) the inhibition is no more of the true competitive type. This result might be due to the presence of a second enzyme, the nature of which is unknown. It may be a butyro-cholinesterase or/and an aliesterase present in low concentration. There is also a possibility that the active groups [20] of the enzyme molecule are different from those of other acetylcholinesterases.

Snake venom esterase is inhibited by choline in a competitive way. Using the same substrate concentration 50 % inhibition is attained at approximately the same choline concentration with both enzymes. In Fig. 3 the results obtained with human erythrocytes are inserted for comparison. The S-shaped curve in this case has been discussed in a previous paper [6].

Methylene blue inhibits the *Helix* blood enzyme in a truely competitive way (Fig. 4). The inhibition of the snake venom esterase seems to be more complicated. With increasing methylene blue concentration the degree of inhibition increases more rapidly at concentrations below 2×10^{-5} M than above. The results obtained with human erythrocytes have been inserted in Fig. 4 for comparison.

Physostigmine and prostigmine. It is important to investigate the time-course of inhibition not only for irreversible enzyme inactivators, but also for certain reversible inhibitors, *e. g.*, physostigmine and prostigmine. When an esterase is incubated with these inhibitors, equilibrium is not attained until about 25 minutes have elapsed; the exact time depends upon the inhibitor concentration used. If the inhibitor and substrate are simultaneously mixed with the enzyme, the period of time required for reaching the equilibrium is about the same as in the "incubation experiments". The initial reaction velocities, however, differ in the two types of experiments; after equilibrium the hydrolysis rates are the same for a given inhibitor concentration. This is illustrated with cobra venom in Fig. 5 for acetylcholine and prostigmine. It confirms the previous results obtained with the electric tissue esterase [18], human erythrocytes [21], and brain homogenates [22]. In Fig. 5 the reaction velocities at equilibrium are exactly the same. The importance of this observation is obvious. If one takes, for instance, the amount of CO_2 evolved in the first 20 minutes as the unit for enzyme activity a distorted picture is obtained. In all cases of this type of enzyme inhibition the complete hydrolysis curve must be analysed.

At constant substrate concentration the degree of esterase inhibition by physostigmine and prostigmine increases relatively rapidly up to a certain point and then continues to increase more slowly (Fig. 6). The relationship

between degree of inhibition and inhibitor concentration is different from that obtained with purified esterase preparations of electric tissue [18].

TEPP. As expected and in accordance with previous results obtained with other acetylcholinesterases, TEPP, an irreversible esterase inactivator, behaves differently from the reversible inhibitors described above. The curves in Fig. 7 show the effect on incubation with TEPP for one hour. The inhibition increases more rapidly with increasing inhibitor concentration than would be expected for a true competitive inhibitor. Both esterases studied seem to be more sensitive to the action of TEPP than are the esterases of electric tissue and erythrocytes.

When the esterases are incubated for about an hour with 10^{-8} *M* TEPP they are almost completely inactivated. When the esterases are mixed simultaneously with acetylcholine and TEPP, *i. e.*, no incubation, a much higher inhibitor concentration is necessary for enzyme destruction. Such results suggest that acetylcholine protects the enzyme against TEPP. Table 4 exam-

Table 4. Protection of the cobra venom acetylcholinesterase by acetylcholine (1.10×10^{-2} M) against the inactivation by TEPP (7.15×10^{-9} M).

	b_{30}
Control (no TEPP)	164.5
Enzyme incubated with TEPP 70 minutes before mixed with acetylcholine	9
Acetylcholine and TEPP simultaneously mixed with the enzyme	163

plifies the results obtained with cobra venom. The protective action of acetylcholine has recently been demonstrated with the electric tissue esterase [18] and the esterases of erythrocytes and brain [21, 22]. The various precautions which must be taken in evaluating the inhibition by irreversible "anticholinesterases", have also been discussed in a recent review [23].

Other esters, choline as well as non-choline, also show this protective action against TEPP inactivation. This will be demonstrated in the second paper [19]. In addition, choline seems to have the same effect although the protection is not as complete as with the other compounds tested. Table 5 shows the results obtained with *Helix* blood. When choline is added to the enzyme prior to TEPP, the hydrolysis rate is between those obtained when the enzyme is incubated with TEPP and choline respectively.

Table 5. Protection of the Helix blood acetylcholinesterase by choline against the inactivation by TEPP. Choline added prior to TEPP.

Choline *M*	TEPP *M*	b_{30}
—	—	166
—	7.15×10^{-9}	9
7.16×10^{-2}	—	87
7.16×10^{-2}	7.15×10^{-9}	57.5

A discussion of the results will be reported in connection with those described in the second paper [19].

SUMMARY

The properties of the esterases of *Helix pomatia* blood and cobra venom have been studied with acetylcholine as substrate. The results have been compared with those obtained with other esterases of the same type. Both enzymes are of the acetylcholinesterase type.

Some general characteristics of the acetylcholine hydrolysis by the action of cobra venom are reported. The venoms from two Viperidae species are inactive and do not contain esterase inhibitors. Fresh solutions of cobra venom do not show the same activity as old ones. Direct proportionality between acetylcholine hydrolysis and concentration of cobra venom is obtained.

Both the *Helix* blood and cobra venom esterases are precipitated by ammonium sulphate and can be thereby separated from non-active material.

The inhibition of the acetylcholine hydrolysis has been studied in detail with choline, methylene blue, physostigmine, prostigmine, and TEPP as inhibitors.

Choline and methylene blue cause a shift of the optimum acetylcholine concentration. For choline and *Helix* blood this shift is such that a small activation is observed at high acetylcholine concentration, probably due to the presence of a second esterase. Physostigmine and prostigmine do not give this shift in contradistinction to the results obtained with other acetylcholinesterases. The inactivation by TEPP is independent of substrate concentration.

The esterase inhibition as function of inhibitor concentration has been studied. In each case the results have been analysed graphically. In some cases the results differ from those obtained with other acetylcholinesterases. Generally, however, the degree of inhibition is of the same order of magnitude as for other acetylcholinesterases. The inhibition of both esterases by physo-

stigmine and prostigmine is a slow process. In the presence of these inhibitors the enzymic hydrolysis of acetylcholine attains an equilibrium which is the same whether or not the enzyme has been incubated with the inhibitor prior to the addition of acetylcholine. Acetylcholine and to a certain degree choline protects both enzymes against TEPP action. The latter compound is an irreversible enzyme inactivator.

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Comparison between the Acetylcholinesterases of *Helix* Blood and Cobra Venom. II. The Hydrolysis of Certain Choline and Non-Choline Esters

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The acetylcholinesterases (aceto-cholinesterases) of various sources (*e. g.*, nervous tissues, erythrocytes and electric tissue) split acetylcholine at a much higher rate than the butyryl ester which in some cases is not split at all. The other type of acetylcholine hydrolysing enzymes is the cholinesterases (butyro-cholinesterases [1]) of certain blood plasmata which split butyrylcholine at a higher rate than acetylcholine [2–4]. Both types of esterase split non-choline esters, the enzyme affinity of which is much lower than that of the choline esters; for details, see a recent review [5]. For the hydrolysis of non-choline esters the same relationship exists between hydrolysis rate and the acyl radical of the substrate, *i. e.*, the acetates bear much the same relationship to the acetylcholinesterase as do the butyrates to the plasma cholinesterase [6,1,7].

The main esterase of *Helix pomatia* blood belongs to the group of acetylcholinesterases [3,8,9]. It has the same specificity pattern, the same activity-acetylcholine concentration relationship and is inhibited by the same enzyme inhibitors as the other enzymes of this group. The *Helix* esterase also splits non-choline esters, *e. g.*, acetic acid esters of ordinary alcohols. In certain respects, however, it differs from the other acetylcholinesterases [10]. Similar properties have been found for the cobra venom esterase. Thus this enzyme splits, in addition to acetylcholine, other esters containing acetyl groups [11–14]. Moreover, it shows the same specificity pattern and gives the same characteristic activity-substrate concentrations relationship as the other acetylcholinesterases [3]. The specific anticholinesterases also inhibit the cobra venom esterase; this has been discussed in detail in Part I of this study [10].

In the present paper the enzymic hydrolysis of certain esters, choline esters as well as non-choline esters, will be discussed. The hydrolysis of triacetin and acetylsalicylcholine is reported in some detail.

METHODS

The esterase activity was measured by the Warburg manometric method [9] and is expressed in b_{30} values. The enzyme preparations have been described in the first paper of the present study [10]. The substrates used are listed in Table 1. They were dissolved in a bicarbonate Ringer's solution. As inhibitors, choline chloride and tetraethyl pyrophosphate (pure) were used.

Results were analysed graphically as described in the previous paper [10], and according to the procedure of Lineweaver and Burk [15].

RESULTS

Specificity

Helix blood. The hydrolysis of various esters, choline as well as ordinary esters, by *Helix* blood [3,8,9] gives essentially the same substrate pattern (Table 1) as found for the acetylcholinesterases of erythrocytes and nervous and elec-

Table 1. Enzymic hydrolysis of certain esters by the blood of Helix pomatia and the venom of Naja naja. Helix blood: 25 µl; cobra venom: 0.1 mg per 2.00 ml reaction mixture.

Substrate	Molarity × 100	*Helix* blood	Cobra venom
Acetylcholine chloride	1.10	159	187.5
Propionylcholine chloride	1.02	143	151
Butyrylcholine chloride	0.95	39.5	4.5
DL-Acetyl-β-methylcholine chloride	1.02	43	78
Benzoylcholine chloride	0.82	4.5	0
N-Acetyl-*p*-aminobenzoyl choline chloride *	0.66	0	0
Salicylcholine chloride *	0.72	0	3
Acetylsalicylcholine chloride	0.66	30.5	233
Acetylsalicylic acid (Na-salt)	1.11	12	5
Acetylaneurin chloride hydrochloride	0.53	45	68
Triacetin	9.20	79.5	84.5
Tributyrin	6.62	16	2
Ethyl acetate	22.7	4	4.5
Methyl *n*-butyrate	19.6	3	0

* Synthesised according to Euler *et al.* [17]

Table 2. Competition experiments. Acetylcholine (ACh), 1.10 × 10^{-2} M; butyrylcholine (BuCh), 9.50 × 10^{-3} M; acetylaneurin (AAn), 5.28 × 10^{-3} M; triacetin (TA), 9.20 × 10^{-2} M.

Substrate(s)	*Helix* blood	Cobra venom
ACh	159	187.5
BuCh	39.5	4.5
AAn	45	68
TA	79.5	84.5
ACh + BuCh	155	76.5
ACh + AAn	168.5	135.5
ACh + TA	134.5	117
BuCh + AAn	63.5	12.5
AAn + TA	63	86.5

tric tissues. Acetyl-β-methylcholine is split at a lower rate and gives an optimum at a higher concentration (pS_{opt} 1.35) than is given by acetylcholine (pS_{opt} 2.7). Benzoylcholine is hardly split at all. Propionylcholine is hydrolysed at a somewhat lower rate than acetylcholine, and butyrylcholine at a much lower rate. Among the non-choline esters the hydrolysis rate of acetylaneurin is especially noticeable. Tributyrin, methyl butyrate, and ethyl acetate are split at very low rates or not at all. The blood splits triacetin of high concentration at a higher rate than acetylcholine of the same molar concentration, a fact explained by the different activity-substrate concentrations relationships of the two substrates.

Interestingly acetylsalicylcholine is split at a relatively high rate, whereas salicylcholine is not split at all and acetylsalicylic acid is split at a relatively low rate. An explanation for this may be that the affinity of the acetylsalicylcholine for the enzyme has been changed relative to those of the two separate esters. Probably there is a mutual action of the two ester groups in acetylsalicylcholine. It has been found previously [9] in competitions experiments, at various substrate concentrations, that acetylcholine and acetylsalicylcholine are split by the same enzyme. Moreover, it has been shown that both ester linkages of acetylsalicylcholine are split by the *Helix* blood. It has now been demonstrated that the acetate link is the most rapidly split of the two. There is a possibility that a second enzyme hydrolyses the choline ester link. Preliminary experiments suggest that the hydrolysis of tributyrin is brought about by a separate esterase present in *Helix* blood in a comparatively low concentration.

Competition experiments with certain esters, the results of which are recorded in Table 2, indicate that the hydrolysis of acetylcholine, butyrylcholine, acetylaneurin, and triacetin by *Helix* blood is probably due to one and the same enzyme. In the case of butyrylcholine and eventually also acetylaneurin the possibility may be that a butyro-cholinesterase or/and an aliesterase in addition to the acetylcholinesterase participate in the enzymic hydrolysis.

Cobra venom. Like other acetylcholinesterase, the cobra venom esterase splits in addition to acetylcholine other acetyl esters, an observation first made by Bovet-Nitti [11]. Zeller and his co-workers [14] also found that this enzyme is not a "true" or "specific" cholinesterase. It has been proposed to call this enzyme an "acetylase" [11] or "acetylesterase" [14]. The substrate pattern is, however, much the same as that of acetylcholinesterases [3]. The enzymic hydrolysis of certain esters are shown in Table 1. Moreover, it will be demonstrated in the present paper that although the snake venom esterase differs in certain respects from the other acetylcholinesterases it has many properties in common with them, *e. g.*, inhibition by compounds generally considered to be specific anticholinesterases.

The substrate specificity of the cobra venom esterase is much the same as that of the *Helix* blood esterase (Table 1). The following points are noteworthy. The characteristic relationship between the hydrolysis rates of acetyl-, propionyl-, and butyrylcholine is still more pronounced than for the *Helix* blood esterase. Thus, butyrylcholine is hardly split at all by the cobra venom, in contradistinction to the *Helix* blood. There is no indication of the existence of an aliesterase or butyro-cholinesterase in cobra venom in addition to the acetylcholinesterase. Moreover, acetyl-β-methylcholine is split but not benzoylcholine, in accordance with Mendel's method [18] for the differentiation of the two types of cholinesterases. The characteristic difference between the activity-substrate concentration relationships of acetylcholine and triacetin hydrolysis has also been demonstrated for the cobra venom [3].

This esterase splits acetylaneurin at a relatively high rate (36 % of the acetylcholine rate, as compared with 28 % for *Helix* blood). This rate of hydrolysis is the highest ever obtained for this substrate with any acetylcholinesterase preparation.

To support the idea that only a single enzyme is responsible for the hydrolysis of various esters, some competition experiments have been carried out. The results are found in Table 2.

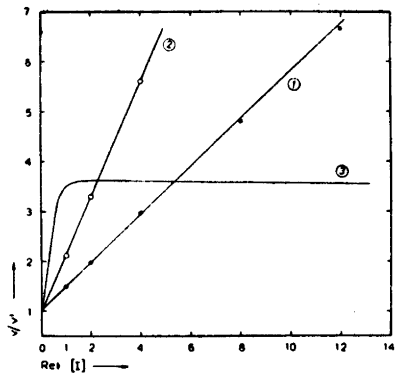

Fig. 1. Enzyme inhibition by choline chloride as function of inhibitor concentration. Substrate: 0.092 M *triacetin. v = velocity in the absence, v' = velocity in the presence of choline, expressed in μl CO_2 evolved in 30 minutes (b_{30}). Relative inhibitor concentration (Rel. [I]), 1 = 1.79 × 10^{-2} M. pI_{50} = negative logarithm of molar inhibitor concentration giving 50 % inhibition.*

Enzyme	pI_{50}
1. Helix blood	*1.45*
2. Cobra venom	*1.82*
3. Erythrocytes (human), aliesterase free	

The hydrolysis rate of acetylsalicylcholine is higher than for acetylcholine of high concentration. By contrast, the rates of hydrolysis of acetylsalicylic acid (*cf.* Zeller *et al.*[14]) and salicylcholine are only about 1/50 that of acetylcholine. As will be described later on, there is no evidence for the presence of a second esterase. It will be demonstrated that the acetate link alone of acetylsalicylcholine is split by the venom.

Hydrolysis of triacetin

Both esterases give the same relationship between triacetin concentration and hydrolysis rate. Triacetin of high concentration (> 0.1 *M*) is split at a higher rate than acetylcholine of the same high concentration. This non-choline ester is split by the acetylcholinesterase and not, even partly, by a second enzyme. Thus the enzymic hydrolysis of triacetin by this esterase is inhibited by choline (Fig. 1). The snake venom esterase seems to be inhibited more strongly than the *Helix* blood esterase. The I_{50} values obtained with 9.2 × 10^{-2} *M* triacetin are 1.52 × 10^{-2} and 3.58 × 10^{-2} *M* choline. The curves shown in Fig. 1 are typical of a competitive inhibitor [16], but the results

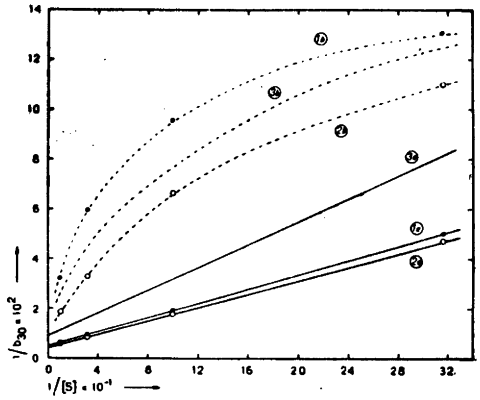

Fig. 2. Effect of choline chloride on the enzymic hydrolysis of triacetin as function of substrate concentration. Results analysed by the Lineweaver-Burk procedure. a) Controls; b) 3.58 × 10^{-2} M choline present.
1. Helix blood; 2. Cobra venom; 3. Erythrocytes (human), aliesterase free.

obtained in experiments with constant choline concentration and different substrate concentration and analysed by the Lineweaver-Burk procedure (Fig. 2) argue in favour of a more complicated mechanism. The inhibition of triacetin hydrolysis by choline may be due to steric hindrance. According to a recent theory of cholinesterase action, triacetin is attracted only to Centre II [19] (the esteratic site [20]) of the enzyme molecule, whereas choline is attracted to Centre I (the anionic site) by electrostatic attraction. The results in Fig. 2 are in accordance with this idea of the enzyme mechanism, for they show that the inhibition is not of the true competitive type.

The results obtained with an aliesterase free preparation of erythrocyte acetylcholinesterase are inserted in Figs. 1 and 2; they will be discussed in a paper to be published in due course.

Hydrolysis of acetylsalicylcholine

The enzymic hydrolysis of acetylsalicylcholine by *Helix* blood has recently been studied in detail [9]. The substrate optimum is much higher than for acetylcholine (pS_{opt} 1.35 and 2.7 respectively). Both ester linkages are split and the acetate linkage seems to be the more dapicly split. The choline ester link may be split by a butyro-cholinesterase.

As pointed out above acetylsalicylcholine is split at a high rate by the cobra venom. In Fig. 3 the total hydrolysis of this substrate of various concentrations is demonstrated. For the four lowest concentrations studied the hydrolysis stops when the calculated values for the hydrolysis of one

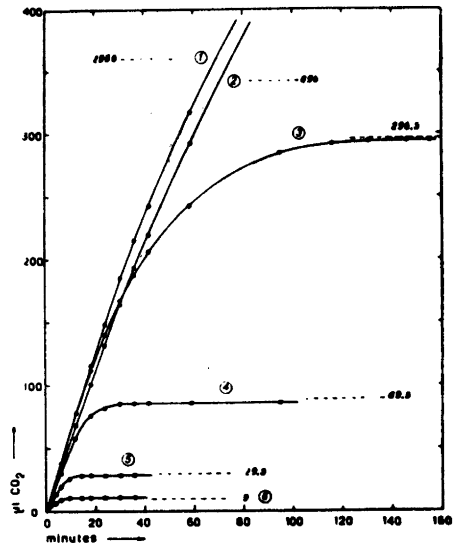

Fig. 3. Total (enzymic + spontaneous) hydrolysis of acetylsalicylcholine of various concentrations by cobra venom (0.1 mg per 2.00 ml reaction mixture). Dotted lines and numbers in µl CO_2 refer to calculated values for total hydrolysis of one ester linkage. Molar substrate concentrations:

1	*2*	*3*	*4*	*5*	*6*
6.62×10^{-2}	1.99×10^{-2}	6.62×10^{-3}	1.99×10^{-3}	6.62×10^{-4}	1.99×10^{-4}

ester linkage are reached. This link is the acetate link. Since after complete hydrolysis, the solution gives a violet colour with ferric chloride which is characteristic of a free phenolic —OH group and which is also obtained with salicylcholine, but not with acetylsalicylic acid (as sodium salt). The presence of the choline link has rendered the acetate link in acetylsalicylcholine more susceptible of enzymic hydrolysis than that in acetylsalicylic acid.

The substrate optimum for acetylsalicylcholine hydrolysis by cobra venom is somewhat higher (pS_{opt} 2.0) than for the corresponding acetylcholine hydrolysis (pS_{opt} 2.5). The hydrolysis rate is definitely depressed by high concentrations of acetylsalicylcholine (Fig. 4). Choline inhibits the hydrolysis and the results are similar to those obtained with acetylcholine. Hence, choline causes a shift of optimum acetylsalicylcholine concentration to higher concentration. At substrate concentrations lower than 10^{-2} *M*, choline inhibits the enzymic hydrolysis of acetylsalicylcholine in a true competitive way. The result suggest that both the choline part and the acetate link of this ester is attracted to the enzyme, in the same way as for acetylcholine.

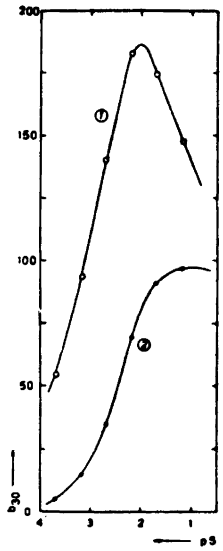

Fig. 4. Activity-pS curves for the enzymic hydrolysis of acetylsalicylcholine by cobra venom in the absence (1) and presence (2) of choline chloride (1.43 × 10^{-2} M).

Experiments with tetraethyl pyrophosphate (TEPP)

TEPP inhibits the enzymic hydrolysis of triacetin by human erythrocytes [19]. Similar results have been obtained with the esterases of *Helix* blood and cobra venom respectively. The concentration of TEPP which gives 50 % inhibition of the triacetin hydrolysis (cobra venom) is 1.73 × 10^{-9} *M*; the corresponding value for the acetylcholine hydrolysis is 4.35 × 10^{-9} *M*. It has been demonstrated in experiments similar to those described previously with acetylcholine [10] that triacetin protects the enzyme against TEPP (Table 4). Choline also protects, using either acetylcholine [10] or triacetin as substrates. The results obtained with triacetin and *Helix* blood are tabulated in Table 3. The enzyme was incubated with choline prior to the addition of TEPP and

Table 3. Protection of the Helix blood acetylcholinesterase by choline against the inactivation by TEPP tested with triacetin as substrate (0.092 M). Choline added prior to TEPP.

Choline *M*	TEPP *M*	b_{30}
—	—	178.5
—	7.15 × 10^{-9}	15.5
7.16 × 10^{-2}	—	79
7.16 × 10^{-2}	7.15 × 10^{-9}	59.5

Table 4. Protection of the cobra venom acetylcholinesterase by certain esters against the inactivation by TEPP (7.15×10^{-9} M).

Substrate	Acetyl-salicyl-choline	Acetyl-aneurin	Tri-acetin	Tri-propionin
Molarity × 100	0.66	0.53	9.20	7.69
Control (no TEPP)	156.5	53	80	13
Enzyme incubated with TEPP 70 minutes before mixed with acetylcholine	3	2	12.5	3
Substrate and TEPP simultaneously mixed with the enzyme	153	45.5	66.5	11

the hydrolysis rate compared with controls. It should be pointed out that the protection is not as good as that obtained in experiments with erythrocyte haemolysate [19].

In addition to triacetin, other esters protect acetylcholinesterases from inactivation by TEPP. Table 4 illustrates some results with acetylsalicylcholine and acetylaneurin and cobra venom. Tripropionin also seems to protect against TEPP, although the hydrolysis values are comparatively low.

A series of other compounds have been shown to protect the cobra venom esterase against TEPP action (to be published). Amongst others butyrylcholine is a protector. In addition to being an esterase inhibitor this choline ester is a TEPP protector. The b_{30} values obtained in one experiment with acetylcholine are as follows: control (acetylcholine alone), 188; incubation with TEPP, 3.5; incubation with butyrylcholine, 153; incubation with butyrylcholine prior to TEPP, 97.5. This confirms a very recent observation made by Cohen *et al.* [21] with brain acetylcholinesterase and diisopropyl fluorophosphate (the publication appeared during the preparation of this paper).

DISCUSSION

The fact that enzymes are proteins makes it inadvisable to expect complete identity between similar enzymes from different sources. The terms "species specificity" and "organ specificity" are as applicable to enzymes as to proteins in general. Proteins are classed together in groups with certain characteristics. This is also appropriate to many enzymes. The characteristics of the acetylcholine splitting enzymes so far tested have made it possible to

classify them into two types. Both types can easily be separated from the aliesterase, lipases, phosphatases, and other enzymes capable of hydrolysing ester linkages of various kinds, but future work will certainly demonstrate that within each group there exist enzymes which in certain respects differ from each other. This is already established for the phosphatases. It is a question only how far the experimenter can go in the refinement of his technique in order to trace these differences.

Certainly there exist many acetylcholinesterases [22] (aceto-cholinesterases) and the second class of cholinesterases (butyro-cholinesterases) also contains esterases which differ from each other in one or another respect [23]. The general properties of the esterases of *Helix* blood and cobra venom are those of acetylcholinesterase. In certain respects, however, they differ from the enzymes of nerve tissues, electric tissue, and erythrocytes. For instance, differences have been observed in the mechanism of action of certain esterase inhibitors.

It is still far too early to suggest that the physiological function of all these esterases is the same. For instance, what is the function of the esterase in cobra venom? For the time being there is no answer. Is there any connection between the toxic effect of cobra venom and its high acetylcholine splitting activity? Can this activity explain the different pharmacological effects of the highly esterase active venoms from Colubridae species and the non-active venoms from Viperidae species? Is the cobra venom a natural solution of the esterase coming from the glands in which it is produced? Can the solution of these problems help to solve the general problem of the function of the choline ester splitting enzymes. In itself this latter problem is part of the whole problem of the function of acetylcholine.

SUMMARY

The enzymic hydrolysis of certain choline esters and non-choline esters has been studied using the blood of *Helix* blood and cobra venom.

The substrate patterns for both esterases are similar to those of acetylcholinesterases from other sources. Acetylaneurin is split in both cases at a comparatively high rate, in contradistinction to other esterases of the same type.

The cobra venom contains only an acetylcholinesterase and is free from aliesterase activity. The presence of a butyro-cholinesterase or/and an aliesterase in addition to the acetylcholinesterase in the *Helix* blood is suggested.

Triacetin is hydrolysed by both the acetylcholinesterases. This hydrolysis is inhibited by choline, the mechanism of action of which is supposed to be a steric hindrance at the active Group II of the enzyme molecule.

Acetylsalicylcholine is hydrolysed at a relatively high rate, although both salicylcholine and acetysalicylic acid are split at a very low rate or not at all. With the cobra venom esterase the acetate link alone is split. This hydrolysis is inhibited by choline.

Tetraethyl pyrophosphate (TEPP) inhibits irreversibly the enzymic hydrolysis of non-choline esters. These esters protect the enzyme from inactivation by TEPP.

The plurality of the esterases splitting acetylcholine has been discussed.

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On the Properties of Rhodanese

Partial Purification, Inhibitors and Intracellular Distribution

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In 1932, K. Lang described an enzyme, that in the presence of a suitable sulfur donor was able to convert cyanide to thiocyanate. As sulfur donors only thiosulfate and colloidal sulfur were found to be effective. The enzyme was named rhodanese and found to be present in high amounts in liver tissue from different animals. Lang also tried to purify the enzyme and obtained a 20-fold purification. Cosby and Sumner [2] reported a 100-fold purification from beef liver, but without giving any specific activity data for their purified material. Here will be described a method for purification of rhodanese which gives preparations about 150-fold more pure than the starting material (beef liver) and about 5 times more active than preparations obtained according to Cosby and Sumner. In connection with the purification experiments, the intracellular distribution of the enzyme has been studied.

METHODS

The rhodanese activity was assayed by a modification of Cosby and Sumners procedure. The system consisted of 2 ml of substrate pH 7.4 containing 0.05 *M* cyanide, 0.05 *M* thiosulfate and 0.05 *M* phosphate, and 0.5 ml of the rhodanese solution. The time of reaction was 5 minutes and the temperature 20°. The reaction was stopped by adding 2.5 ml of ferric nitrate-nitric acid [2], and the test sample was diluted to 30 ml and filtered if necessary. 30 minutes after the addition of ferric nitrate, the extinction at 460 mμ was determined in the Beckman spectrophotometer and the thiocyanate formed was obtained from a standard graph. One rhodanese unit (RU) was taken as the amount

* A preliminary report of this work was presented before the XVIII International Physiological Congress, Copenhagen, 1950.

of enzyme which under the given conditions produced 10 μ equivalents of thiocyanate. The specific activity for the different preparations is expressed as rhodanese units per mg protein, the latter determined by the turbidimetric method of Bücher [3] in the purification experiments and obtained from nitrogen determinations in the distribution studies. In the inhibition studies the enzyme was incubated with a solution of the inhibitor adjusted to pH 7.4 and containing 0.01 *M* phosphate. 30 minutes after adding the enzyme the activity was determined in the usual way. In a control test the enzyme was incubated with 0.01 *M* phosphate buffer pH 7.4.

In the enzyme distribution studies a modification of the method of Hogeboom, Schneider and Pallade [4, 5] was used. Adult guinea pigs were killed by exsanguination and the liver removed and chilled in cracked ice. All the operations during the fractionation were carried out at + 4°. A sample of liver was homogenized in 9 volumes of 30 % sucrose, and the homogenate centrifuged for 10 minutes at 1 100 g. The sediment was washed once by resuspending in half the original volume of sucrose and recentrifuging at the same speed, and the final sediment was suspended in 30 % surcrose. The supernatants were combined and centrifuged for 20 minutes at 19 000 g, to sediment the mitochondria. The sediment was washed twice by resuspending in half the original volume of sucrose and resedimented as before. The mitochondria were finally suspended in 30 % sucrose and the supernatants were combined. Since recent work [6] has indicated that it is possible that "microsomes" are artifacts obtained from mitochondria during the homogenization, the separation of "microsomes" from the supernatant was not undertaken. All of the activity determinations on these fractions and the parent homogenate were carried out at the same time after dilution with 30 % sucrose.

PURIFICATION OF ENZYME

In preliminary experiments different tissues from different animals were assayed for activity. As beef liver was most active, we decided to use it as the starting material. The results are shown in Table 1.

Extraction: Different extraction procedures were tried. The best one was as follows: The liver (fresh or frozen material gave the same results) was disintegrated in a Turmix blendor with 2.5 volumes of water, and 0.2 volumes of a 20 % lead acetate solution was added. When the suspension was centrifuged, a clear supernatant was obtained. If the lead acetate addition was omitted, turbid extracts with the same activity were obtained. However, only about 55 % of the total activity in the liver was extracted, the rest seemed to be bound to insoluble particles. No improvement in yield was obtained by

Table 1. Rhodanese activity for different tissues.

Tissue	Activity in RU/g fresh weight
Beef liver	81
» »	99
» »	76
» kidney	27
Horse liver	32
» kidney	20
Sheep liver	39
Guinea pig liver	55
» » »	41
» » »	52
Salivary gland from beef	3.1

using a larger volume of water, by repeated extractions of the precipitate or by using salt solutions as extracting solvents. Mincing the liver in an ordinary meat mincer gave much smaller yields than homogenizing it in the Turmix blendor. The extraction procedure given by Cosby and Sumner gave only about 50 % of the yield and purity obtained by our procedure. They denaturated the hemoglobin in their extracts with ethanol and chloroform according to Tsuchihashi, but since this procedure seemed to modify rhodanese and since it was possible to obtain preparations essentially free from hemoglobin in other ways, we avoided this step. Autolysis of liver brei in the presence of ethyl acetate destroyed the enzyme, but active autolysates could be prepared by the aid of chloroform or toluene, however with no improvement in yield or purity.

Ammonium sulfate fractionations: When ammonium sulfate fractionation of the extract was tried, it was found that when the fractionation was carried out at pH 5 or higher, the purificatic n was only 3-fold, but when the pH was lowered a much better result was obtained. At a lower pH more of the inert proteins were denatured and precipitated on the addition of ammonium sulfate to 10 % saturation. It was necessary, however, to separate this precipitate from the solution before increasing the salt concentration further, otherwise it carried down a considerable part of the activity. The optimum purification was obtained at pH 3.8; at this pH the fractionation must be carried out in the cold room. The enzyme was precipitated between 40 and 55 % saturation of ammonium sulfate and the precipitate was dissolved in a 0.05 *M* solution of sodium thiosulfate and Na_2HPO_4 to a concentration of 120 RU per ml. To it was added 1.3 volumes of 80 % saturated ammonium sulfate solution, brought to pH 8.1 with ammonia. The inactive precipitate was centrifuged off and discarded, and the enzyme precipitated from the supernatant by addition of

25 g ammonium sulfate per 100 ml original solution. The precipitate was dissolved in distilled water.

Acetone fractionation. After dialysing the ammonium sulfate fractionated enzyme against distilled water until free from salt, fractionation with organic solvents was tried. It was found that ethanol fractionation gave poor yields but methanol or acetone gave better results. To the dialysed enzyme solution was added 0.05 volumes of 0.1 *M* acetate buffer pH 4.63 and acetone to 35 % by volume. The inactive precipitate was centrifuged off and discarded, and the enzyme precipitated by raising the acetone concentration in the supernatant to 50 %. All these operations were carried out at 0° to — 5°. The precipitate now contained rhodanese purified 50-fold from the first extract and about 150-fold from the liver. The results from a typical purification experiment are shown in Table 2.

Table 2. Purification of rhodanese.

Preparation	Total activity RU	Specific activity RU/mg protein
Beef liver	152 000	0.25
Lead acetate extract	86 600	0.82
First ammonium sulfate ppt	35 200	12.6
Second » » »	30 200	25.4
After dialysis	24 400	27.2
Acetone ppt	12 800	41.8

Properties of purified material. A 1 % solution of the purified material was nearly colorless, only a faint yellow tinge attributable to traces of hemoglobin remained. The absorption spectrum determined in the Beckman spectrophotometer showed only the usual protein band at 2 800 Å and a very low Soret band. No change in the spectrum could be detected after adding thiosulfate to 0.01 *M* concentration. Electrophoresis in the Tiselius apparatus at pH 5.4 showed 4 different fractions, 2 moving toward the anode and 2 moving toward the cathode. At this pH the rhodanese moved toward the anode. The two anodically migrating peaks made up about 30 % of the total area, which indicated that the enzyme was far from pure. The iso-electric point of the enzyme must be lower than pH 5.4, therefore attempts to carry out electrophoresis at a lower pH were made. They proved however unsuccessful, as the enzyme was inactivated during the dialysis.

STABILITY OF RHODANESE

Experiments on the heat stability of rhodanese were carried out with the crude extract. It was found that the enzyme could withstand 45° for 5 minutes without any inactivation. In the presence of thiosulfate the stability was extended to 55°. Only small amounts of foreign proteins were however denaturated below these temperatures. The results are shown in Table 3.

Table 3. Heat stability of rhodanese *.

Temperature (°)	Remaining activity in % Without thiosulfate	In 0.01 *M* thiosulfate
40	99	—
45	90	96
50	63	92
55	4	87
60	0	1

* Lead acetate treated extract from beef liver heated for 5 min. at the indicated temperature

The enzyme in the crude extract was stable for 30 min. at room temperature from pH 4.5 to pH 10.0, while pH 4.0 and 10.5 each gave about 40 % inactivation. In the cold room (+ 4°) the stability was extended down to pH 3.5. The crude extract could stand dialysis against distilled water without inactivation. With the most purified preparations, however, a prolonged dialysis gave a loss in activity, which was always accompanied by a decrease in pH and a precipitate in the dialysis bag. The activity of different rhodanese preparations was not increased by the addition of boiled preparations of the enzyme or of preparations inactivated at a high or low pH. Attempts to split off any prosthetic group by precipitating the enzyme with ammonium sulfate at an acid or basic pH were unsuccessful. Hence no evidence for the presence of a dissociable prosthetic group in rhodanese was obtained.

INHIBITORS

The effect of different enzyme inhibitors was studied on a partly purified preparation.

Metal enzyme inhibitors: The only compound found to be active was cyanide [7] as shown in Table 4 a. Cyanate and acetonitrile gave no inhibition. The possibility that the enzyme contains an active metal group, which is inhibited by cyanide, cannot be excluded. However the lack of inhibition with

other metal enzyme inhibitors makes this assumption more unlikely. This is in contrast to the results recently published by Saunders and Himwich [8]. They found rhodanese to be inhibited by cysteine and sulfide and concluded that rhodanese is a metal enzyme. The discrepancy between their results and ours may be due to the different conditions used in the test system. It has also been claimed [9] that rhodanese is inhibited by taurocholate, which was explained as due to a reaction between taurocholate and an iron containing active group in the enzyme. The inhibition obtained with taurocholate in our experiments can, however, be explained by the denaturating effect of this compound on proteins in general [10].

Carbonyl group reagents: Cyanide is also known to react with carbonyl groups and the effect of carbonyl group reagents on rhodanese was therefore studied, Table 4 b. Sulfite and hydroxylamine gave a strong inhibition, while semicarbazide and phenylhydrazine were less active and thiosemicarbazide gave no inhibition at all. It has been shown [11, 12] that phenylhydrazine can inactivate sulfhydryl enzymes by oxidizing their sulfhydryl groups. When rhodanese was incubated with the carbonyl group reagent in the presence of cysteine, no inhibition was in fact obtained. The lack of inhibition with thiosemicarbazide, which itself contains a sulfhydryl group, is also significant. The presence of an active carbonyl group in the enzyme could thus be excluded. But also the inhibition with cyanide could be prevented by the presence of a tenfold excess of cysteine or thiourea. That the inhibition of rhodanese with cyanide could be prevented by cystine, was recently shown by Saunders and Himwich [8]. Cyanide reacts however with cystine under formation of thiocyanoalanine and cysteine [13]. Hence cyanide was removed from their system and cysteine formed and the experimental conditions thus not so well chosen. In our experiments the cysteine was dissolved in the presence of cyanide in order to prevent any formation of cystine. Also the sulfite inhibition could be partially prevented by cysteine or thiourea. The protecting effects of these compounds against cyanide or sulfite inhibition is difficult to explain, as cyanide and sulfite do not inhibit but on the contrary activate sulfhydryl enzymes. Lang has already shown [7] that cyanide must be added to the enzyme in the absence of thiosulfate in order to obtain any inhibition. The same is true for sulfite (Table 4 e), and thus no inhibition is obtained in the test system by the sulfite formed during the reaction, as thiosulfate is present in the substrate. As thiosulfate in solution contains sulfhydryl groups, its protecting properties may be due to these, as cysteine and thiourea protect in the same way.

Table 4 a. Effect of metal enzymes inhibitors.

Inhibitor	Concentration * *M*	Inhibition %
Sulfide	0.1	0
Cysteine	0.1	4
Dithiodiethylcarbaminate	0.1	1
Azide	0.05	6
Cyanide	0.005	100
»	0.0005	24
Acetonitrile	0.05	6
Cyanate	0.05	0
Fluoride	0.1	0
Pyrophosphate	0.1	5
Taurocholate	0.01	53
»	0.001	26

* The indicated concentration in Tables 4 a, 4 b and 4 c refers to the incubation, the inhibitor was diluted 5-fold in the test system.

Table 4 b. Effect of carbonyl group reagents.

Reagent	Concentration *M*	Inhibition %
Cyanide	0.005	100
Cyanide + 0.05 *M* cysteine	0.005	0
Cyanide + 0.05 *M* thiourea	0.005	6
Sulfite	0.0005	99
Sulfite + 0.005 *M* thiosulfate	0.0005	0
Sulfite + 0.05 *M* cysteine	0.005	20
Sulfite + 0.005 *M* thiourea	0.0005	42
Hydroxylamine	0.0005	96
Hydroxylamine + 0.05 *M* cysteine	0.0005	6
Phenylhydrazine	0.005	98
Phenylhydrazine + 0.05 *M* cysteine	0.005	0
Semicarbazide	0.05	63
Semicarbazide + 0.05 *M* cysteine	0.05	5
Thiosemicarbazide	0.05	0

Sulfhydryl reagents: The effect of different compounds, known to react with sulfhydryl groups in proteins, was then studied. Rhodanese was found to be very sensitive to small amounts of H_2O_2 and I_2, less active were $K_3Fe(CN)_6$ and alloxan. (Table 4 c.) The enzyme was also destroyed by incubation with ascorbic acid. Cystine showed no inhibition while iodoacetate, *p*-chloromercuribenzoate and iodosobenzoate gave about 50 % inhibition in 0.001 *M*

Table 4 c. Effect of sulfhydryl reagents.

Reagent	Concentration *M*	Inhibition %
H_2O_2	10^{-4}	100
»	10^{-5}	17
I_2	10^{-5}	98
»	10^{-6}	27
$K_3Fe(CN)_6$	0.001	28
Alloxan	0.01	47
Ascorbate	0.04	95
Cystine	*	0
Iodoacetate	0.001	52
Iodoacetate + 0.005 *M* cysteine	0.001	5
p-Chloromercuribenzoate	0.001	61
p-Chloromercuribenzoate + 0.005 *M* cysteine	0.001	0
Iodosobenzoate	0.001	63
»	0.0001	31
Iodosobenzoate + 0.005 *M* cysteine	0.001	0

* Saturated solution in 0.01 *M* phosphate buffer pH 7.4.

solution. The effect obtained with the latter three compounds is weak, considering the inhibition they usually give with other sulfhydryl enzymes. Hence these results do not merit the conclusion that rhodanese is a sulfhydryl enzyme. Supporting evidence is however obtained from the protecting effect of cysteine against the inhibition with these compounds, as shown in Table 4 c. This is in accordance with the conclusions arrived at by Saunders and Himwich [8] from the inhibition obtained with iodoacetate.

INTRACELLULAR DISTRIBUTION

The purification experiments had indicated that part of the enzyme was bound to insoluble matter. Differential centrifugation experiments on sucrose homogenates of guinea pig liver also show that the activity of the homogenate was equally distributed between the mitochondrial fraction and in the supernatant. Only small amounts appeared in the nuclear fraction. The results from a typical experiment are shown in Table 5.

Here an interesting fact is apparent. The sum of activity of the nuclei, mitochondria and supernatant is greater than the original homogenate. This effect was repeatedly obtained in different experiments. Similar results for a transaminase [14] have recently been described but no explanation for this effect

Table 5. Distribution of rhodanese and rhodanese activity in fractions isolated from homogenates of guinea pig liver.

Fraction	% activity of homogenate	RU/g protein
Homogenate	100	103
Nuclei	7.0	97
Mitochondria	67.5	438
Supernatant	71.5	86

The sum of activity in nuclei, mitochondria and supernatant is 146 % of the activity in the original homogenate.

was attempted. The increase in total activity during the fractionation was obtained by washing the mitochondria with sucrose as shown by the following experiment.

Guinea pig liver was homogenized and the nuclei sedimented as before (without any washing) and their activity and the activity in the supernatant (S_1) was determined. The sum of these two activities was now found to be equal to the activity in the original homogenate. The mitochondria were then sedimented from S_1 and washed twice as before, and their activity (M) and the activity in the supernatant (S_2) was assayed. The sum of the activity in M and S_2 was now found to be about 130 % of that in S_1.

It was also found that the rhodanese activity for a cell free sucrose homogenate was only 30—50 % of that obtained in a water homogenate of the same liver, even though sucrose did not inhibit rhodanese. The rhodanese activity in homogenates with most of the mitochondria present in an intact form is thus lower than that obtained after disrupting the mitochondria. Saunders and Himwich [15] have shown that the rhodanese activity in intact cells is only a small fraction of that obtained after disintegration of the cells. They explained this as due to the small permeability of the cell membrane to thiosulfate ions. It therefore seems reasonable to ascribe the low rhodanese activity in intact mitochondria to permeability effects. It has in fact been claimed that the mitochondria are surrounded by a membrane [16–18] but this has recently been denied by Harman [19]. In any case the complex structure of the mitochondria makes the presence of permeability effects possible.

From the preceeding discussion it can be seen that our data for the intracellular distribution of rhodanese has only a relative significance, as a large part of the total activity in liver is not accounted for. It is presumeably localized in the mitochondria. It is clearly established from the high specific activity of the mitochondria as compared with the activity of the homogenate

that a large part of the rhodanese is associated with these entities (see Table 4). Since recent work by Harman [6] and Still and Kaplan [20] has shown that enzymes are transferred from the mitochondria to the supernatant during homogenization, the validity of data attained from such distribution studies is doubtful. As a consequence we have not carried out these investigations further.

SUMMARY

Rhodanese has been partially purified and some of its properties have been studied. It is inhibited by cyanide but not by the other metal enzyme inhibitors studied. The cyanide inhibition could be prevented by cysteine. Studies with inhibitors suggested that rhodanese is a sulfhydryl enzyme, but no evidence was obtained for the presence of an active carbonyl group. The intracellular distribution of rhodanese has been studied in sucrose homogenates and the enzyme found to be mainly associated with the mitochondria.

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ADDENDUM

Since this paper was submitted for publication we have noticed the appearance of a paper by Ludewig and Chanutin [21]. Studying the distribution of different enzymes in isotonic sucrose homogenates of rat liver, they found the main part of rhodanese activity in the mitochondria.

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Selbstdiffusion in Bleisilikaten

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Verbindungen zwischen Bleioxyd und Kieselsäure haben Bedeutung als Bestandteil vieler Gläser und ihre Bildung aus den Komponenten, sei es oberhalb [1], sei es unterhalb des Schmelzpunktes [2,3] ist der Gegenstand zahlreicher Untersuchungen gewesen.

Bei der Reaktion im festen Zustand ist zu einer vollständigen Erfassung der Vorgänge die Kenntnis der Beweglichkeit der reagierenden Partikel nötig. Hierzu ist die Methode der radioaktiven Indikatoren das geeignete Verfahren.

Zunächst wurden in vorliegender Arbeit die Diffusionskonstanten für Blei in Bleimetasilikat $PbO \cdot SiO_2$ und Bleiorthosilikat $2PbO \cdot SiO_2$ gemessen, während die reaktionskinetische Auswertung nach ergänzenden Untersuchungen (der elektrischen Leitfähigkeit, der etwaigen Beweglichkeit von Silicium u.s.w.) für eine spätere Mitteilung geplant ist.

DIE VERSUCHSMETHODIK

Es wurden die Silikate durch Reaktion im festen Zustand dicht unterhalb des Schmelzpunktes gebildet und aus ihnen Presstabletten (von 8 mm Durchmesser und 1,5 mm Dicke) hergestellt, die zum Erreichen hoher Dichte bei möglichst hoher Temperatur getempert, dann aber auf die Temperatur des jeweils geplanten Versuches gebracht und bei dieser einige Zeit gehalten wurden.

Der radioaktive Indikator ThB (Pb-212), aus der Emanation eines »hochemanierenden» Radiothoriumpräparates nach O. Hahn an einem elektrisch geladenen Platinblech niedergeschlagen, wurde auf geeignete Weise dem Diffusionsmedium zugefügt und die Diffusion verfolgt nach verschiedenen Methoden, die weiter unten näher beschrieben werden.

DIE PRÄPARATE

Bei der Herstellung des *Bleimetasilikates* wurde folgender Weg beschritten (nachdem die Rekristallisation des glasigen Metasilikates unbefriedigende Präparate ergeben hatte).

Analytisch reines PbO und analytisch reines (zur Konstanz geglühtes) SiO_2 wurden sorgfältig gemischt und zu Pastillen gepresst, die eine Stunde auf etwa 710° gebracht wurden, wobei Reaktion erfolgte unter starker Schrumpfung der Pastillen. Die Pastillen wurden gemörsert, neu gepresst und diesmal 6 Stunden auf etwa 730° gebracht, wobei mitunter feuchte Flecken (offenbar auf Bildung geringer Mengen eutektischer Schmelzen zurückzuführen) auftraten, die allmählich aber wieder verschwanden. Gleichzeitig ging die gelbe Farbe des Reaktionsgemisches in weiss über. Nach nochmaligem Ummörsern wurden die Tabletten im Laufe einer Stunde auf 745° und danach auf 750° gebracht, bei welcher Temperatur sie 24 Stunden gehalten wurden. Danach wurde das Silikat fein gepulvert und einer Schmelzpunktsbestimmung mittels der Methode der Differentialthermoanalyse [4] unterzogen (Vergleichssubstanz KCl : Fp = 770°).

Es ergab sich für $PbO \cdot SiO_2$: Fp = 767 ± 2° C, was gut mit den Angaben der Literatur [5] übereinstimmt.

Die Dichte des Pulvers wurde pyknometrisch unter Verwendung einer von 165 bis 185° siedenden Petroleumfraktion im Mittel zu 6,26 bestimmt, was wiederum gut mit neueren Literaturangaben übereinstimmt [6]. Das Röntgendiagramm nach Debye-Scherrer gab deutliche Metasilikatlinien.

Analog wurde bei der Herstellung und Untersuchung des *Orthosilikates* verfahren. Hier war die erste Erhitzung 4 Stunden auf 650°, die zweite 12 Stunden auf 700° und die dritte 12 Stunden auf 725° C. (Jedesmal nach Mörsern und Umpressen der Pastillen.)

Nach der ersten Erhitzung war die dunkelgelbe Farbe in hellgelb übergegangen; nach der zweiten war die Farbe des Pulvers weiss; nach der dritten grauweiss, eine Färbung, die auch nach weiteren Erhitzungen beibehalten wurde.

Unter dem Polarisations-Mikroskop waren keine glasigen Bestandteile zu sehen, die Schmelzpunktsbestimmung ergab 740 ± 2° C (in der Literatur [5] 743 ± 3°), die Dichtebestimmung im Mittel 7,40. (Hier war keine Literaturangabe zugänglich, aus dem Biltz'schen [6] Theorem der Volumenadditivität mit Berücksichtigung der gefundenen Dichte des Metasilikats folgt ein Wert von 7,42.)

Die zu den Diffusionsversuchen verwendeten Tabletten hatten beim Metasilikat im allgemeinen Dichten zwischen 5,2 und 5,5 (max.: 88 % Raumerfüllung), beim Orthosilikat Werte zwischen 6,8 und 7,0 (max.: 94 % Raumerfüllung).

BESTIMMUNG DER DIFFUSION

Wie an anderer Stelle ausgeführt [7], gibt es über einen weiten Grössenordnungs-Bereich hinweg stets mindestens eine Methode, den Selbstdiffusionskoeffizienten in festen Stoffen zu messen. Bei Bleiverbindungen bieten sich die von Hevesy [8] eingeführten Methoden der α-Strahlungsabsorption sowie der Absorption der Rückstossstrahlung dar, die zweckmässigerweise durch die in der Empfindlichkeit dazwischen liegende »Kontaktmethode» [9] ergänzt werden.

Im folgenden werden diese drei Methoden im einzelnen bezüglich der Auswertungsverfahren und der hier verwendeten experimentellen Anordnungen besprochen wie auch die direkte Ermittlung der nach der Diffusion eingetretenen Konzentrationsverteilung mittels Abschleifen dünner Schichten.

Vorausgeschickt sei ein Hinweis auf die Tatsache, dass das Verhalten des Elementes Blei bei den vorliegenden Indikatorversuchen angezeigt wird durch die Strahlung der radioaktiven Folgeprodukte des Bleiisotopes ThB (mit 10,6 h Halbwertszeit), nämlich hauptsächlich des Wismutisotopes ThC (mit 60,5 min Halbwertszeit), das seinerseits dualen Zerfall erleidet und sich rasch ins Gleichgewicht mit seinen Folgeprodukten ThC′ (Po-212) und ThC″ (Tl-208) setzt.

ThC ist der Träger der Rückstosstrahlung, die durch die resultierende Aktivität des ThC″ bestimmt werden kann; ThC′ ist der Träger der weitreichenden α-Strahlung, deren Absorption in den vorliegenden Versuchen benutzt wird; ThC und ThC″ sind die Träger der harten β-Strahlung (und γ-Strahlung), die die Hauptmenge der in üblichen Zählrohren registrierten Impulse ausmacht.

Aus all diesem geht hervor, dass bei unsern Versuchen vor den Messungen stets eine gewisse Zeit zur Einstellung des radioaktiven Gleichgewichts (gegeben durch die Nachbildung von ThC) veranschlagt werden muss, wenn jenes Gleichgewicht durch einen Vorgang (wie Diffusion oder Verdampfung) gestört worden war, an dem sich ThB (Blei) und ThC (Wismut) in verschiedenem Ausmasse beteiligen.

Zu dieser Einstellung wurde nach Aufdampfungen meist etwa 12 Stunden, nach Diffusionen meist etwa 3 Stunden gewartet, welch letzterer Wert sich als ausreichend erwies, da hier das Mengenverhältnis Blei/Wismut im allgemeinen nicht sehr erheblich verschoben wurde, (was durch laufende Messung der Aktivität kontrolliert werden konnte).

1. Die Bestimmung der Selbstdiffusionskonstanten auf Grund der *Absorption von α-Rückstosstrahlung* ist die empfindlichste der zur Verfügung stehenden Methoden und erlaubt, Diffusionskonstanten zwischen etwa 10^{-14} und 10^{-18} cm^2/sec zu messen. Dies beruht auf der ausserordentlich geringen Reichweite der Rückstosstrahlung in festen Stoffen (Grössenordnung 10^{-6}—10^{-5} cm).

Die Ausgangsbedingung ist die der sehr dünnen radioaktiven Schicht, die auf dem Probekörper durch Kondensation aus der Dampfphase erzeugt wird. Hierzu wird das in Thoriumemanation aktivierte Platinblech durch direkten Stromdurchgang erhitzt und so das ThB zum Verdampfen gebracht. Eine einfache hierzu verwendete Apparatur ist an anderer Stelle [10] beschrieben.

Nach Einstellung des radioaktiven Gleichgewichts ThB/ThC wird eine Messung der harten Eigenstrahlung der Tablette am Geiger-Müller Zählrohr mit 64-fachem Untersetzer vorgenommen, um die Möglichkeit zur Kontrolle etwaiger Verdampfungsverluste im Laufe des Diffusionsversuches zu haben.

Dies ist ein wichtiges Prinzip für Diffusionsmessungen mit der »dünnen aktiven Schicht» als Ausgangsbedingung: Die weiche Strahlung kann durch Diffusion, aber auch

durch Verdampfung abnehmen; die harte Strahlung dagegen nur durch Verdampfung, und daher ist ihre Messung geeignet zur Kontrolle von etwaigen, das Versuchsresultat fälschenden Verdampfungsverlusten, für die notfalls eine Korrektur bei der Auswertung eingeführt werden kann.

Die Ermittlung der Rückstossaktivität vor und nach den jeweiligen Diffusionsabschnitten wurde folgendermassen durchgeführt:

Eine bis zwei »Diffusionstabletten» wurden zusammen mit einer »Standardtablette» (einer Tablette, die nicht zur Diffusion gelangte, aber sonst gleichermassen aktiviert war) in die Ausbohrungen einer geerdeten Metallplatte eingesetzt. Auf die Grundplatte wurde ein Ring aus Isolationsmaterial aufgesetzt in dessen Durchbohrungen eine der Tablettenanzahl (2—3) entsprechende Zahl Metallstempel so eingehängt wurden, dass sie nur etwa 1 mm von der Oberfläche der betreffenden Tablette entfernt waren.

Die Metallstempel wurden mit Hilfe einer aufgelegten Metallplatte auf etwa -300V gebracht und sammelten so die aus der Tablette austretenden Rückstossatome des ThC — Folgeproduktes ThC″ (3,1 m Halbwertszeit) an ihrer Oberfläche. Diese »Exposition» wurde 15 Minuten lang durchgeführt, meist bei vermindertem Druck, was zweckmässig, aber nicht nötig ist. In dieser Zeit hat sich die maximale Menge ThC″ auf den Stempeln gesammelt, die nun am Zählrohr abwechselnd während 10—15 Minuten gemessen werden. Man erhält so die Abfallskurven für ThC″, die, auf eine »Nullzeit» extrapoliert, Werte für die Rückstossaktivität ergeben. Bei kleinen Rückstossaktivitäten ist zwecks Erreichung ausreichender Genauigkeit die Messung über einen längeren Zeitraum (z. B. 10 Minuten) nötig, wobei jeweils nur ein Stempel an einem Zähler gemessen werden kann.

Die so gewonnenen Werte der jeweiligen Rückstossaktivität, bezogen auf die Anfangsaktivität und als Funktion der Diffusionszeit angegeben, führen zu Werten für die Diffusionskonstante, wie nachstehend gezeigt.

Die mathematische Behandlung des Diffusionsvorganges (seinerzeit durch Fürth [8] vorgenommen) ergibt folgenden Ausdruck für die Aktivität der nach einer gewissen Diffusionszeit aufgefangenen Rückstossatome relativ zur Aktivität vor der Diffusion (selbstverständlich unter Berücksichtigung des ThB-Zerfalles):

$$A = \psi\left(\frac{a}{2\sqrt{Dt}}\right) - \frac{1}{\sqrt{\pi}\left(\frac{a}{2\sqrt{Dt}}\right)}\left(1 - \exp\left(-\frac{a^2}{4Dt}\right)\right)$$

(a: = Reichweite der Rückstossstrahlung in dem untersuchten Stoff.)

Diese Funktion wird zweckmässigerweise wiedergegeben in einer graphischen Darstellung, wie z. B. in Fig. 1, die zur Berechnung der Diffusionskonstante benutzt werden kann.

2. Die Bestimmung der Selbstdiffusionskonstanten auf Grund der *Absorption von α-Strahlung* hat auf Grund der grösseren Reichweite der α-Strah-

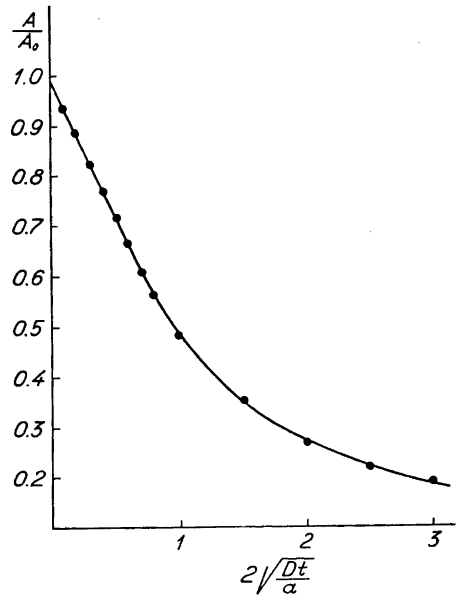


Fig. 1. Graphische Darstellung zur Ermittlung der Diffusionskonstante D (bei bekannter Versuchszeit t und Rückstossreichweite a) aus der Änderung der relativen Aktivität A/A_0 bei der Rückstossmethode.

lung in festen Stoffen (für den hier verwendeten Strahlungsanteil etwa 10^{-3} cm) eine geringere Empfindlichkeit; der Anwendungsbereich liegt zwischen etwa 10^{-13} und 10^{-9} cm^2/sec.

Die Ausgangsbedingungen (Aufdampfung der dünnen Schicht) sind die gleichen wie bei der Rückstossmethode. Noch wichtiger als dort ist bei den hier vorliegenden höheren Versuckstemperaturen die Kontrolle etwaiger Verdampfung. Allgemein gelangten hier Tablettpaare zur Diffusion, bei denen die Tabletten mit den (gleich stark) aktiven Seiten aufeinandergelegt wurden, was die unerwünschte Verdampfung der aktiven Schicht erschwert. Die radioaktiven Messungen wurden in einem Goldblattelektroskop kleinen Kammervolumens durchgeführt und auf eine »Standardtablette» bezogen, die einerseits Aufschluss über den ThB-Zerfall und Schwankungen in der Wirksamkeit des Elektroskopes, andererseits auch über den geringen Anteil Ionisation gab, der durch härtere Strahlung (hauptsächlich β-Strahlung) hervorgerufen und nicht durch Diffusion geschwächt wurde. Dieser Anteil (in den auch die »Isolation» oder »Nulleffekt» einging, herrührend von der Einwirkung von Strahlung im Messzimmer) ist offenbar von den gemessenen Aktivitäten abzuziehen, um ein korrektes Mass für die Wirkung der α-Strahlung zu erhalten.

Die Verminderung der α-Aktivität durch Diffusion führt zu einem Wert für die Diffusionskonstante [8], wobei allerdings ziemlich umständliche Rech-

nungen durchzuführen sind, die wir im folgenden kurz skizzieren wollen mit Rücksicht darauf, dass die Methode bei der wachsenden Anzahl nun zur Verfügung stehender künstlich-radioaktiven α-Strahler steigende Verwendung finden könnte.

Der Ausdruck für die im Elektroskop gemessene Aktivität als Funktion der Diffusionszeit lautet [9]:

$$A = \psi\left(\frac{a-b}{2\sqrt{Dt}}\right) - \frac{1}{\sqrt{\pi Dt}} \int_0^{a-b} \varphi(x) \exp\left(-\frac{x^2}{4\,Dt}\right) dx$$

Hierbei ist a die Reichweite der α-Strahlen im Bleisilikat und b eine Strecke im Bleisilikat, die der Luftschicht zwischen Präparat und Elektroskop und der das Elektroskop verschliessenden Aluminiumfolie äquivalent ist. Der Abstand zwischen Präparat und Elektroskop wurde nach dem Vorgång von Hevesy [8] so gewählt, dass nur die α-Strahlen des ThC″ (Reichweite in Luft 8,62 cm), nicht aber die des ThC′ (Reichweite in Luft 4,79 cm) das Elektroskop erreichen.

Um das Integral in obiger Formel auswerten zu können muss die Verminderung $\psi(x)$ der integralen Ionisation gemessen werden. Diese Werte sind für verschieden gewählte Werte von x (etwa von 10^{-4} bis 10^{-3} cm im vorliegenden Fall) in den Integranden einzusetzen, wonach der Wert des Integranden für verschiedene x-Werte aufgetragen und das Integral graphisch (etwa durch Ausschneiden und Wägen) ausgewertet werden kann.

Im vorliegenden Fall war die Kurve für die integrale Ionisation eine Gerade (wohl auf ungenügende Kanalisation der α-Strahlung zurückzuführen, da der theoretische Verlauf eine Verzögerung der Ionisationsverminderung kurz vor der Reichweite zeigt), was zu willkommenen Vereinfachungen in der Berechnung führte, da dann das Integral rechnerisch zu:

$2Dtc\ (1\text{-}\exp(\text{-}(a-b)^2/4Dt))$ ausgewertet werden konnte. ($(a-b)$ war im vorliegenden Fall bei $PbO \cdot SiO_2$: $1{,}16 \cdot 10^{-3}$ cm, also $c = 0{,}86 \cdot 10^3\ cm^{-1}$; bei $2PbO \cdot SiO_2$: $1{,}00 : 10^{-3}$ cm, $c = 1{,}00 \cdot 10^3\ cm^{-1}$).

Mit den so gewonnenen Werten für das Integral kann A für beliebige Werte von Dt (hier zwischen 10^{-8} und 10^{-6} cm^2 gewählt) ermittelt werden. Dies führt zu den in Fig. 2 wiedergegebenen Kurven, die zur Auswertung der Diffusionsversuche benutzt wurden, wobei bei $PbO \cdot SiO_2$ (Tablettendichte 5,25) sowohl die graphische als auch die rechnerische Integralauswertung angewendet wurden, die sich nicht nennenswert voneinander unterschieden.

3. Wie schon seinerzeit von Hevesy [8] festgestellt, klafft zwischen den Anwendungsbereichen der beiden Absorptionsmethoden eine Lücke bei etwa 10^{-14} bis 10^{-12} cm^2/sec. Dies führte uns seinerzeit — bei der Aufgabe, die Selbstdiffusion von Blei in PbO zu untersuchen — zur Einführung der *Methode des Aktivitätsüberganges bei idealem Kontakt* [11] die in ihrem Anwendungsbereich gerade diese Lücke deckt. Der Grundgedanke mag nicht neu sein (Groh und Hevesy [12] stellten einen ähnlichen Versuch bei metallischem Blei an, allerdings mit negativem Erfolg), neu ist aber die grosse Anwendungsmöglichkeit der

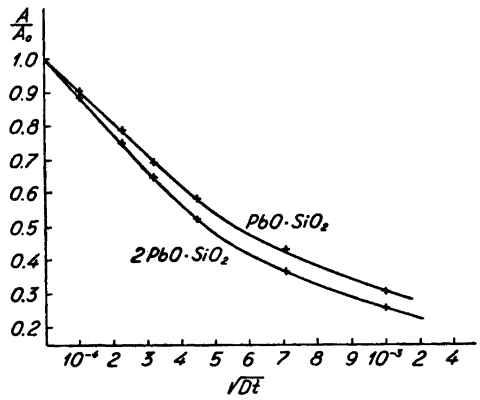


Fig. 2. Graphische Darstellung zur Ermittlung der Diffusionskonstante D in Bleimetasilikattabletten von der Dichte 5,25 und Bleiorthosilikattabletten von der Dichte 6,9 aus der Änderung der relativen Aktivität, gemessen mit dem in vorliegender Arbeit verwendeten α-Elektroskop.

Methode, die sich ausser bei den Bleisilikaten bei so verschiedenen Stoffen wie Ag_2SO_4 [12], PbO, ZnO, $ZnO \cdot Fe_2O_3$, CaO, $CaO \cdot SiO_2$, $2CaO \cdot SiO_2$ zu bewähren scheint.

Die Ausführung des Versuches und die benutzte Apparatur ist an anderer Stelle beschrieben [9]; hier ist daher nur eine kurze Beschreibung der Besonderheiten bei der Herstellung der homogen aktiven Bleisilikattabletten nötig:

ThB wird mit mittelstarker Salpetersäure vom exponierten Gold- oder Platinblech gelöst und die Lösung im Platintiegel zur Trockne gedampft. Einige Tropfen destillierten Wassers ergeben dann eine neutrale Lösung, in die das fein gepulverte Bleisilikat (etwa 2 g) eingetragen und verrührt wird. Anschliessend wird die Mischung unter dem Wärmestrahler getrocknet und dann im Tiegelofen einige Stunden auf etwa 700° erhitzt, danach im Mörser fein zerrieben und nochmals einige Stunden auf die gleiche Temperatur gebracht zur völligen Homogenisierung, die durch Aktivitätskonzentrationsbestimmung von Parallelproben innerhalb der Fehlergrenze nachgewiesen werden konnte. Hiernach wurden aus dem aktiven Bleisilikat Tabletten von 8 mm Durchmesser und 1,5—2 mm Dicke gepresst, die über Nacht auf etwa 700° erhitzt, dann allmählich auf die Versuchstemperatur hinabgebracht worden. Im Diffusionsversuch wurden diese Tabletten mittels Federdruck gegen inaktive Tabletten gepresst und auf die Versuchstemperatur erhitzt. Nach dem Versuch wurden die Tabletten wieder voneinander getrennt und die Aktivitäten der vordem inaktiven Tabletten am Zählrohr gemessen.

Die Messungen ergaben nach entsprechender Wartezeit (zwecks Einstellung des ThB/ThC Gleichgewichtes) Aktivitäten, die bei idealem Kontakt mit der Wurzel aus der Diffusionszeit zunahmen und aus denen die Diffusionskonstante gemäss

$$D = A^2/t \cdot \pi/c^2 \cdot q^2$$

ermittelt wurde (c_2 ist die ursprüngliche Konzentration in Impulsen/cm³, q die Kontaktfläche in cm²).

4. Als letzte hier verwendete Untersuchungsmethode sei die der *direkten Untersuchung der* durch Diffusion eintretenden *Konzentrationsverteilung* mittels Abschmirgeln dünner Schichten [7] besprochen.

Die folgende Ausführung stellte sich als bequem und einigermassen zuverlässig heraus:

Auf eine rauhe runde Glasplatte von etwa 2 cm Durchmesser, die durch einen Messingring mittels Schrauben auf einer Messingunterlage befestigt war, wurden etwa 5 mg feines Karborundum-Schleifpulver gebracht und das Ganze gewogen. Auf dieser Unterlage wurden von der aktiven Seite der Tablette mit mässigem Fingerdruck und rotierenden Bewegungen Fraktionen von 1 bis 5 mg Gewicht abgeschmirgelt. Nach jeder Abschmirglung wurde die Tablettenfläche behutsam von anhaftendem Pulver befreit, und das abgeschmirgelte Pulver in die Mitte der Schleifplatte gebracht. Tablette und Schleifplatte wurden gewogen und am Zählrohr auf Radioaktivität untersucht. Ausserdem wurde in regelmässigen Abständen die Höhe der Tablette mit einer »Indikatoruhr» an mehreren Punkten gemessen, um zu kontrollieren, dass parallel zur Oberfläche abgeschliffen worden war. Aus den Wäge- und Messdaten ergeben sich die Konzentrationen der einzelnen Fraktionen (etwa in Impulsen/10^{-4} cm Tablettenhöhe) sowie deren Verlauf als Funktion des Abstandes von der ursprünglichen aktiven Oberfläche. Man erhält je eine Kurve für die Tablette und das abgeschmirgelte Pulver, die im allgemeinen gut übereinstimmen.

Trägt man den Konzentrationsverlauf als $\ln c = f(x^2)$ auf, so erhält man bei durch eine Konstante bestimmten Diffusionsvorgängen Gerade, aus deren Neigung diese Konstante ermittelt werden kann.

$$\left(D = x_2 - x_1/t \cdot 4 \ln \frac{c_1}{c_2}\right)$$

DIE VERSUCHE UND IHRE ERGEBNISSE

Die Erhitzung der Tabletten auf die Diffusionstemperatur geschah in mit Chromnickelband bewickelten Öfen aus Supremaxglas, deren Temperatur durch Vorschalten von Eisenwasserstofflampen auf $\pm 2°$ konstant gehalten werden konnte.

Die Temperatur wurde mittels kalibrierten Pt/Pt-Rh Thermoelementen bestimmt und nötigenfalls mit einem schreibenden Millivoltmeter registriert.

Bei allen Versuchen wurde die Diffusionszeit in mehrere Abschnitte geteilt um durch schrittweise Verfolgung der Diffusion Ausfchluss über die zeitliche Konstanz des Diffusionskoeffizienten zu erhalten. Die Länge der Diffusionszeiten ist durch die Lebensdauer von ThB (Halbwertswert 10,6 Stunden) begrenzt.

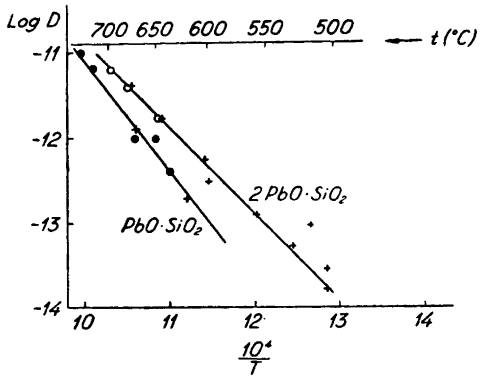


Fig. 3. Die Diffusionskonstante D von radioaktivem Blei in Bleimetasilikat und Bleiorthosilikat als Funktion der Temperatur T.

1. Die Diffusionskonstanten bei hohen Temperaturen

Bisher ist die Bleisilikatbildung hauptsächlich im Temperaturbereich zwischen etwa 600 und 700° C untersucht worden. Daher ist die Kenntnis der Diffusionskonstanten in den Bleisilikaten bei diesen Temperaturen von hauptsächlichem Interesse für die reaktionskinetische Behandlung.

Unsere Messungen in diesem Temperaturbereich sind in üblicher Weise ($\log D = f\ (1/T)$) in Fig. 3 dargestellt.

Punkte bezeichnen Werte, die nach der Methode der α-Strahlungsabsorption, Kreuze solche, die nach der »Kontaktmethode» erhalten wurden.

Die beiden Methoden stimmen in ihren Ergebnissen gut überein; die Temperaturabhängigkeit des Diffusionskoeffizienten (in $cm^2\ sec^{-1}$) lässt sich folgendermassen wiedergeben:

Blei in Bleimetasilikat: $D = 8{,}5 \cdot 10^1 \exp\ (-59500/RT)$
Blei in Bleiorthosilikat: $D = 2 \cdot 10^{-1} \exp\ (-47000/RT)$

Die Werte für das Temperaturinkrement dürften schätzungsweise eine Genauigkeit von 5 % haben, die präexponentiellen Faktoren sind naturgemäss erheblich unsicherer.

Bei den hier betrachteten Temperaturen liegt also normalerweise die Diffusionskonstante für Blei im Orthosilikat höher als im Metasilikat, was sich gut mit der bevorzugten Bildung von Orthosilikat [3] bei der Reaktion zwischen Bleioxyd und Kieselsäure verträgt.

2. Anomalien in der Temperaturfunktion des Diffusionskoeffizienten

Wir müssen nun auf einige Erscheinungen zu sprechen kommen, die im Laufe der Diffusionsmessungen innerhalb begrenzter Temperaturbereiche auftraten. Es zeigten sich nämlich sowohl bei Bleimetasilikat bei etwa 585° als auch bei Bleiorthosilikat bei etwa 620° »Spitzen» oder relative Maxima für die Werte des Diffusionskoeffizienten.

Diese Erscheinungen sind reproduzierbar und durch mehrere Versuche sowohl nach der α-Absorptionsmethode wie nach der Kontaktmethode belegt.

Die Werte für das Metasilikat sind in Fig. 4 wiedergegeben (beim Orthosilikat ist das Verhalten etwa das gleiche, aber nicht durch soviel Punkte belegt).

Beim Versuch die Natur dieses Phänomens zu klären, mussten verschiedene Möglichkeiten untersucht werden:

Zunächst könnte man an »Einfrieren» hoher reversibler Fehlordnung denken, da die Tabletten ja ursprünglich oberhalb 700° gesintert worden waren.

Doch waren die meisten Tabletten schon bei niedrigeren Temperaturen (ca 600—650°) angewendet worden, ehe sie zu den fraglichen Versuchen gelangten, und orientierende Messungen der elektrischen Leitfähigkeit gaben keinen Anhalt für eine langsame Einstellung der reversiblen Fehlordnung in diesem Temperaturgebiet. Aufschlussreich in diesem Zusammenhang sind auch die Ergebnisse eines in Fig. 4 enthaltenen Diffusionsversuches bei 567° ($10^4/_I = 11{,}9$).

Hier gibt ein Tablettpaar, nach der Hochtemperatursinterung 24 Stunden auf etwa 580° gehalten, einen hohen Diffusionswert, ein anderes Paar, nach der Sinterung 4 Stunden auf 500° gehalten, einen tiefen Wert. Dies spricht offenbar gegen »eingefrorene» Fehlordnung, die sich im zweiten Fall ebenfalls hätte bemerkbar machen müssen. —

Ein anderer Gedanke, der sich sofort ergibt, ist der einer Umwandlung des Diffusionsmediums im fraglichen Temperaturbereich. Hedvall [13] hat viele Beispiele angegeben, bei denen im Temperaturbereich von Umwandlungen verschiedener Art Anomalien in der Ausbeute von Reaktionen auftreten, an denen feste Stoffe beteiligt sind.

Dabei müssen offenbar sekundäre Veränderungen des festen Stoffes wie etwa Rissbildungen in Betracht gezogen werden; erst nach dem Ausscheiden solcher Möglichkeiten kann ein »Hedvall-Effekt» im engeren Sinne vorliegen.

Bei den vorliegenden Diffusionsversuchen lässt nun sich der Diffusionsvorgang in (mindestens) zwei Teilvorgänge aufspalten, die wir als

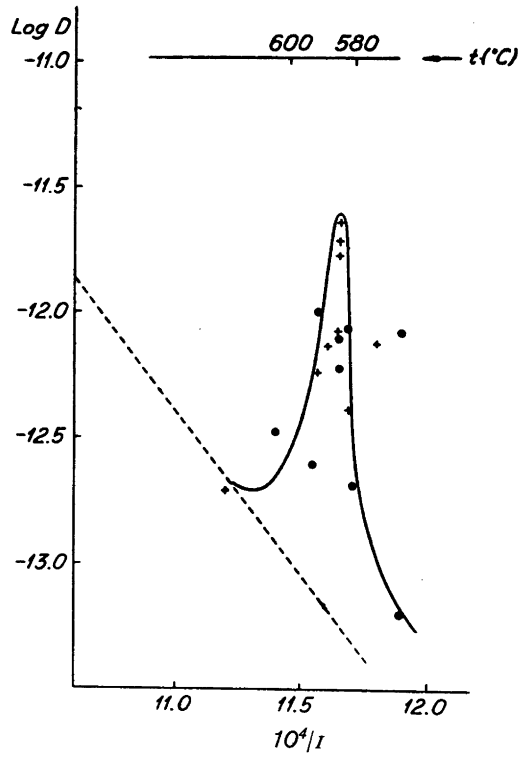


Fig. 4. Diffusionsanomalien bei Bleimetasilikat.

Gitterdiffusion einerseits und als Diffusion auf inneren Grenzflächen (kurz »Kanaldiffusion») andererseits bezeichnen.

Dies ergab sich sowohl aus dem Aktivitätsverlauf als Funktion der Eindringtiefe bei den »Abschleifungsversuchen» — wie weiter unten ausgeführt — als auch aus »radioautographischen» Aufnahmen der Tablettenoberfläche nach sukzessiven Abschleifungen.

Der Anteil der »Kanaldiffusion» ist jedoch bei den hier betrachteten Versuchen *nicht so*viel höher als bei anderen Temperaturen als dass so die hohen Diffusionswerte erklärt werden könnten.

Dies lässt Vorgänge im Kristallgitter als verantwortlich für die anomal hohe Beweglichkeit erscheinen, und es wurde daher versucht, mit den uns zur Zeit zur Verfügung stehenden Mitteln näheren Aufschluss über diese Vorgänge zu erhalten, wie im folgenden besprochen.

3. Die Diffusionskoeffizienten bei tieferen Temperaturen

Beim *Orthosilikat* lässt sich auch unterhalb 620° mit den Methoden der α-Absorption bezw. des Aktivitätsüberganges bei idealem Kontakt ein geradliniger Verlauf $\log D = f(1/T)$ feststellen (Fig. 3).

Beim *Metasilikat* liegt indessen der „normale" Wert des Diffusionskoeffizienten so niedrig (10^{-13} $cm^2sec/^1$), dass die α-Absorptions-Methode für genaue Messungen zu unempfindlich ist. Auch die Kontaktmethode war hier bei den seinerzeit zur Verfügung stehenden Aktivitäten nicht sehr empfindlich und hat bisher keine klare Entscheidung über die Diffusion bei tiefen Temperaturen zugelassen.

Es verbleibt die Methode der Absorption der Rückstosstrahlung, mit der zahlreiche Versuche durchgeführt wurden.

Die Diffusionswerte sind stark von der Vorbehandlung des Materials abhängig, wobei irreversible Fehlordnungserscheinungen offenbar eine Rolle spielen.

Im allgemeinen tritt schon bei der Aufdampfung der dünnen radioaktiven Schicht sowie in den ersten Augenblicken der Diffusion eine unverhältnismässig rasche Ausbreitung der Radioaktivität ein, was sowohl die nötige Anfangsbedingung der »unendlich» dünnen Schicht beeinträchtigt als auch die Konstanz des Diffusionskoeffizienten.

Unter Berücksichtigung dieser Umstände lässt sich zur Zeit nur die vorläufige Aussage treffen, dass eine markante Richtungsänderung der $\log D = f(1/T)$-Kurve nicht mit Sicherheit festzustellen ist.

4. Die Untersuchung der Silikate nach der Methode der thermischen Differentialanalyse

Es kann auf eine kürzlich erscheinene Veröffentlichung verwiesen werden [4] betreffend der Methodik, die jedoch im vorliegendem Fall vielleicht zu unempfindlich war.

Beim Bleimetasilikat konnte kein thermischer Effekt festgestellt werden. Beim Bleiorthosilikat dagegen wurde beim Erhitzen ein thermischer Effekt bei $625 \pm 10°$ gefunden, beim Abkühlen ein solcher bei $530 \pm 10°$.

Krakau und Vachrameev [14] gaben die Andeutung einer Umwandlung bei 620° mittels Erhitzungskurven an; Geller, Creamer und Bunting [5] dagegen konnten keinen solchen Effekt finden (benutzten allerdings die verhältnismässig geringe Erhitzungsgeschwindigkeit 1,8° C/min).

Zusammenfassend ist zur Frage der Umwandlungen bei den hier untersuchten Bleisilikaten zu bemerken, dass zwar Andeutungen vorliegen (zumindest beim Orthosilikat), dass jedoch weitere Ergebnisse von Diffusionsversuchen, thermischen Analysen (Hochtemperaturthermostat) und Röntgenanalysen (Heizkamera) abzuwarten sind.

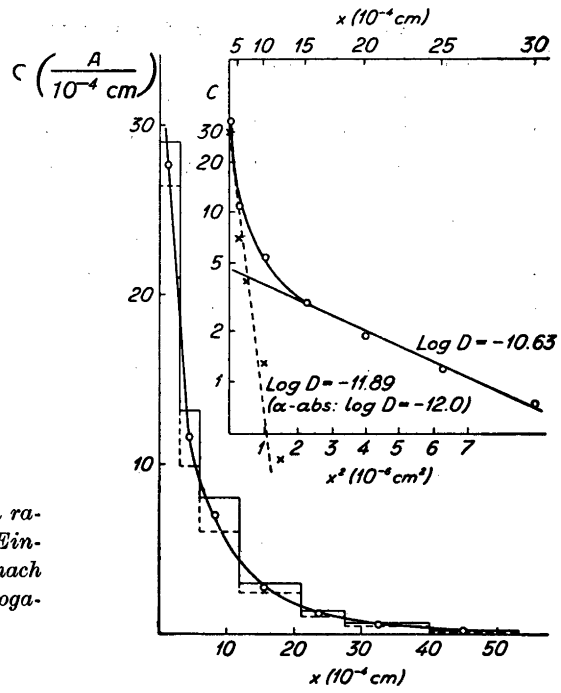


Fig. 5. Der Verlauf der Konzentration radioaktiven Bleies (C in willkürlichen Einheiten) in einer Bleimetasilikattablette nach erfolgter Diffusion in linearer bezw. logarithmischer Darstellung.

5. Die Aufspaltung des Diffusionsvorganges in gleichzeitig verlaufende Transportvorgänge

Die Analyse der Tablette nach erfolgter Diffusion mittels Abschleifen dünner Fraktionen führte durchgehend zu dem Ergebnis, dass die Konzentrationsverteilung in der logarithmischen Darstellung nicht durch eine Gerade darzustellen ist, sondern durch eine zusammengesetzte Kurve, die sich in 2 Geraden aufspalten lässt.

Ein Beispiel, die Analyse einer Metasilikattablette, die im Laufe eines α-Absorptionsversuches 14,25 Stunden auf 650° erhitzt worden war, ist in Fig. 5 wiedergegeben.

Links, in der linearen Darstellung, die Konzentration des radioaktiven Indikators als Funktion der Eindringtiefe. Dabei sind sowohl die Messungen an der Tablette (ausgezogene Stufen) als am abgeschliffenen Pulver (gestrichelte Stufen) eingetragen.

Rechts, in der logarithmischen Darstellung, die resultierende zusammengesetzte Kurve, aus der sich zwei annähernd gerade Linien erhalten lassen, die zwei Diffusionskonstanten entsprechen, deren kleinere ($\log D = -11{,}89$)

nahe bei dem mittels der α-Strahlungsabsorptionsmethode erhaltenen (log $D = -12{,}0$) Werte liegt.

Es liegt nahe, diesen Wert als die Konstante für die Gitterdiffusion anzusprechen, während der höhere Wert (log $D = -10{,}63$) einer Diffusion auf inneren Grenzflächen zuzuordnen ist. —

Diese Auffassung wird durch zwei weitere Befunde gestützt:

Erstens nimmt die Aktivität bei den Absorptionsmethoden mitunter im Anfang rasch ab, um erst dann eine Abnahme entsprechend einem konstanten Diffusionskoeffizienten zu zeigen.

Diese Erscheinung verschwindet nach einer verhältnissmässig kurzen Erhitzung, die daher bei den meisten α-Absorptionsversuchen vor Beginn des eigentlichen Versuches durchgeführt wurde, da die Gitterdiffusionskonstante gemessen werden sollte.

Die Aktivitätsabnahme ohne Vorbehandlung zeigt einen Verlauf, der sich durch Zusammensetzen zweier Kurven erhalten lässt, entsprechend zwei Diffusionskonstanten, die sich um den Faktor 20 voneinander unterscheiden.

(Um diesen Faktor nämlich liegen durchschnittlich die Werte für den Koeffizienten der »Kanaldiffusion» höher, wie aus Abschleifungen bei verschiedenen Versuchen erschlossen.) —

Zweitens lassen sich lokale Anhäufungen der radioaktiven Substanz auch radiographisch nachweisen, wie aus Fig. 6 hervorgeht.

Der homogenen Schwärzung einer frisch aufgedampften Tablette (A) stehen die Inhomogenitäten nach der Diffusion gegenüber (B), die nach Abschleifen — bis über die »Reichweite» der Gitterdiffusion hinaus — klar hervortreten (C).

(Expositionen auf Kodak-Röntgenfilm ca 1 Stunde (A und B) bzw. 12 Stunden im Falle C, da hier das Präparat durch radioaktiven Zerfall erheblich schwächer geworden war.) —

Quantitativ ist zur Aufspaltung des Diffusionsvorganges folgendes zu sagen (wobei ein Austausch »Kanal»-Gitter unberücksichtigt bleibt):

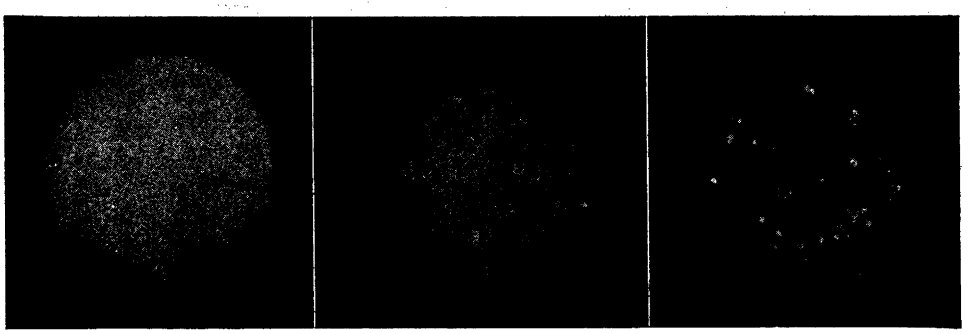
Die Temperaturinkremente für beide Diffusionsarten sind etwa die gleichen, ein Befund, der sich auch bei einigen in Untersuchung befindlichen Oxyden (CaO, ZnO, $ZnO \cdot Fe_2O_3$) und Silikaten ($CaSiO_3$) ergeben hat.

(Ähnliche Erscheinungen sind auch bei Metallen beobachtet worden [15].)

Der präexponentielle Faktor, in erster Hand mit der Fehlstellenkonzentration zu identifizieren, liegt bei der Kanaldiffusion um etwa den Faktor 20 höher als bei der Gitterdiffusion.

Die radioaktive Substanz verteilt sich zu ungefähr gleichen Teilen auf die beiden Diffusionsmöglichkeiten.

Daraus könnte man den Schluss ziehen, dass das wirksame »Volumen» der Kanäle etwa 1/20 des Gesamtvolumens beträgt, ein überraschend hoher



A B C

Fig. 6. Radioautographien von Bleimetasilikattabletten:

A: nach der Aufdampfung
B: nach erfolgter Diffusion
C: B nach Abschleifung einer Schicht von ca $25 \cdot 10^{-4}$ *cm.*

Wert verglichen mit dem geometrischen Anteil der inneren Oberflächen in dem dicht gesinterten Material. Offenbar sind die »Kanäle» nicht als Röhren im geometrischen Sinne aufzufassen, sondern als Zonen, in denen Gradienten der Fehlstellenkonzentration vorliegen, wie das z.B. allgemein bei gesinterten Metallpulver angenommen wird [16].

Selbstverständlich wäre es wünschenswert, reine Fälle von Gitterdiffusion (und Grenzflächendiffusion) zu untersuchen, etwa an Einkristallen.

Doch scheint uns die ausführliche Behandlung der Vorgänge an Sinterkörpern gerechtfertigt mit Rücksicht auf die tatsächlichen Verhältnisse bei Festkörperreaktionen, insbesonders solchen zwischen Pulvern.

ZUSAMMENFASSUNG

Verschiedene Methoden zur Messung der Selbstdiffusion in Bleisilikaten werden in ihren experimentellen Einzelheiten besprochen.

Im Temperaturbereich von etwa 550—700° C gilt für die Diffusion von radioaktiven Blei in Presstabletten von:

Bleimetasilikat: $D = 8{,}5 \cdot 10^{1} \exp(-59500/RT) \mathrm{cm}^2\ \mathrm{sec}^{-1}$
Bleiorthosilikat: $D = 2 \cdot 10^{-1} \exp(-47000/RT) \mathrm{cm}^2\ \mathrm{sec}^{-1}$

Es ergaben sich Anomalien (relative Maxima) mit bis um den Faktor 30 erhöhten Werten für die Diffusionskonstante. Diese Maxima liegen bei Blei-

metasilikat bei etwa 585° C, bei Bleiorthosilikat bei etwa 620° C und scheinen auf Vorgänge im Kristallgitter zurückzuführen zu sein.

Kristallographische Umwandlungen bei den fraglichen Temperaturen konnten zunächst nicht mit Sicherheit nachgewiesen werden, doch wurde beim Bleiorthosilikat mittels thermischer Differentialanalyse ein Effekt bei 625° C beobachtet in Übereinstimmung mit Literaturangaben.

Bei den untersuchten gesinterten Presstabletten überlagern sich zwei Diffusionsvorgänge, die als Gitterdiffusion bzw. als Diffusion auf inneren »Grenzflächen» («Kanaldiffusion») anzusprechen sind.

Herrn Prof. J. A. Hedvall danke ich für sein Interesse an der Untersuchung, *Statens Tekniska Forskningsråd* für die Bereitstellung von Mitteln.

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Studies Related to Pristane

V. The Constitution of Zamene

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The saturated hydrocarbon, pristane, was discovered in 1917 by Tsujimoto [1] in the liver oil of the basking shark. He found that fractional distillation gave a crude pristane which had an iodine number of 4.4, whilst refining with sulphuric acid gave the hydrocarbon free from unsaturated material.

In 1923, Toyama [2] investigated the pristane content of six Japanese elasmobranch fish oils. He found that the pristane fractions, which had been purified by distillation only, still had considerable iodine numbers.

Fish	Iodine number
Cetorhinus maximus Gunner	2.7
Scymnorhinus licha Bonaterre	9.8–11.3
Centrophorus spec.	15.9
Zameus squamelosus / *Centroscymnus owstonii* (mixed oil)	10.6
Chlamydoselachus anguineus Garman	22.2 / 81.6

In 1935, Tsujimoto [3] showed that this unsaturation was due to an olefine hitherto unknown. He isolated this new hydrocarbon, which he named "zamene", by addition of bromine followed by removal of the unchanged pristane by vacuum distillation. Zamene was regenerated from its dibromide with zinc and shown to have the composition $(CH_2)_n$ and the following constants:

$$d_4^{20} = 0.7948$$
$$n_D^{20} = 1.4470$$
$$\text{I. N.} = 66.2$$

Catalytic hydrogenation gave a saturated hydrocarbon which Tsujimoto considered to be identical with pristane. He held the opinion that both pristane and zamene were C_{18}-hydrocarbons. In the previous four communications of this series [4–7] it was shown that pristane is the C_{19}-hydrocarbon, 2,6,10,14-tetramethyl-pentadecane or *nor*-phytane. As mentioned in the third communication the *nor*-phytane structure of pristane was supported by a preliminary investigation of zamene. Zamene was oxidised with peracids and the oxide hydrolysed to the corresponding α-glycol which was separated from Pristane by means of partition and distillation. Splitting of the α-glycol with lead tetra-acetate gave formaldehyde and a liquid ketone whose physical constants resembled those of 2,6,10-trimethylpentadecan-14-one, the classical degradation product of phytol [8–10]. We were, however, unable to isolate crystalline derivatives of this important degradation product and, therefore, were led to a detailed reinvestigation of this degradation of zamene.

In this reinvestigation we met with unexpected difficulties. The starting material was, as in our preliminary investigation, the foreruns of basking shark liver oil. Fractional distillation gave a crude pristane fraction which was contaminated with zamene and the fatty alcohols, cetyl, stearyl and oleyl alcohols. The bulk of these fatty alcohols was removed by several crystallisations at — 15° although some oleyl alcohol remained in the crude pristane. To remove the final traces of alcohol sodium was pressed in, the jelly of alcoholates filtered off and the filtrate distilled over sodium. This procedure is the same as that used by Tsujimoto [1] and by us in our preliminary investigation. The more intensive this pretreatment the worse was the purity of the product on oxidation with peracid and hydrolysis to α-glycol. On distillation of the expected α-glycol at 10^{-4} mm, the products distilled over a range of at least 70°, the lowest boiling fraction analysed for one oxygen per C_{19}, the middle fraction, which boiled between 120—130° at 10^{-4} mm gave analytical figures which corresponded approximately to $C_{19}H_{40}O_2$ but on lead tetraacetate titration [11] gave α-glycol values of only 20—30 %. Preparative oxidation of the middle fraction with lead tetra-acetate gave carbonyl compounds of differing boiling points.

As any pronounced inhomogeneity of the za ene fraction was highly improbable some rearrangement must have taken place in the reactions we have mentioned. The more prolonged each treatment the more these side reactions would tend to predominate. Lüttringhaus, Sääf, Sucker and Borth have shown in a paper [12] published in 1945 that the double bond of a mono-olefine may be displaced as a result of distillation over metallic sodium. As soon as we heard of this paper (which owing to war conditions was only reprinted in 1949) we repeated our experiments with the exclusion of the treatment with metallic

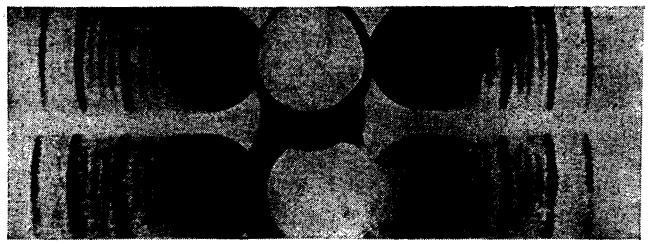

Fig. 1. Debye-Scherrer diagrams of the semicarbazone of 2,6,10-trimethyl-pentadecan-14-one. I from Phytol II from Zamene.

sodium. To remove the remaining fatty alcohols, the crude pristane was treated with excess 3,5-dinitro-benzoyl chloride and pyridine in the cold and then for 3 hours at 80°. The mixture of pristane, zamene and dinitro-benzoates was separated by chromatography on alumina. As will be seen from the experimental section, pristane is eluted more readily than zamene but the difference in adsorptivity on ordinary activated alumina is insufficient for a preparative separation on a small scale. The pristane-zamene mixture was oxidised with OsO_4 according to Criegee [13].

The osmic ester was decomposed with mannitol-potassium hydroxide and the α-glycol obtained from zamene purified by partition and distilled at 10^{-4} mm. On this occasion, we obtained only a small forerun (< 10 %), the rest distilling at 118—122° (air bath temperature) practically without residue. This α-glycol was a viscous oil, $n_D^{20} = 1.4582$, $d_4^{20} = 0.887$, $M_{D\,obs.} = 92.49$, $M_{D\,calc.} = 92.99$, and analysing for $C_{19}H_{40}O_2$. As will be seen from the experimental section the analytical figures tend to preclude a difference of $\pm CH_2$.

The elementary formula $C_{19}H_{40}O_2$ was confirmed fully by lead tetra-acetate oxidation which gave formaldehyde — characterised as its dimedone derivative — and 2,6,10-trimethyl-pentadecan-14-one characterised as its semicarbazone, m. p. 64°. The semicarbazone of the same ketone, prepared by ozonolysis of phytol, melted at 65.5° (older statements in the literature gave m. p.'s 64.5—67° [8–10]), mixed m. p. 65°. For purposes of further identification, Debye-Scherrer diagrams of the two semicarbazones were taken and, as may be seen from Fig. 1, were identical.

EXPERIMENTAL

The starting material was foreruns from industrial distillation of basking shark liver oil most kindly placed at our disposal by Chem. Eng. Alf Brautaset, Jahres Kjemiske Fabrikker A/S, Sandefjord. About 63.5 % of the crude oil boiled between 105—115°

on fractionation at 0.2—0.5 mm using a 70 cm column. This fraction was left for some days at — 17° C and the solid fatty alcohols removed by filtration at that temperature (loss about 8 %). The crude pristane fraction had an iodine number (Wijs) of 4.0 and analysed as follows:

C	84.94	H	14.53	100—(C + H)	0.53
»	84.61	»	14.72	100—(C + H)	0.67

150 g of this crude oil was shaken in a closed vessel for 43 hours at room temperature with 15 ml anhydrous pyridine and 6 g 3,5-dinitrobenzoyl chloride. The mixture was then heated for 3 hours at 80° C, shaken 3 times with 5 % $NaHCO_3$ solution, then with water, and the pristane ester mixture dried over anhydrous $MgSO_4$. On chromatography on activated alumina and elution with petroleum ether, the first half of the eluate contained 122 g, of oil, $n_D^{20} = 1.4386$ and the remainder contained 17 g of oil $n_D^{20} = 1.4391$. Both these fractions were rechromatographed on activated alumina in petroleum ether. The first fraction was separated into 7 equal parts and the second into 3. The iodine numbers were as follows:

Fraction	1a	1b	1c	1d	1e	1f	1g
I. N.	0.34	0.92	1.12	1.13	1.20	1.18	1.23
Fraction	2a	2b	2c				
I. N.	0.54	2.58	11.9				

The iodine numbers clearly demonstrate that chromatographic separation of zamene and pristane would be practicable with oils somewhat enriched in zamene.

82 g of this chromatographically purified pristane-zamene mixture with iodine number 1.04 was treated with 1 g osmium tetroxide in 50 ml anhydrous ether. After 2 days 1 ml of pyridine was added and the dark precipitate separated from the solution. Both the precipitate and filtrate were decomposed in methylene chloride with 1 % potassium hydroxide containing 10 % mannitol. The precipitate gave only a small quantity of pure pristane (C + H = 100.1). The filtrate was partitioned three times between petroleum ether and 92 % methanol. The hypophasic part was distilled at 10^{-4} mm. Forerun, below 90°, 13 mg. Fraction boiling 118—122°, 119 mg $n_D^{20} = 1.4582$, $d_D^{20} = 0.887$. No residue.

Calc. for	$C_{18}H_{38}O_2$ (286.5)	C = 75.46,	H = 13.37 %
	$C_{19}H_{40}O_2$ (300.5)	C = 75.93,	H = 13.42 %
	$C_{20}H_{42}O_2$ (314.5)	C = 76.37,	H = 13.46 %
Found		C = 75.87,	H = 13.09 %
		C = 75.86,	H = 13.21 %
		C = 76.17,	H = 13.50 %
	Mean	C = 75.97,	H = 13.27 %

42 mg of the glycol, $C_{19}H_{40}O_2$ was treated with 10 ml $N/10$ lead tetra-acetate in acetic acid [14]. The formaldehyde was expelled by means of a stream of nitrogen at 40—50° for 4 hours and absorbed in a solution of dimedone in water. On standing, crystals of the dimedone derivative appeared, m. p. 190° (corr.). Mixed m. p. with a synthetic sample showed no depression. Debye-Scherrer diagrams of the two dimedone derivatives proved their identity.

The oxidation solution was diluted with water, extracted with ether and the ethereal extracts washed until neutral, dried and distilled at 0.001 mm, air bath temperature: 75°. As may be observed from the table the physical constants of the distillate are in good agreement with those reported in the literature for 2,6,10-trimethyl-pentadecan-14-one.

Table 1. Physical constants of 2,6,10-trimethyl-pentadecan-14-one. (All data recalculated to 20° C.)

Author	n_D^{20}	d_4^{20}	R_D^{20} (calc. = 85.33)
Willstätter *et al.* from phytol [8]	1.44434	0.844	84.50
» » » » » [9]	1.44516	0.834	85.65
Fischer » » » » [10]	1.4450	0.8355_5	85.49
» » » synthetic [10]	1.4453	0.8349_5	85.61
Fischer and Löwenberg » [15]	1.4452	0.8369_5	85.44
Heilbronn *et al.* » [16]	1.44315	0.83965	84.73
This paper from zamene	1.4450	0.8392	85.09

The distillate gave a crystalline semicarbazone with semicarbazide hydrochloride which on recrystallisation from methanol gave colourless needles m. p. 64°. The semicarbazone of 2,6,10-trimethylpentadecan-14-one, obtained by ozonolysis of phytol, melted at 65.5°, mixed m. p. 65°. Debye-Scherrer diagrams *cf.* Fig. 1.

SUMMARY

The constitution of the olefine hydrocarbon, zamene, found in some elasmobranch fish oils, as 2,6,10,14-tetramethyl pentadec-l-ene was established by degradation to 2,6,10-trimethyl-pentadecan-14-one and formaldehyde.

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Studies Related to Pristane

VI. Synthesis of Digeranyl with some Remarks on the Physical Constants of Crocetane

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The aliphatic diterpene "Digeranyl" or 2,6,11,15-tetramethylhexadeca-tetra-2,6,10,14-ene, and its perhydro compound "Crocetane" were — as already mentioned in the first paper of this series [1] — synthesised for the purpose of comparison of the latter with pristane. Digeranyl was synthesised by treating geranyl bromide with magnesium in the same manner as in the classical synthesis of squalene of Karrer and Helfenstein [2]. Although the synthesised diterpene and its perhydro compound had the correct composition, the physical constants of the latter cast doubt upon the homogeneity of these products. Crocetane had been synthesised twice before. Fischer and Löwenberg [3] used the Würtz synthesis from tetrahydrogeranyl bromide [3]. Karrer and Golde [4] started from the carotenoid pigment crocetine. They converted its tetradecahydro compound into the ω,ω'-glycol by Bouveault-Blanc reduction. The ω,ω'-glycol, in turn was reduced, via its dibromide, to crocetane, with Zn-Cu in acetic acid at 150°. As will be seen from Table 1, these two syntheses of crocetane gave preparations that differed markedly in their physical constants. [All values recalculated to 20° according to Egloff: *Physical constants of hydrocarbons.* N. Y. 1939.]

Table 1. Physical constants of crocetane.

Author	d_4^{20}	n_D^{20}
From crude "Digeranyl"	0.8036	1.4480
Karrer and Golde	0.8025	1.4492
Fischer and Löwenberg	0.7887	1.4402
From citral [1]	0.7858	1.4405
From purified "Digeranyl"	0.7888	1.4410

Hydrogenation of our crude digeranyl gave a saturated hydrocarbon, $C_{20}H_{42}$, whose constants agree closely with those given by Karrer and Golde. (*cf.* first and second row of Table 1.)

In order to get some insight into the cause of these anomalies the same hydrocarbon was synthesised from citral in a manner similar to that used by Fischer and Löwenberg. Details of our method are given in the first communication of this series [1]. As may be seen from the fourth row of Table 1 the physical constants of our product were very close to the ones given by Fischer and Löwenberg.

Using the conditions given by Karrer and Helfenstein [2] the digeranyl synthesis gave a forerun of over 35 % of aliphatic monoterpenes and a residue of about 10 % of higher terpenes which distilled at about 190° at 0.05 mm Hg; that is very close to the boiling point of the aliphatic triterpene, squalene. Establishment of these side reactions led to the possibility that they might have some influence on the diterpene fraction.

Since natural or synthetic squalene may be readily purified by the facile crystallisation of the hexahydrochloride, we attempted to isolate crystalline tetra derivatives from the diterpene of analogous structure: digeranyl. Dry hydrochloric acid in ether solution gave no crystalline addition compounds, at first, but on seeding the reaction mixture with squalene hexahydrochloride, ready crystallisation of a tetrahydro-chloride, m. p. 110°, occured. The tetrahydrochloride which crystallises in thin leaflets, is apparently isomorphic with squalene hexahydrochloride. The yield in 4 preparations amounted to only 13 % of theory.

Treatment of this tetrahydrochloride with pyridine followed by warming to 150° with quinoline regenerated a digeranyl which was purified by chromatography on alumina and distillation at 95° C (air bath temperature) 10 mm Hg. The dispersion of this purified digeranyl is given in the experimental section. The digeranyl regenerated from the crystalline tetrahydrochloride has a lower density and refractive index than the crude starting material:

	d_4^{20}	n_D^{20}	R_D^{20}
crude "digeranyl"	0.8547	1.4884	92.59
digeranyl from hydrochloride	0.8347	1.48124	93.56

and a small exaltation in molecular refraction: R_D calc. 92.69.

Hydrogenation of this regenerated digeranyl gave a saturated hydrocarbon whose physical constants are given in the last row of Table 1. These constants are identical within the limits of experimental error, with the earlier determinations of Fischer and Löwenberg [3] and of our analogous preparation from citral.

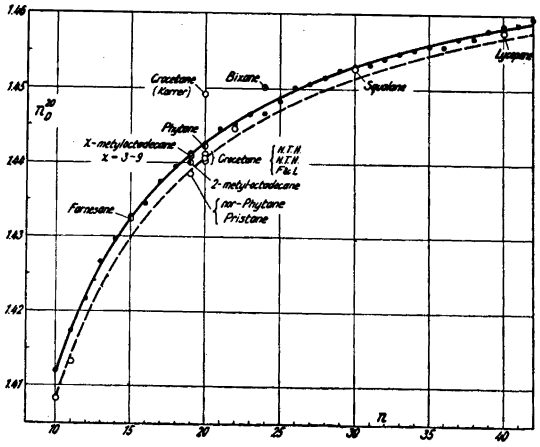


Fig. 1. Refractive index of higher alkanes.

——— *calculated for n-alkanes, according to Francis* [6].

— — — *calculated for 2,2′-dimethylalkanes, according to Francis* [6].

The identity of crocetane from purified "digeranyl" with that prepared according to Fischer and Löwenberg was put beyond any question of doubt by the infra-red measurements of Pliva and Sörensen given in the fourth communication of this series [5].

Further proof for the correctness of the data given in the last three rows of Table 1 for crocetane may be deduced from Fig. 1 which gives the refractive indices of the higher alkanes.

The curves are drawn according to the equations of Francis [6] for normal alkanes and for 2,2′-dimethylalkanes. The observed values are in good agreement with the calculated ones, the sole exception being Karrer's value for crocetane.

The empirical rules given by Francis for the refractive index of higher alkanes are simple. For normal alkanes Francis gives the general formula:

$$n_D^{20} = 1.4736 - \frac{0.62}{n}, \quad n > 10$$

Higher 2-methyl alkanes show depressions in density and refractive index of such an order that these constants coincide with those for the normal n-1 alkanes. 2,2′-dimethylalkanes exhibit still greater depressions and obey, according to Francis, the empirical relationship:

$$n_D^{20} = 1.4731 - \frac{0.65}{n}$$

Methyl groups removed by three carbon atoms or more from the end of the alkane chain cause small exaltations in n_D^{20} — of the order of + 0.0004 per methyl group. The excellence of these simple empirical rules for higher methyl substituted alkanes may be seen

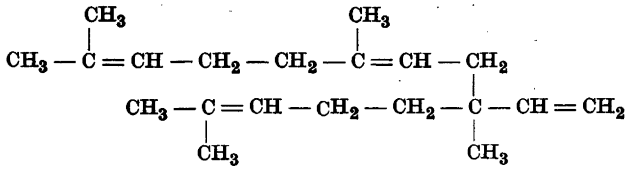
from Table 2 which correlates some of the data determined in connection with the pristane problem with some data used by Francis.

Table 2. Refractive index of methyl-substituted higher alkanes.

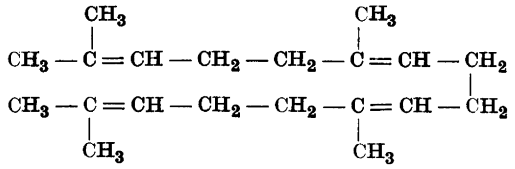
Compound	Calculation	Refractive index = n_D^{20} Calc.	Found
C_{18}	2-Methylheptadecane (= n-C_{17})	1.4371	1.4384
C_{19}	2-Methyloctadecane (= n-C_{18})	1.4392	1.4399
C_{19}	3 to 9-Methyloctadecane (= n-C_{19} + 1 side-Me)	1.4414	1.4406 1.4413
C_{19}	*Nor*-phytane (= 2.2′-dimethyl + 2 side-Me)	1.4397	1.4386
C_{20}	Phytane (= n-C_{19} + 3 side-Me)	1.4422	1.4422
C_{20}	Crocetane (2.2′-dimethyl + 2 side-Me)	1.4414	1.4406 1.4411
C_{24}	Bixane (= n-C_{24} + 4 side-Me)	1.4493	1.4502
C_{30}	Squalane (= 2,2′-dimethyl + 4 side-Me)	1.4530	1.4528
C_{40}	Lycopane (2,2′-dimethyl + 6 side-Me)	1.4592	1.4589

It is obvious that uncontrolled side reactions are also the cause of the deviations recorded for Karrer's crocetane, since there can be no doubt concerning the constitution of crocetin — the starting material in Karrer's and Golde's synthesis. Owing to the high cost of crocetin, Karrer and Golde were forced to repeat the Bouveault-Blanc reduction three times, side reactions at this stage are, however, improbable. We must, therefore, presume that the side reactions took place in the Zn-Cu reduction at the comparatively high temperature of 150° C. The four chlorine atoms were readily given off by the tetrahydrochloride of "digeranyl" on treatment under Rosemund-conditions. The resulting hydrocarbon was saturated but the refractive index of $n_D^{20} = 1.4695$ shows at once that these conditions must have caused cyclisation.

After our experimental work had been completed and partially published, there appeared three syntheses of digeranyl from geranyl chloride by Barnard and Bateman [7] in connection with their studies on the reactions of allylic Grignard compounds. These authors obtained digeranyl I and isodigeranyl II as by-products in the reaction between the Grignard compound from geranyl chloride and carbon dioxide or ethyl chloroformate. They were also obtained in the reaction of the Grignard compound with silver bromide or with 1 mole geranyl chloride.



II

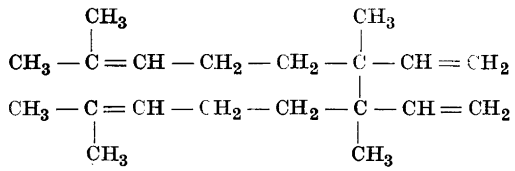


I

Barnard and Bateman were able to separate the two *iso*mers by fractional distillation, the ratio of *iso*digeranyl to digeranyl was always about 2 : 1. The constitution of the two compounds was established on the basis of their infrared spectra and by oxidative degradation. The only physical constant recorded is the refractive index, *viz.* *iso*digeranyl $n_D^{20} = 1.4821$; digeranyl $n_D^{20} = 1.4837$. It is remarkable that both values are higher than the one found by us for digeranyl regenerated from the crystalline tetrahydrochloride, and, furthermore, both are lower than the value obtained by us for the whole diterpene fraction. Barnard and Bateman found no other isomers, in the diterpene fraction, and do not mention any mono- or tri-terpene reaction products. They suppose that in the three reactions studied by them only allylic ions participate.

The monoterpene fraction in our experiments had physical constants that are in agreement with a 1 : 1 mixture of myrcene and dihydromyrcene. If one also takes into consideration the 10 % yield of a triterpene fraction one is led to the conclusion that allylic radicals participate in our reaction. The monoterpene mixture results from disproportionation of the geranyl radical itself, whilst the triterpene is obtained by an interchange of radical character between mono- and diterpene.

Participation of radical mechanisms should also give us a third isomer III[8] It ought to be mentioned in this connection, that the physical constants of the



diterpene fraction are highly dependant upon the ratio of geranyl bromide to magnesium used under the conditions of Karrer and Helfenstein's squalene synthesis (see Table 3).

Table 3. Physical constants of whole diterpene fraction in relation to amount of magnesium present.

Geranyl bromide: Mg	n_D^{20}	d_D^{20}	R_D
2 : 1	1.4973	—	—
»	1.4967	—	—
»	1.4970	0.8824	90.96
1 : 1	1.4838	0.846	92.73
»	1.4884	0.8547	92.53
»	1.4889	0.8570	92.43
1 : 2	1.4846	0.8476	92.68

The yield of digeranyl-tetrahydrochloride, however, remained constant in all cases, at about 13 % of the diterpene fraction. These observations are in agreement with earlier statements [9, 10] regarding the effect of small variations in reaction conditions upon the course of Grignard reactions.

It is highly probable that our diterpene fraction contains isomers other that I—III for the following reason. Digeranyl I from the tetrahydrochloride is perhydrogenated at a speed comparable to that of squalene. The crude diterpene fraction is hydrogenated at the same rate at the beginning but after about 2/3 of the theoretical amount of hydrogen has been taken up, the rate falls off and practically stops after 3/= have been hydrogenated. The fact that this phenomenon, which is quite reproducible, is not due to the presence of poisoning impurities was clearly shown by the addition of squalene some hours later. The hydrogen consumption started again at the rate which is normal for this triterpene. Since I—III are devoid of hindered double bonds, one may presume the presence of other (cyclised) isomers.

EXPERIMENTAL

Geranylbromide. Prepared from linalool by treatment with PBr_3 in pyridine solution in a manner analogous to that given by Karrer and Helfenstein [2] for the preparation of farnesyl bromide. B. p. 75—78°/1.6—1.8 mm $n_D^{20} = 1.5021$.

Crude digeranyl. Geranyl bromide was treated with varying relative quantities of magnesium (turnings) in anhydrous ether in a manner analogous to the squalene synthesis of Karrer and Helfenstein (*ibid.*), and the reaction product fractionated. A halogen free liquid fraction, which amounted to about 1/3 of the material and distilled at 65°/12 mm

was collected. n_D^{20} = 1.4629 (myrcene 1.4716, dihydromyrcene, n_D^{20} 1.4514). The diterpene fraction, which amounted to about 55 % of theory, distilled at 118—121°/0.5 mm (147—149°/2.6—3 mm). The rest gave a fraction amounting to 8—10 % which boiled at 190°/0.05 mm; squalene boiling at 195° at the same pressure. The physical constants of the diterpene fraction, as a function of the quantity of magnesium used, are given in Table 2.

$C_{20}H_{34}$ (274.5)	Calc.	C 87.50	H 12.50
	Found	» 87.2, 87.6	» 12.10, 12.84

Digeranyl tetrahydrochloride. A solution of 38 g crude digeranyl in anhydrous ether was saturated with dry hydrogen chloride at — 10° C. The solution turned dark but no crystallisation ensued. On seeding the solution with a few crystals of squalene hexahydrochloride immediate crystallisation occured. m. p. was 110° after two recrystallisations from ether. The yields from four different preparations of digeranyl were 17, 12.4, 11.4 and 13.1 % of theory.

$C_{20}H_{38}Cl_4$ (420.3)	Calc.	C 57.14	H 9.11
	Found	» 57.07	» 9.22

Digeranyl from the tetrahydrochloride. 4.8 g of digeranyl tetrahydrochloride was refluxed in pyridine in an atmosphere of CO_2 for 14 hours in a manner analogous to the procedure given by Heilbron, Kamm and Owens [11] for the regeneration of squalene from its hexahydrochloride. Since this treatment proved to be insufficient the material was heated for further 4 hours at 150° in quinoline. The hydrocarbon was isolated in the usual way and distilled at 95—100°/10^{-4} mm (air bath temperature).

$C_{20}H_{34}$ (274.5)	Calc.	C 87.50	H 12.50
	Found	» 87.1	» 12.6

Table 4. Dispersion of digeranyl from tetrahydrochloride.

$$d_4^{20} = 0.8347 \quad R_\lambda \text{ calc.} = \frac{90.725 \cdot \lambda^2}{\lambda^2 - 1.0554 \cdot 10^6} \quad \lambda_0 = 1027 \text{ Å}$$

λ	n_λ^{20}	$R_{\lambda,\text{ obs.}}$	$R_{\lambda,\text{ calc.}}$
6678.1	1.47732	92.91	92.92
5895.9	1.48124	93.56	93.57
5875.7	1.48132	93.57	93.58
5015.6	1.48814	94.70	94.70
4921.9	1.48914	94.87	94.86
4713.1	1.49154	95.26	95.25
4471.5	1.49476	95.79	95.78

Crocetane. 0.87 g digeranyl regenerated from the tetrahydrochloride was hydrogenated with 17 % PtO_2 on silica without solvent at 90° C, filtered and distilled at 95°/0.001 mm. The dispersion of this distillate — *cf.* Table 5 — gave the correct value for

the dispersional constant λ_0 for saturated hydrocarbons. In agreement with this result further purification with 100 % sulphuric acid followed by distillation over metallic sodium had no effect on the dispersional data.

Table 5. Dispersion of crocetane from digeranyl tetrahydrochloride.

$$d_4^{20} = 0.7888,\ R_{\lambda,\ \text{calc.}} = \frac{92.383 \cdot \lambda^2}{\lambda^2 - 0.7866 \cdot 10^6}\ \lambda_0 = 886.9\ \text{Å}$$

λ	n_λ^{20}	$R_{\lambda\ \text{obs.}}$	$R_{\lambda\ \text{calc.}}$
6678.1	1.43834	94.02	94.04
5895.9	1.44100	94.52	94.52
5875.7	1.44108	94.53	94.53
5015.6	1.44561	95.38	95.37
4921.9	1.44624	95.49	95.48
4713.1	1.44782	95.79	95.77
4471.5	1.44982	96.16	96.16

Perhydro squalene. Squalene is hydrogenated slowly. The most suitable conditions are those used by Heilbron, Hilditch and Kamm [12] and by Chapman [13], that is, in the absence of solvent and at a high temperature (150 to 200° C). Using platinum catalysts, at ordinary temperature the hydrogen uptake in most solvents is very slow and incomplete. In acetic acid or mixtures of solvents in which acetic acid is the major component, hydrogenation is rapid and complete. To test the reproducibility of the dispersional data for higher alkanes, 3 preparative hydrogenations in acetic acid with PtO_2/SiO_2 catalysts and one without solvent using Ni at 100° and 150 atm., were carried out.

All hydrogenation products were purified with 100 % H_2SO_4, washed carefully with sodium hydroxide solution and with water and distilled over liquid sodium. As may be seen from Table 6 the reproducibility of the dispersional constants is good. Refractive index figures vary by some 3 units in the fourth decimal place; our values being intermediate between those of Tsujimoto [14], $n_D^{20} = 1.4525$, and those of Chapman [13], $n_D^{20} = 1.4532$.

Table 6. Dispersion of Squalane. I—III Pt, IV Ni-Catalyst.

$$R_{D,\ \text{theory}} = 140.74,\quad R_{\lambda=\infty,\ \text{theory}} = 137.51,\quad \lambda_{0\ \text{theory}} = 885.2\ \text{Å}$$

$I\ d_4^{20} = 0.81078$	$\lambda_0 = 889.6$	$R_{\lambda=\infty} = 137.58$	$II\ d_4^{20} = 0.81026$	$\lambda_0 = 887.2$	$R_{\lambda=\infty} = 137.67$
λ	n_λ^{20}	$R_{\lambda,\ \text{obs.}}$	$R_{\lambda,\ \text{calc.}}$	n_λ^{20}	$R_{\lambda,\ \text{obs.}}$	$R_{\lambda,\ \text{calc.}}$
6678.1	1.45009	140.06	140.07	1.45001	140.13	140.14
5892.9	1.45277	140.78	140.79	1.45267	140.84	140.86
5875.7	1.45289	140.81	140.81	1.45277	140.87	140.88
5015.6	1.45755	142.06	142.05	1.45745	142.13	142.11
4921.9	1.45823	142.24	142.23	1.45812	142.31	142.29
4713.1	1.45986	142.68	142.67	1.45976	142.75	142.72
4471.5	1.46187	143.22	143.25	1.46179	143.29	143.31

III $d_4^{20}=0.81061$ $\lambda_0=886.8$, $R_{\lambda=\infty}=137.58$ IV $d_4^{20}=0.81072$, $\lambda_0=885.9$ $R_{\lambda=\infty}=137.63$

λ	n_λ^{20}	R_λ, obs.	R_λ, calc.	n_λ^{20}	R_λ, obs.	R_λ, calc.
6678.1	1.44989	140.04	140.05	1.45017	140.09	140.10
5892.9	1.45257	140.76	140.77	1.45287	140.82	140.81
5875.7	1.45265	140.78	140.79	1.45297	140.84	140.83
5460.7	—	—	—	1.45483	141.34	141.35
5015.6	1.45732	142.03	142.02	1.45764	142.10	142.07
4921.9	1.45801	142.22	142.19	—	—	—
4713.1	1.45959	142.64	142.63	—	—	—
4471.5	1.46167	143.20	143.21	1.46195	143.25	143.26
4358.3	—	—	—	1.46302	143.54	143.56

SUMMARY

One of the components of the diterpene mixture that ensues when geranyl bromide is coupled with magnesium has been isolated as its crystalline tetrahydrochloride. The hydrocarbon which is regenerated from this tetrahydrochloride is digeranyl (I), since on hydrogenation a hydrocarbon $C_{20}H_{42}$, which is identical with crocetane — 2,6,11,15-tetramethylhexadecane, is formed. The physical constants of this hydrocarbon are in accordance with those recorded by Fischer and Löwenberg in 1929. It is demonstrated that these are the correct values for crocetane — the data in the literature for this hydrocarbon being somewhat conflicting.

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Microbiological Determinations of Amino Acids in Foodstuffs. II

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The present paper in continuing the work started in 1949 describes the results obtained by the microbiological determination of eighteen amino acids in the foodstuffs which are commonly used for animal nutrition in this country.

MATERIAL AND METHODS

Preparation of material. The materials were produced by the following Swedish institutions and factories: *sweet blue lupine* by Svalövsfilialen, Uppsala; *brewer's yeast* by Kärnbolaget, Stockholm; *sulfite yeast* by Jästbolaget, Stockholm; *fish meal*, mainly from herring by the Cod meal factory of Gothenburg; *fish meal* mainly from cod and cod scraps by H. Fors, Ltd., Stockholm; *molasses* by Mälardalens Lantmannaförbund, Uppsala; *linseed cakes* and *peanut cakes* by Svenska Lantmännens Riksförbund, Stockholm; *hog blood* from the local slaughter house; *hog hoof meal* by Scan's slaughter house, Tomelilla. All materials were selected by the National Animal Experimental Station, Uppsala. They were ground and freed from lipids as previously described [1]. Hydrolysates of tryptophan were prepared with barium hydroxide in nickel containers according to Miller and Ruttinger [2], the method which at present gives the best results. Acid hydrolysates, used for the determinations of the other amino acids, were prepared by suspending 1 g of the sample in 10 ml of 2 *N* HCl and autoclaving at 15 pounds pressure for 10 hours [3].

Methods of analysis. Microorganisms, basal media, ranges of standard curves, and incubation times are given in Table 1. The procedures followed for the cultures and inoculum have been described in previous papers [6,7]. A Cannon automatic dispenser was used for the serial pipettations. A casein hydrolysate was always included as an extra control in the determinations of the amino acids. For each amino acid several separate assays were carried out. In each series, five assay levels were used. The amino acids used as standards were dried *in vacuo* at room temperature and kept *in vacuo* in a desiccator containing silica gel. DL-forms of isoleucine, phenylalanine, threonine, and valine were employed. The natural isomers of the others were used. Constant moisture values were obtained by drying at 100° for 30 hours.

Table 1. Experimental conditions for the microbiological analysis.

Amino acid	Medium	Micro organisms	Standard curve γ per 2 ml	Incubation time, hours
Alanine	Sauberlich and Baumann [4]	*L. citovorum* (8081)	0—80	72
Arginine	Steele *et al*,[5]	*L. mesenteroides* P-60	0—40	72
Aspartic acid	» » »	» » »	0—40	72
Cystine	» » »	» » »	0—5	72
Glutamic acid	» » »	» » »	0—80	72
Glycine	» » »	» » »	0—20	24
Histidine	» » »	» » »	0—20	72
Isoleucine	Sauberlich and Baumann [4]	*L. citrovorum* (8081)	0—15	72
Leucine	Steele *et al.*[5]	*L. mesenteroides* P-60	0—20	72
Lysine	» » »	» » »	0—40	72
Methionine	» » »	» » »	0—10	72
Phenylalanine	» » »	» » »	0—10	72
Proline	» » »	» » »	0—20	72
Serine	» » »	» » »	0—20	72
Threonine	Sauberlich and Baumann [4]	*S. lactis* (4790)	0—20	48
Tryptophan	Steele *et al.*[5]	*L. mesenteroides* P-60	0—5	72
Tyrosine	» » »	» » »	0—20	72
Valine	» » »	» » »	0—20	72

RESULTS AND DISCUSSION

In Table 2 the nitrogen values and the content of crude protein of the materials are recorded. Recent total nitrogen values for peanut meal [8] and scleroproteins [9] are in agreement with the values of this investigation. The figure for brewer's yeast is higher than that given by Horn *et al.* [8]. The low nitrogen content of sulfite yeast as compared with brewer's yeast may be stressed. In order to make sulfite yeast edible for animals, it has been found necessary to mix it with brewer's yeast. Accordingly, it can be supposed that the nitrogen value of commercial samples of pure Torula yeast cultivated on sulfite lye may be lower that that given in Table 2.

In preliminary assays a comparison was made between the results obtained with the microorganisms, the basal medium of Henderson and Snell [10], the ranges of standard curves, and the incubation times used in the previous paper [1], and the results obtained during the corresponding experimental conditions as given in Table 1. On the whole, lower blank values and, in several cases, steeper standard curves were obtained on the medium of Steele

Table 2. Nitrogen and crude protein content of the foodstuffs. Percentages calculated for ash- and moisture-free material.

Material	N per cent	Crude protein N × 6.25	Ash per cent	Moisture per cent
Sweet blue lupine, whole meal	6.3	39.4	8.5	9.2
Brewer's yeast, dried	10.5	65.5	6.8	12.3
"Sulfite yeast", dried mixture of *Torula* and brewer's yeast	4.1	25.6	8.5	9.2
Whole fish meal, mainly from herring	14.0	87.5	23.2	6.0
Whole fish meal, mainly from cod	14.8	92.5	15.4	9.3
Molasses	1.8	11.2	7.5	24.8
Pig blood	16.0	100.0	1.1	80.3
Linseed cakes, whole meal	6.6	41.2	5.5	10.0
Peanut cakes, whole meal	9.4	58.7	6.1	6.8
Pig hoofs, whole meal	15.0	93.5	12.0	7.1

et al. [5]. In some cases the microbiological data was a little higher when determined during the experimental conditions given in Table 1. The necessary number of microorganisms could be reduced from five to three. Accordingly, the last mentioned medium was prefered.

To facilitate a comparison with previous data of the amino acid composition in foodstuffs the microbiological values of the present investigation were calculated as described in the last paper [1]. The values are given in Table 3. The question as to whether the different amino acids in the foodstuffs are

Table 3.

1 = Values expressed as percentage for ash- and moisture-free material.
2 = Values expressed as percentage in crude protein (total nitrogen in hydrolysate × 6.25).
3 = Amino acid nitrogen in percentage of total nitrogen in hydrolysate.

Amino acid	Sweet blue lupine			Brewer's yeast			Sulfite yeast			Fish meal from herring			Fish meal from cod		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Alanine	1.2	3.8	3.7	4.5	7.9	7.7	2.1	8.2	8.0	5.1	7.0	6.8	3.6	5.1	5.0
Arginine	4.2	13.3	26.6	1.5	2.6	5.3	1.1	4.4	9.9	5.9	8.1	16.1	4.0	5.7	11.3
Aspartic acid	3.9	12.7	7.9	6.2	11.0	7.3	3.2	12.8	8.5	8.2	11.2	7.5	8.7	12.2	8.1
Cystine	0.15	0.5	0.3	0.4	0.7	0.5	0.06	0.2	0.2	0.68	0.9	0.7	0.93	1.3	1.0
Glutamic acid	7.8	24.4	14.5	6.2	11.0	6.5	2.8	11.3	6.8	9.7	13.4	7.9	15.0	20.2	12.0
Glycine	1.8	5.7	6.7	3.1	5.5	6.4	1.3	5.4	6.3	5.4	7.4	8.6	4.0	5.8	6.7
Histidine	1.0	3.1	5.3	1.5	2.6	4.7	0.60	2.4	4.1	2.0	2.8	4.8	2.1	2.8	4.8
Isoleucine	1.5	4.6	3.1	3.1	5.5	3.7	0.98	3.9	2.6	4.1	5.6	3.8	5.3	7.6	5.1
Leucine	2.1	6.5	4.3	4.0	7.0	4.7	1.8	7.4	5.0	5.6	7.8	5.2	7.2	10.2	6.9
Lysine	1.2	3.8	4.5	3.3	5.9	7.1	1.2	4.9	5.9	6.9	9.5	11.3	6.7	9.5	11.3
Methionine	0.18	0.6	0.03	0.9	1.6	0.9	0.28	1.1	0.7	2.3	3.1	1.8	2.1	2.9	1.7
Phenylalanine	1.1	3.3	1.8	2.5	4.4	2.3	1.0	4.0	2.2	3.0	4.1	2.2	3.2	4.6	2.5
Proline	1.6	5.0	3.8	2.8	5.1	3.8	1.2	4.9	3.8	4.2	5.8	4.4	4.1	6.0	4.5
Serine	1.9	6.1	4.6	3.6	6.4	5.3	1.8	7.4	6.2	4.1	5.6	4.7	3.5	4.9	4.1
Threonine	0.99	3.1	2.5	1.7	3.1	2.3	1.6	6.4	4.7	3.1	4.3	3.2	4.3	6.1	4.5
Tryptophan	0.29	0.8	0.7	1.1	1.7	1.4	0.24	0.9	0.7	0.75	0.9	0.7	0.73	0.8	0.7
Tyrosine	0.97	3.1	1.5	1.6	2.9	1.4	0.68	2.8	1.3	2.1	2.9	1.4	2.3	3.2	1.5
Valine	1.6	5.0	3.7	3.6	6.4	4.8	1.8	7.4	5.5	5.1	7.0	5.2	4.7	6.7	4.9
NH_3-N			8.5			14.0			12.5			8.1			7.0
Total	33.5	105.4	104.0	51.6	91.3	90.1	23.7	95.8	94.9	78.2	107.4	104.4	82.5	115.6	103.6

present in sufficient amounts to satisfy the synthesis of body proteins in animals cannot be solved by merely analyzing the amino acids. But on the basis of a comparison with the amino acids found in the hog's blood in this investigation and by Block and Mitchell [11] in muscle tissue from different animals, it may be possible to draw some conclusions with regard to the nutritional values of the other foodstuffs in Table 3.

As could be anticipated, molasses contains very small quantities of essential amino acids. Non-essential amino acid nitrogen amounts to about 85 per cent of total nitrogen. With regard to the content of essential amino acids in the other analyzed foodstuffs, the *arginine* value of sulfite yeast is a little low. The same holds for *histidine* on hog hoof, *isoleucine* in sulfite

Table 3 (continued).

1 = Values expressed as percentage for ash- and moisture-free material.
2 = Values expressed as percentage in crude protein (total nitrogen in hydrolysate × 6.25).
3 = Amino acid nitrogen in percentage of total nitrogen in hydrolysate.

Amino acid	Molasses			Pig blood			Linseed cakes			Peanut cakes			Pig hoof		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Alanine	0.50		3.5	0.86	1.0	1.0	1.1	2.8	2.8	1.4	2.9	2.8	4.5	4.7	4.6
Arginine	0.11		1.6	3.7	5.2	10.0	3.7	9.9	19.7	5.1	10.0	20.0	8.8	9.2	18.4
Aspartic acid	0.50		2.3	14.0	16.1	10.1	4.5	11.8	7.8	7.8	15.11	10.2	10.0	10.5	6.9
Cystine	0.25		0.1	1.2	1.4	1.5	0.33	0.9	0.6	0.44	0.9	0.6	3.6	3.7	2.7
Glutamic acid	5.2		21.0	9.8	10.7	6.3	8.2	21.6	12.9	11.0	21.7	12.8	18.0	17.8	10.5
Glycine	0.20		2.0	4.8	5.5	6.4	2.5	6.7	7.8	2.9	5.7	6.7	8.8	9.2	10.7
Histidine	0.03		0.4	8.1	9.2	15.5	1.0	2.7	4.5	1.4	2.9	4.8	1.9	1.9	3.3
Isoleucine	0.32		1.5	1.0	1.2	0.8	2.1	5.6	3.7	2.3	4.6	3.1	5.3	5.5	3.7
Leucine	0.13		0.7	12.0	13.7	9.2	2.4	6.3	4.2	3.3	6.6	4.4	4.7	4.8	3.2
Lysine	0.072		0.6	8.1	9.2	10.8	1.3	3.5	4.1	1.6	3.2	3.8	4.3	4.5	5.3
Methionine	0.038		0.2	1.1	1.2	0.7	0.55	1.5	0.9	0.42	0.8	0.5	1.2	1.3	0.8
Phenylalanine	0.038		0.1	6.5	7.3	3.8	1.5	3.9	2.1	2.3	4.6	2.4	3.2	3.4	1.8
Proline	0.082		0.5	4.3	4.9	3.7	1.8	4.8	3.6	2.6	5.2	3.9	5.3	5.5	4.2
Serine	0.15		0.9	7.5	8.4	7.0	2.4	6.3	5.3	3.3	6.6	5.5	9.1	9.6	7.9
Threonine	0.10		0.5	2.0	2.3	1.7	1.5	3.9	2.9	1.3	2.6	1.9	4.1	4.2	3.1
Tryptophan	0.046		0.03	1.5	1.5	1.4	0.6	1.4	1.2	0.55	1.4	0.8	0.6	0.6	0.5
Tyrosine	0.13		0.4	2.2	2.5	1.2	0.82	2.2	1.1	1.6	3.2	1.5	5.3	5.5	2.6
Valine	0.17		0.9	9.7	10.7	7.9	2.3	5.9	4.4	2.9	5.7	4.3	6.6	6.8	5.0
NH_3-N			7.6			3.0			12.7			11.5			8.6
Total	8.06		44.8	98.4	112.0	102.0	38.6	101.7	102.3	52.2	103.7	101.5	105.3	108.7	103.8

yeast and hog blood, *leucine* in hog hoof, *lysine* in blue lupine, sulfite yeast, linseed cakes, peanut cakes and hog hoof, *methionine* in all of the foodstuffs with exception of the fish meals, *phenylalanine* in blue lupine, sulfite yeast, fish meal from herring, linseed cakes and hog hoof, *threonine* in blue lupine, brewer's yeast, hog blood, linseed cakes, and peanut cakes, *tryptophan* in blue lupine, sulfite yeast, fish meals, and hog hoof.

For two amino acids, hydroxyproline and hydroxylysine, which may be more commonly present in protein of animal or plant origin, only chemical methods are available. The presence of hydroxyproline in the foodstuffs were analyzed by means of two-dimensional paperchromatography with the modifications used in this laboratory [12,13]. With the usual amounts of hydrolysate,

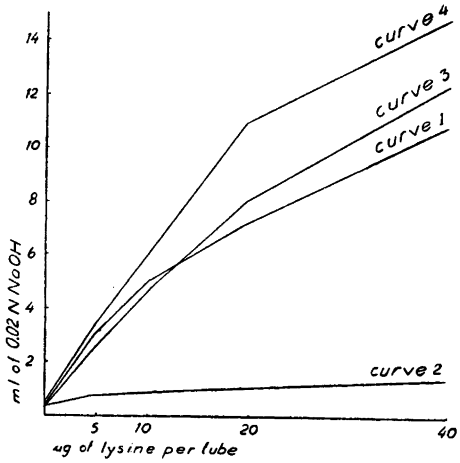

Figure 1. Curve 1 standard curve for lysine. Curve 2 obtained with corresponding amounts of hydroxylysine. Curve 3 is obtained with standard amounts of lysine plus addition of equal amounts of hydroxylysine for each point. Curve 4 obtained with standard amounts of lysine and a tenfold increase of hydroxylysine.

0.5 to 1.0 mg, there was no trace of a hydroxyproline spot on the papers. From previous analysis on whole meals and muscle meals of cod [14] it is known that hydroxylysine is present in hydrolysates from this fish. An attempt was made to determine whether hydroxylysine influenced the microbiological lysine values obtained with *L. mesenteroides* P-60. Assays were run parallel with hydroxylysine alone, and added in different quantities to lysine. The results are given in Fig. 1. It is obvious that hydroxylysine is not an antagonist to lysine. Further, it can be said that the amounts of hydroxylysine present, for instance, in fish have no influence upon the lysine determinations. When a large excess of hydroxylysine, 200 to 400 µg, is added to the medium, an extra growth effect is observed. At present it can not be decided whether this effect can be ascribed to the hydroxy-amino acid or depends upon impurities in the preparation.

Recent data, from the literature, for the amino acid content of the foodstuffs investigated in this paper or in closely related materials is given in Table 4. Mainly, only values obtained by microbiological methods have been recorded.

In general, there is a comparatively good agreement with the values obtained in this investigation. The sweet blue lupine has not been previously analyzed. A comparison with the sweet yellow lupine [1] shows several differences in the amino acid distributions between the two species. The yeast values of Horn *et al.*[15–22] in Table 4 have been obtained on brewer's yeast while the values of Block and Mitchell [11] and those of Block and Bolling [33] are given for yeast without any particular specification of the source of material. The values obtained for brewer's yeast and sulfite yeast in the present investigation

Table 4. Amino acid content of some foodstuffs (literature values).

1 = Values expressed as percentage for ash- and moisture-free material.
2 = Values expressed as percentage in crude protein (total nitrogen in hydrolysates × 6.25).

Amino acid	Yeasts			Fish meal	Blood meal	Linseed meal		Peanut meal		Cattle horn	
	1	2		2	2	2		1	2	1	2
	(15–22)	(11)	(23)	(23)	(11)	(11)	(23)	(15–22)	(11)	(9)	(23)
Alanine											
Arginine	2.3	3.1–5.3	4.3	5.6	3.7	8.4	6.2	7.8	9.9	10.7	10.4
Aspartic acid										7.7	3.0
Cystine		0.9–1.1	1.3	1.0	1.8	1.9	1.9		1.0	12.1	7.3
Glutamic acid										13.8	18.0
Glycine									5.6		10.0
Histidine	0.8	2.3–3.1	2.8	2.4	4.9	1.5	1.5	1.3	2.1	1.0	1.0
Isoleucine	2.4	5.5–6.2	6.0	4.8	1.1	4.0	3.4	2.4	3.0	4.3	4–5
Leucine	3.3	6.1–8.5	7.3	10.0	12.2	7.0	7.5	4.3	7.0	8.3	15.0
Lysine	3.5	6.7–8.1	6.0	5.7	8.8	2.5	2.5	1.9	3.0	3.6	3.2
Methionine	0.5	1.7–2.0	2.0	3.0	1.5	2.3	3.0	0.52	1.2	0.5	
Phenylalanine	1.8	2.9–4.6	4.1	4.8	7.3	5.6	5.6	3.3	5.4	3.2	4.0
Proline										8.2	
Serine											
Threonine	2.5	5.1–6.0	5.0	5.0	6.5	5.1	5.1	1.9	1.5	6.1	5–6
Tryptophan		1.2–1.5	1.8	1.2	1.3	1.5	1.9		1.1		1.5
Tyrosine		3.4–3.7	4.8	2.8	3.7	5.1	5.1		4.4	5.6	4–6
Valine		4.6–5.9	5.3	4.0	7.7	7.0	5.8		8.0	5.5	5.0

generally falls within the limits given by Block and Mitchell. A comparison between the two sources of yeast shows smaller amounts of sulphur-containing amino acids in sulfite yeast.

The figures for the fish meal in Table 4 are taken from Block and Bolling [23]. They do not specify the origin of the material. By and large, there is a good agreement as compared with the amino acid values given for the two fish meals in Table 3, but some differences could be anticipated since discrepancies already had been observed in a comparison of the amino acid composition of the fish meals from herring and from cod. With regard to the unknown blood meal analyzed by Block and Mitchell [11] and the hog blood of the present investigation, the amino acid values coincide fairly well. From the nutritional point of view, the low isoleucine and methionine values of these materials may

be emphasized. The amino acid values for linseed and peanut cakes of the present investigation and for linseed meal and peanut meal quoted in Table 4 are of the same order of size. The amino acid content of pig hoofs has not previously been investigated. For comparison, available data on cattle horn are given. It is obvious that as a potential source of essential amino acids, pig hoof meal may be quite as good as cattle horn.

SUMMARY

Ten foodstuffs commonly used in animal nutrition in Sweden have been analyzed by microbiological methods for eighteen possible amino acids. Where comparison is possible the results agree fairly well with those obtained by other microbiological methods on similar materials.

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The Partition of Organic Compounds Between Higher Alcohols and Water

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In a recent paper (Collander [1]) the partition of organic compounds between *iso*-butanol and water was examined. Now it has been thought of interest to study the solvent properties of some higher alcohols also, in order to establish the influence of the length of the hydrocarbon chain on these properties. The alcohols chosen for this study were, in the first place, prim. *n*-octyl alcohol and oleyl alcohol. (The last-named substance is of special interest in this connection as it has been recommended by K. H. Meyer and Hemmi [2] as a suitable model for the plasma membrane lipoids.) Some experiments were carried out also with *iso*-amyl alcohol, but they are not reported here in full.

Table 1 contains the partition coefficients referring to the solvent systems octyl alcohol/water and oleyl alcohol/water.

The first column gives the empirical formulae and the names of the solutes studied. The order of the compounds is determined, firstly, by the number of carbon atoms in the molecule, secondly, thirdly, and fourthly by the numbers of nitrogen, oxygen, and hydrogen atoms respectively. Halogen compounds are listed immediately after the corresponding halogen-free compounds.

The second column gives the organic solvent used: Oc = octyl alcohol (B.D.H.), Ol = oleyl alcohol (a commercial product, probably not very pure, from Deutsche Hydrierwerke, Rotleben, Germany).

The third column gives the temperature in degrees centigrade. If not reported, the temperature was between 17 and 22° C.

The fourth and fifth columns give the concentration of the solute in the alcoholic and aqueous phase respectively. All concentrations are expressed as millimols per liter solution.

The last column gives the partition coefficients defined as the ratio of the total concentration of the solute in the alcoholic phase to its total concentration in the aqueous phase. Also the partition coefficients referred to in the text always mean *concentration in the organic phase/concentration in the aqueous phase*, never the inverse value. In the case

of acids and bases with a dissociation constant greater than 10^{-3} the partition of the undissociated molecules has been calculated on the assumption that only undissociated molecules are soluble in the organic solvent. The values arrived at in this way are marked with *M*.

The partition coefficients listed are for the most part thought to be correct within limits of about 20 per cent.

Table 1. Partition of organic compounds between octyl and oleyl alcohols and water.

Solute	Solvent	Temp.	$C_{alcohol}$	C_{water}	Part. coeff.
CH_3I Methyl iodide	Oc	19	2 090	42.4	49
CH_4O Methanol	Oc	19	200	1 300	0.15
CH_2O_2 Formic acid	Oc	20	247	842	0.29
» » »	Ol		103	877	0.12
CH_5N Methylamine	Oc	20	260	970	0.27
CH_3NO_2 Nitromethane	Oc	20	1 500	1 250	1.2
C_2H_6O Ethanol	Oc	20	426	891	0.48
$C_2H_4O_2$ Acetic acid	Oc	20	423	859	0.49
» » »	Ol		180	831	0.22
$C_2H_3BrO_2$ Bromoacetic acid	Oc	20	590	248	2.4 (*M* 2.6)
$C_2H_4O_3$ Glycolic acid	Oc	19	77.6	1 014	0.077
» » »	Ol	19	19	968	0.020
C_2H_7ON Ethanolamine	Oc	19	94.0	1 926	0.049
C_3H_6O Acetone	Oc	20	506	867	0.58
$C_3H_6O_2$ Methyl acetate	Oc	20	780	526	1.5
» Propionic acid	Oc	20	880	482	1.8
» » »	Ol		453	558	0.81
$C_3H_5BrO_2$ α-Bromopropionic acid	Oc	20	901	121	7.4 (*M* 8.3)
$C_3H_8O_2$ Methylal	Oc	19	1 350	1 390	1.0
$C_3H_4O_3$ Pyruvic acid	Ol		102	878	0.12
$C_3H_6O_3$ Lactic acid	Oc	20	177	752	0.24
» » »	Ol		59	957	0.062
$C_3H_4O_4$ Malonic acid	Ol		48	978	0.049 (*M* 0.052)
C_3H_5N Propionitrile	Oc	18	1 420	1 290	1.1
C_3H_9ON *iso*-Propanolamine	Oc	19	227	2 001	0.11
C_4H_8O Methyl ethyl ketone	Oc	19	631	351	1.8
$C_4H_{10}O$ *iso*-Butanol	Oc	20	2 591	381	6.7
» Ethyl ether	Oc	20	3 290	486	6.8
$C_4H_8O_2$ Ethyl acetate	Oc	20	645	141	4.6
» Dioxane	Oc	20	319	840	0.38
» *n*-Butyric acid	Oc	20	628	102	6.2
» » »	Ol		667	230	2.9
$C_4H_7BrO_2$ α-Br-n-butyric acid	Oc	19	1 028	46.8	22 (*M* 26)

Solute	Solvent	Temp.	$C_{alcohol}$	C_{water}	Part. coeff.
$C_4H_{10}O_2$ Ethylene glycol monoethyl ether	Oc	19	238	810	0.29
» 2,3-Butylene glycol	Oc	18	150	1 250	0.12
$C_4H_6O_3$ α-Oxy-*iso*-butyric acid	Oc	20	195	447	0.44
» » » » »	Ol		128	890	0.14
$C_4H_4O_4$ Maleic acid	Ol		51	459	0.11 (*M* 0.13)
$C_4H_6O_4$ Succinic acid	Oc	20	132	513	0.26
$C_4H_6O_5$ Malic acid	Oc	20	26.4	483	0.055
» » »	Ol		17.2	976	0.018
$C_4H_{11}N$ *n*-Butylamine	Oc	20	1 294	271	4.8
» Diethylamine	Oc	19	816	299	2.7
$C_4H_{11}O_2N$ Diethanolamine	Oc	19	26.4	704	0.037
$C_4H_4N_2$ Ethylene cyanide	Oc	20	75.0	625	0.12
$C_4H_{10}N_2$ Piperazine	Oc	17	15.1	224	0.067
$C_5H_8O_4$ Glutaric acid	Ol		100	903	0.11
» Dimethylmalonic acid	Ol		254	515	0.49
$C_6H_{12}O_2$ Caproic acid	Ol		984	21.7	45
$C_6H_{10}O_4$ Adipic acid	Oc	20	148	121	1.2
$C_6H_8O_6$ Tricarballylic acid	Ol		30.4	996	0.030
$C_6H_8O_7$ Citric acid	Oc	20	11.5	609	0.019
$C_6H_{15}O_2N$ Di-*iso*-propanol-amine	Oc	19	34.0	222	0.15
$C_8H_{18}O$ prim. *n*-Octanol	Oc	25	6 360	4.51	1 410
$C_9H_{16}O_4$ Azelaic acid	Oc	20	155	4.15	37 (*M* 40)

CONCLUSIONS

According to Frumkin[3] the partition coefficient k equals $e^{W/RT}$, in which W denotes the energy that can be gained by the transport of one mole of the solute from the one phase to the other. Frumkin did not express any views about the nature of this energy, but to-day it seems plausible to assume that it depends principally on the formation and breaking of hydrogen bonds between the solute and the solvent molecules. If this is the case, it is only natural that the greater the difference as to the hydrogen bondedness of the two phases of the solvent systems examined the greater is the difference between two solutes as to their partition coefficients. Tables 2 and 3 give some examples of this.

Table 2 shows that in the butanol/water system the partition coefficient increases by 1.8—2.8 times within each homologous series of solutes from one member to the next. In the octanol/water system the corresponding increase is a little greater and in the oleyl alcohol/water system still somewhat greater.

Table 2. Increase of the partition coefficient within homologous series of solutes in different alcohol/water systems.

	Butanol / Water	Octanol / Water	Oleyl alcohol / Water
Acetic acid — Caproic acid	2.8	—	3.8
Br-acetic acid — Br-butyric acid	2.8	3.2	—
Methyl acetate — Ethyl acetate	2.8	3.1	—
Malonic acid — Dimethylmalonic acid	2.6	—	3.1
Methylamine — Butylamine	2.5	2.6	—
Methanol — *iso*-Butanol	2.4	3.5	—
Succinic acid — Adipic acid	1.9	2.1	—
Glycolic acid — Oxy-*iso*-butyric acid	1.9	2.4	2.7
Diethanolamine — Di-*iso*-propanolamine	1.9	2.0	—
Ethanolamine — *iso*-Propanolamine	1.8	2.2	—

Similarly Table 3 shows that when a hydroxyl group is introduced into a solute molecule the longer the hydrocarbon chain of the alcohol used as a non-aqueous solvent the more reduced will be the partition coefficient of the solute.

As previously stated (Collander [4]) the partition coefficients in different solvent systems composed of monohydric alcohols and water are mutually correlated in such a way that, if k_1 denotes the partition coefficient of a given solute in one solvent system and k_2 that of the same solute in another of the solvent systems here in question, then the following equation is approximately valid:

$$\log k_1 = a \cdot \log k_2 + b \qquad (1)$$

In this equation a and b are two constants characterizing the solvent systems in question.

Table 3. Influence of a hydroxyl group on the partition coefficient in different alcohol/water systems.

	Butanol / Water	Pentanol / Water	Octanol / Water
Diethylamine — Diethanolamine	4.8	—	8.5
Propionic acid — Lactic acid	4.3	—	7.6
Acetic acid — Glycolic acid	3.6	5.4	6.3
Tricarballylic acid — Citric acid	2.9	4.4	—
Succinic acid — Malic acid	2.6	4.6	4.8
Malic acid — Tartaric acid	2.2	2.3	—

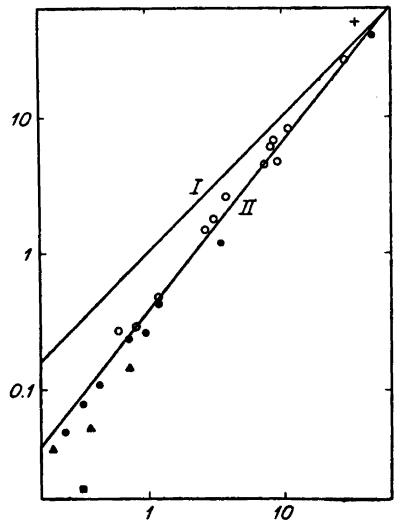

Fig. 1. Abscissa: partition coefficient in the iso-*butanol/water system, ordinate: partition coefficient in the octanol/water system. Line I represents the equation* $k_{octanol} = k_{butanol}$, *line II the equation (3). + molecule containing no hydrophilic group, o molecule containing one hydrophilic group, ●, ▲, and ■ molecules containing two, three, or four such groups, respectively.*

Thus the organic acids whose partition in the *iso*-butanol/water and *iso*-pentanol/water systems has been studied all fit fairly exactly the equation

$$\log k_{iso\text{-}pentanol} = 1.17\ k_{iso\text{-}butanol} - 0.17 \qquad (2)$$

On the other hand, a contemplation of Fig. 1 showing the correlation between the partition coefficients in the *iso*-butanol/water and the prim.-octanol/water systems reveals the fact that although the equation

$$\log k_{octanol} = 1.24 \log k_{butanol} - 0.42 \qquad (3)$$

is approximately valid, there is, never-the-less, a distinct difference between the behaviour of those compounds which contain one hydrophilic group only in their molecule and those which contain two or more such groups. The points representing the former (*i. e.*, the fatty acids, the α-bromo-substituted fatty acids, the monohydric alcohols, the alkyl acetates, the dialkyl ethers, and the alkylamines) are almost all situated more or less above line II representing equation (3) while the points representing the compounds with two or more hydrophilic groups in their molecule are all situated below this line.

The reason for this is not quite clear, but the following hypothesis may perhaps be considered possible: — When an organic compound dissolves in a relatively hydrophilic solvent, like butanol, numerous hydrogen bonds are formed between the solute and the solvent molecules. When, however, the

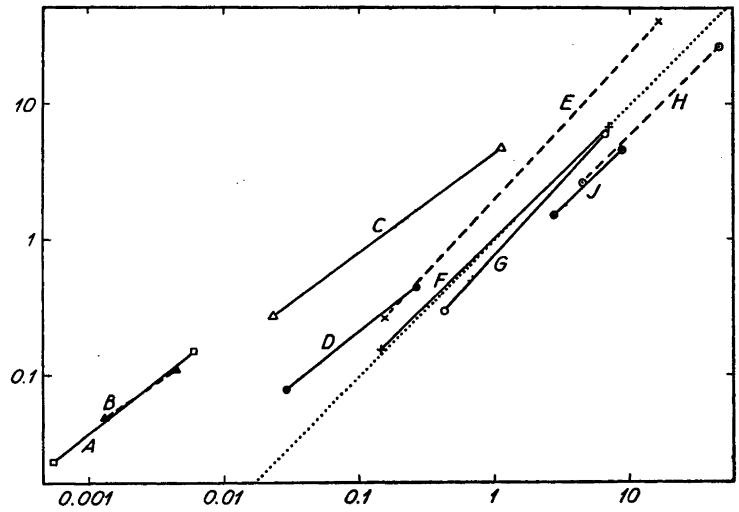

Fig. 2. Abscissa: partition coefficient in the ethyl ether/water system, ordinate: partition coefficient in the octanol/water system. A dialkanolamines, B monoalkanolamines, C alkylamines, D α-hydroxy-substituted fatty acids, E dicarboxylic acids, F monohydric alcohols, G fatty acids, H α-bromo-substituted fatty acids, I alkyl acetates.

same substance dissolves in a less hydrophilic solvent, like octanol, hydrogen bonds are to a considerable extent formed between pairs of solute molecules. (In the case of fatty acids this tendency to form double molecules in hydrophobic solvents has been well known for a long time.) The double molecules thus formed are always more hydrophobic than the original single molecules. If, however, the solute molecule contains many hydrophilic groups, in most cases probably only one such group per molecule will be engaged in the formation of molecule pairs. The other hydrophilic groups thus remain unaltered and the increase of the alcohol/water distribution coefficient will therefore be relatively smaller than in the case of molecules containing one hydrophilic group only. (The behaviour of methyl iodide, the only compound studied which is without a single hydrophilic group, seems, however, not to harmonize very well with this hypothesis.)

If the partition coefficients found in the system oleyl alcohol/water are plotted against those in the iso-butanol/water system, we get a graph virtually very like Fig. 1. For the sake of saving space it will not be reproduced here.

A saturated solution of ethyl ether in water at 25° C contains about 5.7 g ether per 100 g solution, while a saturated aqueous solution of prim. octanol at the same temperature contains only about 0.059 g octanol per 100 g solution

(Butler *et al.*[5]). In view of this one would feel tempted to regard octanol as a much less hydrophilic substance than ethyl ether. Such a conclusion would not, however, be consistent with the results visualized by Fig. 2, viz., that strongly hydrophilic solutes are somewhat more soluble in octanol than in ether, while more hydrophobic substances are about as soluble in ether as in octanol. (For the sake of clearness, only a selection of the substances studied in the ether/water and the octanol/water systems has been included in this graph.)

Another thing seen from Fig. 2 is that bases (alkylamines, mono- and dialkanolamines) are relatively more soluble in octanol than in ether. This is probably due to the octanol being (just like *iso*-butanol; *cf.* Collander [1]) of a more acidic nature than ether.

SUMMARY

The partition of some fifty organic compounds (acids, bases, and non-electrolytes) in the solvent systems *iso*-amyl alcohol/water, octyl alcohol/water, and/or oleyl alcohol/water has been determined and compared with their previously studied partition in the *iso*-butanol/water, and ethyl ether/water systems.

The partition in one alcohol/water system can be calculated approximately from that in another such system, using the equation $\log k_1 = a \log k_2 + b$ where k_1 and k_2 denote the partition coefficients in the two solvent systems, while a and b are constants. It should, however, be noted that the "constant" b has not quite the same value in the case of solutes containing one hydrophilic group as in the case of solutes containing two or more such groups. This difference is hypothetically explained as a consequence of the formation of intermolecular hydrogen bonds between the solute molecules in non-aqueous solutions.

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The Preparation of Highly Purified Spinach Leaf Catalase

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The enzyme catalase has now been prepared in crystalline form from many different sources: the liver of beef, horse, man, guinea pig and lamb, the erythrocytes of beef, horse and man, the kidney of horse and the cells of the bacterium *Micrococcus lysodeikticus*. (For references, see Herbert and Pinsent [1] and Bonnichsen [3, 2]). In general, catalase from animal sources is quite stable and convenient to handle, but in sharp contrast with this, the catalase of green plants has been found by numerous investigators to be very labile. Only in the work of Embden [4] has a relatively stable plant catalase extract been reported. However, this investigator used as a source of catalase pumpkin seedling cotyledons, from which it would be difficult to prepare enough material for isolation or for chemical characterization of the enzyme.

As a result of the lack of a readily available source of stable plant catalase, our knowledge of the nature of the enzyme is very scanty. We know only that it rises and falls suddenly several days after germination (Embden [4], Zeile [5], Holman [6]), that chloroplasts contain most of the activity of the leaf (Neish [7]), that the enzyme is inhibited by cyanide and gives a faint hematin spectrum (Zeile [5], Keilin and Hartree [8]), and that it is stable between pH 6.8 and 8.2 (von Euler, Myrbäck and Myrbäck [9]).

The present study was undertaken (a) to attempt to find a convenient source of stable plant catalase, (b) to purify the enzyme as much as possible, (c) to obtain information as to the chemical nature of the enzyme.

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EXPERIMENTAL

A. Estimation of catalase activity

All assays for catalase activity described in this paper were made by the rapid titration method of Bonnichsen, Chance and Theorell [10]. Essentially, this is a modification of the von Euler and Josephson [11] technique, and is based on the titration of H_2O_2 by $KMnO_4$ before the addition of catalase and at frequent intervals after its addition. In our experiments, from 0.01 to 0.2 ml of the extract was assayed, depending on the activity of the particular sample. The aliquots for titration were removed at 15 and 30 seconds from the active preparations, and at 1 and 3 minutes from the less active ones. From the titration data, a monomolecular k value was calculated according to the expression

$$k = \frac{1}{t} \log_{10} \frac{x_0}{x_t}$$

where x_0 is the titer at time zero, x_t the titer at time t and t the time in minutes which the enzyme has acted on the H_2O_2.

The relative purity of each fraction was expressed in terms of the *Kat.f.* of von Euler and Josephson [11], where

$$Kat.f. = \frac{k}{\text{gms. enzyme used in the test}}$$

To obtain the dry weight of the proteins per ml of sample used, 1 ml of the material was dialyzed two times against 2L of distilled water for 24 hours. The sample was then pipetted on to a tared watch glass, dried overnight at 105° C and weighed on an analytical balance.

B. Preparation of the enzyme

Spinach leaves were chosen as a starting material because of the ready availability of large quantities of this product, and because preliminary tests showed the presence of active catalase in crude extracts. 75 kilograms of freshly harvested spinach * were washed in running tap water aad stored in a refrigerated room maintained at + 4° C. About 3 days after harvest, the leaves were transferred to large stainless steel vats containing commercial acetone chilled to — 15° C in a freezing room. After at least two hours of storage under the

* Purchased from T. Wistrand, Lindholmen.

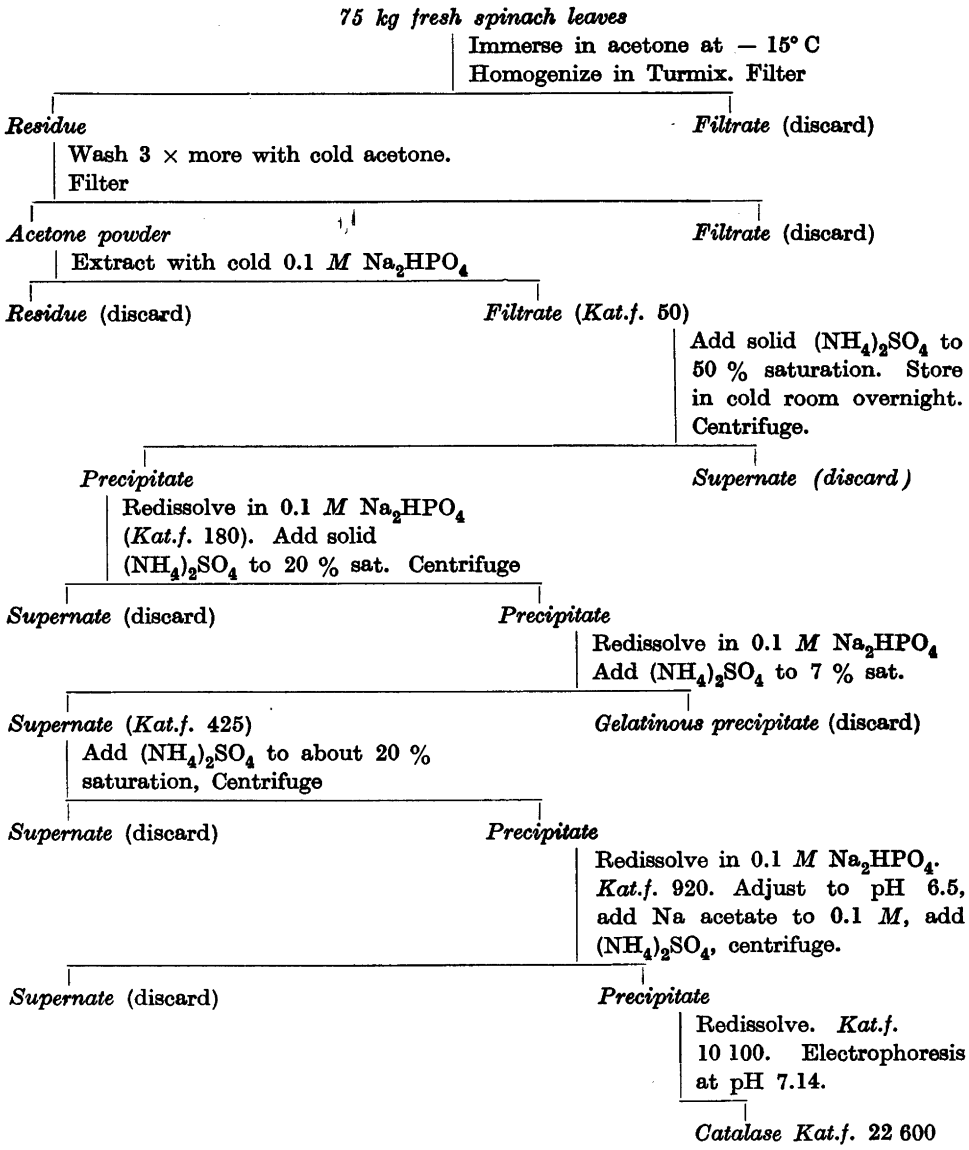


Fig. 1. Scheme for the fractionation of spinach leaves to yield a highly purified catalase.

cold acetone, samples of the leaves were removed, placed in a large Turmix apparatus, covered with some of the cold acetone, and blended vigorously for 30 seconds. Since the leaves were rendered very brittle by the cold acetone,

even the brief blending treatment sufficed to reduce them to a fine slurry. The slurry was decanted into another large stainless steel tub kept in the cold room. After the entire 75 kilograms of spinach had been blended, the slurry was stirred vigorously, filtered through a perforated stainless steel funnel lined with fine muslin, and the green filtrate discarded. The precipitate was once more treated with about 20 liters of cold acetone, again filtered through muslin, and the filtrate again discarded. The residue was then transferred to large glass funnels lined with two layers of filter paper, washed several more times with cold acetone, and allowed to drain for several hours in the cold room. The partially-dried residue was then spread out on large sheets of filter paper in a well-ventilated hood and allowed to dry overnight. The result of this treatment was about 6 kilograms of greenish powder. Occasional grayish lumps in the powder reduced readily to the powder form when rubbed between the hands.

The further fractionation of this acetone powder can be followed by reference to Fig. 1. The powder was first placed in a stainless steel tub, mixed with 10L of cold 0.1 M Na_2HPO_4, and stirred vigorously with a motor stirrer for one hour. The slurry was then filtered by gravity in the cold room through two layers of filter paper. The residue of the filtration was washed at least three more times with additional portions of the cold Na_2HPO_4. The total volume of the filtrate at this point was approximately 25 liters, and had a *Kat.f.* of about 50. The residue of this extraction still contains considerable catalase, which continues to be slowly extracted by large volumes of buffer. For the sake of convenience, however, the extraction was halted when the main bulk of the catalase had been obtained in the extract.

The 25L of extract were then half-saturated with $(NH_4)_2SO_4$ by the addition of the solid salt, and the solution permitted to stand in the cold room overnight. The next morning, a surface layer of lipoidal material was skimmed off and discarded, and the remainder of the solution centrifuged for 30 minutes at 2 000 rpm in an International Serum Centrifuge having a capacity of 13 L. Almost all of the catalase activity was in the precipitate, and the brownish supernatant liquid was therefore discarded. The ammonium sulfate precipitate was then redissolved in 0.1 *M* Na_2HPO_4, allowed to stand overnight in the cold room, and recentrifuged. The supernatant liquid was saved, and the precipitate was again mixed with cold Na_2HPO_4, centrifuged, and the supernatant liquid combined with the first supernatant. The *Kat.f.* at this point was 180 and the total volume about 4L.

To the clear supernatant, saturated $(NH_4)_2SO_4$ was now added dropwise with vigorous stirring. At about 12 % saturation with the ammonium sulfate, a silkiness developed. This was deposited by prolonged centrifugation, and

proved to be colorless rhomboidal crystals devoid of catalase activity. The nature of this material was not further investigated. Ammonium sulfate was again added dropwise, and at about 20 % saturation, a copious precipitate deposited. This was sedimented by centrifugation for 1 hour at 3 000 rpm. in a water-cooled Collatz centrifuge. The supernatant liquid was practically without activity and was discarded.

The precipitate was redissolved in cold 0.1 *M* Na_2HPO_4 and once again subjected to ammonium sulfate fractionation. This time, at about 7.5 % saturation, a copious gelatinous precipitate deposited which was devoid of catalase activity, and was removed by centrifugation. The *Kat.f.* of the resulting clear solution was 425. More ammonium sulfate was then added, and at about 20 % saturation a fine precipitate appeared which contained almost all of the catalase activity. The *Kat.f.* of this material was 920. The material also showed a faint absorption band at about 630 mμ when viewed through a hand spectroscope. Its absorption spectrum, as obtained in a model DU Beckman spectrophotometer, showed strong peaks at 275 and 330 mμ and weaker maxima at 405 and 625 mμ.

This catalase solution was now adjusted to pH 6.5 and made 0.1 *M* with respect to sodium acetate by the addition of the solid salt. Cautious fractionation with saturated ammonium sulfate resulted in the deposition of several precipitates, ranging in *Kat.f.* from 1 200 to 10 100. A total of 19 ml of the most active material was obtained, with a protein concentration of 24.6 mg per ml.

Various techniques were now tried on small aliquots of the enzyme in an attempt to achieve further purification of this material. Some of the techniques failed to purify at all and others gave such poor yields of the purified material as to be impractical as preparative procedures. Among the techniques surveyed were fractionation by cold ethanol, cold acetone, alcohol-chloroform, K_2HPO_4, $(NH_4)_2SO_4$, and $(NH_4)_2SO_4$ plus sodium acetate; also adsorption on charcoal, solid $Ca_3(PO_4)_2$ column, $Ca_3(PO_4)_2$ gel, and several ion-exchange resins. It was therefore decided to subject the material to electrophoretic analysis, with a view to use electrophoresis as a preparative method.

A preliminary run was made with catalase of *Kat.f.* 920 which had been dialyzed for two days against two changes of pH 7.14 phosphate buffer of ionic strength 0.1. Four electrophoretic components appeared, two major colored components and two minor uncolored components. All migrated toward the anode. After 4 hours of electrophoresis (18 milliamperes, 330V), it was possible to obtain samples enriched in each of the colored constituents. These were assayed for catalase activity and were also subjected to spectrophotometric investigation. The faster moving fraction had a *Kat.f.* of 797

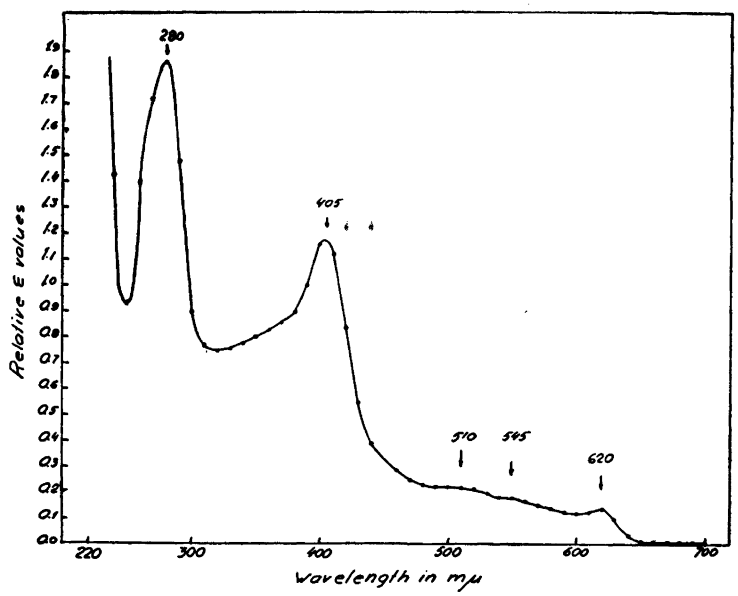
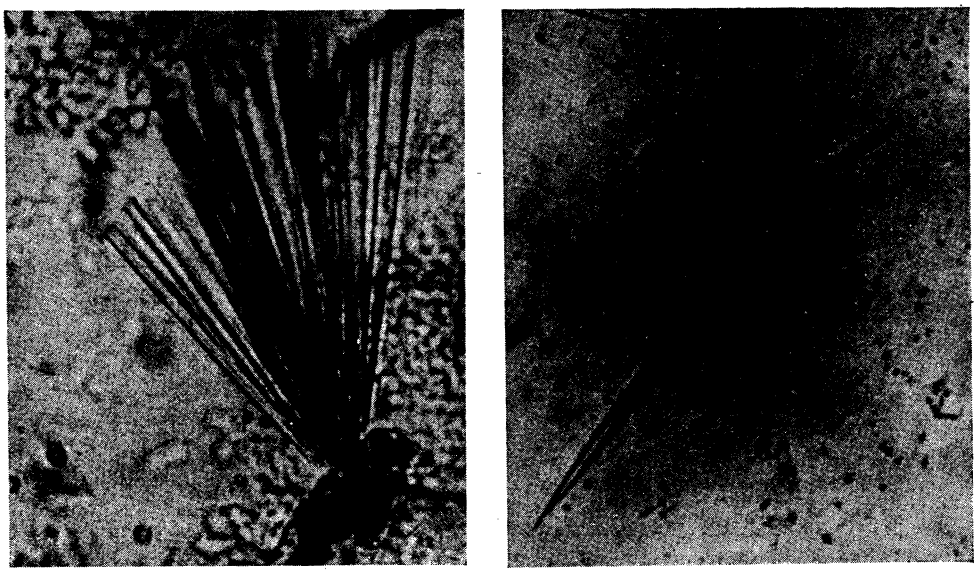


Fig. 2. The absorption spectrum of electrophoretically prepared spinach leaf catalase. Kat. f. = 23 600. Concentration of the enzyme approximately 0.5 mg per 1 ml.

and a ratio $\frac{E_{280}}{E_{405}}$ of 2.61. The slower moving component had a *Kat.f.* of 1 548 and a ratio $\frac{E_{280}}{E_{405}}$ of 1.97. Obviously, the slowermoving component was enriched in catalase.

Another electrophoretic run was then made with the catalase of *Kat.f.* 10 100 which had been dialyzed for 2 days against pH 7.14 phosphate buffer of ionic strength of 0.1. Five fractions were collected, three on the anodic side, one in the bottom cell, and one from the cathodic side. They were each assayed for catalase activity and were also subjected to spectrophotometric analysis in the Beckman spectrophotometer. The results of these analyses are shown in Table 1. It is clear that the cathodic fraction is greatly enriched in catalase. An absorption spectrum of this material is shown in Fig. 2. It shows peaks at 280, 405, 510, 545 and 620 mμ and has a ratio $\frac{E_{280}}{E_{405}}$ of 1.54. This ratio is still considerably higher than that found in blood and liver catalases, in which the 405 mμ peak is actually higher than the 280 mμ peak.

Attempts were now made to crystallize the enzyme. The purified catalase was first concentrated to one-third its volume by placing it in a cellophane



A *B*

Fig. 3. Crystals of the presumptive plant catalase together with the amorphous material which also precipitates (440 x). A = occasional clusters of needles. B = abundant isolated needles or needle fragments.

Table 1. Catalase activity and spectrophotometric characteristics of electrophoretically obtained spinach catalase fractions.

Fraction	Kat.f.	Ratio E_{280}/E_{405} mμ
− 2	23 600	1.54
Bottom cell	9 020	2.07
+ 2	12 100	2.07
+ 1	7 320	2.37
+ (top cell)	2 620	2.54

dialysis bag and blowing air gently over the surface of the bag. The concentrated material was then dialyzed in the cold room against 0.1 *M* Na_2HPO_4 to which small increments of saturated ammonium sulfate were added every few hours. The solution became quite silky at an ammonium sulfate concentration of about 12 %. It was permitted to stand in the cold room for two days, and the fine precipitate sedimented in the Sorvall high-speed centrifuge. The precipitate contained numerous clusters of needle-like crystals (Fig. 3), together with a greater quantity of amorphous material. When the precipitate was redissolved in 0.1 *M* Na_2HPO_4, it gave a good catalase spectrum and had

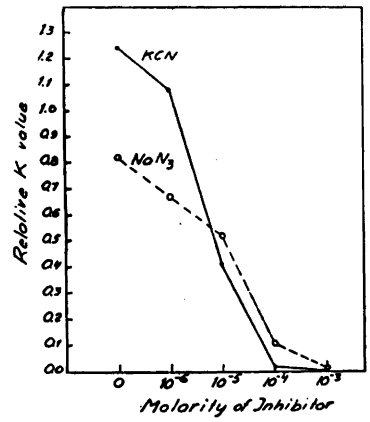


Fig. 4. The effect of KCN and NaN_3 on the activity of spinach leaf catalase.

a *Kat.f.* of 18 600. Other precipitates obtained from the mother liquor had similar or lower *Kat.f.* values.

Although it is highly probable that the crystals portrayed in Fig. 3 are actually crystalline plant catalase, this cannot be stated with certainty, since some amorphous material was present in all the fractions obtained. Thus far, all attempts to separate the crystals from the amorphous matter by differential centrifugation and differential solubilities have been unsuccessful.

C. Experiments with the purified enzyme

Small aliquots of the purified catalase were employed for various studies designed to yield information as to the nature of the enzyme.

1. *Inhibition studies.* Heme-containing enzymes are typically inhibited by cyanide and azide. Our experiments with spinach catalase showed clear inhibition by KCN and NaN_3. As seen from Fig. 4, a KCN concentration of 5×10^{-6} *M* produces a 50 % inhibition of the enzyme, and 10^{-4} *M* produces essentially complete inhibition. NaN_3 is somewhat less effective, half inhibition being produced by 2×10^{-5} *M* and complete inhibition by 2×10^{-4} *M*. Na diethyldithiocarbamate, a typical copper-enzyme inhibitor, is without effect on the enzyme.

2. *Identification of the prosthetic group.* The prosthetic group of heme containing enzymes can in most cases be removed by dilute HCl in acetone. After such a splitting, recombination experiments and identification of the prosthetic group can be attempted. 1 ml of the spinach enzyme was treated with 3 ml of cold 0.1 % HCl in acetone. The white protein was centrifuged down and redissolved in 1 % $NaHCO_3$. It proved to be devoid of catalase

activity, and could not be reconstituted by the addition of the prosthetic group or of hematin.

The acetone was then removed from the supernatant liquid by blowing a stream of air over it. This resulted in the deposition of a red-brown precipitate, leaving behind a colorless aqueous layer. The precipitate was dissolved in pyridine. To 1.4 ml of the pyridine solution were added 0.3 ml of 1N NaOH and 2.3 ml of H_2O. The mixture was then placed in a Beckman cuvette, and after the addition of crystals of $Na_2S_2O_4$, a sharp absorption peak at 557 mμ appeared. This indicated that the prosthetic group of the plant catalase is protohemin. The fact that the aqueous layer was colorless indicates the absence of bile pigments from the catalase preparation.

3. *Iron assay.* The iron content of the enzyme was determined by the sulfosalicylic acid colorimetric method. To 1 ml of enzyme solution were added 1 ml of concentrated H_2SO_4 and 3 drops of concentrated H_2O_2. The material was digested over a flame, being cooled occasionally to permit the addition of more H_2O_2. After digestion was complete, the solution was cooled and 5 ml of distilled water added. The preparation was then concentrated to 1/3 volume by boiling, decanted into a 10 ml volumetric flask and 0.2 ml of 20 % sulfosalicylic acid added. Concentrated NH_4OH was then added dropwise until the solution became yellow. It was then made up to volume and the absorption at 424 mμ measured in a Beckman spectrophotometer. The sample assayed 0.049 % iron, which is roughly half the value obtained for a pure 4-hematin catalase.

4. *Stability of the enzyme.* The enzyme is completely destroyed by being heated at 60° C for 10 minutes. It is stable in the cold between pH 5.3 and 8.9, being destroyed rapidly at lower pH values and slowly at higher pH values. The activity of the enzyme over a 30 second period is optimal between pH 5.3 and 8.0, falling off rapidly at more alkaline and acid pH values.

DISCUSSION

The experiments described in this paper show clearly that stable plant catalase can be conveniently prepared and considerably purified by conventional techniques. The unambiguous crystallization of this enzyme should now be only a matter of time. The *Kat.f.* of 23 600 obtained for highly-purified spinach catalase is roughly one-fourth that obtained for bacterial catalase and highly-active animal catalase. Preliminary kinetic data obtained by Dr. B. Chance on a very impure sample of our material also indicate a k' (Bonnichsen, Chance and Theorell [8]) roughly 20 % that of other catalases. These facts, coupled with the low iron content (0.049 %) and low ratio of absorpt-

ion at 405 mμ compared with 280 mμ suggests that plant catalase may have less than 4 hematin groups per molecule.

Considerable physiological significance may possibly be attributed to plant catalase in view of the fact that it is apparently localized in the chloroplasts (Neish [5]). Since it has been suggested that the oxygen-releasing system of photosynthesis is a heavy metal enzyme, and since catalase contains a heavy metal and releases oxygen, it may conceivably be involved in the photosynthetic release of oxygen. The possible role of catalase in the mechanism of photosynthesis was recently re-evaluated by Tamiya [12]. Since, also, the catalase content of various plant tissues is known to shift rapidly at the inception of growth and in response to illumination, catalase may somehow be importantly involved in the control of plant growth and form.

SUMMARY

The catalase of spinach leaves has been prepared in a stable, highly purified form. The method of preparation involves reducing the leaves to an acetone powder, extracting with 0.1 *M* secondary phosphate, several ammonium sulfate fractionations, and finally preparative electrophoresis. The maximum *Kat.f.* obtained was 23 600. The spectrum shows peaks at 280, 405, 510, 545 and 620 mμ. The ratio E_{280}/E_{405} is 1.54. The preparation has 0.049 % iron and has a protohemin type of prosthetic group. The enzyme is stable between pH 5.3 and 8.9 and is rapidly destroyed at temperatures above 60° C.

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Studies on a Proteolytic Enzyme from Seeds of Peas

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The proteolytic enzymes from different plants have, in most cases, rather low hydrolytic activities. Exceptions from this rule are ficin, papain, bromelin and a few other enzymes which, however, are not present in the seeds of the plants. Very few proteolytic enzymes from seeds have been isolated probably because of the low activity of the enzymes from this source. In the seeds the protein breakdown during germination is not very rapid, *e. g.* it takes about 15 days before the reserve proteins have been broken down in seeds of peas. In the alimentary canal of animals the food must be digested in a few hours. Perhaps this is the reason why strong proteolytic enzymes are easier to isolate from the animal kingdom.

A few proteolytic enzymes with low activity from seeds have been studied. Linderstrøm-Lang and co-workers [1,2] reported a proteinase from malt. Very closely related to this enzyme is a proteinase from seeds of wheat which have been studied by Mounfield [3] and Balls and Hale [4]. Hale succeded in purifying this enzyme by precipitation with $(NH_4)_2SO_4$ and acetone [5]. The enzyme preparations from malt and wheat contain, however, at least two proteolytic enzymes, one proteinase with its optimal activity at pH 4.1 and one dipeptidase with optimal activity at pH 8.5. Linderstrøm-Lang [2] succeded in separating the two enzymes by storage of the enzyme solution for a short time. The dipeptidase was then inactivated. Balls and Hale [4] obtained enzyme preparations from wheat with an activity of about 1/400 of that of crystallized papain.

The protein breakdown during germination has been intensely studied from different aspects. Seeds from plants belonging to the *Leguminosae* have been used in most cases. Many attempts have also been made to isolate proteolytic enzymes from these seeds. In 1874 von Gorup-Besanez prepared a proteolytic enzyme from shoots of vetch, *Vicia sativa* [6]. He extracted the material with glycerol, filtered and precipitated the proteins with absolute ethanol. After repeated precipitations with ethanol a preparation with proteolytic activity

was obtained. A solution of the enzyme obtained in this way could dissolve a gel of fibrin. Any determination of the activity by the more usual methods was never done.

In 1919 Fischer [7] investigated the proteolytic activity in dried plant material from different *Leguminosae* plants. The enzyme preparations at each experiment contained of 10 g dried plant material. As substrate he used 3 g legumin, *i. e.* much more "enzyme" than substrate. No blank determinations can be found in his papers. Thus his methods and results are not very convincing.

In 1924 Blagoveschenski [8] investigated the proteolytic activity of 5 % NaCl extracts of young shoots from *Leguminosae* and other different plants. Globulins from different seeds served as substrate because Blagoveschenski believed that the globulins from the mother plant are broken down faster by the proteolytic enzyme than other substrates. Blagoveschenski and Melamed in 1934 investigated the proteolytic enzymes in seeds of *Vicia sativa* and *Phaseolus aureus* [9]. The authors used crude glycerol extracts from the seeds. No attempts to purify the extracts were made. No absolute activities, based on the content of enzyme nitrogen in the digestion solutions can be found in their paper.

In 1939 Davis [10] reported the presence of a proteolytic enzyme in lima beans, *Phaseolus lunatus*. In some experiments he used glycerol extracts from the seeds, and in some cases he extracted the pods with water. The water extract was fractionated by $(NH_4)_2SO_4$ precipitation and the fraction between 40 % and 80 % saturation was used for the digestion experiments. Davis' experiments show that a proteolytic enzyme can be extracted from seeds of lima beans.

In 1946 Labarre and Pfeffer [11] investigated the proteolytic activity in extracts from seeds of *Vicia Faba*, but never tried to isolate the enzyme. The seeds which had been germinated between 0 and 14 days were extracted with a buffer solution of pH 7.5 and the extract filtered. The extract then contained the enzyme in addition to large amounts of other proteins. Free amino groups were determined according to van Slyke immediately after extraction and after two hours at 40° C, *i. e.* the autolysis was measured. They found the highest rate of autolysis after 8 days of germination.

The most successful attempts so far to isolate proteolytic enzymes from leguminosae seeds were made in 1946 by Mergentime and Wiegand [12]. They extracted green peas with water and then the pH was brought to pH 4.0, by which procedure part of the globulins (legumin) is precipitated and discarded. The supernatant solution was used as enzyme solution. They found the pH optimum of this proteolytic enzyme to be pH 5.5 when acting on casein. Only

a few values (here called enzyme-N) of the nitrogen content of the enzyme solutions are published. These values show, however, that the enzyme solutions were very concentrated and the ratio $\frac{\text{enzyme-N}}{\text{substrate-N}}$ during the digestions was about 1/4, which is very high. Their method of purification is unsatisfactory because all globulins and low-molecular nitrogen products are not removed by the method used. Another striking thing is that they did not succeed in isolating any protein fraction from peas which was free of proteolytic activity and which could be digested by fresh pea enzyme.

It can be seen that a great number of attempts to isolate proteolytic enzymes from seeds or shoots of *Leguminosae* plants have been made. However, no satisfactory determinations of absolute activities have been carried out. The experiments which are described in this paper have been performed in order to obtain more information about these enzymes, *i. e.* their absolute activities and their possible purification.

EXPERIMENTAL

I. Glycerol extracts of germinated seeds

A. Extraction of the enzyme

Seeds of yellow peas, *Pisum sativum*, (Field variety, "Torsdagsärt II") were used. Previous investigations at this Institute [13] have shown that the protein breakdown is most intense 6—10 days after the beginning of the germination. This is in agreement with the results of Labarre and Pfeffer [10]. Thus the seeds were allowed to germinate for 7—8 days before the enzyme was extracted.

The seeds were placed in water overnight in order to swell and then allowed to germinate between wet filter papers in Petri dishes. After 8 days they were washed several times with water and then ground for 6 minutes in a Waring blendor with 50 % glycerol. The weight of the extraction solution was twice that of the wet peas. The extraction was carried out at + 4° C for 20 hours. The extract was filtered through a piece of linen cloth and then centrifuged for 30 minutes at 3 500 r.p.m. (radius 5 cm). The first measurements were performed with enzyme solutions prepared in this way.

B. Preliminary determinations of the proteolytic activity

Commercial edestin (La Roche) was used as substrate because it can be obtained in large amounts and it is a rather homogeneous protein. The edestin was dissolved to 2.5 % in a buffer containing 0.038 *M* HAc and 0.011 *M* NaAc.

The pH of the substrate solution was 4.6. This pH value was chosen because edestin has a high solubility at this pH but is precipitated when the pH is increased. Later experiments have shown that the pH optimum of the proteolytic enzyme isolated from seeds of peas is above pH 6, where edestin is insoluble. It is, however, very difficult to pipette solutions containing precipitated edestin and therefore a pH value was chosen where clear solutions of the substrate can be obtained, and the activity determinations can be performed with satisfactory accuracy.

The breakdown of the substrate was followed by formol titration according to Sørensen. The method used was that indicated by Mounfield [3]. The titration was carried out with $N/20$ NaOH. Previous potentiometric titrations on the substrate solution showed no buffer capacity in the pH-range 8—9.

In all experiments described here 15.0 ml substrate solution were mixed with 2.0 ml enzyme solution. 5.0 ml of this mixture were pipetted into three 25 ml Erlenmeyer flasks and 2 drops of toluene were added to each flask. Two flasks were placed in a thermostate at + 40° C and the third one was titrated immediately. In later experiments the digestion was carried out in one flask only, and 5.0 ml were withdrawn after different times of digestion. The results from the activity determinations of an enzyme solution prepared in the way described above is shown in Table 1.

Table 1. Digestion experiment using 2.5 % edestin at pH 4.6 as substrate solution. Temperature + 40° C. Enzyme solution: 50 % glycerol extract from seeds of peas, germinated for 8 days. The enzyme solution contained 2.52 mg N/ml. 2 ml enzyme solution and 15 ml substrate solution were digested. Formol titrations were carried out on 5 ml digestion solution.

Flask	Digestion solution	Time h	Titer ml $N/20$ NaOH	Time h	Titer ml $N/20$ NaOH	Increase in formol titration ml $N/20$ NaOH	Time h	Titer ml $N/20$ NaOH	Increase in formol titration ml $N/20$ NaOH
A	Edestin + 50 % glycerol	0	3.65	6.2	3.64	— 0.01	23.2	3.62	— 0.03
B	Buffer pH 4.6 + enzyme	0	3.46	6.2	3.51	0.05	23.2	3.54	0.08
C	Edestin + enzyme	0	4.05	6.2	4.25	0.20	23.2	4.64	0.59

According to Table 1 the breakdown in flask C can easily be measured after about 6 hours at + 40° C. It can also be seen that the rate of breakdown decreases with time. In flask B, containing enzyme and buffer only, a small increase in the titer was observed, probably due to the fact that the enzyme solution contained large amounts of protein other than enzyme which are broken down. In flask A the titer decreased with time because fresh edestin solution was used. In later experiments the substrate solution was placed at 40° C over night before it was used for digestion experiments. After that no change could be observed in the titer of the substrate. In the activity determinations the changes in the titers of the flasks A and B must be taken into consideration. Thus, the real change in flask C after 6.2 hours digestion was 0.16 ml $N/20$ NaOH. These corrections have been made in all experiments described in this paper. They are, however, very small when purified enzyme solutions are used.

C. *The unit of proteolytic activity*

It is very difficult to choose a suitable activity unit for an enzyme of this kind which has a very low activity and also is very impure. Preliminary experiments with crude extracts and also with purified enzyme solutions have shown that the rate of breakdown is proportional to enzyme concentration. During the first stage of the digestion experiment the curve of breakdown is practically linear. The results are shown in Figure 1 and Figure 2. If these facts are taken into consideration, an activity unit defined by Northrop (14) but somewhat modified can be used. A correction has been made for the dilution of the enzyme. The formula is:

$$\left[A\right]^{40^\circ}_{\substack{\text{pH 4.6}\\ \text{Edestin}\\ 2.5\ \%}} = \frac{\dfrac{\text{milliequivalents NaOH}}{\text{ml. digestion solution}}}{\text{hours} \times \dfrac{\text{mg. enzyme-N}}{\text{ml. digestion solution}}}$$

From Table 1 the following value of the activity of the enzyme used is obtained:

$$A = \frac{\dfrac{0.16}{5} \times \dfrac{1}{20}}{6.2\ \dfrac{2 \times 2.52}{17}} = 0.87 \times 10^{-3}$$

The activity unit used here does not take into consideration the fact that the breakdown decreases with time. Thus the measurements must be made as soon as possible after the start of the experiment. But Table 1 shows that the

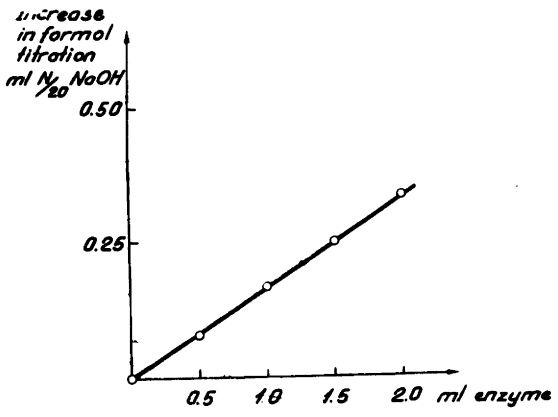

Fig. 1. The dependence of edestin breakdown on enzyme concentration. 15 ml 2.5 % edestin, pH 4.6, x ml enzyme solution and (2−x) ml H_2O were digested at + 40° C for 4 hours. The enzyme solution contained 0.760 mg N/ml. Formol titrations were carried out on 5.0 ml.

breakdown cannot be easily measured before about four hours if crude glycerol extracts are used. It is thus difficult to obtain the initial rate due to the low activity of the enzyme. Preliminary experiments have shown, however, that the error is not very large if the measurements are made within 6 hours from the start.

D. *Purification experiments on the glycerol extracts*

The glycerol extracts contain large amounts of proteins with no enzyme activity. Some methods to purify the enzyme were tried. The activities of the different fractions are found in Table 2.

1. *Dialysis.* The glycerol extracts were dialysed against water through a cellophan bag. A dense precipitate was obtained which after dissolving in 50 % glycerol had no measurable activity. The supernatant solution was still active.

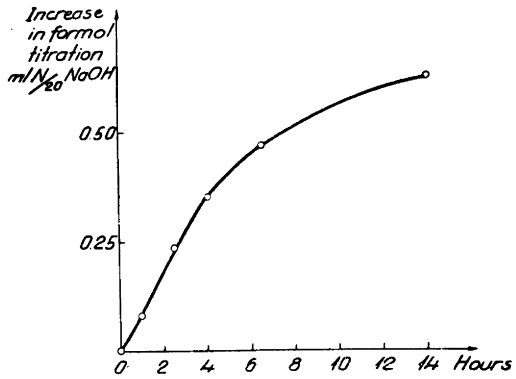

Fig. 2. The breakdown of edestin by pea proteinase as a function of time. 33 ml 2.5 % edestin, pH 4.6, and 4 ml enzyme solution, containing 0.760 mg N/ml were digested at + 40° C. After different times of digestion 5.0 ml were withdrawn with a pipette and titrated with N/20 NaOH and 4 ml 35 % formaldehyde.

2. *Ultrafiltration.* The dialysed solution was ultrafiltrated through a collodion membrane in order to concentrate the enzyme[15]. The solution which passed through the membrane had no measurable activity but the remaining fraction was still active. In most cases the solutions were concentrated so that the volume left after filtration was about 1/5 of the initial volume.

3. *Precipitation with $(NH_4)_2SO_4$.* Dialysed glycerol extracts were precipitated at pH 5 at + 4°C with solid $(NH_4)_2SO_4$ at different concentrations. The precipitates were centrifuged down and dissolved in water. The solutions obtained were dialysed against water in order to remove all ammonium sulphate, since traces of this salt precipitate edestin. Upon dialysis to the salt-free state dense precipitates were obtained. These precipitates were dissolved in 50 % glycerol and the proteolytic activity of the solutions measured. It was, however, very low. The supernatant solution from the dialysis showed high activity.

The results from the purification experiments described above are found in Table 2. All the enzyme solutions were investigated in the electron microscope in order to see if any bacteria were present. This was never the case.

Table 2. Determination of the proteolytic activity of enzyme preparations from seeds of peas at pH 4.6. Temperature + 40° C. Substrate 2.5 % edestin. The experiments were performed in the way described in legend of Table 1.

Enzyme preparation	Nitrogen content of the enzyme solutions mg *N*/ml	Time h	Increase in the titer ml. *N*/20 NaOH	Activity × 10^3
A. Glycerol extract no. 1	2.52	6.2	0.16	0.87
B. » » » 2	2.24	4.8	0.19	1.5
C. » » » 3	6.12	4.3	0.19	0.61
D. Solution B, dialysed	0.408	7.0	0.15	4.5
E. Solution D, ultrafiltrated	3.13	7.0	0.59	2.3
F. Solution C, dialysed Precipitated with $(NH_4)_2SO_4$ · 0—80 % fraction	1.17	6.3	0.67	7.7
G. Glycerol extract no. 6, treated as solution F. 0—30 % fraction	0.141	6.2	0.09	8.7
H. As solution G. 30—60 % fraction	1.22	6.2	0.76	8.5
I. As solution G. 60—80 % fraction	0.760	6.2	0.54	9.7

The values of the activities from Table 2 show that the crude glycerol extracts have a proteolytic activity of the magnitude 1×10^{-3}. The dialysed glycerol extract no. 2 has an activity about 3 times higher than the undialysed extract. Thus a great deal of protein with no proteolytic activity is removed by dialysis. Ultrafiltration of the dialysed extract gives a solution with lower activity, *i. e.* inactivation occurs by ultrafiltration. It is, however, possible to concentrate the enzyme with this method. Precipitation with $(NH_4)_2SO_4$ of the dialysed extracts gives an enzyme preparation with high activity. In the experiments described here about the same activity was obtained independently of the amount of $(NH_4)_2SO_4$ used. Probably the enzyme is precipitated together with those proteins which have no proteolytic activity. In the last experiment in Table 2, the enzyme was distributed in the following way; solution G contained 5 %, solution H 65 % and solution I 30 % of the enzyme. In many cases the enzyme solutions were dried in vacuum at low temperature over $CaSO_4$. The white powder obtained in this way had the same proteolytic acitivity as before the drying procedure.

II. Water extracts of germinated seeds

The experiments above show clearly that a proteolytic enzyme from germinated seeds of peas can be extracted with 50 % glycerol, and the enzyme can be concentrated about ten times. It is, however, difficult to purify the enzyme more because glycerol extracts hold too many nitrogen products other than enzyme which contaminate the enzyme even after fractionation with $(NH_4)_2SO_4$. Some experiments with water extracts of the germinated seeds were performed which showed that the enzyme could be extracted with water, and about the same activities as by glycerol extraction were obtained. After precipitation with $(NH_4)_2SO_4$ of the dialysed extract between 0—80 % saturation and subsequent dialysis of the dissolved precipitate, the activity obtained was about 15—17 $\times 10^{-3}$, *i. e.* somewhat higher than before. Further purification by precipitation with $(NH_4)_2SO_4$ to different concentrations was tried but with no success.

III. Water extracts of unripe seeds

In order to get more active enzyme solutions, some experiments with unripe seeds were carried out. Earlier unpublished investigations by the present author have shown that during the first stage of ripening about 60 % of the total nitrogen is of lowmolecular nature in the seeds. The ripe seeds contain only 17 % lowmolecular nitrogen, and during germination this value

never is higher than 45 %. Thus, if the unripe seeds contain the same proteolytic enzyme as that which can be extracted from the germinated seeds, then the unripe seeds must be the best material for the isolation of the enzyme, because most of the nitrogen can be removed by dialysis from the extracts. As a working hypothesis it was considered that the proteolytic enzymes from seeds can be used by the plant both for hydrolytic and synthetic purposes. In the unripe seeds, where 60 % of the total nitrogen is of lowmolecular nature, the protein synthesis is very intense, and thus the proteolytic enzymes work as synthetic enzymes. If these enzymes are extracted and used in digestion experiments of the kind described above, they should have hydrolytic activity, because water is in excess.

Seeds of peas of the same variety as used in earlier experiments were harvested three weeks before the usual time of harvesting when the seeds are ripe. The seeds were separated from the pods and allowed to dry. After two months they were placed in water overnight and then macerated in a Waring blendor for 6 minutes, frozen down to — 16° C and dried in vacuum over $CaSO_4$. The dry powder was then extracted with water. 1.5 g dried seed material and 25 ml H_2O were used at each experiment. As can be seen from Table 3, enzyme preparations from unripe seeds are more active than those from germinated seeds. This fact, however, probably is due to the low concentration of high molecular nitrogen products other than enzyme in the water extracts of the unripe seeds *i. e.* probably very little of the enzyme is adsorbed on nitrogen products from the seeds. When using the enzymes with the highest activity the ratio $\frac{\text{enzyme-N}}{\text{substrate-N}}$ was about $\frac{1}{300}$.

It is clear from the results above that for further purification of the proteolytic enzyme from pea seeds dialysed water extracts from unripe peas should be used. Such experiments were not carried out in the present study because of insufficient amounts of unripe seeds.

IV. Determination of the pH optimum

For further characterization of the enzyme the pH optimum for a few substrates was determined. The substrates used were gelatin, legumin, and vicilin, two of which, gelatin and vicilin, are soluble in the whole pH range studied. It is important not to use buffer systems with buffer capacity in the pH range 8—9 if the breakdown is measured by formol titration. This difficulty was avoided by using unbuffered solutions. The pH of the substrate solutions was adjusted with *N* HCl and *N* NaOH. The solution was then heated for a few hours to 40° C, and the pH measured with a glass electrode

Table 3. Proteolytic activities of enzyme preparations from unripe seeds of peas. The digestion conditions are the same as in Table 2.

Enzyme preparation	Nitrogen content of the enzyme solution mg N/ml	Time h	Increase in formol titer ml $N/20$ NaOH	Activity $\times 10^3$
No. 1				
a. 0.2 M NaCl extract	1.54	3	0.70	13
b. 1 a dialysed, supernatant	0.077	3.3	0.22	74
c. 1 b, precipitated with $(NH_4)_2SO_4$ 0—80 % fraction	0.099	3	0.33	94
No. 2				
a. 0.2 M NaCl extract	1.23	19.7	1.60	5.6
b. 2 b dialysed, supernatant	0.082	19.7	0.60	31
c. 2 b, precipitated with $(NH_4)_2SO_4$, 0—80 % fraction	0.064	19.7	0.63	42
No. 3				
a. Water extract	1.11	4.1	0.26	4.8
b. 3 a, dialysed supernatant	0.092	5.5	0.22	37
c. 3 a, dialysed precipitate dissolved in water	0.164	4.1	0.01	1.3
No. 4				
a. Water extract	2.07	3.6	0.38	4.3
b. 4 a, dialysed	0.100	3.6	0.30	71
No. 5 Water extract, dialysed	0.130	3.3	0.32	63
No. 6 Water extract, dialysed	0.159	3.3	0.32	52
No. 7 Water extract, dialysed	0.142	5.6	0.37	40

before and after the digestion experiment. The difference never exceeded 0.1 pH unit. Blank determinations were performed only at pH 3, pH 6.5 and pH 8. No change in the titer of the blanks were observed. The substrate solutions used are described below.

1. *Vicilin.* A dried preparation of vicilin from peas was used. It was homogeneous by ultracentrifugation and electrophoresis. The concentration used was 2.5 % in 0.2 *M* NaCl 5 ml of the vicilin solution were pipetted into 25 ml. Erlenmeyer flasks and the pH adjusted to the desired value as described above. 0.4 ml of dialysed enzyme solution from unripe seeds was pipetted into each flask. 0.4 ml H_2O was pipetted into each blank. The enzyme solution contained 0.077 mg N/ml. Formol titrations were carried out on 2.0 ml of the digestion solution after 0 and 18.7 hours digestion at + 40° C. In Fig. 3 the increases in formol titration are recalculated to 5 ml of digestion solution.

2. *Legumin.* 8 g commercial legumin (Merck) probably inhomogeneous, were suspended in 300 ml 0.2 *M* NaCl. The legumin solution was then treated as in the preceding experiment, but 15 ml were pipetted into each flask. 2 ml of enzyme preparation containing 0.856 mg N/ml, from germinated seeds were pipetted to each flask. Titrations were carried out on 5 ml after 0 and 7 hours digestion at + 40° C.

3. *Gelatin.* A stock solution of granular gelatin (Eimer and Amend, N.Y. U.S.A.) in water was made up. The gelatin concentration was 4 %, and a little crystal of thymol was added in order to avoid infection. Two experiments were carried out in the same way as described for legumin. In the first experiment the enzyme used was from germinated seeds, containing 0.856 mg N/ml. The digestion time was 5 hours. In the second experiment an enzyme preparation from unripe seeds, containing 0.077 mg N/ml, was used. The digestion time was 17.5 hours.

The results of these experiments are shown in Fig. 3. The pH optimum 6.7 is the same for the enzyme from germinated and unripe seeds when digesting gelatin. When vicilin is used as substrate the optimum is more acid, or about pH 6.0—6.3, which has been checked by several experiments. When digesting legumin, the optimum is close to pH 7. It is, however, important to remember that the legumin used was not homogeneous.

DISCUSSION

The experiments described above show that it is possible to extract a protein fraction with proteolytic activity from seeds of peas. It is possible to purify the enzyme by dialysis, ultrafiltration and precipitation with $(NH_4)_2SO_4$,

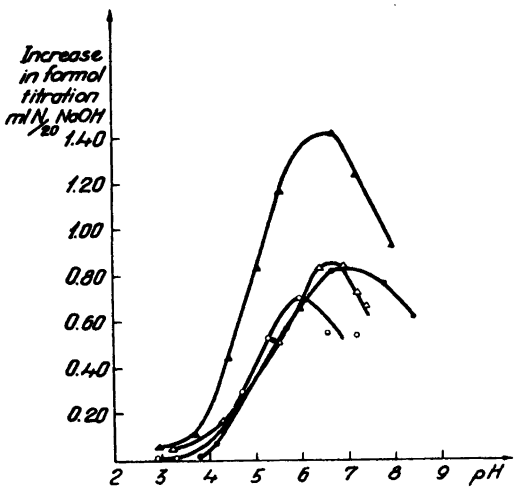

Fig. 3. Determination of pH optimum for pea proteinase.
▲ = *gelatin + enzyme from unripe seeds.*
△ = *gelatin + enzyme from germinated seeds.*
● = *legumin + enzyme from germinated seeds.*
○ = *vicilin + enzyme from unripe seeds.*

i. e. removing nitrogen products other than enzyme. By all the purification experiments, however, part of the activity is lost, probably depending on inactivation. By dialysis of an extract against water, about 30 % of the total activity is lost, and it seems as if still more is lost by precipitation with $(NH_4)_2SO_4$. As has been said above, part of the enzyme probably is adsorbed to other proteins in the solutions. The possibility that an activating substance is removed during the purification experiments has not been investigated. From Table 3 it can be seen that the precipitate after water dialysis is still active. If the precipitate is dissolved in 0.2M NaCl and dialysed once more, the globulin fraction is then inactive. Mergentime and Weigand [12] did not succeed in preparing a pea protein which was free of proteolytic enzymes and which could be digested by fresh pea enzyme. The present author has shown that the two pea globulins vicilin and legumin are digested by pea proteinase. Mergentime and Wiegand [12] found the pH optimum for pea proteinase to be pH 5.5 for casein splitting, *i. e.* one pH unit lower than the value determined in this paper for gelatin splitting. This difference is probably due to the different substrates used.

The pea proteinase has a low proteolytic activity. It is very difficult to compare the activity of this weak enzyme with activities of stronger enzymes. However, a comparison with the activity of crystalline pepsin will be made. From Northrop's determinations of the activity of crystalline pepsin [14] the activity value $A = 11$ can be calculated when edestin was used as substrate and the same activity unit as in the present paper is used. Thus pepsin is about 100—200 times more active than the best pea proteinase preparations

from unripe seeds. It is, however, important to remember that the determinations of the activity of pea proteinase were performed at pH 4.6, *i.e.* not at the pH-optimum of the enzyme. From the values in Fig. 3 and Table 3 it can be seen that the activity of pea proteinase when splitting gelatin at pH 6.7 is about the same as when splitting edestin at pH 4.6 ($A = 89 \times 10^{-3}$ compared with $A = 74 \times 10^{-3}$ for the same enzyme preparation). Northrop's values give $A = 100 \times 10^{-3}$ for pepsin when splitting gelatin at its pH optimum. Thus pea proteinase and pepsin have about the same activity when digesting gelatin, but pepsin is much stronger when edestin is used as substrate. It is clear that we are here dealing with two different types of proteolytic enzymes. A comparison with the activity of wheat proteinase can also be done. Unpublished investigations by the present author have shown that a water extract of ground seeds from wheat, germinated for 6 days, had a proteolytic activity $A = 4.6 \times 10^{-3}$ when splitting edestin at pH 4.1, the pH optimum of this enzyme. The enzyme was purified by dialysis against water and precipitated with $(NH_4)_2SO_4$. The fraction between 40—80 % saturation had the activity $A = 47 \times 10^{-3}$, *i. e.* the activity was of the same order of magnitude as the activity of pea proteinase from unripe seeds.

The nature of the pea proteinase is not yet clear. No determinations of the dipeptidase activity have been carried out. The possibility that the proteinase is accompanied by a dipeptidase in the same way as in seeds of wheat and malted barley is under investigation.

SUMMARY

1. A protein fraction with proteolytic activity has been isolated from seeds of peas. The highest activity is obtained when unripe seeds are used.

2. The pH optimum of the pea proteinase was determined to be pH 6.7 when splitting gelatin.

3. Crystalline pepsin is 100—200 more active than the best pea proteinase obtained when edestin is used as substrate. The activity of pea proteinase is of the same order of magnitude as the activity of wheat proteinase.

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X-Ray Camera for Continous Recording of Diffraction Pattern-Temperature Diagrams

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In the course of work on the structure of phthiocerane [1] it became necessary to study crystal structure changes with temperature in a series of methylsubstituted long chain hydrocarbons. This was done by taking X-ray photographs at a series of different temperatures, but this procedure proved very tedious and time-consuming, and the need was felt for an X-ray camera capable of automatically recording diffraction pattern-temperature diagrams. A simple form of such an apparatus can be obtained by allowing the diffraction pattern from the specimen to fall through a horizontal slit on to a cylindrical photographic film with the axis parallel to the slit. If the film cylinder moves in such a way that a certain angular position always corresponds to a certain temperature of the specimen, heating or cooling the latter at a constant rate will give a continous recording of the diffraction pattern as a function of the temperature. Thermal expansion of the lattice is thus easily followed, and the occurrence of a transition to a new crystal form will be evident as a displacement of the lines in the diagram. The diffraction pattern disappears on melting, and the melting point is therefore also recorded. The synchronism between temperature changes and the rotation of the film cylinder can be accomplished in different ways. In the first instrument constructed the EMF of a thermocouple in the goniometer thermostat was counteracted by the potential of a slide-wire potentiometer whose movable contact was turned with the film cylinder. Any difference in potential caused a deflexion of the mirror of a sensitive galvanometer placed in series with the thermo-couple and the slide-wire, light thereby falling on one of the cathodes of a double photocell, which in turn via an electronic amplifier and relays caused a small motor geared to the film cylinder to turn the latter with the attached slide-wire arm until the potential of the slide-wire and the thermo-couple balanced each other.

The photocell-relay system was sensitive to changes in temperature of 0.15 °, and the device worked satisfactorily for a long period, but the camera was not fully automatic as the temperature calibration had to be performed by hand. This was done by removing the stop for the primary beam for about one second, thus causing a mark on the film.

It has recently been possible to use the recording camera in conjunction with a Speedomax G type temperature recorder.* With the aid of the latter instrument the temperature calibration has been made fully automatic and it has also been possible to obtain the temperature synchronization in a more simple manner. In the Speedomax recorder the angular position of the shaft carrying the arm of the main potentiometer depends on the temperature at the measured point, and the desired movement of the film cylinder can therefore be obtained by linking the movement of the film drum in the camera to that of the potentiometer shaft of the recorder, provided that the balancing motor of the latter is able to cope with the extra load. As this appeared to be the case, the camera was redesigned and the movement of the film is now obtained from the potentiometer of the recorder via a synchronous link system employing two "Magslip" motors **. Direct mechanical coupling would have been very awkward in the present case.

DESCRIPTION OF THE APPARATUS

Goniometer thermostat

Fig. 1 shows two sections through the thermostat, which is machined from a piece of copper rod 5 cm in diameter and 5 cm long. The upper part of the thermostat has a hollow mantle through which oil from a separate bath is circulated. When specimens deposited on glass plates are investigated the plate is clamped in a slot in the threaded copper rod *r* which is then screwed into the threaded central hole of the thermostat. The slot is made in such a way that the side of the glass plate carrying the specimen comes along a diameter of the rod. The rod *r* is provided with a knurled bakelite head *b* and is locked in position by the knurled nut *c*. By means of the handle *d*, which engages into a longitudal slot on rod *r*, the latter can be prevented from turning when nut *c* is tightened. Rod *r* is provided with a central hole *a* into which a small Anschütz type thermometer can be inserted. Specimens

* Made by Leeds and Northrup Co., Philadelphia, U.S.A.; obtainable from AB Max Sievert, Ulvsunda.

** Made by Muirhead and Co., Ltd., Beckenham, Kent, England, obtainable from Ingeniörsfirma Hugo Tillquist, Stockholm.

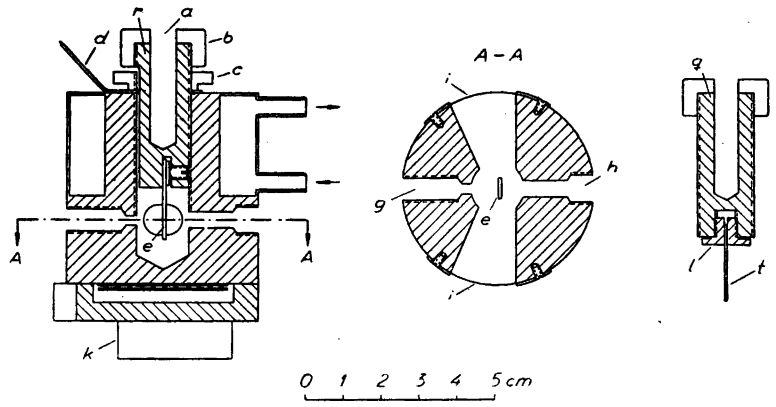

Fig. 1. Goniometer thermostat. For explanation of letters see text.

contained in Keesom-capillaries or extruded cylindrical specimens are mounted on the rod shown in Fig. 1 *q*. The small tube or rod holding the specimen is inserted into a small inset *l* as shown and attached by means of a trace of shellack.

The X-rays pass through two sector-shaped slots *s* whose outer openings are covered by thin cellophan films *i*. A threaded hole *g* is provided for a miniature lamp which throws light on the specimen while the initial adjustments are being made. On the opposite side is another threaded hole *h* which takes an iron-constantan thermo-couple. The thermo-couple, which measures the temperature at a point close to the specimen, is connected to the Speedomax recorder.

The thermostat is mounted on a goniometer head of fairly sturdy construction (cf Fig. 2) which makes it possible to set the specimen in the correct position with respect to the primary X-ray beam.

Arrangements for heating (or cooling) the specimen at a controllable rate

Over the temperature range 10—100° the heating or cooling of the specimen at a controlled rate is accomplished by circulating oil (transformer oil), through the goniometer thermostat from a separate bath. The oil is pumped by a small gear-wheel pump immersed in the oil to the goniometer thermostat via flexible oil-resistant tubing. Fairly flexible tubing is necessary in order not to interfere with the rocking motion of thermostat. The oil bath is provided

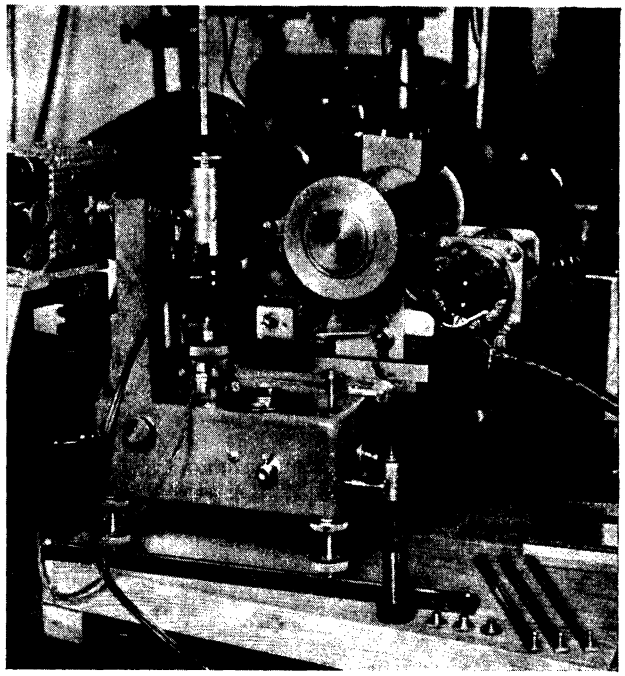

Fig. 2. Goniometer with thermostat and camera for continous recording of diffraction pattern-temperature diagrams. In the lower front part of the photograph are shown copper rod with insets for cylindrical specimens and three slits of different width.

with an electric heater controlled by a contact thermometer driven by a small synchronous motor. The latter is fed from an RC-generator whose frequency can be varied from 8 to 64 cycles/second, thus allowing the motor speed to be varied in the ratio 1 : 8. The motor (Bodine type KYC *) requires an input power of 8 watts at 115 volts (at 50 cycles). The oil is vigorously stirred by a separate motor, and when the thermometer motor runs with 50 cycles current the temperature of the bath is changed by one degree every five minutes. The motor driving the contact thermometer is of the reversible type, and when it is desired to record the diffraction pattern on cooling the specimen from an elevated temperature the motor is reversed, and the heating system used to balance the cooling provided from a coil of copper tubing immersed in the bath. The circulation rate of the coolant (usually tap water) is manually adjusted to a suitable value.

* Made by Bodine Electric Company, Chicago, U.S.A., obtainable through Ingeniörsfirman Sandblom and Stohne, Stockholm.

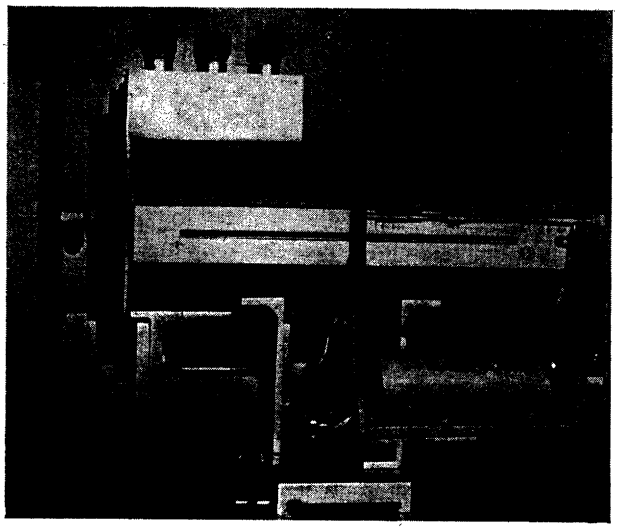

Fig. 3.

For temperatures above 100° (up to 150°) direct electric heating of the goniometer thermostat is used. The small heater *f* (Fig. 1) interposed between the thermostat and the insulating (bakelite) support *k* is fed from a variac type transformer driven by a synchronous motor. The device has proved quite satisfactory although the heating curves deviate more or less from straight lines. It would appear that the oil bath could be used also at temperatures above 100° by substituting a silicone oil for the transformer oil. Silicone oil also has the advantage of a smaller change in viscosity with temperature, but the high cost has so far prevented its use.

Camera and synchronous link system

A removable film drum 5.9 cm in diameter and 13.5 cm long (for 13 × 18 cm films) rotates behind a horizontal slit in the housing shown in Figs. 2 and 3. A series of exchangeable slits 0.5, 1, 1.5 and 2 mm wide respectively is provided (some of these are shown in the lower right hand part of Fig. 2). The drum shaft is supported by ball bearings at both ends (one bearing is mounted in the removable lid) and is driven by a 2'' Magslip motor through gears having the ratio 7.5 : 1. An identical motor serving as transmitter in the synchronous link is connected to the main potentiometer shaft of the Speedomax recorder through a system of gears having the ratio 1 :11.25. The method

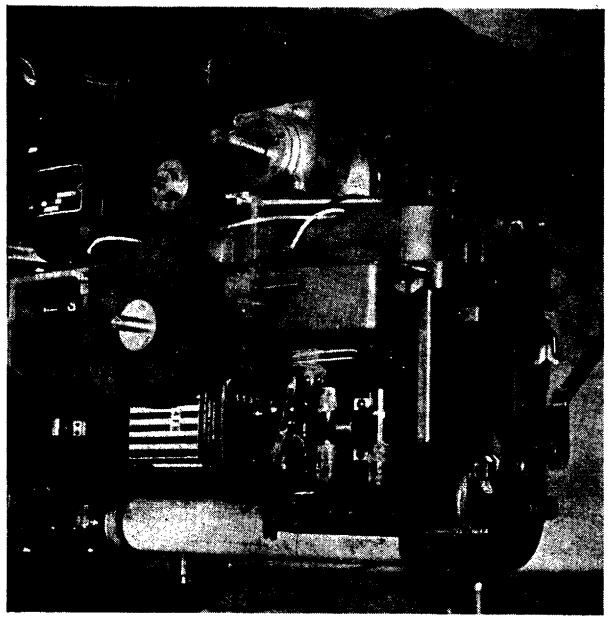

Fig. 4. Method of mounting Magslip transmitter motor in the Speedomax recorder. The contact disc for the temperature marking device is visible behind the large gear wheel on the main potentiometer shaft.

of mounting the transmitter motor is shown in the photograph of Fig. 4. In order to minimize backlash it might be preferable to use spirally cut gears instead of the straight ones shown.

With the gear ratios used a temperature interval of 1 ° corresponds to 1.6 mm on the film. The alignment error of the Magslip motors is less than one angular degree. With the gear ratio of 1 : 11.25 this will correspond to an error in the temperature measurement on the film of less than 0.05 °, and is hence negligible. Gear backlash, if present, will of course increase the alignment error.

Temperature marking device

A mark on the film at a certain temperature can be made by removing the stop for the primary beam for about one second. This can be done manually at any time by means of the small handle shown to the right in Fig. 3, and is done automatically at every 10 ° by means of the magnet shown in the lower part of Fig. 3 and the associated electrical circuit shown in Fig. 5. The magnet consists of a telephone type relay whose moving part has been provided with

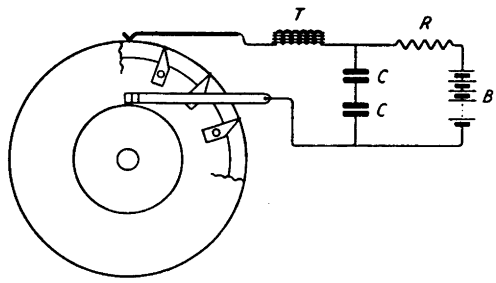

Fig. 5. Circuit used for the automatic temperature marking. T = telephone type relay, 2000 ohms resistance, normal working current 8 mA, B = 40 volts dry cell battery, R = 0.4 megohm, C = electrolytic condenser 500 mF, 12 volts D.C. working.

an extension resting on the stop for the primary beam. When the magnet is energized, the stop is moved towards the side. A small spring returns it to the original position as soon as the magnet is deenergized. In order to limit the marking time to about one second and to prevent chattering, the current for the magnet is obtained from the discharge of a condenser. The circuit is closed by a series of adjustable contacts on a disc mounted on the main potentiometer shaft of the recorder (cf Fig. 4). The contacts are empirically adjusted to the correct position. After one temperature marking has been made the marking device is inoperative until the condenser has been recharged to about 20 volts. In order to prevent a possible jerky motion of the recorder potentiometer arm from causing more than one marking at one temperature, the condenser is charged at a slow rate from a battery in series with a large resistance. With the arrangement of Fig. 5 it was found that the leakage current of the electrolytic condensers was about 40 μA. The two series-connected condensers therefore become charged to a maximum of 24 volts. The low current drain allows the battery to be permanently connected in the circuit.

As an example of the use of the apparatus*, Fig. 6 shows the automatically recorded diffraction pattern-temperature diagram for *n*-heptacosane ($C_{27}H_{56}$)**. This hydrocarbon is orthorhombic at room temperature, but as shown by Müller [2], long chain hydrocarbons undergo a reversible transition to a hexagonal crystal structure a few degrees below the melting point. According to Piper, Chibnall *et al.* [3] *n*-heptacosane melts at 59.0—59.1 °, and the

* Further examples will be given in forthcoming communications from this laboratory.

** The author is indebted to Professor A. C. Chibnall, Cambridge, England, for a specimen of this hydrocarbon.

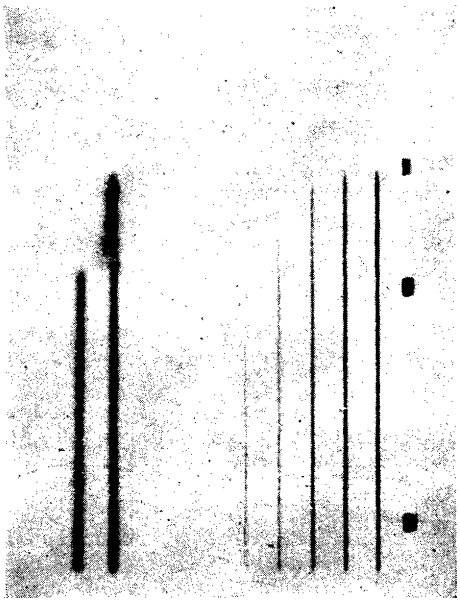

Fig. 6. Diffraction pattern-temperature diagram for n-*heptacosane* ($C_{27}H_{56}$).

transition occurs at 52.8—53 °. The corresponding temperatures read from the recorded diagram of Fig. 6 are 59.2 ° and 51.5 ° respectively. As usual for powder and oriented powder photographs of long chain compounds, the diffraction pattern consists of a series of 00*l* reflexions (corresponding to the long crystal spacing) and a series of *hk*0 reflexions, the latter making up the so-called side spacing pattern. In Fig. 6 the strong *11*0 and 200 reflexions of the orthorhombic form are very prominent. The 200 reflexion disappears at the transition and at the place of the previous *11*0 reflexion there appears two reflexions situated close together [2]. As the temperature is increased and the full hexagonal symmetry is attained these two reflexions unite to a single strong reflexion. A feature not previously noticed is that there is a slight shortening in the long spacing just around the transition point.

Apart from the recording of transition temperatures and melting points, the diagrams also give continous records of the thermal expansion of the lattice. On the whole, the continously recorded diagram gives a much clearer picture of the changes taking place within the crystal than a series of separate photographs taken at different temperatures, and no transitions are missed.

The temperature markings in Fig. 6 are of different strength (that for 40 ° is missing). This is caused by the rocking motion of the goniometer thermostat, which screens off the primary X-ray beam near one of the end positions of

the movement. The diagram in Fig. 6 was obtained with a rate of heating of 1 ° per 5 minutes. The slit width was 2 mm. This combination gives sufficient exposure in most cases when nickel-filtered copper radiation from a Philips-Müller tube is used, and the tube run at 30 kV and 20—30 mA. Smaller slits of course decrease the exposure and as a rule necessitate a lower rate of change of the temperature of the specimen*. As the melting and transition points can be read off with sufficient accuracy when using the larger (1.5 and 2 mm) slits, the latter are almost always used. The diffraction pattern-temperature diagrams for many long chain compounds with sharp and accurately known melting points have been recorded, and it has been found that the melting point can be determined from the diagrams with an accuracy of about 0.3 °.

An apparatus of the type described should prove useful also for phase analysis of organic as well as inorganic mixtures, including alloys. The temperature range of the camera can of course be considerably extended. As described, the apparatus is capable of recording reflexions over Bragg angles up to 15 ° only. For larger angles a different goniometer thermostat must be used. It would appear to be a comparatively easy matter to change a Weissenberg camera such as the Buerger type made by LKB-Produkter, Stockholm, into a temperature recording camera. A slit is already provided by the layer line slit supplied, and the film motion could be obtained by a Magslip motor linked to the potentiometer of the temperature recorder. The friction of the film carriage, which is considerable in the instrument mentioned, could be overcome either by a slight redesign or by interposing a capstan type torque amplifier [4] between the Magslip motor and the film carriage.

The recording speed of the camera is essentially that of the temperature recorder which in the case of the Speedomax G recorder employed is 4 seconds across the scale of 150 °. Provided that a sufficiently powerful source of X-rays is available, it might thus be possible to study crystal structure changes under processes involving very high heating or cooling rates.

The author is indebted to Mr. S. Liliedahl, former instrument maker of the Institute of Physiology, Uppsala, for the construction of the goniometer and the goniometer thermostat, to Mr. P. Olsson of this Institute for the construction of the recording camera and accessories, and to Diplomingenjör J. Björkman for the design and construction of the variable frequency generator. The purchase of the Speedomax recorder and the redesign of the apparatus was made possible by a grant from *Statens naturvetenskapliga forskningsråd.*

* In the first instrument the exposure could if necessary be increased by compressing the temperature scale by sending more current through the potentiometer slide-wire. In the present form of the apparatus the temperature scale can only be altered by changing the gear ratio between the film drum and the Magslip motor.

SUMMARY

An X-ray diffraction camera for the continous recording of diffraction pattern-temperature diagrams is described. The apparatus has been designed mainly for the study of polymorphism in long chain compounds, but should prove useful also for other purposes, *e.g.* for the study of thermal expansion of crystals and for phase analysis of mixtures.

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A Highly Active Horse Erythrocyte Catalase

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Purified erythrocyte catalase preparations from various laboratories generally give Kat.F. values of from 50 000—65 000 [1–3]. Agner [4], however, has succeeded in isolating a sample from human red cells by a method utilizing electrophoretic separation in the final purification step which gave a value of 100 000. The human erythrocyte catalase of Herbert and Pinsent [3] gave a Kat.F. of only 60 000. This discrepancy in activity suggests that catalase preparations may be composed of molecules of varying activities and that under certain conditions it should be possible to prepare material having higher activity than the usual Kat.F. 60 000. This possibility has been realized in working with horse erythrocyte catalase and a preparation showing a Kat.F. approaching 120 000 has been obtained. Some of the properties of such material are presented here.

EXPERIMENTAL

Crude horse erythrocyte catalase was prepared by the method of Bonnichsen [2] with some modification of procedure. Initially, sufficient of the ethanol-chloroform mixture was added (from 1 to 1.3 volumes) to washed red cells, previously diluted with 2 volumes of distilled water, to effect a rapid removal of the hemoglobin as in the procedure of Herbert and Pinsent [3]. The final ethanol fractionation step prior to crystallization was omitted since all of the catalase was found to precipitate at the step designed to remove impurities. A series of crystalline fractions were prepared by slowly increasing the $(NH_4)_2SO_4$ concentration. The initial fractions appeared to be contaminated with varying amounts of amorphous material. The more soluble fractions were composed only of crystalline material and were readily recrystallized. If dilute solutions were slowly recrystallized from $(NH_4)_2SO_4$ some of these fractions often formed relatively large crystals which dissolved only very slowly upon suspension in distilled water. A microphotograph of such a suspension is shown in Fig. 1. These crystals resemble those obtained upon storage of relatively concentrated aqueous catalase solutions in the cold [2,5].

* Rockefeller Foundation Fellow 1950–1951.

Two attempts to utilize the $Ca_3(PO_4)_2$ adsorption method of Herbert and Pinsent [3] for the preparation of horse erythrocyte catalase were unsuccessful. This method was, however, found to be applicable to human erythrocytes as described by these workers.

The absorption spectrum of the catalase preparations was determined in a Beckman spectrophotometer using $M/150$, pH 6.8 phosphate as solvent. Hemin was determined as alkaline-pyridine-hemochromogen and iron as ferric sulfosalicylate in ammoniacial solution.

Kat.F. determinations were carried out by both the method of von Euler and Josephson [6] and of Bonnichsen, Chance and Theorell [7]. These methods gave very comparable results, the values usually agreeing within 5 %. Because of its ease and rapidity of performance most activity measurements were carried out as recommended by the latter workers.

EXPERIMENTAL RESULTS

A single preparation of catalase was utilized in these studies. It represented the final crystalline fraction obtained from 14 liters of washed horse erythrocytes and amounted to 12 ml of an 0.89 % solution. This amount represents approximately 5 % of the usual yield.

Activity: When first tested this catalase preparation gave an average Kat.F. of 117 000. It gave a first order reaction constant at 18° C of 6.64×10^7 liter $\times$ mole $^{-1} \times$ sec^{-1} when assayed by the rapid method of Bonnichsen, Chance and Theorell [7]. This may be compared to these workers' value of 3.5×10^7 at 22° C for horse erythrocyte catalase of Kat.F. 61 000. Consequently our catalase showed a Kat.F. of 116 000 by this method if the temperature coefficient of this reaction, which is low [7], is neglected. The average value obtained by the method of von Euler and Josephson [6] was 118 000.

Enzyme lability: After standing for 18 days in the refrigerator the preparation gave a first order reaction constant of 4.88×10^7 mole $\times$ liter$^{-1} \times$ sec $^{-1}$. This decrease in activity of 27 % is an example of the well-known lability of catalase in dilute solution. Another example of this type of lability is seen upon dilution of the solution for assay. A typical result of the assay of the 0.89 % catalase at various times after ten-fold dilution is as follows:

Time after dilution (min.)	K_1 *
Initial	1.29×10^{-2}
5	0.87×10^{-2}
15	0.88×10^{-2}

* Given by 0.1 ml of a 1—10 dilution of the above 0.089 % solution under the usual conditions of the test [7].

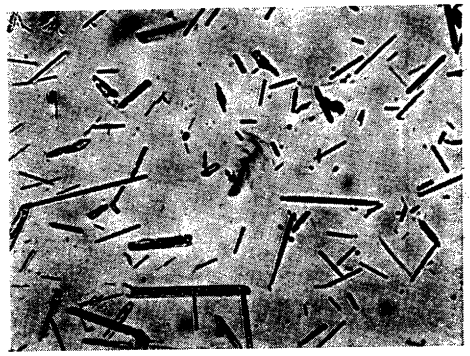

Fig. 1. Photomicrograph of catalase crystals from ammonium sulfate (magnification 130 ×).

Approximately 30 % of the activity was lost between the initial and 5 minute assay. The activity then remains quite constant. This suggests that only a portion of the catalase is very labile or that the denaturation reaction that takes place rapidly attains an equilibrium. As the activity of the 0.89 % solution decreased with age it appeared to be less sensitive to this dilution effect. Dilution with glass distilled water or bovine serum albumin solution did not abolish or retard this destruction.

Since the initial activity determination cannot be conducted in less than 30—60 seconds after dilution, it is likely that the first reaction rate constant is also low. Hence the original Kat.F. value of this preparation must be regarded as minimal. A truer picture of the activity of such potent but labile catalase preparations will require a rapid assay which circumvents the high dilution step.

Even in the rapid method of assay, the enzyme activity is seen to fall off slightly with time, a decrease in activity of 5—10 % in 45 seconds usually resulting. An example of this is shown by the data of Fig. 2. For this reason the results of the 15, 30 and 45 second titrations were extrapolated to zero time in activity determinations.

Absorption studies: This catalase gave a molar extinction coefficient (β) of 89.4 and 68.8 $\times$ 10^7 $cm^2 \cdot mole^{-1}$ at 405 and 277 mμ respectively. This gives a value of 1.3 for the ratio of the 405 to 277 mμ extinctions. The absorption coefficients are slightly higher than those reported by Bonnichsen [8], particularly at 405 mμ. The above ratio for this workers preparation was approximately 1.26. Increases in this ratio should be synonomous with increased catalase purity. We have obtained two other horse erythrocyte catalase preparations giving a ratio for the above extinctions of 1.31. These fractions were obtained in larger yield than the presently discussed material and gave Kat.F. values of only 84 000 and 94 000. Apparently very pure catalases as judged by absorption data can be obtained which show variable activity.

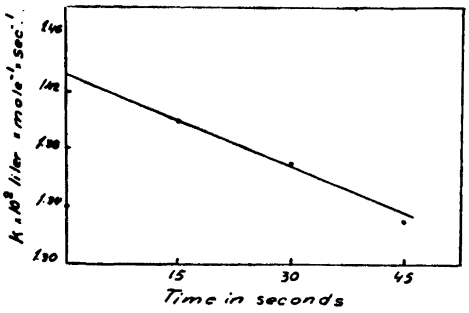

Fig. 2. The decrease in activity of catalase during determination by the rapid method (7).

Hemin and iron content: A hemin content of 1.04 % and an iron level of 0.097 % were found. The hemin value is slightly lower than the values reported by Bonnichsen [8], for horse erythrocyte catalase of Kat.F. 61 000—65 000. This is somewhat surprising in view of the strong absorption at 405 mμ. Other preparations showing ratios of 1.30—1.31 for the extinctions at 405 and 277 mμ have given essentially the same hemin values.

Other studies: A velocity sedimentation and diffusion experiment were performed by Dr. K. O. Pedersen of Uppsala University. The protein showed a single boundry in the ultracentrifuge and gave values for both the sedimentation and diffusion constants which were close to those reported for other catalases [9–11]. More detailed molecular kinetic investigations of horse erythrocyte catalase are being carried out at present.

DISCUSSION

The data presented here suggest that crystalline erythrocyte catalase preparations are composed of molecules of varying activities. A crystalline fraction of low yield was found to give a Kat.F. close to 120 000. Other and somewhat larger yield fractions also representing the more soluble portions of the usual catalase preparations have given activities as high as 97 000. The human erythrocyte catalase of Agner [4] and the bacterial catalase of Herbert and Pinsent [12] are other examples of Kat.F. 100 000 catalases. Brown [13] has also shown by solubility techniques that beef liver catalase contains material of Kat.F. as high as 180 000. Such results may explain why various investigators have obtained catalases showing different activities. It would appear that the catalase preparations studied to date cannot be considered as representative of pure, native catalase, at least from an activity standpoint.

Two reasons can be readily suggested for the variation in activity of different catalase fractions. It is possible that in nature there exist a series of

catalase molecules with widely divergent activities but with close physical and solubility properties. A second alternative is that the rigorous conditions usually employed in preparing catalase may lead to changes in some of the molecules which results in decreased activity. These changes are likely synonomous with solubility changes which allow for the separation of fractions of varying activity. It would appear that the answers to these possibilities would exist in the preparation of catalase by much milder fractionation methods, particularly one that circumvented the Tsuchihashi [14] conditions for the initial removal of hemoglobin by denaturation with an alcohol chloroform mixture.

The high activity catalase studied here was extremely sensitive to destruction on dilution. This destruction is of such a nature as to suggest that only some of the molecules are extremely labile. Present investigations on the denaturation kinetics of catalase have also revealed that this material as well as other crystalline horse erythrocyte catalase preparations are composed of molecules of varying reactivities. The above lability of this catalase preparation along with the known sensitivity to H_2O_2 and the possibility that present fractionation methods tend to give modified catalases suggests that erythrocyte catalase should not necessarily be considered a "rugged enzyme".

SUMMARY

A catalase preparation with a Kat.F. of at least 117 000 has been studied. It gives a first order reaction velocity constant at 18° C of 6.64×10^7 liter $\times$ mole^{-1} $\times$ sec^{-1}. This material has most of the physical properties of other less active erythrocyte catalase preparations. It shows an initial rapid rate of destruction upon dilution in buffers for assay purposes.

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Short Communications

The Base Catalysed Acidolysis of Diethyl Acylmalonates. A New Synthesis of β-Ketoesters of the Type $RCOCH_2CO_2C_2H_5$

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In 1944 Breslow, Baumgarten and Hauser [1] described a method for the preparation of β-ketoesters of the type $RCOCH_2CO_2C_2H_5$. Their starting products were ethyl *t*-butyl acylmalonates, which were decomposed by heating with toluenesulphonic acid. This method seems to be the most promising one for the preparation of β-ketoesters of this type [2]. It has however that weakness that ethyl *t*-butyl malonate is not commercially available. Some attempts to use diethyl acylmalonates in this preparation of β-ketoesters have been made [1,3], but none has been entirely satisfactory. This is due to the fact that the acid catalysed acidolysis of ethyl esters is a very slow reaction [4].

The present author has recently shown [5] that a base catalysed acidolysis of diethyl acylmalonates is readily effected in the preparation of β-diketones from diethyl malonate (1 mole) and an acid anhydride (2 moles). Preliminary experiments to stop this reaction at the β-ketoester stage by using one mole of diethyl malonate per mole of the anhydride resulted in mixtures of the β-diketone and the β-ketoester which could not be easily separated. This can, however, be avoided if the pure diethyl acylmalonate is at first prepared from the acid chloride and diethyl malonate according to Lund [6]. The resulting diethyl acylmalonate can then be acidolysed with the theoretical quantity of the organic acid with the same acyl group as the diethyl acylmalonate used. A mixture of magnesium oxide and copper acetate is a good catalyst. *In all cases studied the yields obtained by this method were practically quantitative.* The method is probably applicable to the preparation of every β-ketoester if the corresponding diethyl acylmalonate can be obtained.

If an organic acid and a diethyl acylmalonate containing different acyl groups are used a redistibution of the acyl groups is possible. This reaction will be studied further.

Experimental: Pure diethyl propionylmalonate (1 mole) was mixed with the corresponding acid (propionic acid 1 mole), 0.1 g of magnesium oxide, and 0.05 g of copper acetate in a round-bottomed flask fitted with a reflux condenser. The mixture was refluxed for 5–8 hours, after which time the vigorous evolution of carbon dioxide had subsided. The mixture was then fractionated under reduced pressure. Ethyl propionate goes over in an almost quantitative yield at first, followed by a little middle fraction consisting of mainly propionic acid, and finally the *ethyl propionylacetate* is collected at 65–80°/10 mm in a yield of 92 %, leaving a little unchanged diethyl propionylmalonate in the distillation flask. If for any reason much middle fraction and residue are obtained they can be mixed and heated again

together with some fresh catalyst. Redistillation of the crude fraction shows that the product is quite pure, b.p. 72°/10 mm.

In the same way the following β-ketoesters were prepared: *Ethyl* iso*butyrylacetate* b.p. 70°/10 mm in 88 % yield, and *ethyl* n-*butyrylacetate* b.p. 86°/10 mm in 97 % yield.

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New Books

A. V. Blom. *Organic Coatings in Theory and Practice.* Elsevier's Polymer Series no. 6. Elsevier Publishing Comp. Inc. New York, Amsterdam, London, Brussels, 1949. 298 pages, fl. 15, 90.

The book is very well written, clear and easily read. According to the preface the book is addressed to the man in the laboratory and to everybody who wishes to gain insight in coating problems. The former may certainly have a source of inspiration in the book while the latter will only obtain informations from it with some difficulties. The book contains interesting remarks on a variety of fundamental questions concerning the applicability of the polymer science to coating problems — collected by a skilled specialist but hardly sufficiently balanced for general use.

Part I gives a clear introduction to Staudinger's macromolecular concepts whereas more recent ideas are not much dealt with. Specially recommended is the treatise on plasticizers where it is attempted to bring about a more general distinction between the various types of plasticizers and extenders.

The treatment of the kinetics of film formation is rather broad and perhaps not quite satisfactory — one could wish a more thorough treatment of the chain reactions in the auto-oxidation of the oils and a more exact treatment of the functions of pro- and antioxygens would have been desirable.

In part II and III rather short descriptions are given of the most common raw materials of natural and synthetic origin. The properties of chief importance for the various types of laquers and varnishes are well illustrated, cf. the interesting treatment of compositions derived from bitumen and cellulosederivatives.

The description of the linear synthetic polymers appears to be limited to rather physical aspects.

Part IV and V concern the film formation and contain *i. a.* and attempt to classify solvents and thinners. The formulae attached to the rate of evaporation occurs rather isolated and chosen at random.

Part VI gives a picture of the properties of the pigments and the final part VII contains some remarks on the testing procedures, chiefly concerning the physical background of the methods.

The book contains valuable references to German and English literature, but is a cause of irritation that items are limited to a quotation in stead of a brief outline (*cf. e.g.* "spot test of Oliensis"?? p. 59).

The book gives a good picture of difficulties and possibilities of high polymer chemistry — still a young and unsettled science. *H. Leth Pedersen*

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Spectrophotometric, Magnetic and Titrimetric Studies on the Heme-linked Groups in Myoglobin

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Hemoglobin (Hb) and its derivatives have been subjected to a great deal of chemical and physical studies. Myoglobin (Mb), however, has not been so extensively investigated. This is partly due to the fact that Mb was not isolated and definitely characterized as different from Hb until 19 years ago. Its similarity to Hb in many respects, such as iron content, light absorption in the visible, and biological function, has probably induced a temptation to think that only minor differences could exist between Mb and Hb.

There are, however, some facts that make this assumption doubtful. Mb has a much greater affinity for oxygen and the pH-dependence of this is much smaller [1]. The molecular weight of Mb is only one fourth that of Hb [2]. One Mb-molecule thus contains just one iron atom.

The brown ferriHb (metHb, Hb^+) shows a change in absorption spectrum in alkaline solution giving a red colour. This change is reversible and follows a monovalent dissociation curve. On the basis of spectrophotometric measurements by Austin and Drabkin [3] and magnetic measurements by Coryell, Stitt and Pauling [4] the pK of this transition was found to be 8.12. The latter workers found that the magnetic moment changed from $\mu_{eff} = 5.80$ Bohr magnetons for the neutral ferriHb to $\mu_{eff} = 4.47$ for the alkaline form (HbOH). The first value corresponds closely to the theoretical one for 5 unpaired electrons, while the second can be ascribed to 3 unpaired electrons only if a rather big orbital contribution is assumed. It was also observed that the paramagnetic susceptibility decreased somewhat with increasing acidity. This was interpreted as the effect of a heme-linked histidine group with $pK = 5.3$ (Coryell and Pauling [5]).

The corresponding transition from neutral (Mb^+) to alkaline ferriMb (MbOH) has been known since 1934, when one of us [6] pointed out that the change

in colour of Mb^+ takes place at a higher pH than for Hb^+. Bowen has recently made measurements of the light absorption of ferriMb in the visible and at different pH [7]. However, he did not attempt to find the pK of this transition but simply stated taht "at pH-values above 7.... the absorptions are so affected by variations of pH that they are difficult to duplicate". Thus no comprehensive absorption data are available.

Taylor [8] in 1939 measured the magnetic susceptibility of ferriMb and ferroMb, assuming CO-ferroMb to be diamagnetic. He found $\chi_m = 14\,200$, resp. $12\,400 \times 10^{-6}$ cgs. "A very dilute solution of ferriMb" at high, but undefined pH, gave the value $\chi_m = 8\,000 \times 10^{-6}$. Taylor's results checked so closely with the corresponding susceptibilities in the case of Hb that he concluded "that magnetic interactions do not occur between the four hemes of hemoglobin". In view of the uncertainty of some of Taylor's values a more detailed investigation of the magnetic properties of myoglobin seemed to be of interest. He made no attempts to study the pK of the transition from Mb^+ to MbOH or to determine whether the dissociation constant found for Hb at low pH ($pK^1 = 5.3$) likewise obtains for Mb. Magnetic and spectrophotometric measurements on the fluoride compound of ferriHb has given valuable information on its hemelinked groups and similar studies for Mb^+ are indicated.

TECHNIQUE

The spectrophotometric determinations were made with a Beckman apparatus. An optical depth of 1 cm was employed for the dilute solutions and a 0.016 cm cell for the more concentrated solutions that were subjected to both magnetic and spectrophotometric study.

Most of the pH-values were determined with a Pt-H_2-electrode in a volume of about 1 ml with bubbling hydrogen gas and one drop of octanol to avoid foaming. The glass electrode was used in some cases for dilute Mb^+ solutions.

The magnetic measurements were performed with the micro-apparatus designed by the authors and described elsewhere [9]. The apparatus was calibrated with a nickel chloride solution ($\chi_{NiCl_2,\ 20^\circ C} = 4434.10^{-6}$ cgs), so that the constant k of the equation

$$\Delta\varkappa = kp \tag{1}$$

was found to be equal to $2.154\ (\pm 0.008) \cdot 10^{-11}$ cgs/gμ. To obtain p from the readings s, the specific gravities of the protein solution and buffer must be known. The molecular constant can be calculated from $\Delta\varkappa$ if the concentration of heme-iron is known. These values are easily obtained from the amounts

of stock solutions mixed, the partial specific volume of Mb ($V_{sp} = 0.743$ ml/g) and the tabulated specific gravities of the solutions employed.

The molecular susceptibility of the iron is obtained in the following way:

A sample containing a g Mb/ml (dry weight) and c g iron/ml is subjected to magnetic measurement at the temperature T ° K against a buffer containing the same concentration of salt and acid or base as the sample. The measurement gives p. The susceptibility of the buffer is found to be $\varkappa_b$ by a measurement relative to water.

A diamagnetic compound of Mb (MbCO is convenient) in a water solution containing d g Mb/ml is also meausred. This gives p_{MbCO} and according to equation (1)

$$\varkappa_{MbCO,\ H_2O} - \varkappa_{H_2O} = k \cdot p_{MbCO} \tag{2}$$

Assuming that Wiedemann's law is applicable we get

$$\varkappa_{MbCO} = \frac{k \cdot p_{MbCO}}{V \cdot d} + \varkappa_{H_2O} \tag{3}$$

where V is the specific volume of Mb. If the Mb in the original samle (a g Mb/ml) was made diamagnetic and the sample measured in this state, the value p_{diam} would be obtained. This should give

$$\varkappa_{Mb,\ diam} = \frac{k \cdot p_{diam}}{V\ a} + \varkappa_b \tag{4}$$

Since the diamagnetism is independent of the temperature the results of equations (3) and (4) are equal. Hence

$$p_{diam} = \frac{a}{d}\ p_{MbCO} + \frac{Va}{k}\ (\varkappa_{H_2O} - \varkappa_b) \tag{5}$$

p_{diam} is the diamagnetic correction and it is the difference $p_T = p - p_{diam}$ that is due to the paramagnetism of the iron (c g/ml). This paramagnetism is assumed to follow Curie's law. As all of our measurements have been made within the temperature range 17—23 °C the value is corrected to 20 ° C,

$$\Delta\varkappa_{20°C} = k \cdot p_T \frac{T}{293} \tag{6}$$

The molar susceptibility of the heme-iron at 20° C is obtained by

$$\chi_{Fe,\ 20°C} = k \frac{55.85}{c} (p - p_{diam}) \frac{T}{293} \qquad (7)$$

MATERIAL

The Mb was preparated with slight modifications according to the method described by Theorell[10]. 65 kg of horse muscle were employed and the material was recrystallized three times, the last time in fractions. The crystals formed at 73 % saturated ammonium sulfate were taken as fraction 1 and those at 80 % as fraction 2.

Fraction 1 weighed 90 g and
» 2 » 100 g

The iron content of both fractions was 0.285 %. This value is much lower than the 0.34 % found by Theorell in 1932[10]. More recently Bowen[7] has reported values of the iron content of crystallized horse Mb varying between 0.30 and 0.34 %. Electrophoretic study of our material at pH 6.8 showed two uncoloured fractions in addition to Mb. These impurities were eliminated by electrophoresis on a preparative scale and 100 mg of purified Mb were thus obtained. This sample had an iron content of 0.34 % in agreement with the value found earlier by Theorell. Fractions 1 and 2 were, however, utilized for the magnetic and spectrophotometric measurements without purification. The uncoloured impurities could not influence the adsorption measurements in the visible but the magnetic measurements, however, could be influenced by the impurities if they contained iron. Since the spectrophotometric determination of hemin (as pyridine hemochromogen) gave, within the limits of error, the same values as those calculated from the iron content, we concluded that no foreign iron was present. The molar data for the susceptibilities and the extinctions have been calculated from the hemin content.

PRELIMINARY SPECTROPHOTOMETRIC MEASUREMENTS ON FERRIMYOGLOBIN *

Studies of the absorption of ferri-Mb in the visible at different pH-values were carried out after dissolving the protein in buffers prepared according to Clark[21]. The pH was defined within one tenth of a unit in this way. The ionic strength (μ) varied from 0.02 to 0.35.

* The experiments in this paragraph were carried out in collaboration with dr Margit Béznak during her stay in Stockholm 1949.

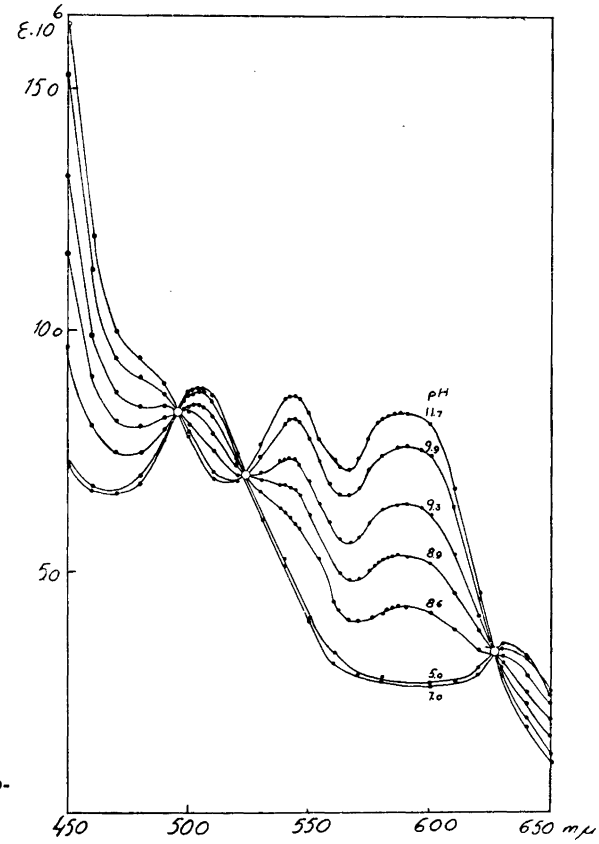

Fig. 1. Absorption curves for ferrimyoglobin at different pH.

Contrary to Bowen's [7] experience we found that the variations of light absorption with pH could be readily reproduced. The curves of Fig. 1 show the molar extinction coefficients in the region 450—640 mμ with pH as parameter. All the curves intersect at three isosbestic points with λ = 495, 523 and 626 mμ. The pH dependence is especially large at 590 mμ, where a flat minimum for the neutral form corresponds to a flat maximum for the alkaline form. This wavelength is thus very suitable for quantitative studies of the transition, and all of the measurements for determination of pK have been made at this wave length.

Below pH 7 practically only Mb^+ and above pH 11 only MbOH are present. If the extinction coefficients at 590 mμ are taken from Fig. 1, and the expressions $\log \frac{\varepsilon - \varepsilon_{pH5.0}}{\varepsilon_{pH11,7} - \varepsilon}$ calculated and plotted with pH as abscissa, a straight line of unit slope is obtained. This shows that the transition has the character

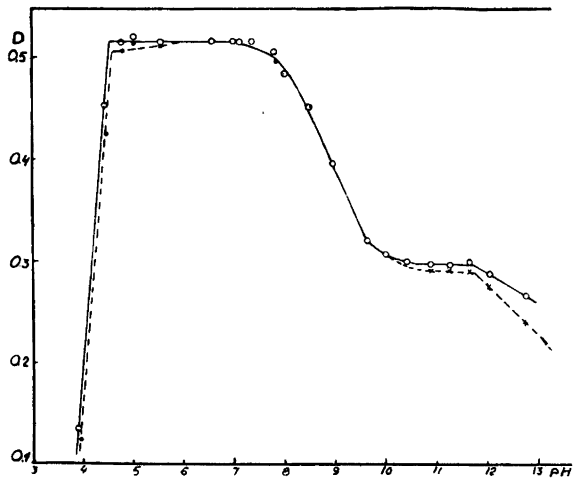

Fig. 2. Stability of ferrimyoglobin at different pH values. Extinction, D, at 409 mμ measured
immediately ○
after 1 hour ●
after 2 hours ×

of a monovalent dissociation. From the graph the pK is found to be about 9.

The limits of stability of ferri Mb in alkaline and acid solutions could also be determined spectrophotometrically. It was found convenient to make these measurements in the region of the Soret-bands, 410 mμ for acid and 414 mμ for alkaline Mb. Fig. 2 illustrates how the extinction coefficient at 409 mμ varies with pH and time. The discontinuity of the slope of the curves shows that there is a sudden change in the stability at pH 4.60 and 11.75. It is also seen that a slow change occurs below pH 6 and above pH 10.

All measurements on native ferri Mb thus must be made within the pH-region 4.60—11.75. Mb can be stored for a long time in a neutral solution but only for a few hours near the limits fo stability.

The data of Fig. 3 for the spectrophotometric studies on solutions outside of the pH stability range indicate that a profound change in the hematin-protein bonds occurred. It seems very interesting that the pH range of stability can be defined so exactly. The explanation for this phenomenon may be the following: If we assume that the first steps of splitting or denaturation processes are reversible with speeds dependent on pH, stability will obtain as long as the destructive processes are slower than the reverse ones. Increas-

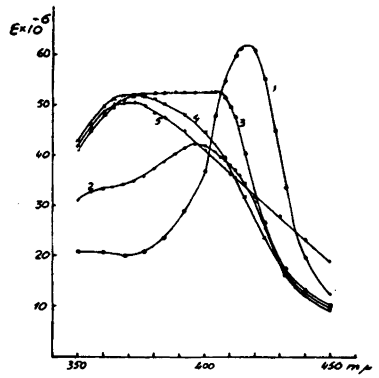

Fig. 3. Absorption curves of ferri-myoglobin in the Soret region of different pH.
Curve 1. pH 12.9 immediately ○
» *2. pH 12.9 2 days* ●
» *3. pH 4.1 immediately* △
» *4. pH 3.4* » ×
» *5. N/10 HCL* » +

ing the concentration of the hydrogen or hydroxyl ions will increase the velocity of destruction. As soon as this velocity exceeds the reverse by an infinitesimal amount the first step product will accumulate and be destroyed in a second step by a following irreversible reaction or reactions. It seems probable to us that analogous processes may be operating in the heat denaturation of proteins.

DETERMINATION OF THE DIAMAGNETISM OF THE PROTEIN

This determination was made on Mb from fraction 2 that had been transferred into diamagnetic ferrous Mb carbonmonoxide (MbCO). Two samples of Mb in aqueous solution were saturated with carbon monoxide. They were reduced with a tenfold excess of sodium hydrosulphite in an atmosphere of CO whereupon MbCO was rapidly formed. Two samples of water were treated in exactly the same way and all four samples were measured against water in the magnet. The difference between the two mean values of p gave $p_{\text{MbCO}} = -192$ gμ, which together with the dryweight of Mb ($d = 0.0735$ g/ml) has been used to obtain the diamagnetic corrections from equation (5).

The mass susceptibility of the protein is calculated by means of equation (3) multiplied by the specific volume of the protein.

$$\chi_{\text{MbCO}} = V \cdot \varkappa_{\text{MbCO}} = -0.591 \cdot 10^{-6} \text{ cgs}$$

This value is about what can be expected for a protein and thus confirms our previous conclusion that foreign iron was essentially absent.

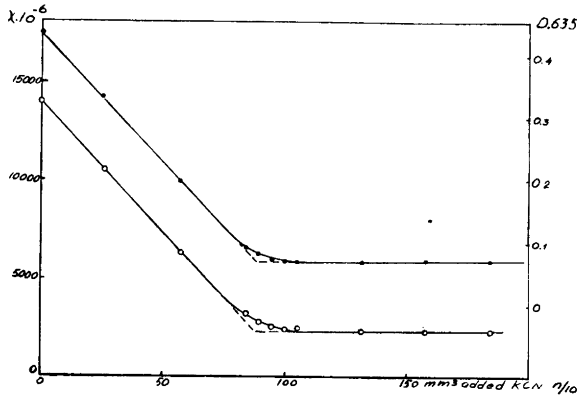

Fig. 4. Magnetic (○) and spetrophotometric (●) titration of Mb^+ with KCN at pH 6.93.

FERRIMYOGLOBIN CYANIDE

When cyanide is added to Mb^+ the solution becomes bright red. The absorption at 630 mμ disappears and a single band appears with its maximum at 540 mμ, $\varepsilon = 9.25 \cdot 10^6$ cm²/M. A solution of Mb^+, $8.51 \cdot 10^{-3}$ M according to spectrophotometric determination of the pyridine hemochromogen, was titrated at pH 6.93 (Phosphate buffer 0.1 *M*) both magnetically and spectrophotometrically (635 mμ) with KCN 0.1 *N*. The results are seen in Fig. 4. The concentration of Mb^+ found by this titrations agreed with the pyridine hemochromogen value within 2 %.

The dissociation constant

$$K_{Mb,\,CN} = \frac{[Mb^+]\,[CN^-]}{[MbCN]}$$

could be estimated from the values obtained near equimolarity. The ionization constant of HCN was taken as 2×10^{-9}. Two points on the magnetic curve gave 4.6×10^{-7} and 3.0×10^{-7} one point on the spectrophotometric curve 3.2×10^{-7}, average $= 3.6 \times 10^{-7}$. This value is 10 times higher than the value (3.6×10^{-8}) found by Coryell, Stitt and Pauling for ferrihemoglobin cyanide [4].

The paramagnetic molar susceptibility of MbCN was found to be $2340 \cdot 10^{-6}$ cgs.

SIMULTANEOUS SPECTROPHOTOMETRIC AND MAGNETIC MEASUREMENTS ON FERRIMYOGLOBIN

In order to ascertain whether the transition in light absorption and the expected change in magnetism are parallell phenomena, measurements on portions of the same samples were carried out simultaneously in the spectrophotometer and in the magnet. A solution containing 0.0828 g/ml of Mb from fraction 1 was prepared and divided into portions of 2.5 ml each. Then 0.25 ml of mixtures of solutions of hydrochloric acid or sodium hydroxide in sodium chloride were added in order to obtain different pH-values at a constant ionic strength ($\mu = 0.10$). The buffer capacity of the concentrated Mb-solution was sufficiently large to obviate pH changes. In the calculation of the ionic strength the contribution of Mb has not been regarded.

Each sample was divided into three portions, which were used for magnetic, spectrophotometric and pH measurements respectively. The extinction coefficient was determined just before and after the magnetic measurement and the mean value employed. A small drift was observed in only a few cases. These results are tabulated in Table 1.

Table 1. Measurements on fraction 1. Ionic strength $\mu = 0.1$.

pH	$\chi_{Fe,\ 20^\circ C}$ 10^{-6} cgs	$\varepsilon_{590\ m\mu}$ 10^6 cm^2/mol
6.55	13 590	2.66
6.92	13 760	2.71
7.96	13 710	3.23
7.97	13 710	3.03
8.09	13 800	3.44
8.22	13 320	3.56
8.36	12 950	3.90
8.47	12 800	4.49
8.48	12 920	3.97
8.83	12 510	4.91
8.94	12 500	5.08
8.96	12 190	5.28
9.07	11 870	6.05
9.13	12 490	6.11
9.63	11 700	7.10
9.78	11 390	7.25
10.06	11 260	7.60
10.21	11 110	7.73
10.23	11 190	7.76
10.40	11 060	7.87
10.79	11 030	8.00
11.30	11 150	8.11
11.80	11 150	8.11
9.35		6.55

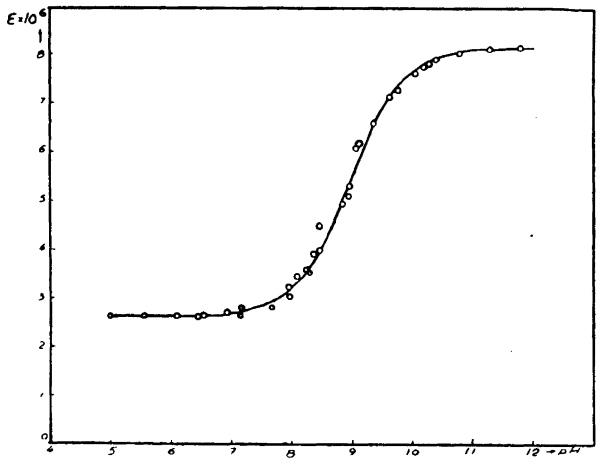

Fig. 5. Transition $Mb^+ \rightleftharpoons MbOH$. measured spectrophotometrically at 590 mμ.

The curve of Fig. 5 shows how the molar extinction coefficient varies with pH from $\varepsilon_{590} = 2.66 \cdot 10^6$ cm²/mole for Mb^+ to $\varepsilon_{590} = 8.11 \cdot 10^6$ cm²/mole for MbOH. The pK of the dissociation has been found graphically to be equal to 8.95 ($\pm$ 0.01). A theoretical curve for this pK has been drawn in the figure.

The magnetic susceptibility values are plotted against pH in Fig. 6. Since they are less reliable than the absorption data, the most probable values of pK and the two asymptotic susceptibilities have been calculated in a manner similar to that employed by Coryell, Stitt and Pauling in the case of Hb[4]. If we write Mb^+ for the neutral form and MbOH for the alkaline form of Mb, the equilibrium can be illustrated by the formula

$$Mb^+ + OH^- \rightleftharpoons MbOH$$

or

$$Mb^+ \cdot H_2O \rightleftharpoons MbOH + H^+$$

The following equation is obtained for the equilibrium constant

$$pK = pH + \log \frac{x}{1 + x} \tag{8}$$

where x is the actual mole fraction of Mb^+ and is related to the measured susceptibility (χ) by the equation

$$\chi = x \cdot \chi_{Mb^+} + (1 - x)\, \chi_{MbOH} \tag{9}$$

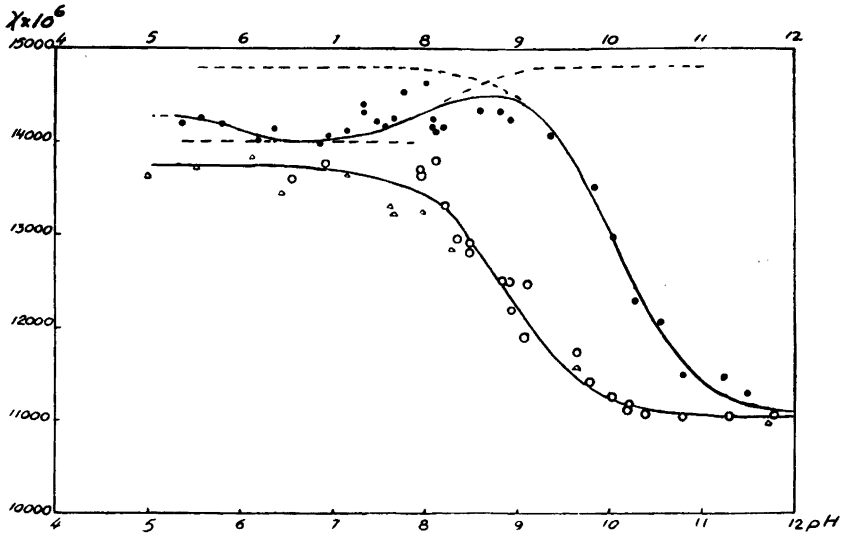

Fig. 6. Paramagnetic susceptibilities at different pH of ferrimyoglobin, fraction 1 (○) and fraction 2 (△), and of ferrimyoglobin with N/1 fluoride (●).

where χ_{Mb^+} and χ_{MbOH} are the asymptotic susceptibility values. From table 1 the approximative values $\chi_{Mb^+} = 13\,670 \cdot 10^{-6}$ cgs and $\chi_{MbOH} = 11\,090 \cdot 10^{-6}$ cgs are calculated. With these as starting point and by means of equations (8) and (9) and the method of least square the parameters pK, χ_{Mb^+} and χ_{MbOH} are then determined by successive approximations. p$K = 8.90$ ($\pm$ 0.04), $\chi_{Mb^+} = 13\,740$ ($\pm$ 90) $\cdot 10^{-6}$ cgs, and $\chi_{MbOH} = 11\,040$ ($\pm$ 40) $\cdot 10^{-6}$ cgs, where all the errors are estimated errors.

The behaviour at low pH-values was studied on a solution containing 0.0657 g/ml of Mb^+ from fraction 2. A very small amount of Mb^+ was denatured when the acid was added to obtain the three points below pH 6.2. A few control magnetic measurements were also taken on this sample at higher pH. The results are shown in Table 2, and the corresponding points have been plotted in Figs. 5 and 6. Both susceptibilities and extinctions are constant within the limits of error between pH 5 and 7.5. As mean values we obtained $\chi_{Mb^+} = 13\,640$ ($\pm$ 60) $\cdot 10^{-6}$ cgs and $\varepsilon_{Mb^+} = 2.63 \cdot 10^6$ cm²/mole, in good agreement with the above results. The two sets of experiments for Mb^+ give as mean values at low pH $\chi = 13\,690$ ($\pm$ 90) $\cdot 10^{-6}$ cgs and $\varepsilon_{590} = 2.64 \cdot 10^6$ cm²/mole.

Table 2. Measurements on fraction 2. Ionic strength $\mu = 0.1$.

pH	$\chi_{Fe,\ 20°C}$ 10^{-6} cgs	$\varepsilon_{590\ m\mu}$ 10^6 cm^2/mol
5.00	13 650	2.59
5.54	13 690	2.62
6.11	13 830	2.62
6.43	13 440	2.62
7.16	13 610	2.64
7.64	13 290	2.67
7.66	13 230	2.81
7.96	13 240	3.01
8.27	12 820	3.53
9.63	11 550	
11.73	10 970	

THE INFLUENCE OF IONIC STRENGTH UPON pK

Three sets of absorption measurements were performed on dilute solutions of Mb^+ from fraction 2. The different pH-values were obtained by means of buffers containing glycine and sodium hydroxide and were measured for each solution. The lowest pH-values, where glycine alone was used, are approximative since the potentials were not stable. The ionic strengths were adjusted by additions of sodium chloride. Table 3 shows the experimental data. The values of pK obtained for the three sets of experiments are

$\mu = 0.02$	pK = 8.92
0.10	8.95 (from the preceeding paragraph)
0.20	9.01
2.0	9.17

Table 3. The variation of $\varepsilon_{590\ m\mu}$ with pH at different ionic strengths.

$\mu = 0.02$		$\mu = 0.20$		$\mu = 2.0$	
pH	$\varepsilon \cdot 10^{-6}$	pH	$\varepsilon \cdot 10^{-6}$	pH	$\varepsilon \cdot 10^{-6}$
5.48	2.73	6.36	2.67	5.13	2.66
7.60	2.91	8.17	3.45	5.97	2.71
8.09	3.10	8.83	4.92	7.97	3.08
8.81	5.11	9.73	7.35	8.73	4.26
9.35	6.63	10.63	7.99	9.05	4.97
10.23	7.96	11.51	8.19	9.26	5.78
11.61	8.13			9.70	6.90
				11.37	8.28
				11.39	8.19

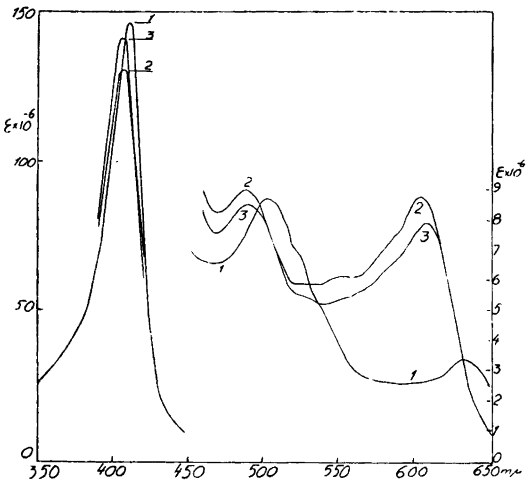

Fig. 7. Light absorption curves for ferrimyoglobin pH 7.0 (1)
» fluoride pH 6.9 (2)
» » pH 5.4 (3)

pK as a function of μ is quite well approximated by the equation

$$pK = pK_0 + a \frac{\sqrt{\mu}}{1 + \sqrt{\mu}} \qquad (10)$$

with a = 0.56 and $pK_0 = 8.84$. If instead one employs

$$pK = pK_0 + a\sqrt{\mu}$$

the equation found by Austin and Drabkin [3] to be valid for ferri Hb, one obtains a = 0.19 and a poorer approximation. A comparison between this value and a = 0.6 for Hb^+ thus shows that the pK of Mb^+ is far less dependent on μ than is Hb.

MEASUREMENTS ON THE FLUORIDE COMPOUND OF FERRIMYOGLOBIN

The addition of fluoride to a neutral solution of Mb^+, gives rise to a pronounced change in the absorption spectrum, as is seen from Fig. 7. This indicates the formation of Mb^+ fluoride, with absorption bands in the visible at 495 and 610 mμ.

A series of samples containing Mb^+ from fraction 2 and potassium fluoride (1 M) was measured in the magnet. The results are given in Table 4. The first five values (pH 5.36—6.35) were obtained by adding acetate buffers containing

Table 4. Magnetic measurements with added fluoride, $c_F = 1.00$ M.

pH	$\chi_{Fe,\ 20°C}$ 10^{-6} cgs	pH	$\chi_{Fe,\ 20°C}$ 10^{-6} cgs
5.36	14 220	8.08	14 230
5.54	14 270	8.08	14 130
5.80	14 160	8.19	14 150
6.20	14 020	8.34	14 510
6.35	14 130	8.62	14 270
6.85	13 980	8.81	14 310
6.93	14 090	8.93	14 240
7.16	14 110	9.36	14 050
7.36	14 400	9.84	13 530
7.36	14 330	10.03	12 980
7.47	14 210	10.28	12 270
7.56	14 190	10.53	12 020
7.66	14 240	10.81	11 680
7.77	14 520	11.23	11 420
8.02	14 640	11.50	11 290
8.06	14 150		

sodium chloride to maintain constant ionic strength in the sample ($\mu = 1.25$). If only acetic acid or hydrochloric acid was added instead of the buffer a small amount of the myoglobin was precipitated. In the other samples the pH was adjusted with acetic acid or sodium hydroxide. Under these conditions the ionic strength was, within a few per cent, equal to 1.03.

The susceptibility values have been plotted in Fig. 6. The curve has a maximum at pH about 8.7 and a minimum at pH about 6.8, which suggests the possibility of three different MbF compounds to be designated as I, II and III, with pK-values in the regions 6, 8 and 10 respectively.

Between pH 9.3 and 12 there are practically only MbF_{III} and MbOH present. The data in this pH-range are treated in the same way as those without added fluoride. This gives $pK_{III} = 10.06$ ($\pm$ 0.03), $\chi_{MbOH} = 11\,040$ ($\pm$ 20) $\cdot$ 10^{-6} cgs, in excellent agreement with the value found without fluoride, and $\chi_{MbF_{III}} = 14\,790$ ($\pm$ 30) $\cdot$ 10^{-6} cgs, which is very close to the theoretical value for 5 unpaired electrons.

The pK of the equilibrium between MbF_{II} and MbF_{III} is evaluated from the data between pH 6.8 and 8.9. Corrections are made for the MbOH present. This gives $pK_{II} = 8.11$ ($\pm$ 0.15) and $\chi_{MbF_{II}} = 14\,000$ ($\pm$ 130) $\cdot$ 10^{-6} cgs.

The pK_I is estimated from magnetic measurements to be 6.0 $\pm$ 0.5, and $\chi_{MbF_I} = 14\,240 \cdot 10^{-6}$ cgs.

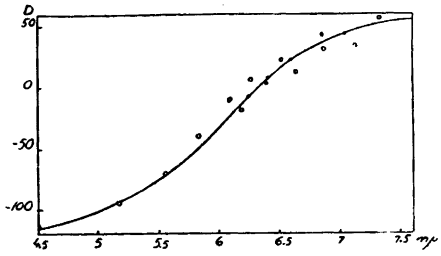

Fig. 8. Change in optical density of ferrimyoglobin fluoride at 610 mμ with change in pH.

It is worth noting that the spreading of the susceptibility values (both with and without added fluoride) is less than 1 % below pH 6 and above pH 10, but somewhat larger (less than 4 %) between these pH values.

Light absorption measurements have been made to verify the above results. No difference in absorption spectrum has been found between samples with and without added fluoride at pH 11.8. Samples at pH 7.1 and 8.7 showed identical absorption spectra in the visible. However, between pH 5.4 and 6.9 there is, as shown by Fig. 7, a pronounced difference. The transition from neutral to acid solution causes the whole absorption curve to be depressed and the maxima displaced a few mμ toward longer wave lengths. The steep parts to the right of the maxima thus coincide. The maximum in the red is displaced from 610 mμ at pH 6.8 to 614.5 mμ at pH 5.5. The Soret band is altered too but in the opposite direction, from 407 to 406 mμ and the maximum absorption is increased by 10 %. The transition is reversible: When an acid sample is neutralised the spectrum changes in the reverse manner.

The pK of the transition between pH 7 and 5 has been determined spectrophotometrically in the following way. Samples containing Mb^+ (c=0.132 mM) and KF (c = 0.90 M) in buffers of various pH were prepared. Phosphate buffers were employed above pH 5.8 and acetate buffers below this value. The total ionic strength varied between 1.0 and 1.2. As these solutions in a 1 cm layer have optical densities at 610 mμ larger than 1, and the change to be studied was about 0.15 it was convenient to compare them with a neutral solution of pure Mb^+ with a density of about 1. Two sets of measurements were carried out. The results are shown in Fig. 8. The pK was found to be 6.03 ($\pm$ 0.03). The theoretical curve for this pK and n = 1 is also shown in the latter figure.

The pK_{I} and the pK_{III} are thus both magnetically and spectrophotometrically operable. The pK_{II}, on the contrary, is found to be spectrophotometrically inoperable.

A spectrophotometric shift corresponding to our pK_{I} for MbF has been observed by Haurowitz for HbF [12]. He found that HbF behaves like an

indicator that changes its colour between pH 5.4 and 6.2. Coryell, Stitt and Pauling [4] in their investigation of the magnetic properties of the HbF did not observe any changes in the region from pH 5.4 to 6.9. However, no determinations were made between these pH values, so that nothing can be said about the possibility of a slight decrease of the susceptibility in this region. Moreover, two single determinations at pH 5.2 and 5.4 both gave the value of 5.89 Bohr magnetons, a value that cannot be said with certainty to be different from our value 5.80 at pH 5.4. It seems probable from the close analogy in the spectral behaviour of MbF and HbF that a slight shift in magnetic susceptibility is likely to occur around pH 6 in HbF as well as in MbF.

The dissociation constant K_{MbF} is calculated from

$$pK_{Mb,\,OH} = 8.84 \ (= pK_0) \text{ and } pK_{III} = 10.06 \ (c_{F^-} = 1 \ M)$$

and from the following equation:

$$K_{Mb,\,F} = \frac{K_{Mb,\,OH} \cdot [F^-]}{K_{III} - K_{Mb,\,OH}} = 10^{-1.19} = 0.065 \qquad (11)$$

This value is at least 4 times higher than $K_{Hb,\,F}$ according to Lipmann [13] and Coryell, Stitt and Pauling [4]. Some spectrophotometric titrations of Mb^+ with fluoride at pH between 7.99 and 5.9 were carried out (at the isosbestic point 626 mμ for Mb^+ and MbOH in experiments above pH 7). (See Table 5 and Figs. 9, 10 and 11.) The ionic strength was close to 0.15 in all these experiments. From the straight lines in Fig. 9 and 10 the K_{app} is obtained by the equation $K_{app} = \frac{m}{b}$ where m is the slope and b the intercept on the ordinate, according to Lewis [14]. Three more experiments were carried out between pH 7 and 8 and μ approximately = 1. The correction term for the influence of ionic strength was calculated from the data obtained to be $\frac{0.44\sqrt{\mu}}{1+\sqrt{\mu}}$. This

Table 5. Ionic strength = 0.

pH	$-\log K_{app,\,Fe,\,F}$
5.91	2.26
6.42	2.02
6.90	1.86
7.32	1.90
7.57	1.87
7.99	1.78

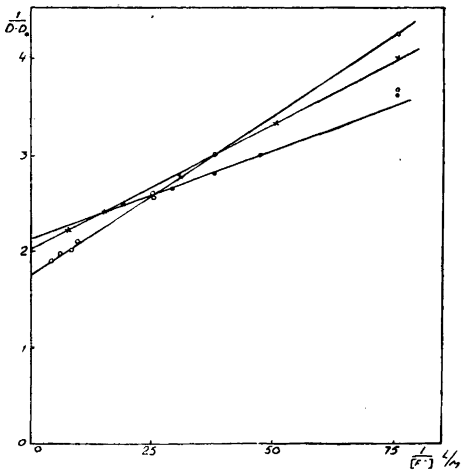

Fig. 9. Equilibrium data of $Mb^+ + F^- \rightleftharpoons MbF$ *measured at 610 mμ. Ionic strength ~ 0.15*
pH = 6.90 ○
pH = 6.42 ×
pH = 5.91 ●

value is not far from the correction term found for the $pK_{Mb,\,OH}$. In Fig. 11 and Table 5 all values are corrected to ionic strength 0.

Lewis [14] has made an attempt to calculate the values of the dissociation constants of methemoglobin fluoride from the known heme-linked groups in methemoglobin with pK 5.3, 6.65 and 8.0 and spectrophotometric experiments with fluoride + methemoglobin. Since he seems to have overlooked Haurowitz' paper, no use was made of the spectrophotometric transition in HbF that occurs around pH 6. Rather complicated formula were used by Lewis. This seemed unnecessary in the present investigation, where the value of this pK was determined experimentally.

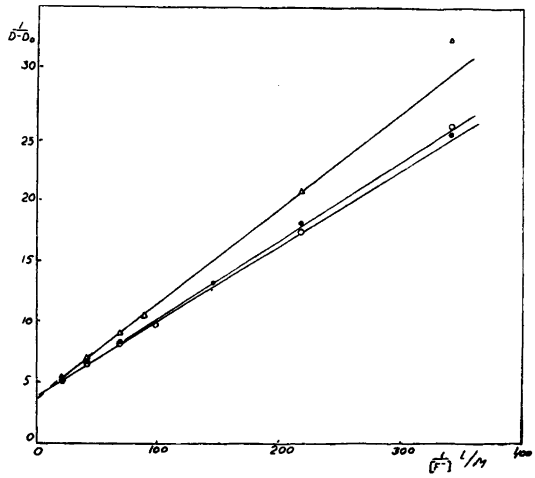

Fig. 10. Equilibrium data of $Mb^+ + F^- \rightleftharpoons MbF$ *measured of 626 mμ*
pH = 7.32 ○
pH = 7.51 ●
pH = 7.99 △

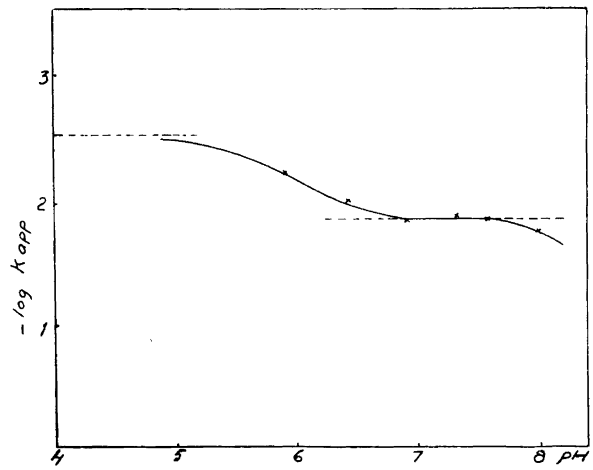

Fig. 11. — log of apparent dissociation constants for myoglobin fluoride at different pH.

Our case, however, was complicated by the absence of any detectable transition in Mb^+ corresponding to $pK = 5.3$ in Hb^+, and by the inaccuracy in the value of the pK around 8.1 in MbF. Furthermore, experimental data on the redox potential in the system ferro-ferrimyoglobin are still lacking outside the pH region 5.9—7.4 (Taylor and Morgan [15]). For these reasons it seemed premature to apply the method of Lewis to the data hitherto available. The use of simplified methods nevertheless permits us to draw certain conclusions concerning the pK:s of the heme-linked groups in ferrimyoglobin.

By equation (11) we have calculated:

$$K_{\mathrm{Mb,\ F}} = \frac{[\mathrm{Mb^+}]\ [\mathrm{F^-}]}{[\mathrm{MbF}]} = 10^{-1.19} \tag{12}$$

It is seen from Fig. 11 that another level with constant values of $pK_{\mathrm{app,\ MbF}}$ is reached in the pH-region 6.9—7.6. This seems to indicate that a $K_{\mathrm{HMb,\ F}}$ is operating essentially alone in this pH region

$$K_{\mathrm{HMb,\ F}} = \frac{[\mathrm{HMb^+}]\ [\mathrm{F^-}]}{[\mathrm{HMbF}]} = 10^{-1.83} \tag{13}$$

$$K_{\mathrm{H,\ MbF}} = \frac{[\mathrm{MbF}]\ [\mathrm{H^+}]}{[\mathrm{HMbF}]} = 10^{-8.11} \tag{14}$$

is determined magnetically. From equations (12)—(14) we can compute

$$K_{H,\,Mb} = \frac{[Mb^+]\ [H^+]}{[HM^+]} = \frac{K_{H,\,MbF} \cdot K_{Mb,\,F}}{K_{HMb,\,F}} = 10^{-7.42} \qquad (15)$$

Towards lower pH-values the $pK_{app,\,MbF}$ (Fig. 11) is increasing again, but because of the spectrophotometrically determined

$$K_{H,\,HMbF} = \frac{[HMbF]\ [H^+]}{[H_2MbF]} = 10^{-6.03} \qquad (16)$$

the next level should not be reached until below pH 5.5. At these low pH values the etching effect of HF prevents accurate spectrophotometric determinations. If we assume that the second proton exerts the same influence as the first an approximate value is obtained for

$$K_{H_1Mb,\,F} = \frac{[H_2Mb^+]\ [F^-]}{[H_2MbF]} = 10^{-2.5} \qquad (17)$$

in analogy with equation (15) thus

$$K_{H,\,HMb} = \frac{[HMb]\ [H^+]}{[H_2Mb]} = 10^{-5.3} \qquad (18)$$

The three dissociation constants $pK_{H,\,HMb} = 5.3$, $pK_{H,\,Mb} = 7.4$ and $pK_{Mb,\,OH} = 8.84$ could possibly be determined by redox potential measurements. Unfortunately Taylor and Morgan [15] confined their work to the region pH 5.9—7.4, where according to our present results no change in the slope $\frac{dE_0'}{dpH}$ could be expected.

TITRATION EXPERIMENTS

(In collaboration with Å. Åkeson)

It was shown in 1934 [1] that the oxygen and carbon monoxide equilibria with myoglobin are very little affected by changes in pH. This fact indicates a profound structural difference between myoglobin and hemoglobin, the latter giving a very strong "Bohr effect".

Therefore it was of great interest to carry out titrations on ferromyoglobin, with the iron held by essentially ionic bonds, and CO-myoglobin, with essen-

tially covalent bonds, in analogy with the differential titrations German and Wyman [16] made on hemoglobin and oxihemoglobin. We preferred to work with the CO-derivative rather than with oximyoglobin, since ferromyoglobin is more easily autooxidized than hemoglobin.

Ferromyoglobin was prepared by reducing 4 to 5 ml of an 8 to 10 % solution of Mb in cellophane tubes by the addition of sodium dithionite in slight excess. The cellophane tubes were sealed immediately and put into a large flask containing 5 liters of water that was freed of oxygen by alternating evacuation and flushing with oxygen-free hydrogen gas. The flask was sealed by a stopper containing an outlet and inlet tube, and the air rinsed out by letting hydrogen through the system. After two days dialysis, during which the myoglobin became diluted two-fold, 3 ml of the solution were transferred to the titration vessel through a hole in the stopper. During this procedure a rapid stream of hydrogen was passed through the vessel. The hole was stopped, and after all oxygen had disappeared (no O_2Mb-bands visible in the spectroscope) 0.1 *N* hydrochloric acid was added from a microburette with magnetic stirring to pH around 5.0. The titration with 1 *N* NaOH was initiated when the potentials of the glass and hydrogen-Pt-electrodes were perfectly stable. The hydrogen was washed free of oxygen by passing it through Fiesers solution (sodium anthraquinone-β-sulphonate + sodium hyposulphite) and silver sulphate.

The titrations of CO-myoglobin were carried out in the same way with the exception that 2 % CO was added to the stream of hydrogen gas. In this way it was possible to avoid the formation of ferrimyoglobin. In Fig. 12 the results of one set of experiments with ferromyoglobin (dots) and CO-myoglobin (full drawn line) are shown. It is seen that the titration curves for the two compounds coincide exactly. A control experiment with Hb and HbCO gave the same differences as were observed by German and Wyman for titrations on Hb and HbO_2. The lack of a Bohr effect in myoglobin thus coincides, as expected, with the similarity in titration curves for ferro- and CO-myoglobin.

Some attempts were made to titrate ferro-versus ferrimyoglobin. These, however, were not entirely successful. It is necessary to let an aliquot of the ferro-myoglobin autoxidize to ferrimyoglobin in order to get strictly comparable results. A pH of around 5 and comparatively long time, in one case up to six days, were required in order to reach complete oxidation. Under these conditions the ferrimyoglobin is not entirely stable. The transition of a p*K* 8.9 could always be nicely demonstrated, but conclusions about other heme-linked groups could not be drawn.

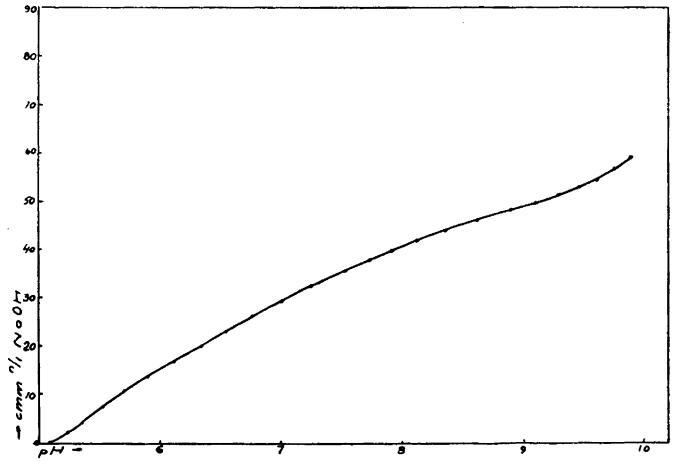

Fig. 12. Titration of 6.41 γ-equivalents of ferromyoglobin (●) and CO-myoglobin (full drawn curve).

DISCUSSION

1. Magnetic and spectral properties

A comparison of the magnetic data now available for ferriperoxidase (from horseradish, "P.O.OH")[17], ferrihemoglobin[4] and ferrimyoglobin in their alkaline form (see Fig. 13 and Table 6) reveals striking differences. Column II, table 6, gives the number of Bohr magnetons calculated from the experimental susceptibility values by the equation

$$\mu_{exp} = 2.84 \sqrt{\chi_{Fe} \cdot T} \text{ Bohr magnetons}$$

Column IV gives the differences between the experimental and the theoretical values for the numbers of unpaired electrons indicated in column III $\mu_{teor} = \sqrt{n(n+2)}$. The values found are nearest to 1 odd electron for P.O.OH, 3 for HbOH and 5 for MbOH, but the differences are still too large to allow for definite conclusions. A strong connection between magnetic and spectral properties would seem very probable, and has in general been found to obtain in the case of the hemoproteins. The three compounds discussed here show in spite of the differences in magnetic properties very similar spectra (see Fig. 14). One difference, however, should be pointed out. Both P.O.OH and HbOH have absorption maxima at 575 mμ, but in addition to this HbOH has an inflection at 595—600 mμ. MbOH has a broad band in the whole region 575—

Table 6.

I	II	III	IV	V
	μ_{exp} Bohr magnetons	Possible numbers of unpaired electrons	$\mu_{exp} - \mu t_{heor}$ Bohr magnetons	$pk_{Fe,\ OH}$ ionic strength = 0
P. O.$^+$	5.44	5	− 0.48	
P.O.OH	2.66	3,1	− 1.11, 0.93	~ 11
Hb$^+$	5.80	5	− 0.12	
HbOH	4.77	5,3	− 1.45, 0.60	7.88
Mb$^+$	5.68	5	− 0.24	
MbOH	5.11	5,3	− 0.81, 1.24	8.84

600 mμ that seems to be formed through the confluence of bands around 575 and 600 mμ. The absorption at 600 mμ thus for the alkaline ferric compounds increase parallel to the ionic character of the bond.

Coryell and Stitt [18] found that the paramagnetic susceptibility of alkaline ferriHb increased when ethanol was added to the solution. The asymptotic change was $3\,800 \cdot 10^{-6}$ cgs (highest ethanol concentration used ≤ 20 %). This would bring the total paramagnetic susceptibility up to the neighborhood of our value of alkaline ferriMb (without ethanol!). It is interesting to note that Coryell and Stitt observed a broadening towards the red of the 575 mμ absorption band of alkaline ferriHb upon the addition of ethanol.

The addition of ammonia to alkaline ferriHb caused a decrease in paramagnetic susceptibility down to $3\,700 \cdot 10^{-6}$ cgs [18]. This value is very close to the χ_M of P.O.OH[17]. Coryell and Stitt could see no change in the spectroscope but we found in spectrophotometric measurements that the addition of ammonia to HbOH causes the inflection at 600 mμ to disappear and lowers the 575 mμ band so that the spectrum becomes very similar to that of P.O.OH. The correspondence between magnetic and spectral properties is thus very striking in these cases. We hope to be able to give more details on this item in the near future.

The spectrum of HbOH in the region mentioned is so strictly intermediate between those of P.O.OH and MbOH that one would be inclined to think that all four iron atoms in the HbOH are not necessarily alike and held by bonds with three odd electrons. For instance two could be held by covalent bonds with one odd electron and two by ionic bonds with five odd electrons. The magnetic data [4] would be well compatible with such an assumption.

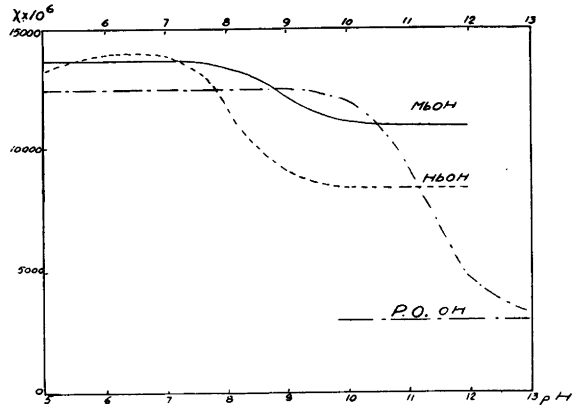

Fig. 13. Paramagnetic susceptibilitius at different pH of ferrimyoglobin, ferrimyoglobin and ferriperoxidase.

2. Heme-linked groups

In Table 7 the available data for the heme-linked groups in ferrihemoglobin, ferrimyoglobin and their fluoride compounds are summarized. The first row of values shows close analogies between hemoglobin and myoglobin. In fact the small differences are far from significant. Lewis' value pK for HbF appears to be too low. Haurowitz found the spectrophotometric shift between pH 5.4 and 6.2, indicating a value of 5.8 to be more likely. Furthermore, the attachment of the fluoride would probably increase the value of the pK more than 0.05—0.20.

According to Coryell and Pauling [5] this heme-linked group in hemoglobin is ascribed to a histidine residue which "is restrained by the configuration of the hemoglobin molecule to a relatively unfavorable position for electrostatic coordination with the iron atom" as suggested by Conant [20]. This assumption may need some further confirmation; but we can say from our present data that the same chemical configuration as in hemoglobin seems to be present in myoglobin on one side of the hemin disc. This is the same side where oxygen is supposed to be attached to the iron, thus substituting the losely bound histidine. Perhaps this mechanism is of importance in explaining the peculiar ability of ferrous iron in hemoglobin and myoglobin to attach oxygen reversibly without being oxidised to the ferric state.

The second row of values in Table 7 shows considerable differences between the pK of ferrihemoglobin and ferrimyoglobin and their fluoride compounds. The pK_2 for hemoglobin is explained by a histidine residue being attached to

Table 7.

pK-values of heme-linked groups in ferrimyoglobin, ferrihemoglobin and their fluorides.

	Hb+		Mb+		HbF		MbF	
pK_1	5.3—5.45 (5, 22)	Mo Pi Si	5.3	Mi Pi? Si	5.5—5.8 (14, 12)	Mi? So	6.0	Mo So
pK_2	6.65 (21, 22)	Mi Po Si	7.4	Mi P? Si	6.9 (14)	Mi? Si?	8.1	Mo Si
pK_3	7.88 (3, 4)	Mo So To	8.84	Mo So To	—		—	

M = magnetically
P = potentiometrically
S = spectrophotometrically
T = titrimetrically

o = operable
i = inoperable
( ) = references

the iron on the other side of the hemin by covalent bonds in oxyhemoglobin and by ionic bonds in Hb and Hb+. This group is responsible for the "Bohr effect" [5]. The absence of this effect in myoglobin and the above mentioned differences in pK seem to exclude the possibility of histidine being the analogous heme-linked group in myoglobin. The chemical nature of this group in myoglobin is still obscure. It can be said, however, that this group seems to be more negative in character than histidine, since the dissociation constants of the component between iron and the negative ions OH^-, CN^- and F^- are all larger by a factor of ten:

$$K_{\mathrm{Mb,\ OH}} = 10^{-5.16} \text{ and } K_{\mathrm{Hb,\ OH}} = 10^{-6.12},\ K_{\mathrm{Mb,\ CN}} = 10^{-6.44} \text{ and}$$

$$K_{\mathrm{Hb\ CN}} = 10^{-7.44},\ K_{\mathrm{Mb,\ F}} = 10^{-1.19} \text{ and } K_{\mathrm{Hb,\ F}} = 10^{-2.33}$$

(the last value is taken from reference (4)). The dissociation constants of the hydrogen ions (pK_2 in Table 7) are accordingly lowered by the same factor in myoglobin compared with hemoglobin.

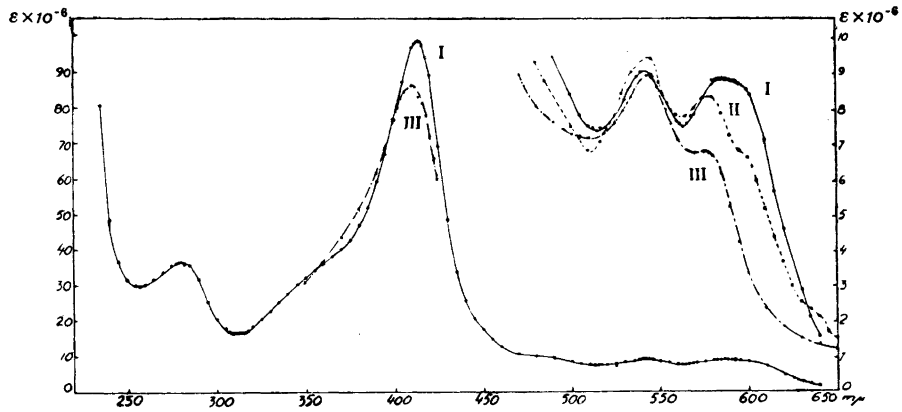

Fig. 14. Lightabsorption curves of MbOH (I, ——), HbOH (II, - - - -) ref. (19) and P.O.OH at pH 12.0 (III, — · — · —).

SUMMARY

1. Ferrimyoglobin has a well-defined pH-stability range. Rapid destruction occurs at pH < 4.60 or > 11.75.

2. The transition from neutral to alkaline ferrimyoglobin follows a monovalent dissociation curve. Its pK value, extrapolated to zero ionic strength, has been determined spectrophotometrically (p$K = 8.84$) and magnetically (p$K = 8.77 \pm 0.04$), thus one unit higher than for ferriHb. The dependance of pK of the ionic strength is three times less than in the case of ferriHb.

3. The paramagnetic susceptibility of ferrimyoglobin changes from $13\,690 \cdot 10^{-6}$ cgs in neutral solution to $11\,040 \cdot 10^{-6}$ cgs in alkaline. In contradistinction to ferriHb, ferriMb gives constant values between pH 7.5 and 5.

4. The ferriMb-fluoride gives three magnetically operable transitions around pH 6, 8 and 10. The first and last one are spectrophotometrically operable and could thus be determined with high accuracy. The transition at pH 8 is spectrophotometrically inoperable. The dissociation constants of the corresponding heme-linked groups were calculated.

5. Titration experiments revealed that ferromyoglobin, with ionic bonds, and CO-myoglobin, with covalent bonds, give identical titration curves. This explains the nearly complete absence of Bohr effect in myoglobin. The conclusion is drawn that the iron in myoglobin cannot be firmly linked to a histidine residue, as is assumed to be the case in hemoglobin.

6. The nature of the heme-linked groups in myoglobin is discussed. An unknown group of more negative character than a histidine residue must be

present, as judged from the values of the dissociation constants for OH^-, CN^- and F^- that are all ten times higher than in ferriHb.

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A Microosmometer and Its Use for the Determination of the Molecular Weight of Hyaluronic Acid

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A technique for construction, filling and operation of small osmometers is described. Using this technique, the molecular weight of hyaluronic acid prepared from human umbilical cord is estimated at 4.7×10^5.

On the basis of measurements of double refraction of flow and viscosity Meyer and Palmer[1] and Blix and Snellman[2,3] have arrived at estimates of the molecular weight of hyaluronic acid ranging from 2×10^5 to 5×10^5. The purpose of the present investigation is to estimate the molecular weight of the acid from osmotic pressure measurements.

The material used was prepared from human umbilical cord[4].

As the acid is only sparingly soluble, and its viscosity is high when dissolved in water, concentrations of more than 2 weight per cent could not be used for the present purpose. An estimation shows that the expected osmotic pressure of such a solution corresponds to a water column of about 10 mm.

An outer solution of aqueous 0.2 molar sodium chloride and an inner solution consisting of a 2 per cent solution of hyaluronic acid in the outer solution, were used for the measurements.

Because of the preciousness of the acid it was necessary to work with small quantities of the material.

For the purpose of correction for capillary rise of the solution the technique of measuring osmotic pressure by means of toluene described by Güntelberg and Linderstrøm-Lang[5] was adopted. Using this method, measurements are made of the difference between the heights of the toluene columns in osmometer and correction capillary, the latter immersed in toluene.

The osmometer is constructed as follows:

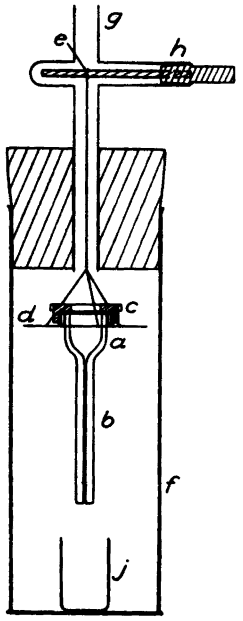

Fig. 1. Microosmometer.

a: bell-shaped cell, b: capillary, c: nut, d: ring, e: metal pin, f: glass cylinder, g: tube, h: ground glass joint, j: sampling tube.

A capillary of 0.88 mm bore, outside diameter approximately 7 mm and length approximately 100 mm is provided at one end with a bell-shaped cell *a*, height about 15 mm, inside diameter about 10.5 mm, thickness of wall about 1 mm. The open end of the cell is ground plane, and a male thread brass ring is pressed down and cemented onto the cell with a zinc oxide-zinc phosphate cement (dental cement) permitting the plane edge of the cell to protrude from the threaded ring by a few tenths of a millimetre. A milled nut *c* with a circular hole of a diameter of about 9.5 mm screws onto the threaded ring. A 50 mm piece of the capillary is cut off, and the cuts are ground plane, the spare capillary being used later for correction for capillarity.

The membrane used is a collodion membrane prepared according to the method described by N. Bjerrum and E. Manegold [6], a collodion solution obtained from British Drug House (BDH) being diluted in the ratio 16 to 100 with equal parts of ethanol and ether, all constituents by volume. After drying for 1—1/4 hours at room temperature (without a fan) the prepared membranes were stored in distilled water covered with toluene. Disks of a diameter of about 13.5 mm are punched out from the central part of the membrane. Immediately before use the disk is fastened in the dried osmometer with a washer of celluloid inserted between nut and membrane.

The osmometer is filled by means of the apparatus shown in Fig. 1.

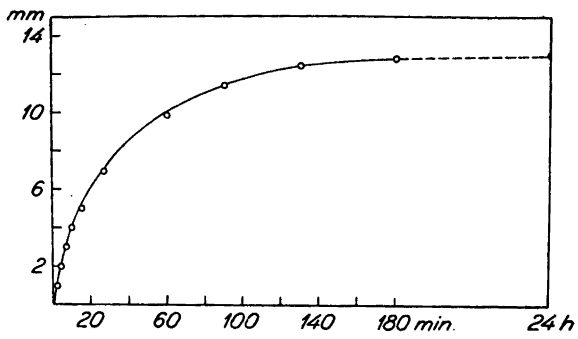

Fig. 2. Difference between heights of menisci in osmometer and correction capillary as a function of time.

The osmometer is turned upside down, and the membrane is covered with a few drops of the outer solution. Still upside down it is suspended in the glass cylinder *f* from a ring *d* hung by a thread to be wound round a metal pin *e* which is inserted in the ground glass joint *h*. By this device the osmometer can be raised and lowered from outside. In the sampling tube *j* about 2 millilitres of hyaluronic acid solution prepared by weight is placed and covered with a layer of about 2 millilitres of toluene. By means of a T-tube with two stopcocks the tube *g* is connected on one side to a glass filter pump and on the other side to a flask of about 1 litre. A manometer with scale division is inserted between the two stopcocks.

The evacuation is begun with both stopcocks open. When the pressure has been reduced to about 5 mm above the pressure of water vapour, the stopcock to the flask is closed, and evacuation is continued for a few minutes. In this way the air is driven out of the osmometer, being swept off with water vapour from the liquid layer on the membrane.

Then the stopcock to the pump is closed, and the osmometer is lowered to the bottom of the aqueous solution of hyaluronic acid. Now the stopcock to the flask is opened, the solution thereby being pressed up into the osmometer. When the osmometer is about 3/4 full, it is raised so much that the opening of the capillary is in the toluene layer where it is held till the osmometer is completely filled. Now the stopcock to the flask is closed, and the pump is stopped, whereafter the stopcock to the pump is opened carefully, allowing the cylinder to be filled with air of atmospheric pressure. Any entrapped bubble of air will then be so small that it can escape through the capillary when the osmometer is turned back into position.

For measurement the osmometer is placed in an optical cell filled with the outer solution covered by a layer of toluene. The optical cell again is placed in a glass walled rectangular container through which water from a thermostat is circulated, thus keeping the temperature of the container constant at 20° C (variations about 0.003° C).

The whole system of container and optical cell can be moved vertically by means of a screw to adjust the boundary line between aqueous phase and toluene layer in the optical cell to the level of that in the osmometer.

Osmometer and correction capillary are clamped close to each other in the same fittings to permit the correction capillary to dip into the outer layer of toluene.

The difference between the liquid levels of osmometer and correction capillary is measured by means of a vertical reading microscope with micrometer eyepiece (100 scale divisions = 5.0 mm). The microscope rotates on a vertical axis, and its vertical displacement is read on a millimetre scale with an accuracy of 0.1 mm by vernier. The heights of menisci in the capillaries are always read on a level with the center line of the eyepiece scale.

It proved to be the most convenient procedure in taking readings of the difference in heights to begin with arbitrary levels of the solution columns near the expected difference, and await establishment of equilibrium.

Check runs of the apparatus were made with a known albumin solution kindly supplied by the Carlsberg Laboratory.

The result of a characteristic experiment: No. 2, Table 1, is illustrated in Fig. 2 showing that osmotic equilibrium was reached in approximately three hours.

The results of five different experiments all made at 20° C are given in Table 1. It should be mentioned that the solutions are prepared by weight, and that the specific gravity values of toluene and sodium chloride solution used are 0.866 and 1.008, respectively.

Table 1.

Experiment	no. 1:	454 000
»	» 2:	470 000
»	» 3:	461 000
»	» 4:	489 000
»	» 5:	467 000
	Mean:	468 000

It will be seen that the result is in good agreement with the highest values found by Blix and Snellman.

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The Separation of Small Amounts of Aromatic Amino Acids

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Tyrosine, phenylalanine and tryptophane can easily be separated from the other amino acids by adsorption on to carbon on account of their high affinity for this adsorbent, and can be displaced by a number of compounds with even greater tenacity for carbon such as benzyl alcohol. Under these conditions, however, difficulties are encountered in the separation of the individual amino acids especially if they are present in small quantities, owing to the narrow contiguous zones in which they appear in the effluent. Tiselius and Hagdahl [1] have described a method to which they have assigned the name "carrier displacement" whereby zones of inert substances of intermediate affinity are interposed between pairs of amino acids to enable the cutting of fractions to be carried out with greater ease. Hagdahl employed the interferometric technique of Tiselius and Claesson [2] for the identification of the fronts of the inert carrier substances. Owing, however, to the fact that the main steps in the refractive index/volume curve which act as markers for the positions of the amino acids, can, within considerable limits, be made of any desired height, solely depending on the concentrations and properties of the substances employed, the extreme sensitivity of this method is not required. The effluent was collected either in the linear, time operated collector of Drake [3], or in an apparatus of the drop Counting type of Moore and Stein [4]. Portions of 0.5, 1.0 or 2.0 ml were collected and 0.05 ml of each examined in a Zeiss dipping refractometer with contact prism for small quantities. The sensitivity of this instrument, 4×10^{-5} units of refractive index is quite adequate to distinguish the grosser steps of the curve, and (*cf.* Fig. 1) will also show up the amino acids as peaks if their concentration approaches the 100 μg level. No attempt was made to regulate the temperature, since such changes as might occur would not alter the position of the steps but only

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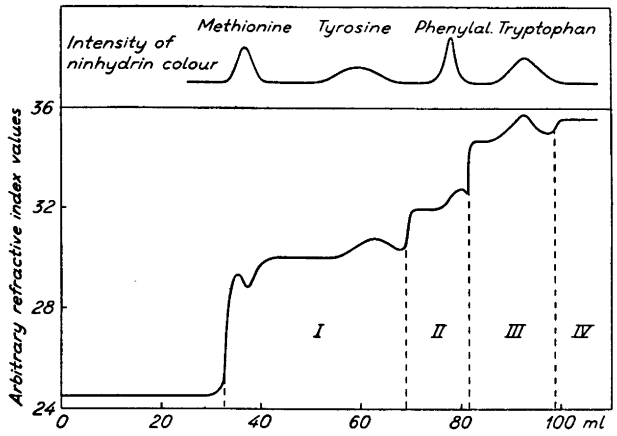

Fig. 1. The separation of aromatic amino acids in 100 μg quantities by carrier adsorption on 14.1 ml of carboraffin supra/super cel 1:3 mixture. I = n-butanol, II = 2-methyl-2-butanol, III = 3-methyl-1-butanol, IV = benzyl alcohol.

their height. The results obtained were sufficiently constant and predictable to permit the inert carriers to be completely ignored in later stages of the work. The separation of any pair of amino acids was proved by the treatment of each fraction with ninhydrin, regardless of the distribution of the carrier substances.

Preliminary work by Hagdahl [5] showed that a system containing certain amyl alcohols does not permit the separation of tyrosine and phenylalanine, although separating these two from tryptophane, and also from other less strongly adsorbed amino acids. The former pair always appeared together in the effluent after 2-methyl-2-butanol and before 3-methyl-1-butanol which emerge in that order. Of the eight isomeric amyl alcohols, only the two mentioned above and 1-pentanol were readily available at the commencement of the work.

Claesson [6] has shown that in an homologous series the degree of adsorption can be correlated with the boiling point, always rising with increases in that function. This has also proved to be the case where isomeric compounds with similar functional groups are concerned. Thus the boiling points of the three amyl alcohols referred to above are respectively 101.8, 130.5 and 138° C., whereas the retention volumes observed in a typical experiment in which 1 % solutions of each were forced through 14.1 ml of a 3:1 mixture of Super Cel and Carboraffin Supra to give a frontal analysis, are 76, 97 and 113 ml.

On the basis of these results it could be deduced that the three amyl alcohols, 2-methyl-1-butanol, (b. p. 128°) 2-pentanol (b. p. 119°) and 3-pentanol (b. p. 115.6°) would be more strongly adsorbed than 2-methyl-2-butanol and less than 3-methyl-1-butanol, and might be useful in the separation of tyrosine and phenylalanine. Subsequent experiments, however, using mixtures of 2 % solutions of 2-methyl-2-butanol with each of these three other isomers showed no separation of the two amino acids concerned.

The correlation between b. p. and position of the adsorption isotherm as measured by the retention volume can also be extended to the isomeric aliphatic acids. A set of retention volumes for 2 % solutions of 2-methyl propionic, *n*-butyric, 3-methyl butyric, *n*-valeric and 4-methyl valeric acids are given in Table 1.

Table 1.

Substance	B. p. °C	Retention volume
2-methyl propionic acid	154.4	9.0
n-butyric acid	163.5	18.5
3-methyl butyric acid	176.7	20.1
n-valeric acid	187.0	27.5
4-methyl valeric acid	207.7	42.0

The figures refer to a displacement experiment with 1 % benzyl alcohol as displacer, using 8.5 ml of the same Super Cel/Carboraffin Supra mixture. The 4-methyl valeric acid was dissolved in 5 % ethanol, as was the benzyl alcohol in this case on account of the low solubility of the acid in water. The effect of this was merely to lower the retention volume, hence the correlation of boiling point with retention volume was still valid.

Tyrosine and phenylalanine were, however, not separated by any of these organic acids, invariably appearing in both frontal and displacement analysis between *n*-butyric and 3-methyl butyric acids. They might be separated by 2-methyl butyric acid, but not by the other isomeric 5-carbon acid pivalic acid (b. p. 163.8°). The former was, however, not readily available, and hence other methods have had to be employed.

Separation of organic acids has been achieved by partition chromatography using heavily buffered columns (Moyle, Baldwin and Scarisbrick [7]) and a similar system has been shown to facilitate the separation of the aromatic amino acids by displacement techniques. In a typical experiment, 104 μg of tyrosine, 120 μg of phenylalanine, 97 μg of methionine and 140 μg of tryptophane were dissolved in 0.1 *M* sodium carbonate/bicarbonate buffer (pH 9.7) and added to a column composed of filters having a total volume of 14.1 ml

and containing 3 parts of Super Cel to 1 of Carboraffin Supra. Through this column which had already been thoroughly saturated with the buffer solution, was passed a mixture of *n*-butanol, 2-methyl-2-butanol, 3-methyl-1-butanol and benzyl alcohol in 1 % concentration in the same buffer. Small peaks appeared in the refractive index/volume curve just before each of the main steps corresponding to the alcohols, (Fig. 1.). Paper chromatographic examination of the fractions representing these portions of the effluent showed that all the methionine was located at the foot of the first step, *i. e.* between the buffer and the *n*-butanol; the tyrosine at interface where the 2-methyl-2-butanol broke through; the phenylalanine was displaced by the 3-methyl-1-butanol, and the tryptophane by the benzyl alcohol. Between the fractions containing the acids the following volumes giving no ninhydrin colour were collected: 6.0, 3.5 and 4.5 ml. These are small but quite adequate to enable a separation to be effected since tailing of the amino acids was negligible.

Quantitative experiments using similar concentrations of the aromatic acids, and also lower amounts down to 20 μg showed that the recovery of the amino acids was of the order of 96 %. The method employed for the estimation of the amino acids was that of Moore and Stein [8] using ninhydrin. It was found that the method could not be employed directly to samples as they left the column since the presence of the small and unpredictable amounts of alcohols effected the colour production by the amino acid. The fractions were evaporated to dryness *in vacuo* and dissolved in water. The colour production was compared with a standard curve prepared from a pure sample of the amino acid concerned dissolved in sodium carbonate/bicarbonate buffer.

For quantitative experiments, it was necessary to purify the carbon and the Super Cel. Both were washed repeatedly with hydrochloric acid and ammonia, and finally extracted with butanol before use. This decreased the amount of soluble material which was extracted by the solvents during a run to a negligible amount.

As yet columns of this volume (10 to 20 ml) have not proved adequate for the separation of tyrosine and phenylalanine from a whole protein hydrolysate (casein hydrolysed with 6 *N* HCl). It will presumably be necessary to effect a group separation first [9] and then employ the above technique.

SUMMARY

1. Figures are given for the retention volumes of two series of compounds, showing that the correlation of boiling point with retention volume can be applied to isomeric substances having similar functional groups.

2. Such a series of isomers having closely similar retention volumes has been utilised in attempts to separate the aromatic amino acids by "carrier displacement chromatography" on carbon.

3. Buffered alkaline solutions of certain pentanols have been proved adequate for the separation of tyrosine and phenylalanine.

4. The recovery of these two amino acids from a column is 96 %.

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The Use of Cation Exchangers for the Quantitative Investigation of Complex Systems

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For the investigation of complex systems and the determination of the complexity constants, in the first place of the mononuclear complexes, especially potentiometric and extinctiometric methods have been applied. In previous papers [1–3] the present author has developed and used different such methods. But the application of those presupposes that suitable electrodes can be found, or that the complexes have absorption bands within available wave length ranges.

Therefore it is very important to search for new and more generally applicable investigation methods. Then it is near at hand to try to use cation exchangers, as acid-resistent, synthetic cation exchangers with reproducible exchange equilibria are available. Some investigations of this kind have been performed by Samuelson [4] and especially by Schubert [5]. Their calculation methods, however, presuppose that only complexes with zero or net negative charges are formed, and the methods then result in a determination of the concentration of free central group in the solutions. The calculation method of Schubert also presupposes that only one complex is formed. Very often, however, these conditions are not fulfilled, and then none of the complexity constants of the system can be determined according to those methods.

It is the aim of the present investigation to develope a method, the applicability of which is not limited by such conditions, and to prove it on a complex system, that has been thoroughly investigated before by potentiometric and extinctiometric methods. As a model system the cupric acetate system was chosen.

The investigation method

As in previous investigations by the present author the ionic strength of the complex solutions was kept constant, I = 1 C, with sodium perchlorate as a neutral salt. The sodium form of a cation exchanger was used, and the distribution of copper at equilibrium between the ion exchanger and the complex solution was determined at different cupric and acetate ion concentrations. The calculations of the complexity constants are based on these determinations.

Deduction of the equations necessary for the calculations

We presuppose that the central group is a divalent cation M^{2+}, and that the ligand is a monovalent anion A^-, as is the case with the model system chosen. According to the Donnan equilibrium the concentration of complexes with zero or negative charges in the cation exchanger is negligible, as the sodium ion concentration is much higher in the resin phase than in the solution. So we have only to take into consideration the taking up of M^{2+} and MA^+ by the sodium form of the ion exchanger.

The following notation is used (*cf.* Fronæus [1]):

C'_M, C'_A = total concentrations of M^{2+} and A^- in the solution before the ion exchanger is added.

C_M, C_A = total concentrations in the solution at equilibrium with the ion exchanger.

(MR_2), (MAR), (NaR) = moles of M^{2+}, MA^+, and Na^+ contained in one unit weight of the exchanger at equilibrium.

$C_{MR} = (MR_2) + (MAR)$

$\varphi = \dfrac{C_{MR}}{C_M}$; $\varphi_1 = (1/\varphi - 1/l_0)/[A^-]$; (l_0 = a constant).

β_j = the complexity constant of the complex MA_j^{2-j} ($j \leqq N$).

$X = 1 + \sum_{j=1}^{N} \beta_j [A^-]^j$; $X_j = (X_{j-1} - \beta_{j-1})/[A^-]$; ($X_0 = X$, $\beta_0 = 1$).

$\bar{n} = (C_A - [A^-])/C_M$; $\bar{n}_R = (MAR)/C_{MR}$

v = the initial volume of the solution.

$v \cdot \delta$ = the volume at equilibrium.

m = the weight of the exchanger.

If we apply the law of mass action to the exchange equilibria:

$$M^{2+} + 2NaR \rightleftharpoons MR_2 + 2\ Na^+$$
$$MA^+ + NaR \rightleftharpoons MAR + Na^+$$

we obtain:

$$\frac{(MR_2)}{[M^{2+}]} = k_0 \cdot \frac{(NaR)^2}{[Na^+]^2} \; ; \; \frac{(MAR)}{[MA^+]} = k_1 \cdot \frac{(NaR)}{[Na^+]} \tag{1}$$

In eq. (1) k_0 and k_1 are presumed to be constants at a fixed value of C_{MR}, as the ionic strength is practically constant in the resin phase and in the solution. Then we put:

$$l_0 = k_0 \cdot \frac{(NaR)^2}{[Na^+]^2} \; ; \; l_1 = k_1 \cdot \frac{(NaR)}{[Na^+]} \tag{2}$$

If the values of C_M are low, compared with the ionic strength, then $[Na^+]$ is approximately constant in all the solutions. For (NaR) we have the expressions:

$$(NaR) = a - 2(MR_2) - (MAR) \tag{3 a}$$
$$(NaR) = a - (2 - \bar{n}_R) \cdot C_{MR} \tag{3 b}$$

Here a is the exchange capacity, and at certain cation exchangers it is a function of pH. From the definition of C_{MR} and eq. (1—2) we obtain:

$$C_{MR} = l_0 \cdot [M^{2+}] + l_1 \cdot [MA^+] \tag{4}$$

This relation is combined with the expressions $[MA^+] = \beta_1[M^{2+}]\,[A^-]$ and $C_M = [M^{2+}] \cdot X$ (*cf.* Fronæus [1]), and then, putting $l_1\beta_1/l_0 = l$, we get:

$$\varphi = l_0 \frac{1 + l \cdot [A^-]}{X} \tag{5}$$

From eq. (2—3) it is evident that at a constant value of a (that is of pH) and so small C_{MR} that the term $(2 - \bar{n}_R) \cdot C_{MR}$ in eq. (3b) can be neglected, the quantities l_0, l_1 and l are constants, and for $\bar{n}_R$ we have the relation:

$$\bar{n}_R = \frac{l \cdot [A^-]}{1 + l \cdot [A^-]} \tag{6}$$

If at greater C_{MR} the term $(2 - \bar{n}_R) \cdot C_{MR}$ cannot be neglected, but C_{MR} is kept constant, l_0 and l_1 are polynomials of $\bar{n}_R$. Then only at rather low values of $[A^-]$ the numerator in the expression for φ is approximately linear in $[A^-]$, and if the coefficients are still called l_0 and l, it is easily shown that in this case they have not exactly the same meaning as before, and eq. (6) is no longer valid.

Thus we see that C_{MR} should be kept constant and as small as possible, so that eq. (5) with constant l_0 and l can be used within a concentration range of $[A^-]$ that is large enough for the calculation of the complexity constants.

At the deduction up till now it has been tacitly assumed that only mononuclear complexes are formed. If also polynuclear complexes are formed, the expression for φ will contain in the numerator and in the denominator terms with $[M^{2+}]$, $[M^{2+}]^2$ etc. as factors. But for $C_{MR} \to 0$ and consequently $C_M \to 0$ these terms cancel, and thus at small C_{MR} eq. (5) is *general* for complex systems of the type M^{2+}—A^-.

For the calculation of the values of C_{MR} and φ we have the relation:

$$C_{MR} = \frac{v}{m} (C'_M - C_M \cdot \delta) \tag{7}$$

C'_M, C_M and δ are determined at the measurements. Because of the swelling of the dried ion exchanger, when it is added to the solution, δ is always < 1. δ is obtained as the quotient between the initial and the equilibrium concentrations of the ligand in solutions with $C_M = 0$. As will be shown below, δ is independent of C_A at a constant ionic strength. It can be assumed to be independent also of C_M at small C_{MR}, but this cannot be proved exactly at the measurements because of the taking up of the complex MA^+ by the exchanger. However, the exchanger takes up about m ml of water from v ml of the solution, $\delta \simeq 1 - m/v$, and at a small value of m/v only an approximate determination of δ is required.

At the measurements, described below, on the cupric acetate system it is shown, how the φ-values, corresponding to different C_A and a constant C_{MR}, are obtained. For a rough approximation of $\bar{n}$ we can use the relation:

$$\bar{n} \simeq -\frac{C_A}{\varphi} \cdot \left(\frac{\partial \varphi}{\partial C_A}\right)_{C_{MR}} \tag{8}$$

obtained from eq. (5) by putting $l \simeq 0$ and $C_A \simeq [A^-]$. Then the $[A^-]$-values are computed from the relation $[A^-] = C_A - \bar{n} \cdot C_M$. The approximation is quite satisfactory for the calculation of $[A^-]$, as C_M is kept small.

Thus we have determined φ as a function of $[A^-]$ with l_0 and l as constants. For the calculation of the complexity constants β_j the product $\varphi \cdot X$ is differentiated twice with respect to $[A^-]$, and from eq. (5) we get:

$$\varphi'' \cdot X + 2\,\varphi' \cdot X' + \varphi \cdot X'' = 0 \tag{9}$$

($\varphi' = \frac{d\varphi}{d[A^-]}$, $\varphi'' = \frac{d^2\varphi}{d[A^-]^2}$ etc.). Putting in the expressions for X, X', and X'', we obtain:

$$\varphi'' + \sum_{j=1}^{N} ([A^-]^j \cdot \varphi'' + 2\, j\, [A^-]^{j-1} \cdot \varphi' + j\,(j-1)\,[A^-]^{j-2} \cdot \varphi) \cdot \beta_j = 0 \quad (10\ a)$$

or abbreviated:

$$\varphi'' + \sum_{j=1}^{N} a_j\, \beta_j = 0 \quad (10\ b)$$

From a graphic representation of φ as a function of $[A^-]$ we can determine φ' and then φ'' in the same way from a graphic representation of φ'. Then φ'' and the coefficients a_j in eq. (10 b) are determined at N values of $[A^-]$, spread out within the concentration range used. Thus we have a system of N equations, from which the complexity constants β_j can be computed.

At the measurements on the cupric acetate system it was found, however, that only β_1 could be determined accurately in this way, and it is very likely that this fact is general. The reason will be discussed below (p. 868) in connection with the calculations. The remaining constants, $\beta_2 \ldots \beta_N$, can be computed according to the following method. $1/\varphi$, which is a monotonously increasing function of $[A^-]$ (as $l < \beta_1$), is easily extrapolated graphically to $[A^-] = 0$. From eq. (5) we have:

$$\frac{1}{l_0} = \lim_{[A^-] \to 0} \frac{1}{\varphi}$$

Here it should be mentioned that l_0 cannot be determined directly at measurements on a solution with $C_A = 0$, unless the solution has the same value of pH as the solutions with $C_A > 0$.

Then the function $\varphi_1 = (1/\varphi - 1/l_0)/[A^-]$ is extrapolated to $[A^-] = 0$. Putting in the expression for X in eq. (5) we obtain:

$$\frac{\beta_1 - l}{l_0} = \lim_{[A^-] \to 0} \varphi_1$$

When l_0 and l have been determined in this way, the polynomial X can be calculated, and from corresponding values of X and $[A^-]$ the complexity constants can be computed from the relation:

$$X = 1 + \sum_{j=1}^{N} \beta_j\, [A^-]^j$$

It is obvious that if we have a complex system of the form $M^{\nu+}$—$A^{\nu-}$, where all complexes have zero or negative charges (that is $l = 0$), the calculation method is considerably simplified. Then l_0 and the complexity constants β_j can be obtained directly from corresponding values of $[A^{\nu-}]$ and the function $1/\varphi$.

THE MEASUREMENTS ON THE Cu^{2+}—Ac^- SYSTEM

In order to prove the method, described above, it was applied to the cupric acetate system, that has been investigated in a previous work [1] by the present author.

Chemicals used. Cupric perchlorate, sodium perchlorate, perchloric acid, sodium acetate and acetic acid were prepared or purified as in previous investigations [1,2], and stock solutions of an ionic strength of 1 C were prepared. The sodium form of the cation exchanger Amberlite IR—105 with a particle size of 20—40 mesh was used. A portion of the exchanger, sufficient for all the measurements, was dried on a water bath and stored in a closed bottle, so that the content of water was kept constant. The exchange capacity was about 2.4 meq. per gram of the dried hydrogen form of the exchanger.

The measurements were carried out in the following way. To v liters of the complex solution

$$\left|\begin{array}{l} C'_M \text{ mC } Cu(ClO_4)_2 \\ C'_A \text{ mC } NaAc \\ 0.5\ C'_A \text{ mC } HAc \\ (1000 - 3C'_M - C'_A) \text{ mC } NaClO_4 \end{array}\right.$$

m grams of the dried cation exchanger were added. The value of the quotient m/v was the same at all the measurements and equal to 40.0 g · l^{-1} and m was about 0.4 g. The solution was shaken with the exchanger for twenty-four hours in a thermostat at 20.0° C, and then the solution was separated from the exchanger and analysed. It was controlled that a longer time of shaking did not affect the distribution of the copper.

The cupric concentration C_M of the solution was determined extinctiometrically. A surplus of 2 C ammonia was added, and the extinction was measured with a Beckman Quartz Spectrophotometer (Model DU) at the wave length 6 200 Å and with a thickness of 1 cm of the absorbing layer. The molar extinction was obtained from solutions with known cupric concentrations. At the ammonia concentration used the molar extinction was independent of the acetate concentration C'_A.

Table 1. Ion exchange measurements on the cupric acetate system.
$M^{2+} = Cu^{2+}$, $A^- = Ac^-$

C'_M mC	C_A mC	C_M mC	$C'_M - C_M \cdot \delta$ mC	$\varphi \cdot 10^3$ $l \cdot g^{-1}$
6.67	20.2	1.47	5.27	89.6
»	39.0	1.86	4.90	65.9
»	68.5	2.14	4.64	54.2
»	106.0	2.49	4.30	43.2
»	162.0	3.05	3.77	30.9
»	215	3.42	3.42	25.0
»	266	3.68	3.17	21.5
»	319	3.93	2.94	18.7
»	423	4.28	2.60	15.2
»	526	4.67	2.23	11.9
10.00	21.3	3.05	7.10	58.2
»	42.0	3.45	6.72	48.7
»	71.4	4.05	6.15	38.0
»	109.1	4.55	5.68	31.2
»	161.0	5.14	5.12	24.9
13.33	19.3	5.11	8.48	41.5
»	39.0	5.84	7.78	33.3
»	68.5	6.33	7.32	28.9
»	109.0	7.03	6.65	23.6
»	159.0	7.69	6.02	19.6
»	216	8.31	5.44	16.4
»	264	9.04	4.74	13.1
»	313	9.49	4.31	11.4
»	417	10.2	3.64	8.9
»	521	10.8	3.07	7.1
20.0	215	14.2	6.40	11.3
»	266	14.7	5.90	10.0
»	318	15.1	5.50	9.1
»	422	15.8	4.80	7.6
»	526	16.4	4.25	6.5

The concentration C_A at equilibrium could be determined with sufficient accuracy only by extinctiometric titration with perchloric acid. These measurements were performed at the wave length 3 000 Å, where the molar extinctions of the complexes are great, while the other ions in the solution practically do not absorb at this wave length. When so much acid has been added that $C_A < C_M$, approximately only the first complex is formed and $[A^-] \ll C_A$.

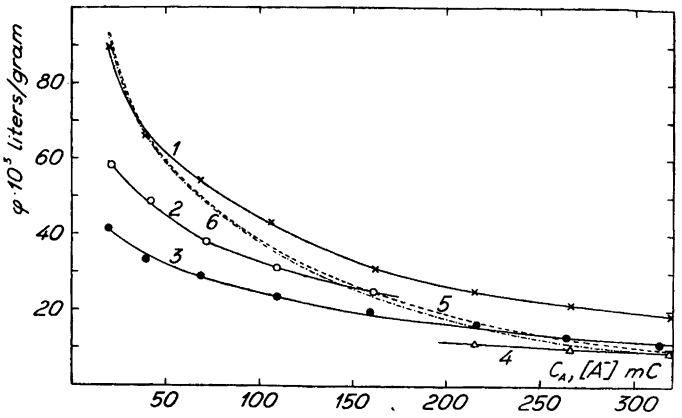

Fig. 1. Fulldrawn curves: φ as a function of C_A at different values of C'_M. 1. $C'_M = 6.67$ mC; 2. $C'_M = 10.00$ mC; 3. $C'_M = 13.33$ mC; 4. $C'_M = 20.0$ mC. — Dashed curves: 5. φ as a function of C_A at $C'_M - C_M \cdot \delta = 5.00$ mC; 6. φ as a function of $[A^-]$ at $C'_M - C_M \cdot \delta = 5.00$ mC.

Then the extinction is almost proportional to C_A, and thus the extinction, corrected for the dilution, is a linear function of the volume of perchloric acid added. After the equivalence point the corrected extinction is constant, and is due to the small extinction of the acetic acid. In this way the acetate concentration could be determined graphically with great accuracy. At very small values of C_M cupric perchlorate was added before the titration.

The values obtained from the distribution measurements on the cupric acetate system are collected in Table 1, the columns 1—3. At the calculation of $C'_M - C_M \cdot \delta$ and φ in the columns 4 and 5 the value $\delta = 0.95$ was used. δ was determined in the way, described above.

In Fig. 1 φ is represented as a function of C_A and in Fig. 2 $C'_M - C_M \cdot \delta$ as a function of C_A, in both cases with C'_M as a parameter. From these curves the values in the columns 2—9 of Table 2 have been obtained. The relation between φ and $C'_M - C_M \cdot \delta$ at a constant value of C_A proves to be practically linear, when graphically represented. So it is easy to determine by graphic interpolation the φ-values corresponding to a constant and low value of C_{MR}, that is of $C'_M - C_M \cdot \delta$. The value $C'_M - C_M \cdot \delta = 5.00$ mC or $C_{MR} = 0.125$ mmoles $\cdot$ g^{-1} was selected, and the φ-values are to be found in column 10 of Table 2. The values of C_M in the column 11 have been calculated from the definition of φ. The $[A^-]$-values in the last column of Table 2 have been determined in the way, described above. The values of $(\partial\varphi/\partial C_A)_{C_{MR}}$ in eq. (8) have

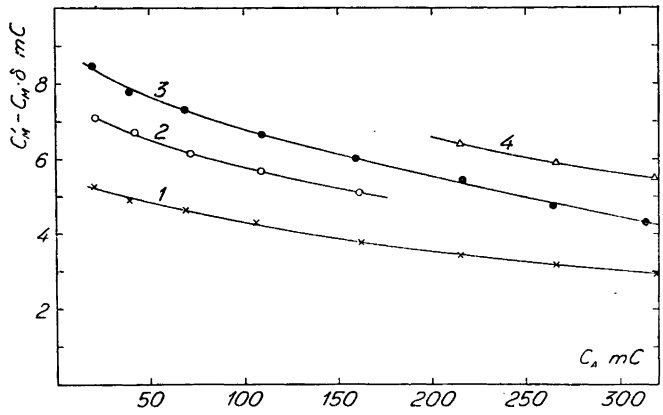

Fig. 2. $C'_M - C_M \cdot \delta$ *as a function of* C_A *at different values of* C'_M. *1.* $C'_M = 6.67$ *mC; 2.* $C'_M = 10.00$ *mC; 3.* $C'_M = 13.33$ *mC; 4.* $C'_M = 20.0$ *mC.*

been obtained from the curve 5 in Fig. 1. It is evident that the difference between C_A and $[A^-]$ is rather small. For the H^+-concentration we have the relation:

$$[H^+] = K_C \cdot \frac{0.5\ C_A}{[A^-]}$$

K_C is the dissociation constant of the acetic acid. As $C_A/[A^-]$ is almost constant at the different C_A, $[H^+]$ is also practically unchanged, and the conditions for

Table 2. Determination of corresponding values of C_A, C_M *and* φ *at a constant value of* $C'_M - C_M \cdot \delta$

C_A	$C'_M = 6.67$ mC		$C'_M = 10.00$ mC		$C'_M = 13.33$ mC		$C'_M = 20.0$ mC		$C'_M - C_M \cdot \delta = 5.00$ mC		
mC	$C'_M - C_M \cdot \delta$ mC	$\varphi \cdot 10^3$ $l \cdot g^{-1}$	$C'_M - C_M \cdot \delta$ mC	$\varphi \cdot 10^3$ $l \cdot g^{-1}$	$C'_M - C_M \cdot \delta$ mC	$\varphi \cdot 10^3$ $l \cdot g^{-1}$	$C'_M - C_M \cdot \delta$ mC	$\varphi \cdot 10^3$ $l \cdot g^{-1}$	$\varphi \cdot 10^3$ $l \cdot g^{-1}$	C_M mC	$[A^-]$ mC
20.0	5.25	89.0	7.15	59.0	8.40	41.0			93.5	1.34	19.4
40.0	4.95	66.5	6.70	48.5	7.90	34.5			66.0	1.89	39.0
70.0	4.60	53.5	6.15	38.5	7.30	28.5			49.5	2.53	68.4
100.0	4.30	44.0	5.75	32.5	6.80	24.5			38.5	3.25	97.5
150.0	3.85	33.0	5.20	26.0	6.15	19.5			26.5	4.72	145
200	3.50	26.3			5.55	16.5	6.60	11.7	19.0	6.58	191
250	3.25	22.5			5.00	13.7	6.05	10.4	13.7	9.12	235
300	3.05	19.7			4.45	12.0	5.60	9.3	10.5	11.9	280
400	2.65	15.7			3.75	9.3	4.95	8.0	7.9	15.8	375
500	2.35	12.7			3.20	7.5	4.40	6.7	6.2	20.2	465

Table 3. φ, φ', and φ'' as functions of $[A^-]$ at $C_{MR} = 0.125$ millimoles/gram.

$[A^-]$ mC	$\varphi \cdot 10^3$ $1 \cdot g^{-1}$	$-\varphi' \cdot 10^3$ $1 \cdot g^{-1} \cdot C^{-1}$	$\varphi'' \cdot 10^3$ $1 \cdot g^{-1} \cdot C^{-2}$
20.0	92.5	2 200	$1.00 \cdot 10^5$
40.0	65.5	780	$2.05 \cdot 10^4$
70.0	49.0	445	5 900
100.0	37.7	315	3 600
150	25.5	190	1 900
200	17.7	130	1 000
250	12.5	78	1 000
300	9.6	38	480
400	7.3	20	75

the use of eq. (5) are fulfilled. Curve 6 in Fig. 1 represents graphically φ as a function of $[A^-]$ at $C_{MR} = 0.125$ mmoles $\cdot g^{-1}$.

In Table 3 the values of the first two derivatives φ' and φ'' at different $[A^-]$ are to found. They have been graphically determined. In Table 4 the coefficients a_j in eq. (10 b) have been computed at $[A^-] = 20$, 100, 200, and 400 mC. a_1 is obtained with great accuracy, especially at $[A^-] = 20$ and 100 mC, but for the other coefficients a_j the relative errors are much greater. However, as the values of φ'' and a_1 are so great at $[A^-] = 20$ mC, compared with the values at the other $[A^-]$, the first complexity constant β_1 is easily calculated from our system of equations (10 b). The value $\beta_1 = 45 \pm 2\ C^{-1}$ is obtained. Approximately the same value is found from eq. (10 b) at $[A^-] = 20$ mC, if the small terms $a_2\beta_2$—$a_4\beta_4$ are neglected. The errors in the values of a_2—a_4 make a determination of the constants β_2—β_4 from the system of equations impossible.

In Table 5, column 2 the function φ^{-1} is computed. The connection between φ^{-1} and $[A^-]$, graphically represented, is at lower $[A^-]$ almost linear, and by

Table 4. Determination of the coefficients a_j in eq. (10 b) at different values of $[A^-]$.

$[A^-]$ mC	a_1 $1 \cdot g^{-1} \cdot C^{-1}$	a_2 $1 \cdot g^{-1}$	a_3 $1 \cdot g^{-1} \cdot C$	a_4 $1 \cdot g^{-1} \cdot C^2$
20	— 2 400	49	6.6	0.3
100	— 270	— 14	7.3	2.4
200	— 60	— 28	2.0	1.8
400	— 10	— 5.4	3.1	5.7

Table 5. Corresponding values of $[A^-]$, φ^{-1}, φ_1 *and the polynomials* X, X_1, X_2, *and* X_3.

$[A^-]$ mC	φ^{-1} $l^{-1} \cdot g$	φ_1 $l^{-1} \cdot g \cdot C^{-1}$	X	X_1 C^{-1}	X_2 C^{-2}	X_3 C^{-3}
0	*7.0*	*195*		*45*	*440*	
20	10.8	190	2.07	53.5		
40	15.3	205	3.67	67		
70	20.4	190	6.38	77	460	
100	26.5	195	10.2	92	470	
150	39.0	215	19.8	125	530	
200	56.5	245	35.5	173	640	1 000
250	80	290	60.0	236	760	1 300
300	104	325	91	300	850	1 350
400	137	325	155	390	860	1 000

extrapolation to $[A^-] = 0$ we get $l_0^{-1} = 7.0$ liter^{-1} · gram. The function φ_1 is practically constant and equal to 195 liter^{-1} · gram · C^{-1} at $[A^-] \leqq 100$ mC. Thus $(\beta_1 - l)l_0^{-1}$ can be determined with great accuracy, and we obtain $\beta_1 - l = 28.0$ C^{-1} or $l = 17 \pm 2$ C^{-1}.

With the values determined of l_0 and l the polynomial X has been calculated in column 4 of Table 5. At the extrapolation of X_1 to $[A^-] = 0$ we find of course the same value of β_1 as before. By extrapolation of X_2 to $[A^-] = 0$ we obtain $\beta_2 = 440 \pm 60$ C^{-2}. The experimental error in this value is substantially dependent on the error in the value of l.

The polynomial X_3 has been calculated for such $[A^-]$ for which the difference $X_2 - \beta_2$ is not small compared with β_2. From the values in the last column of Table 5 the complexity constant of the third complex is determined to $\beta_3 = 1000 \pm 300$ C^{-3}. No value of β_4 can be obtained from the measurements, and this is natural, as according to the potentiometric investigation [1,p.55] only about 10 % of C_M is present as the complex $CuAc_4^{2-}$ at an acetate ion concentration of 400 mC.

Discussion of the results

A comparison between the complexity constants obtained in this investigation and the constants potentiometrically determined shows that within the limits of the random errors the agreement is complete.

	Ion exchange method:	Potentiometric method:
β_1:	45 ± 2 C^{-1}	47 ± 1 C^{-1}
β_2:	440 ± 60 C^{-2}	450 ± 50 C^{-2}
β_3:	1 000 ± 300 C^{-3}	1 150 ± 150 C^{-3}
β_4:		750 ± 200 C^{-4}

This perfect agreement proves that the presumption, on which the use of eq. (5) is based, is fulfilled. Thus at a constant value of C_{MR} the quantities l_0 and l_1 or k_0 and k_1 are constants, so that the activity coefficients in the resin phase seem to be independent of the value of $\bar{n}_R$. It is conceivable that in the exchanger with its high concentration of fixed sulphonate ions there is some complex formation between these and the cupric ions. Then at increasing acetate ion concentration a mere ligand displacement with an unimportant influence on the activity coefficients occurs. On the other hand it is evident from Table 2 that the function φ at every value of C_A is highly dependent on C_{MR}. Thus the activity coefficients in the resin phase are very much affected by a variation of the total cupric concentration C_{MR}, even if this is small in comparison with the exchange capacity.

Concerning the taking up of complex cations by ion exchangers the present author has not found any quantitative data in the literature. In this investigation it has been proved by the determination of the value of the constant l that the complex $CuAc^+$ is taken up by the exchanger used. Further, from eq. (6) the ligand number $\bar{n}_R$ in the resin phase is obtained as a function of the acetate ion concentration of the outer solution.

SUMMARY

A method for the investigation of complex systems by the use of synthetic resin cation exchangers is described. The calculation method is developed for systems of the type M^{2+}—A^-, where the central ion M^{2+} and the first complex MA^+ are taken up by the exchanger, whereas the concentration in the resin phase of the higher complexes with zero or negative charges can be neglected.

From the deduction of the equations it is obvious that it is possible to calculate the complexity constants of all the mononuclear complexes formed, even if also polynuclear complexes occur. For systems, where no cation complexes are formed, e. g. systems of the type $M^{\nu+}$—$A^{\nu-}$, the calculations are considerably simplified.

In order to prove the method it has been applied to the cupric acetate system. The measurements have been carried out at 20° C, and the ionic strength has been kept at 1 C by addition of sodium perchlorate. The complexity constants of the complexes $CuAc^+$, $CuAc_2$, and $CuAc_3^-$ have been computed, and the values obtained are in complete agreement with previous potentiometric determinations. On account of this it is to be expected that the new method will be of great use.

Finally I wish to express my gratitude to Professor S. Bodforss for the facilities he placed at my disposal. My thanks are also due to Mrs. Brita Linderot for her valuable help at the measurements.

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Intramolecular Diazotisation of *o*-Nitro-amines

I. The Reaction between 3-Amino-4,9-dinitroretene and Concentrated Hydrochloric Acid

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During an investigation of the salt-forming properties of 3-amino-4,9-dinitroretene, a compound prepared during the synthesis of 4,9-dinitroretene[1], it was found that the substance reacted with conc. hydrochloric acid * in glacial acetic acid solution to give a product that was not the expected amine hydrochloride. When the reaction mixture was refluxed, a gas was evolved and a yellowish-green substance separated. Purification by chromatography and by repeated recrystallisation yielded pale-yellow prisms, melting at 160—161 °C **, and analysis showed that two atoms of nitrogen had been eliminated and a chlorine atom had entered the retene molecule. In the present paper, this reaction between conc. hydrochloric acid and 3-amino-4,9-dinitroretene is discussed in detail.

First it was established which of the nitrogen containing groups were eliminated in the reaction. The above mentioned product (m.p. 160—161 °C) did not have the properties of an amine, but on reduction it yielded a colourless compound which was proved to be an amine. This indicates that, on treatment with conc. hydrochloric acid, the nitro-amine loses the amino-group and one of the nitro-groups.

The gas evolved in the reaction is odourless, non-inflammable, and does not maintain combustion. It was further investigated in the apparatus, shown on p. 879. In one experiment the gas was conducted directly into the azotometer,

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* Conc. hydrochloric acid mentioned in this paper has a density of 1.18.

** All melting points are approximately corrected.

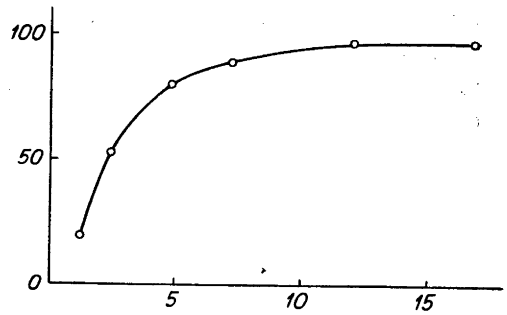


Fig. 1. Relation between yields of the diazonium salt in per cent of the theoretical (ordinate) and amount of conc. hydrochloric acid, expressed as mol HCl/mol of the nitro-amine (abscissa).

in another the gas at first passed through a red-hot tube containing CuO and Cu (charged as for nitrogen analysis according to Dumas). In both cases approximately the same amount of nitrogen/mol of nitro-amine was obtained (see Table 2 on p. 880). Consequently, the gas is probably pure nitrogen, although admixed nitrous oxide cannot be detected in this way.

Thus, on treatment with conc. hydrochloric acid, a glacial acetic acid solution of 3-amino-4,9-dinitroretene gives a product which on warming decomposes with the evolution of nitrogen, and on the basis of this fact, it was assumed that the first-formed product is a diazonium salt. The following experiments supported this assumption. When conc. hydrochloric acid was added to a hot (ca. 100° C) glacial acetic acid solution of the nitro-amine, a faint yellow product (the amine hydrochloride) separated initially, but dissolved in less than half a minute. The yellow solution thus obtained was cooled to room temperature. It reacted with an alkaline solution of β-naphthol forming a red coloured compound, and with cuprous chloride solution or with hypophosphorous acid with evolution of nitrogen. As mentioned above, nitrogen was also evolved if the solution was refluxed.

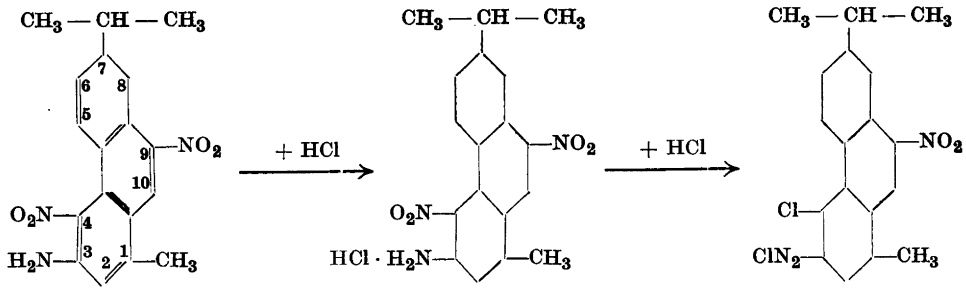
Preliminary experiments showed that the same amount of gas per mol of nitro-amine was evolved when the reaction solution was refluxed for 15—20 minutes or when it reacted with cuprous chloride solution. This tends to indicate that all the gas comes from the diazonium salt and it therefore must consist of pure nitrogen. Consequently, the yield of the diazonium salt can be calculated by measuring the volume of the gas evolved on refluxing the reaction solution. To get an estimate of the relationship between the yield of diazonium salt and the amount of conc. hydrochloric acid used, the following experiments were carried out in the apparatus mentioned above. A known amount of 3-amino-4,9-dinitroretene (ca. 0.170 g) was dissolved in a suitable volume (5 ml) of hot (ca. 100 °C) glacial acetic acid and treated with conc. hydrochloric acid. One or two minutes later, the solution was boiled, and the

nitrogen collected in the azotometer. The results are tabulated on page 880 (Table 3) and in Fig. 1 the yields of diazonium salt are plotted against the amount of added hydrochloric acid. As can be seen from this curve, 7 mols of HCl per mol of nitro-amine gives a yield of about 90 %. It should be emphasized that the time required for the diazotisation reaction to take place at 100 °C seems to be less than one minute, probably as little as 15—30 seconds.

Some experiments were carried out to estimate the speed of the reaction at lower temperatures using an alkaline solution of β-naphthol to detect the presence of the diazonium salt. In a well-stirred suspension of finely powdered nitro-amine hydrochloride (20 mg) in glacial acetic acid (5 ml) and conc. hydrochloric acid (0.5 ml) no diazonium salt was formed during two hours at 25—30 °C, but at a temperature of 35—40 °C, the presence of the diazonium salt could be demonstrated after only 10 minutes.

Some information about the stability of the diazonium salt was obtained by the following experiments. The nitro-amine was dissolved in glacial acetic acid and treated with conc. hydrochloric acid in the usual way. If the solution was refluxed, decomposition was complete in about 10 minutes; at 70 °C about 12 hours were required, while at room temperature, most of the diazonium salt was unchanged even after three days.

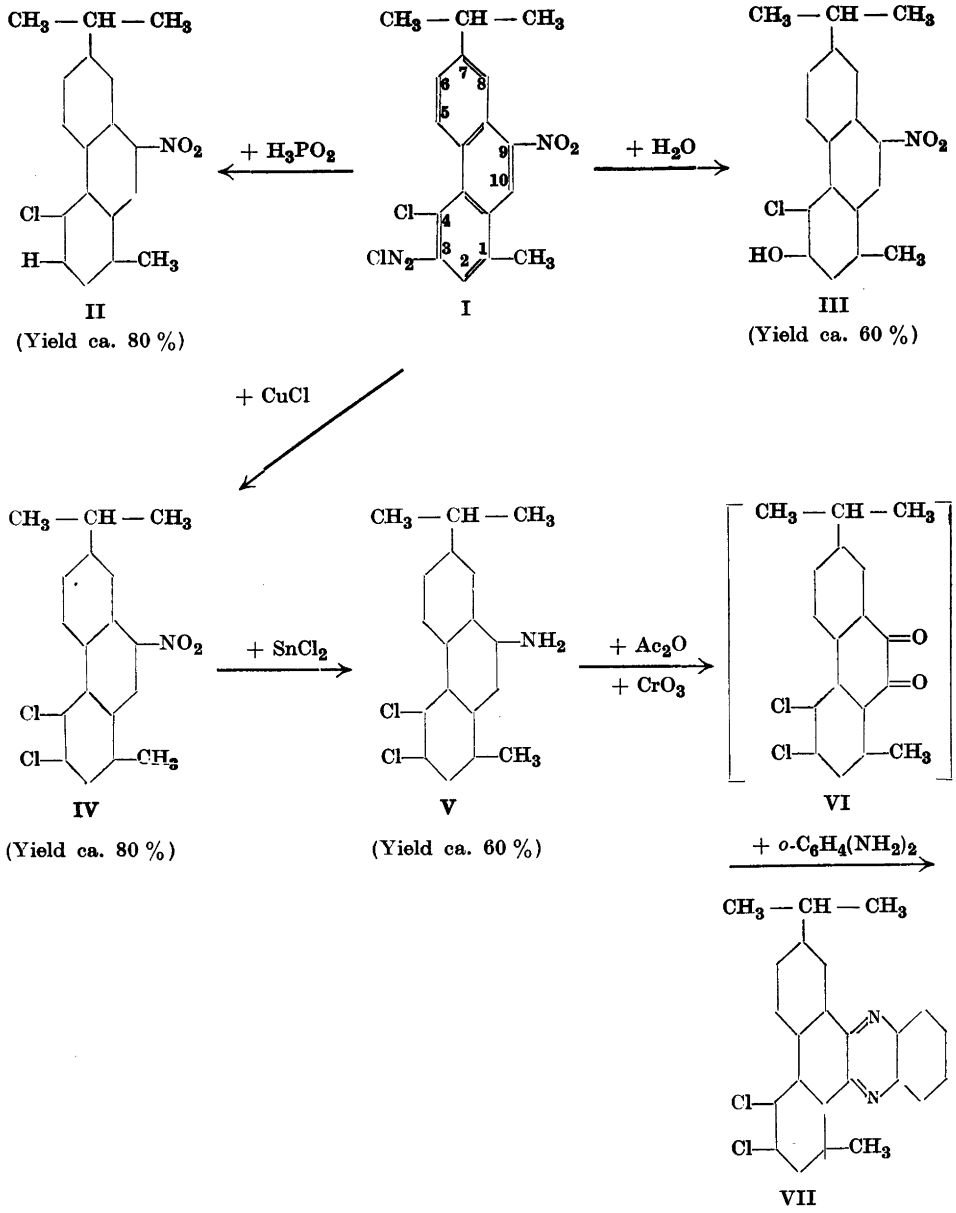
The following reaction scheme seems to be the most probable one for the "intramolecular diazotisation" of 3-amino-4,9-dinitroretene.



On this basis, the yellow product of m. p. 160—161 °C, obtained by refluxing the diazonium salt solution would be expected to be either 3,4-dichloro-9-nitroretene or 3-hydroxy-4-chloro-9-nitroretene. Analyses showed that the former one was obtained, but that 3-hydroxy-4-chloro-9-nitroretene was also present in the crude product.

To establish the empirical formula assumed for the diazonium salt, it was used for the preparation of some simple derivatives of retene (reaction scheme, see p. 875). Analysis figures found for these compounds were in good agree-

ment with the values calculated for the assumed formulae, which therefore must be regarded as correct.



II (Yield ca. 80 %)

I

III (Yield ca. 60 %)

IV (Yield ca. 80 %)

V (Yield ca. 60 %)

VI

VII

Reaction scheme.

The reaction scheme establishes also, to a certain degree, the structural formulae of the diazonium salt (I) and the derivatives prepared from it. From the synthesis of the quinoxaline (VII) we may conclude that the amine (V) obtained by the reduction of 3,4-dichloro-9-nitroretene (IV) loses the amino-group (acetylated) during its oxidation to the quinone. Therefore, the nitro-group in the 9-position remains unchanged during the intramolecular diazotisation, and it is the nitro-group in the 4-position which is replaced by chlorine. The diazonium salt is formed by reaction between the removed nitro-group and the amino-group in the 3-position.

It remains to be proved if the chlorine atom enters the position vacated by the nitro-group (the 4-position) or if it occupies another position in the retene molecule. Information on this point was not obtained from the reaction scheme above, but proof should be obtainable by conversion of the "4-chloro-9-nitroretene" (II) via the amine to the corresponding chlororetene which could be compared with 4-chlororetene which must be obtainable from 4-nitroretene [2]. These investigations will be discussed in a forthcoming communication, but it seems reasonable to assume that the chlorine atom replaces the nitro-group, and the formulae have been written on this assumption.

It should be pointed out that 3,4-dichloro-9-acetylaminoretene was oxidised to the quinone (VI) under milder conditions than are required for other retene derivatives. This oxidation is usually carried out by the addition of chromic acid to a hot (ca. 100 °C) glacial acetic acid solution of the retene derivative, the quinone as a rule being obtained in yields of 30—50 %. In the case of 3,4-dichloro-9-acetylaminoretene, however, no quinone was formed under these conditions, other oxidation products being obtained. However, if aqueous chromic acid was used and the temperature kept at 40 °C, the quinone (VI) was obtained in a probable yield of about 10 %. Because of the low yield and the formation of a large quantity of other oxidation products, the quinone was not isolated in a pure state, but the crude product was allowed to react with *o*-phenylene diamine. The sparingly soluble quinoxaline (VII) which formed, could then be readily isolated.

When the diazonium salt was heated with water, 3-hydroxy-4-chloro-9-nitroretene (III) was obtained although in rather low yield. However, the compound was easily isolated in a pure state by chromatographic purification. The crude product was dissolved in benzene and adsorbed on a column of alumina. Development with benzene gave a distinct red band which moved very slowly and which contained fairly pure 3-hydroxy-4-chloro-9-nitroretene. Washing with acetone removed some impurities but the main band was not eluted although its colour changed from red to paleyellow, a colour change

which could be reversed by the addition of benzene. Acetone, containing a few per cent of conc. hydrochloric acid, proved to be a very good eluant.

It may be mentioned that 3-hydroxy-4-chloro-9-nitroretene can be used as an acid-base indicator. An acidic solution of the compound in dilute ethanol has a pale yellow colour at high concentrations, but is nearly colourless in concentrations suitable for indicators; on the addition of alkali the ethanolic solution turns a blood-red colour which on dilution changes to orange or orange-red. The compound is very sparingly soluble in water, but a very dilute aqueous solution may be obtained by adding an ethanolic solution to water. By using buffers of different pH, the interval for the colour change was determined to be pH 8.5—9.5.

The intramolecular diazotisation described in this paper, takes place with other *o*-nitro-amines. Under the same conditions as those used for 3-amino-4,9-dinitroretene, diazonium salts were formed, apparently in good yields, from 3-amino-4-nitroretene and 1-nitro-2-naphthylamine. With *o*-nitro-amines of the benzene series some experiments were carried out, but no diazonium salt has been obtained as yet. The general applicability of the intramolecular diazotisation will be further investigated.

Investigations of the conditions for intramolecular diazotisation have shown that the hydrochloric acid may be replaced by hydrobromic acid (d, 1.38), without changing the reaction conditions. The diazonium bromide has not been closely investigated, although its presence was indicated by the reactions with alkaline β-naphthol solution (intense red colour) and with cuprous chloride solution (evolution of nitrogen). The addition of hydriodic acid (d, 1.70) to a hot glacial acetic acid solution of 3-amino-4,9-dinitroretene caused an immediate evolution of gas, possibly due to the decomposition of a diazonium iodide initially formed. Owing to the instability of diazonium iodides, these cannot be detected by means of β-naphthol or cuprous chloride. Consequently, the evolved gas was investigated in the apparatus described on page 879, and shown to be nitric oxide in amount corresponding to ca. 1 mol per mol of the nitro-amine. Though the remaining reaction product has not been investigated, it seems safe to assume that no diazonium iodide is formed by treating the nitro-amine with hydriodic acid. The nitro-group in the 4-position is apparently reduced by the hydriodic acid either before or after the exchange reaction. These reactions between *o*-nitro-amines and hydrobromic and hydriodic acids will be further investigated.

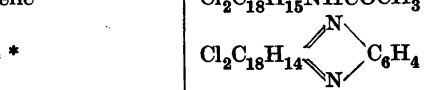
During the investigation of the intramolecular diazotisation attempts were made to use solvents other than glacial acetic acid and it was found that other carboxylic acids (formic, propionic, *n*-valeric, and lactic acids) were useful solvents. With solvents such as ethanol or acetone no diazonium salts were

obtained, the nitro-amine being recovered unchanged and it seems possible that carboxylic acids are the only solvents which can be employed.

The intramolecular diazotisation of *o*-nitro-amines, described in this paper, does not appear to have been reported in the literature. However, it is known that a nitro-group, attached directly to a carbon atom in an aromatic ring can be activated by suitable substitution of groups in the molecule (see, *e. g.*, Degering in *An outline of organic nitrogen compounds* [3]) and the nitro-group is often especially mobile in compounds containing a diazonium-group. Thus it has been observed that many nitro-amines must be diazotised in non-aqueous solution; otherwise the nitro-group is replaced by a hydroxyl-group and a diazo-oxide may be formed [4]. Further, the influence of a diazonium-group is observed in a reaction similar to the intramolecular diazotisation. It has been found that, on diazotisation in a solution containing chloride ions, some aromatic nitro-amines exchange the nitro-group for chlorine. *E. g.*, 1-nitro-2-naphthylamine [5–8], 2,3-dinitro-4-methoxyaniline [9], 2,5-dichloro-4-nitro-aniline [10], 4-chloro-5-nitro-2-aminoacetophenone [11], 4-chloro-3-nitro-2-aminoacetophenone [11], and 4-chloro-2-nitro-3-aminoacetophenone [12]. It may be of interest to establish if intramolecular diazotisation can be carried out with these compounds as well. To date, only 1-nitro-2-naphthylamine has been tested and, as mentioned above, intramolecular diazotisation does indeed take place, the diazonium salt apparently being obtained in very good yield.

Atkinson and Simpson [11] have investigated the diazotisation of 4-chloro-5-nitro-2-aminoacetophenone in the presence of chloride ions. Their observations showed "that a substituted diazonium kation may become involved in an exchange of groups before removal of the diazonium residue, and that slight

Table 1. New compounds prepared in the present investigation.

Name	Formula	M. p. °C
4-Chloro-9-nitroretene-3-diazonium chloride	$Cl(NO_2)C_{18}H_{15}N_2Cl$	—
3,4-Dichloro-9-nitroretene	$Cl_2C_{18}H_{15}NO_2$	160–161
3,4-Dichloro-9-aminoretene	$Cl_2C_{18}H_{15}NH_2$	122–123
3,4-Dichloro-9-acetylaminoretene	$Cl_2C_{18}H_{15}NHCOCH_3$	248–249
3,4-Dichlororetenequinoxaline *		133–134
4-Chloro-9-nitroretene	$ClC_{18}H_{16}NO_2$	133–134
3-Hydroxy-4-chloro-9-nitroretene	$HO(Cl)C_{18}H_{15}NO_2$	170–171
3-Acetoxy-4-chloro-9-nitroretene	$CH_3COO(Cl)C_{18}H_{15}NO_2$	137–138

* See note on page 882.

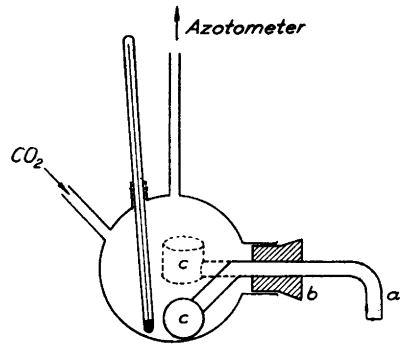


Fig. 2. Apparatus, used for quantitative investigations of the intramolecular diazotisation.

variations in the experimental conditions, such as gentle warming, may suffice to determine whether or not such an interchange occurs". Intramolecular diazotisation cannot proceed by this mechanism as the diazonium cation cannot be formed before the nitro-group is split off. Perhaps the essential step is the reaction of the hydrochloric acid with a compound formed from the nitro-amine and the carboxylic acid used as the reaction medium. As yet we know, however, too little about this transformation to be able to say anything about its mechanism. The investigation of the intramolecular diazotisation reaction will be continued.

EXPERIMENTAL

I. Investigation of the reaction between 3-amino-4,9-dinitroretene and concentrated hydrochloric acid

A. Analysis of the evolved gas

The apparatus used for this investigation is schematically drawn in Fig. 2. In the 50-ml round-bottomed flask, 3-amino-4,9-dinitroretene (ca. 5×10^{-4} mol) was suspended in glacial acetic acid (5 ml). On the glassrod (a), through the rubber stopper (b) was fixed a small container (c) containing a mixture of conc. hydrochloric acid and glacial acetic acid (1.5 ml) corresponding to 0.70 ml of conc. hydrochloric acid. A glass tube connected the flask to an azotometer, containing 50 % aqueous potassium hydroxide. Carbon dioxide was passed through the apparatus until micro bubbles were obtained in the azotometer and the suspension in the flask was heated to about 100° C to obtain a clear solution. By turning the glassrod (a) the acid mixture in the container (c) was added, and, after 1—2 minutes, the reaction mixture was boiled for 15 minutes. While boiling was continued, carbon dioxide was again passed through the apparatus until micro bubbles appeared in the azotometer. The volume, temperature, and pressure of the collected gas was determined. The experiment was repeated, using a modified apparatus in which the gas, before entering the azotometer, was passed through a red-hot tube, charged with CuO and Cu as for nitrogen analysis according to Dumas. The results of these experiments are listed in Table 2 and it can be seen that approximately the same volume of gas was obtained in both cases. This indicates that the gas, which was odourless, non-inflammable, and not able to maintain combustion, consisted of pure nitrogen.

Table 2. The results of the investigation of the evolved gas.

3-Amino-4,9-dinitroretene mol × 10^4	Gas, collected in the azotometer					Remarks
	Temp. °C	Pressure mm Hg	Volume ml	Volume at N.T.P. ml	Vol./mol of nitroamine l	
5.010	21	758	11.88	11.00	22.0	
5.013	22	766	11.75	10.96	21.9	
5.019	18	766	11.48	10.86	21.6	Cu-CuO-tube
5.010	19	746	11.92	10.94	21.8	Cu-CuO-tube

B. The relationship between the amount of concentrated hydrochloric acid and the yield of diazonium salt

In the same way as before a series of intramolecular diazotisations were carried out, using varying amounts of conc. hydrochloric acid. The evolved gas did not pass through the combustion tube in these experiments. The hydrochloric acid was added mixed with 2—5 vol. of glacial acetic acid and the total volume of glacial acetic acid in the reaction mixture was, in each case 5 ml. From the amount of nitrogen collected in the azotometer, the yield of diazonium salt was calculated. The results are listed in Table 3 and in the curve in Fig. 1 (p. 873) the yields of diazonium salt are plotted against the amount of conc. hydrochloric acid added.

Table 3. Yields of the diazonium salt with varying amounts of concentrated hydrochloric acid.

3-Amino-4,9-dinitroretene mol × 10^4	Hydrogen chloride		Nitrogen			Diazonium salt yield of the theoretical	
	12 *M* HCl ml	mol HCl per mol of nitroamine	Temp. °C	Pressure mm Hg	Volume ml	%	% aver.
5.016	0.05	1.2	23	742	2.44	19.6	
5.001	0.05	1.2	23	760	2.34	19.3	19.5
5.004	0.10	2.4	25	742	6.80	54.3	
5.010	0.10	2.4	24	742	6.40	51.2	52.7
4.995	0.20	4.8	23	762	9.77	80.8	
5.007	0.20	4.8	24	742	9.92	79.4	80.1
5.004	0.30	7.2	21	750	10.68	87.3	
5.007	0.30	7.2	23	762	10.92	90.0	88.7
4.992	0.50	12.0	22	755	11.67	96.0	
5.022	0.50	12.0	22	765	11.82	97.9	96.9
5.019	0.70	16.7	18	766	11.48	96.6	
5.013	0.70	16.8	22	766	11.75	97.6	97.1

C. Suitable conditions for the intramolecular diazotisation of 3-amino-4,9-dinitroretene

Conc. hydrochloric acid (5 ml) was added to a stirred solution of 3-amino-4,9-dinitroretene (2.0 g) in hot (ca. 100° C) glacial acetic acid (60 ml). The amine hydrochloride first formed, dissolved immediately yielding 4-chloro-9-nitroretene-3-diazonium chloride and after 1—2 minutes the solution was cooled to room temperature. Diazonium salt solutions, prepared in this way, were used for the syntheses described below.

II. Derivatives prepared from 4-chloro-9-nitroretene-3-diazonium chloride

3, 4-Dichloro-9-nitroretene

3-Amino-4,9-dinitroretene (2.0 g) was diazotised as described above and the diazonium salt solution was added to a boiling solution containing cuprous chloride (10 g) in conc. hydrochloric acid (100 ml). A yellowish-green product separated (2.0 g), which after one crystallisation from glacial acetic acid (12 ml) was dissolved in a mixture of 15 ml of benzene and 10 ml of petroleum ether (b. p. 40—50 °C) and adsorbed on a column of alumina (18 × 3.5 cm). Elution with petroleum ether, containing about 10 % of benzene, yielded a pale-yellow residue (1.70 g) which melted at 157—158 °C, and after one crystallisation from glacial acetic acid gave pure 3,4-dichloro-9-nitroretene (1.50 g) as pale-yellow prisms melting at 160—161 °C. The yield corresponds to 73 % of the theoretical, calculated on the amount of 3-amino-4,9-dinitroretene consumed. The compound is easily soluble in chloroform and benzene, rather soluble in ethanol, glacial acetic acid, and acetone, and slightly soluble in ligroin.

$C_{18}H_{15}Cl_2NO_2$ (348.2)	Calc.	C 62.1	H 4.34	N 4.02	Cl 20.4
	Found	» 62.2	» 4.36	» 4.00	» 20.3

3, 4-Dichloro-9-aminoretene

Finely powdered 3,4-dichloro-9-nitroretene (2.9 g) was suspended in a solution of $SnCl_2 \cdot 2H_2O$ (12 g) in glacial acetic acid (60 ml) saturated with hydrogen chloride and stirred at 20° C for two hours. The nitro-compound dissolved gradually and the white crystalline reaction product which separated was filtered off after some hours and washed with dilute hydrochloric acid and ether then suspended in ether and washed with aqueous alkali. Evaporation of the ether yielded 3,4-dichloro-9-aminoretene (1.6 g) of m. p. 117—119 °C — 60 % of the theoretical. After one crystallisation from ethanol or from ligroin-benzene, the amine was obtained as colourless plates melting at 122—123 °C.

$C_{18}H_{17}Cl_2N$ (318.2)	Calc.	C 67.9	H 5.39	
	Found	» 67.6	» 5.44	

3,4-Dichloro-9-aminoretene was easily diazotised, and the diazonium salt gave a red azo-compound with β-naphthol.

The *hydrochloride* crystallised as white needles (m. p. 226—228 °C, decomp.) when conc. hydrochloric acid was added to a hot ethanolic solution of the amine and the solution allowed to cool.

$C_{18}H_{18}Cl_3N$ (354.7)	Calc.	C	60.9	H	5.11
	Found	»	61.0	»	5.15

Picrate. 3,4-Dichloro-9-aminoretene (0.1 g) and picric acid (0.15 g) were dissolved in ca. 5 ml of hot ethanol. On cooling, the picrate separated as short, yellow needles melting at ca. 176—178 °C (decomp.).

$C_{24}H_{20}Cl_2N_4O_7$ (547.3)	Calc.	C	52.7	H	3.68
	Found	»	52.8	»	3.75

3,4-Dichloro-9-acetylaminoretene. Acetic anhydride (2 ml) was at room temperature added to a solution of 3,4-dichloro-9-aminoretene (2.0 g) in benzene (15 ml). Within one minute, the acetyl derivative separated as thin, white, needle-shaped crystals (2.0 g, 90 %) which were collected after one hour. The compound thus prepared was pure and melted at 248—249 °C.

$C_{20}H_{19}Cl_2NO$ (360.3)	Calc.	C	66.7	H	5.32
	Found	»	66.7	»	5.25

3,4-Dichlororetenequinoxaline *

A solution of CrO_3 (0.8 g) in water (3 ml) was added with stirring to a suspension of 3,4-dichloro-9-acetylaminoretene in glacial acetic acid (10 ml) at 40 °C. After 10 minutes the dark solution was diluted with water and the precipitated product collected, washed with water, and dissolved in hot ethanol containing *o*-phenylene diamine (ca. 0.1 g). On cooling, 3,4-dichlororetenequinoxaline (0.03 g) crystallised as pale-yellow needles, m. p. 132—133 °C. The yield was only ca. 7 %, calculated on the 3,4-dichloro-9-acetylaminoretene consumed. The quinoxaline was crystallised from *n*-propanol and then melted at 133—134 °C.

$C_{24}H_{18}Cl_2N_2$ (405.3)	Calc.	C	71.1	H	4.48	Cl	17.5	N 6.91
	Found	»	71.1	»	4.60	»	17.3	» 6.97

The quinoxaline dissolves in conc. sulphuric acid giving an intense red colour which disappears on dilution with water.

4-Chloro-9-nitroretene

3-Amino-4,9-dinitroretene (2.0 g) was diazotised as described above (C) and 20 ml of hypophosphorous acid (50 %) was added at room temperature. Nitrogen was evolved and a yellow product separated within a few minutes. When the reduction had proceeded

* About the name of this type of compound, see *Acta Chem. Scand.* **2** (1948) 487.

for one hour, the product (1.7 g, m. p. 125—127 °C) was filtered off, washed with dilute acetic acid, dissolved in a mixture of benzene (10 ml) and petroleum ether (10 ml, b. p. 40—50 °C) and adsorbed on a column of alumina (15 × 3.5 cm). Development with a mixture of 5 vol. of petroleum ether and 1 vol. of benzene gave a yellow band, which was eluted with the same solvent. The eluate was decolourised with charcoal and yielded 1.45 g of product of m. p. 130—131 °C. (Yield: 78 %, calculated on 3-amino-4,9-dinitroretene consumed). One crystallisation from *n*-propanol or glacial acetic acid gave pure 4-chloro-9-nitroretene as flat prisms, melting at 133—134 °C, easily soluble in benzene, acetone, and chloroform, and slightly soluble in ligroin and ethanol.

$C_{18}H_{16}ClNO_2$ (313.8)	Calc.	C 68.9	H 5.14	Cl 11.30	N 4.46
	Found	» 68.8	» 5.16	» 11.1	» 4.44

3-Hydroxy-4-chloro-9-nitroretene

3-Amino-4,9-dinitroretene (1.0 g) was diazotised as above (C). Water (5 ml) was added to the diazonium salt solution which was then heated on a boiling water-bath for four hours. More water was then added to the reaction mixture to enssure complete precipitation of the 3-hydroxy-4-chloro-9-nitroretene formed in the reaction. The crude product was dissolved in benzene and adsorbed on a column of alumina (15 × 3.5 cm). Development with benzene gave a sharp red band. Washing with acetone removed some impurities, but the main band was not eluted, although it turned pale yellow in colour. The band was eluted with acetone containing a few per cent of conc. hydrochloric acid, the eluant diluted with water and extracted with ether. Evaporation of the washed ether solution gave a crystalline residue (0.30 g) of almost pure 3-hydroxy-4-chloro-9-nitroretene (m. p. 166—167 °C; yield 32 %). The product was further purified by crystallisation from ligroin-benzene and then formed yellow prisms melting at 170—171 °C, easily soluble in most organic solvents

$C_{18}H_{16}ClNO_3$ (329.8)	Calc.	C 65.6	H 4.89	Cl 10.7	N 4.25
	Found	» 65.5	» 5.01	» 10.6	» 4.32

An alcoholic solution of 3-hydroxy-4-chloro-9-nitroretene turns blood-red on the addition of aqueous alkali. Despite its low solubility in water, the compound can be used as an acid-base indicator. By adding the alcoholic solution to buffers (sodium hydroxide-boric acid) of different pH the interval for the colour change was determined to be pH 8.5—9.5.

3-Acetoxy-4-chloro-9-nitroretene was prepared by refluxing a solution of 3-hydroxy-4-chloro-9-nitroretene (0.1 g), acetic anhydride (2 ml), and sodium acetate (0.1 g) for one hour. On cooling, the acetyl derivative separated as nearly colourless, very thin, short, needles melting at 136—137 °C. Crystallisation from glacial acetic acid yielded the pure compound, m. p. 137—138 °C.

$C_{20}H_{18}ClNO_4$ (371.8)	Calc.	C 64.6	H 4.88
	Found	» 64.6	» 4.92

SUMMARY

A new reaction for *o*-nitro-amines has been investigated and discussed. This reaction, termed in the present paper intramolecular diazotisation, consists the conversion of an *o*-nitro-amine to a chlorine substituted diazonium chloride by treatment with concentrated hydrochloric acid alone. For a closer study of the reaction, 3-amino-4,9-dinitroretene was used, and the product, 4-chloro-9-nitroretene-3-diazonium chloride, was characterised by the formation of several derivatives. The reaction was demonstrated to be applicable to 1-nitro-2-naphthylamine and 3-amino-4-nitroretene. *o*-Nitro-amines of the benzene series do not appear to undergo this reaction.

I wish to express my sincere thanks to Professor Karl Myrbäck for valuable discussions of the problems that have arisen in connexion with the work, and to *Stiftelsen Bokförlaget Natur och Kultur* for financial support.

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The Addition Product of 4,4′-Dimethoxydiquinone and Hydrogen Chloride

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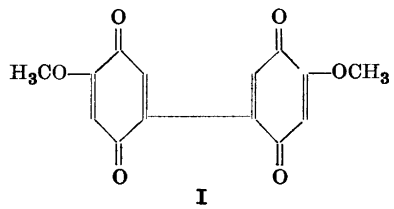
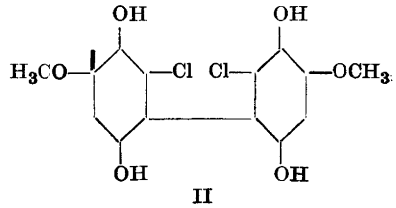
By the addition of hydrogen chloride to 4,4′-dimethoxydiquinone (I) Erdtman [1] obtained a substance possessing the expected composition (*i. e.*, addition of two moles of hydrogen chloride occurred) but very unexpected properties. On attempted recrystallization it decomposed into the original quinone and hydrogen chloride. On acetylation the addition product yielded a diacetate, not a tetraacetate as would be expected if it were dichloro-4,4′-dimethoxydiquinol (II).

Erdtman considered that the chlorine atoms entered the 6,6′-positions, an assumption which, although originally based on an analogy that was later shown to be invalid [2], is nevertheless supported by the Thiele acetylation of 4,4′-dimethoxydiquinone [3].



I



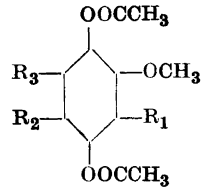
II

The addition product has now been methylated with diazomethane, and, by this reaction also, only two hydroxyl groups could be detected.

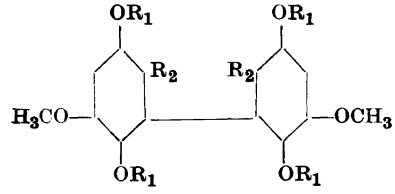
The instability of the addition product is not inconsistent with the diquinol structure (II), since Asp and Lindberg [4] have shown that another quinol, monobromo-2,6-dimethoxyquinol is very unstable and easily decomposes into 2,6-dimethoxyquinone and hydrogen bromide. The fact that only two hydroxyl groups can be acetylated or methylated, however, is not easily understandable on the basis of this structure.

ULTRAVIOLET ABSORPTION DETERMINATIONS

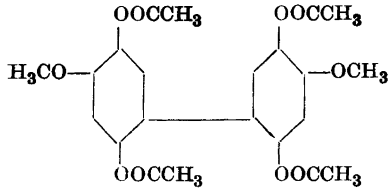
In order to investigate the structure of the addition product, the ultraviolet absorption spectra of this substance and a number of potentially related phenyl and diphenyl derivatives have been examined. The results are summarized in Table 1 and four typical absorption curves are given in Fig. 1.



III $R_1 = R_2 = R_3 = H$
IV $R_1 = Cl$, $R_2 = R_3 = H$
V $R_2 = Cl$, $R_1 = R_3 = H$
VI $R_3 = Cl$, $R_1 = R_2 = H$



VII $R_1 = CH_3$, $R_2 = H$
VIII $R_1 = OCCH_3$, $R_2 = H$
IX $R_1 = CH_3$ $R_2 = Br$



X

XI Diacetate of addition product

XII Dimethyl ether of addition product

Table 1. Ultraviolet absorption spectra. All determinations in absolute ethanol solution.

Substance	Maximum	Inflexion	Minimum
III	272 mμ		247 mμ
IV	270		248
V	280		250
VI	275		250
VII	286		263
VIII	278		261
IX	292		263
X	246	273 mμ	234
XI	234, 288	248, 315	223, 274
XII	237, 295	253, 320	225, 275

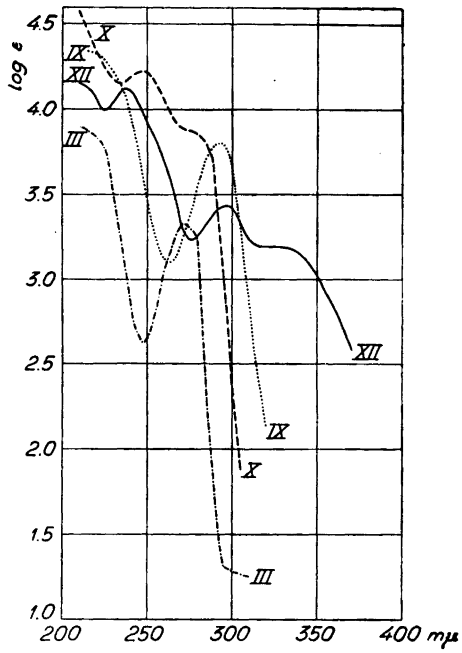


Fig. 1. Ultraviolet absorption spectra for methoxyquinol diacetate (III), 2.2′ di. bromo 3,5,6,3′,5′,6′,hexamethoxydiphenyl (IX), 4,4′-dimethoxydiquinol tetraacetate (X) and the dimethyl ether of the addition product (XII). All determinations in absolute ethanol.

The spectra of III, IV, V and VI are very similar and only that of III is given in Fig. 1. For the same reason, IX has been chosen as representative for VII, VIII and IX, and XII for XI and XII.

Both from Table 1 and Fig. 1 it is evident that the substances XI and XII cannot be aromatic. The spectra do not correspond at all with those of the diphenyls (VII—X), in particular the high absorption at longer wavelengths (log $\varepsilon = 3$ at 350 mμ) has no counterpart at all in the latter.

INFRARED ABSORPTION DETERMINATIONS

In order to confirm the foregoing evidence of the non-aromatic nature of the addition product, the infrared absorption spectrum of its dimethyl ether has been determined (Fig. 2). No hydroxyl band (3400—3800 cm^{-1}) was found but a strong band at 1683 cm^{-1} was present. The region 1660—1830 cm^{-1} is typical for the C = O group absorption band and no other groups are known to appear in this region [5]. For ketones the C = O absorption band lies between 1670 and 1720 cm^{-1}. As the diquinol structure involves two hydroxyl groups but no C = O groups, the infrared spectrum furnishes conclusive evidence against it.

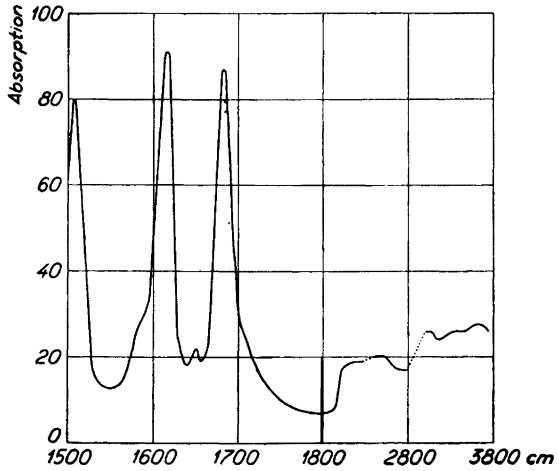
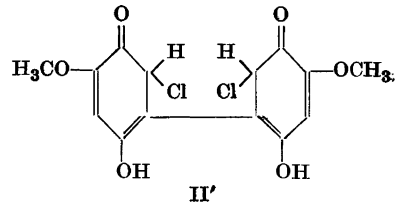


Fig. 2. Infrared absorption spectrum of the dimethyl ether of the addition product.

DISCUSSION

The ultraviolet and infrared absorption spectra show that the product obtained when hydrogen chloride is added to 4,4′-dimethoxydiquinone, or at least the diacetate and the dimethyl ether of this addition product, is non-aromatic and contains carbonyl groups. Different formulas can be suggested fulfilling these requirements, of which structure II′ seems to be the most probable. The six conjugated double bonds will render this structure stable compared with the other non-aromatic isomers. A hydrogen shift would transform this substance into the diquinol (II) which, by analogy with the previously mentioned monobromo-2,6-dimethoxyquinol, would probably not be stable in the presence of acids. The non-aromatic substance (II′), on the other hand, is stable towards acids but very unstable towards bases, as the structure requires.



II′

EXPERIMENTAL

The addition product and its diacetate were prepared according to Erdtman [1]. The preparation of the substances III—VI has been described elsewhere [6]. The substances VII—X were Erdtman's original specimens.

Dimethyl ether of the addition product

To the addition product (2 g) in a solution of acetone (300 ml) was added diazomethane, prepared from nitrosomethylurea (10 g), in ether (150 ml). After 24 hours a yellow

material which had separated was removed by filtration and the solution then concentrated to dryness under reduced pressure. The residue was dissolved in ether (500 ml) and the solution washed with 10 % potassium hydroxide (3 × 50 ml), which as a result turned dark red in colour. The ether solution was then washed three times with water, dried over calcium chloride and concentrated to a small volume (30 ml). Slightly yellow crystals of m. p. 210—211° (uncorr.) were precipitated during the concentration. After one recrystallization from acetic acid-water, 1 : 1, these crystals were almost colorless and had m. p. 214—215°. Yield, 350 mg. For analysis and spectrophotometric determinations the substance was recrystallized four times further from the same solvent. The melting point was unchanged by these recrystallizations.

$C_{16}H_{16}O_6Cl_2$ (375.2)	Calc.	C	51.2	H	4.30	Cl	18.9	OCH_3 33.1
	Found	»	51.2	»	4.44	»	19.0	» 33.2

The ultraviolet absorption determinations were carried out with a Beckman Model DU spectrophotometer. All determinations were made in absolute ethanol.

The infrared absorption determinations were carried out with a Perkin Elmer infrared spectrometer, Model 12 B, with a 60° rock salt prism, using the paraffin oil paste method.

SUMMARY

The dimethyl ether of the addition product of hydrogen chloride and 4,4'-dimethoxydiquinone has been prepared. The ultraviolet spectra of this substance, the corresponding diacetate and a number of related benzene and diphenyl derivatives have been investigated. The infrared spectrum of the dimethyl ether has also been investigated. On the basis of these experiments, a non-aromatic structure for the addition product has been suggested.

The author wishes to thank Professor H. Erdtman of this Institute for suggesting this work and for valuable discussions. His thanks are also due to Professor H. Theorell, Medicinska Nobelinstitutet, Stockholm, for placing facilities for the infrared absorption determination at his disposal and to Dr. K-G. Paul of the same Institute, for a discussion concerning the infrared spectrum.

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On the Sensitivity of Direct Spectral Analysis of Solutions

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During an investigation of trace elements in Finnish ground- and mine waters, the author has attempted to find the most suitable method with regard to sensitivity, accuracy and speed using the available apparatus. It is evident that, when numerous trace elements, as many as possible, are to be determined, the spectral analysis has all advantages when compared *e. g.* with the most frequently used colorimetric methods. The spectral method chosen by the author for direct analysis of dilute solutions and a study concerning the sensitivity obtained by this method compared with other methods will be presented in this paper.

Since the classical publication of Lundegårdh [1] much research work has been made to find out a suitable method for direct spectral analysis of solutions. Several sources of excitation have been experimented. On the basis of the pioneering work of Lundegårdh on the flame spectrographic analysis, many, especially European scientists have used the flame exctitation either alone or combined with spark, as did Bouchetal de la Roche [2]. Of American investigations those of Cholak and Hubbard [3] on spectral analysis with the air-acetylene flame and the recently published work of Gilbert, Hawes and Beckman [4] on the use of Beckman flame spectrophotometer are to be mentioned. The modern flame photometry is the best technique for many purposes, but the chief limitation of this method is that several important elements either cannot be excitated by the flame or have too weak an emission, consequently cannot be determined or have a very low sensitivity.

Thus, the analysis of solutions by pure spectrographic means has further been of continued interest. Many investigators have not carried out the analysis in liquid phase. Fred, Nachtrieb and Tomkins [5] and many others dried drops of solution on the electrodes before excitation. Sloviter and Sitkin [6] impregnated the electrodes with the solution to be analysed and Roux and Husson [7] determined boron by impregnating a cotton pad with the solu-

tion and burning this between the electrodes. Duffendack, Wolfe and Smith [8] and later Duffendack, Wiley and Owens [9] instead of drying used excitation between a solid electrode and the free surface of a liquid, and this procedure has won many supporters. Lundegårdh (*l.c.*) proposed, according to Necke the continous feeding of solution into the analytical gap through a hollow upper electrode, and this method has been further developed by Keirs and Englis [10]. A spark excitation is used between two liquid surfaces in the "dropping electrode" of Lomakin [11] and on the other hand between a solid electrode and a thin film of solution in the method of Rohner [12]. Already Hartley [13] produced the spark from a thin liquid film by taking advantage of the capillarity.

None of these methods seemed to have the characteristics required for the work at hand. The residue methods, even though sensitive enough, were not convenient for very dilute solutions, as the nature waters are, and the continous feeding method lacked the simplicity and sensitivity desired. The "Porous Cup Electrode Technique", a very simple and ingenious continous feeding method by Feldman [14], however, seemed to fulfill all reasonable requirements. The sensitivities given by Feldman were in most cases rather high, in some cases, however, striking low. Therefore, the sensitivity of this technique was at first studied by means of the available apparatus.

Feldman (*l. c.*) uses as the upper electrode a spectrographic graphite rod 0.6 cm in diameter and 3.75 cm in length, drilled along the axis with 0.125-inch drill from one end to within 1.1 ± 0.2 mm from the other end adjusting the drill press with a special jig. The lower electrode is a graphite rod, 0.125 inch in diameter with a flat or pointed end. The solution to be analysed is inserted into the upper electrode by means of a long-nosed pipette and feeds to the analytical gap trough the intact bottom of the rod. There it is dispersed and excited by the high-voltage spark from a Baird spark source with a synchronous interrupter, or by a low-voltage intermittent arc. A single filling, of about 0.32 ml, will permit an exposure of 120 to 240 seconds. Usually exposures of 180 seconds are made. A 5—10 second pre-spark period followed by a rest period is proposed. Sensitivity limits for 45 elements in 10 % sulfuric acid solution (Pb, Ba and Sr in 3 % nitric acid) are given. A preferential adsorption of trace elements by the graphite is not detected. Duplicate samples have given intensity ratios agreeing to within 2 to 5 % of their mean. The analytical accuracy has not yet been systematically estimated by independent methods. No noticeable vertical variation of intensity in the source is observed.

EXPERIMENTAL

English „Ship Carbons", 7 mm in diameter, were used as electrode material. The carbon rods were ignited according to the usual procedure of our spectrographical laboratory 75 seconds by a current of 300 amperes in air to remove impurities. After this treatment the carbons were 6 mm in diameter, very porous and still contained as contaminations much boron, thus indeterminable by means of these electrodes, and traces of Mg, Ca, Si and Ti, which therefore are not considered in this work. The electrodes were like those of Feldman, only the lower end of the drilling was not conical as usually made by a drill, but flat. The purpose of this was to obtain a more equal feeding of solution trough the bottom. A special drill press with a jig was not needed, but the electrodes could be made with a sufficient speed in a lathe by a 3 mm drill, fitted with a steel collar equipped with a set screw at a distance of 3.65 cm from the point of the drill. A flat-pointed carbon rod 3 mm in diameter at the point was used as the lower electrode.

Dilution standards of the elements were made by using reagent grade elements or compounds. As solvent 3 % nitric acid, purified by distillation, was used in most cases, only few elements were analysed in 10 % sulfuric acid solution. In contrary to Feldman the observation was made that a nitric acid solution feeds to the analytical gap much better than a solution in 10 % sulfuric acid, which had in many cases a too weak wetting power, which could be helped only by a wetting agent.

The commercial type of Feussner spark source, made by Heraeus GmbH, Hanau, was used with synchronous interrupter and a resistance of 40 ohms. The full capacitance, 3.33×10^{-3} μf and the inductance marked 1/10 giving the value 8×10^{-6} henry were used. As it can be seen, the most sparklike conditions were effected in order to obtain particularly the spectra of singly ionized atoms.

A Zeiss Qu 24 Spectrograph with a useful wavelength range from 2 200 to 4 600 Å was used. It had a pre-slit optical system consisting of a condenser $f = 80$ cm, a field aperture with horizontal slits of different widths and a lens $f = 160$ cm which formed an image of the condenser on the slit of the spectrograph. The optical system was set sharp for 3 000 Å and left fixed. A slit width of 0.025 mm was used. The exposure time was 180 seconds after a 15-second pre-spark period, the analytical gap 2 mm. Kodak Scientific Plates III-O were used, developed in a 1 : 2 diluted Kodak D-19b developer 3 minutes at a temperature of 18° C, rinsed in a stop bath, fixed in Kodak F-5 fixing bath, washed and dried in air.

The sensitivity limits for different elements are given in Table 1, in the first sensitivity column those of the author, in the second are the figures of Feldman [14], thereafter the values of Cholak and Hubbard [3] by the air-acetylene flame method and in the last column those by Gilbert et alii [4] by Beckman flame photometer.

Thus all elements investigated by the author have been detected in a concentration below 100 ppm, and a great majority has the detection limit 10 ppm or even less. The flame photometer is much more sensitive when alkalies are to be determined, in all other cases the PCE-technique either equals to or exceeds the flame photometry in sensitivity.

DISCUSSION

When comparing the Raies Ultimes of different elements obtained with this technique with those collected by Meggers [16] in a table comprising the

Table 1. Sensitivity limits for different elements in the direct spectral analysis of solutions.

Element	Raie ultime *			Approximative sensitivity ppm			
				PCE-technique		Flame excitation	
	λ	Classification	Total excitation potential	Author	Feldman	Cholak	Gilbert
Ag	3280 . 683	I	3.8	0.02	1	5	2
Al	3961 . 527	I	3.1	0.5	1		No emission
As	2349 . 84	I	6.6	3			
	2288 . 12	I	6.7	3			
	2780 . 197	I	6.7	10	100		
Au	2427 . 95	I	5.1	30			
	2675 . 95	I	4.6	100	100		
B	2497 . 733	I			0.5		5
Ba	2335 . 269	II	11.2	0.5		100	1
	2304 . 235	II	11.2	0.5			
	4130 . 664	II	10.9	4	50		
Be	3130 . 416	II	13.2	0.02			
	2348 . 610	II	5.4	0.02			
	3131 . 072	II	13.2	0.05	0.02		
Bi	3067 . 716	I	4.0	1	5		
Cb	3094 . 183	II	8.0		5		
Cd	2265 . 017	II	14.4	0.2	100	200	500
	2288 . 018	I	5.4	0.2			
Ce	4012 . 388	I	> 8.7	10			
	3942 . 736	II		35	25		
Co	2286 . 156	II	14.3	0.5		1	5
	2363 . 787	II	14.2	0.5			
	3453 . 505	I	4.0	2	2		
Cr	2835 . 633	II	12.6	0.3		0.5	3
	2843 . 252	II	12.6	0.3	2		
Cu	3247 . 540	I	3.8	0.2	0.6	0.5	f
Cs	8521 . 10	I	1.4		15	50	0.1
Fe	2382 . 039	II	13.0	0.5		5	10
	2395 . 625	II	13.0	0.5			
	2599 . 396	II	13.0	0.5	2.5		
Ga	2943 . 637	I	4.3	10	10		1
	4032 . 982	I	3.1	10	10		
Ge	3039 . 064	I	4.9		10		
Hf	2820 . 224	II	9.2		4		
Hg	2536 . 519	I	4.9	10	50	200	50
K	4044 . 140	I	3.1		200	8	0.05

* Data according to Harrison [15]. In flame methods these lines are not necessarily used.

Table 1 (cont.)

In	3256 · 090	I	4.1		10		1
La	3949 · 106	II	9.1	1	5		
Li	3232 · 61	I	3.9	10		0.1	0.05
	6707 · 844	I	1.8		0.1		
Mg	2795 · 53	II	12.0		0.01	5	10
Mn	2576 · 104	II	12.2	0.02		0.3	1
	2593 · 729	II	12.2	0.05	2		
Mo	2816 · 154	II	11.9	1			
Na	3302 · 323	I	3.7	35	35	0.2	0.01
Ni	2287 · 084	II	14.8	4		10	3
	3414 · 765	II	14.2	4	10		
P	2535 · 65	I	7.2	5	80		No emission
Pb	2833 · 069	I	4.4	4	10	100	300
Pt	2659 · 454	I	4.6	1	100		
Pd	3404 · 580	I	4.4		2		50
Re	3460 · 47	I	3.6		10		
Ru	3498 · 942	I	3.5		100		30
Sb	2598 · 062	I	5.8	3	100		No emission
Sn	2839 · 989	I	4.8	5	100		500
Sr	4077 · 714	II	8.7	0.2	0.5	0.2	0.5
Te	2385 · 76	I	5.8	10	1 000		
Th	3538 · 75		> 3.5	35			
	3290 · 59		> 7.31		100		
Ti	3349 · 035	II	11.1		3		
Tl	2767 · 87	I	4.5	3		0.4	1
V	3033 · 822	II		0.3			
	3093 · 108	II	11.2	1	5		
W	2397 · 091	II	> 10.9	3			
	4008 · 753	I	3.4	30	500		
Y	3710 · 290	II	10.0		0.1		
Zn	3345 · 020	I	7.8	4	25	3 000	No emission
Zr	3391 · 975	II	10.7	1			
	3273 · 047	II		3	2		

strongest lines of the second spectra of elements, it can be seen that for all elements having their strongest lines of the second spectra in the spectral region between 2 200 and 4 600 Å, the Raies Ultimes in this technique always are lines of the second spectra, except Ag and Pd. This exception may be explained by the very high excitation potentials of the strongest lines (Ag II 2246.41 21.9 Volts, Pd II 2296.53 19.8 Volts). The Raies Ultimes

Table 2. The strongest lines of Pb, Pt, Mo and V by the PCE-technique.

Element	Line *			Sensitivity ppm	Intensity *	
	λ	Classification	Total Excitation Potential		Arc	Spark
Pb	2833 · 069	I	4.4	4	500R	80R
	4057 · 820	I	4.4	4	2 000R	300R
	2203 · 505	II	14.7	10	50	5 000R
	2614 · 178	I	4.7	10	200R	80
	3639 · 580	I	4.4	10	300	50
Pt	2659 · 454	I	4.6	1	2 000R	500R
	2997 · 967	I	4.2	3	1 000R	200
	2929 · 794	I	4.2	10	800R	200
	2830 · 295	I	4.4	10	1 000R	600
	3064 · 712	I	4.0	35	2 000R	300
Mo	2816 · 154	II	11.9	1	200	300
	2848 · 232	II	11.8	3	125	200
	2890 · 994	II	11.7	10	30	50
	2871 · 508	II	11.7	10	100	100
	3902 · 963	I	3.2	10	1 000R	500R
V	2924 · 025	II		0.3	70R	300R
	3033 · 822	II		0.3	20	90R
	2924 · 644	II		1	60	200R
	3267 · 702	II		1	30	80R
	3271 · 125	II		1	25	50R
	3276 · 124	II		1	50	200R
	3093 · 108	II	11.2	1	100R	400R
	3102 · 299	II	11.1	1	70	300R
	3183 · 406	I	3.9	10	200R	100R
	3183 · 982	I	3.9	10	500R	400R
	3185 · 396	I	3.9	10	500R	400R

* Harrison [15].

of all other elements are lines of the first spectra. Two elements, Be and Cd, have equally sensitive lines both of the first and of the second spectra.

The relative intensity order of lines in many cases differs from the data given in wavelength tables. The elements having a line of the first spectrum as Raie Ultime are more regular, *i. e.* the intensity order is mostly equal to data given by Harrison [15] for arc excitation. Lead and platinum, however, show exceptionally great differences, as may be seen in Table II. The elements,

the strongest lines of which arise from singly ionized atoms are very irregular in this respect. The intensities in general follow the spark excitation data in wavelength tables, but many deviations were observed. As examples. the values of molybdenum and vanadium are given in Table II.

All these phenomena find their explanation in the special excitation technique. When the excitation conditions are altered, great changes in the appearance of spark and spectra will be observed. When the conditions are made more arclike by increasing the inductance, the pink colour of the spark, due according to Feldman (*l. c.*) to the H_α-line, almost disappears and the sparking tone becomes weaker. The spectrum lacks many bands and lines caused by carbon or air, and the background effect is weakened. On the other side, the sensitivity, especially of elements, the Raies Ultimes of which belong to the second spectra, is largely decreased. It is probable that the differences observed in different investigations are caused by different excitation conditions.

The strongest lines are necessarily not the best working lines, as is well known, not even in small concentrations. They may be interfered by lines of other elements, and this is very important to keep in mind when using spectrograph with relatively small dispersion. For instance, the three strongest lines of phosphorus are seldom useful, because iron and manganese interfere. The best lines of chromium are interfered, too. Because, as has been pointed out, the relative intensities of lines in this method are often different from the table values, it is always necessary to determine possible interferences experimentally. In this work, spectra were taken in all doubtfull cases of pure solutions of one element.

The application of this method to quantitative analysis of trace elements in nature waters will be presented in a furthcoming paper.

SUMMARY

The sensitivity limits of the Porous Cup Electrode technique by Feldman in spectral analysis of solutions were investigated using condensed spark excitation. The following sensitivities were obtained: 0.02 - 0.1 ppm Ag, Be, Mn; 0.1—1 ppm Al, Ba, Bi, Cd, Co, Cr, Cu, Fe, Mo, Pt, Sr, V, Zr; 1—10 ppm As, Ce, Ga, Hg, La, Li, Ni, P, Pb, Sb, Sn, Te, Tl, W, Zn; 10—30 ppm Au, Na, Th. It has been stated that the most sparklike excitation conditions produce the greatest sensitivity and that the relative intensities of lines are often different from those of wavelength tables. The PCE-technique was

found to be the best one in regard to detecting the most elements, only alkalies may be better determined by the flame excitation.

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Studies of the Constituents of Crassulaceous Plants

II. Paper Chromatographic Investigation of the Free Sugars of some *Rochea*, *Kalanchoë*, *Cotyledon*, *Aeonium*, *Monanthes*, *Rosularia*, *Altamiranoa*, and *Echeveria* Species

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In a previous communication [1] we reported the results of our paper chromatographic investigations of the free sugars of some *Sedum*, *Sempervivum*, *Echeveria*, and *Crassula* species. Thanks to the assistance of Professor Nannfeldt of the University of Uppsala, we have been able to extend the investigations to cover some species of other genera of the *Crassulaceae*, and 2 further species of the genus *Echeveria*.

We have used the same procedure as before, namely, direct chromatography of the juice pressed out from the plants. Ketoses were detected by means of a special reagent [2] which our experience has indicated produces a bluish-green colour only with ketoheptoses, and is, therefore, especially suited for the detection of these sugars in plants. Aldoses were detected by other reagents.

The results are given in Table 1. Sedoheptulose was detected in all the species investigated, and on most chromatograms the spot produced by this sugar was the predominating one. One or more fermentable sugars (glucose, fructose, sucrose) were also detected in all the species.

According to Richtmyer [3], sedoheptulose has been previously reported to be present in species of *Sedum*, *Bryophyllum*, and *Sempervivum*, and in a previous communication [1] we reported the presence of this sugar also in representatives of the genera *Echeveria* and *Crassula*. As we now have detected sedoheptulose in a further 7 genera, this sugar has been found to occur in species of 12 genera of the family *Crassulaceae*. According to Berger [4], this family is divided into 33 genera, in 5 subfamilies. All the subfamilies are represented in the 12 genera studied. According to Engler and Diels [5] 9 of these genera comprise

about 1 250 species of the family's total number of about 1 300. It may therefore be concluded that sedoheptulose occurs in species of the principal genera of the family *Crassulaceae* and is distributed throughout the family.

EXPERIMENTAL

Juices pressed from fresh plants were used for the experiments. If the juices were not to be used immediately, they were heated to boiling in order to destroy the enzymes. It was impossible to press out any juice from *Monanthes atlantica* because of its morphological structure. The plant was therefore extracted with ethanol, and this extract used after concentration and filtration. About 0.01—0.02 ml of the juice was placed on the paper (Whatman No. 1). The solvent used was ethyl acetate — acetic acid — water (3 : 1 : 3)[6]. The paper was equilibrated with the solvent atmosphere overnight, then the chromatogram was run for 36—48 hours, during which time the solvent ran off the lower edge of the paper. R/-values were therefore unobtainable, but sedoheptulose, fructose, glucose, and sucrose standards were run each time (0.005—0.01 ml of approximately 1 % solution).

As spraying reagents we used:

1. Orcinol and trichloracetic acid in *n*-butanol [2], for the detection of ketoses.
2. Aniline hydrogen phthalate in *n*-butanol [7], and
3. *m*-Phenylenediamine in aqueous ethanol [8], the latter two for the detection of both aldoses and ketoses.

On some occasions when we attempted to demonstrate the presence of glucose, we obtained a long, white trail, which rendered this impossible. This is most likely due to the presence of too high a concentration of salts, as these juices had been concentrated to a rather large extent.

Table 1. Free sugars of some Crassulaceous plants, detected by means of paper partition chromatography. The sugars have not been determined quantitatively, but from the size of the spots and the intensity of the colours we have estimated the quantitative relationship. The predominating sugar is denoted by ++, very weak spots by (+). The genera are arranged in the order described by Berger [4], and the nomenclature follows Berger [4] and Praeger [9].

Species	Sedoheptulose	Glucose	Fructose	Sucrose
Rochea coccinia (L.) DC.	++	+	+	(+)
R. falcata Wendl.	+	+	+	+
Kalanchoë Hildebrandtii Baill.	+	+	++	
K. marmorata Bak.	++		+	(+)
Cotyledon coccinia Cav.	++	(+)	(+)	(+)
Aeonium cuneatum Webb et Berth.	++	+	+	+
A. Haworthii Webb et Berth.	+		+	+
Monanthes atlantica Dall.	+	+	+	+
Rosularia pallida Stapf.	++			+
Altamiranoa elongata Rose	++		+	
Echeveria Scheideckiri X (L.) De Smet.	++		+	(+)
E. scaphylla X Deleuil	++		+	+

SUMMARY

The free sugars of the juice pressed out from some Crassulaceons plants have been studied by means of paper partition chromatography. All the extracts contained sedoheptulose and fermentable sugars. Sedoheptulose has now been shown to occur in all the 5 subfamilies of the family *Crassulaceae*.

The authors are indebted to Professor Nannfeldt, University of Uppsala, for his generous aid in supplying the species referred to above, and to Mrs. G. Knaben, University of Oslo, for assistance in connection with the taxonomy and nomenclature. Thanks are also due to Norsk Varekrigsforsikrings Fond A/S for financial support.

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Retene Investigations

XIX. The Structure of 2-Hydroxyretene (2-Retenol)

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A- and B-hydroxyretene, prepared by E. Wahlforss [1] in 1924, were the first known monosubstituted retene derivatives. As a result of physico-chemical investigations, Fieser and Young [2] considered it probable that in B-hydroxyretene the hydroxyl group occupied the 3- or the 6-position and more recently Campbell and Todd [3] and Karrman [4], have by pure chemical methods decided in favour of the 3-position.

With regard to the structure of A-hydroxyretene, Fieser and Young decided that the hydroxyl group must be in the 2-position. This conclusion was based on the following two observations. A-Hydroxyretene does not couple with diazotized amines and according to Fieser and Young this indicates that the hydroxyl group occupies the 2- or the 8-position. The 8-position is ruled out on the basis of the Dimroth test for α-hydroxyquinones, as 8-hydroxyretene quinone should give a colour reaction with boroacetic anhydride and A-hydroxyretene quinone does not give this reaction.

Fieser and Young have also investigated the reduction potentials of a number of different mono- and dihydroxy derivatives of phenanthraquinone and compared these with the reduction potential of A-hydroxyretene quinone. From this comparison they again concluded that A-hydroxyretene probably had the hydroxyl group in the 2-position.

In the present communication evidence is presented to show that A-hydroxyretene is indeed 2-hydroxyretene. 9,10-Dihydroretene was nitrated to a mononitro derivative [5], known to have the nitro group in the same position as the hydroxyl group of A-hydroxyretene [5]. The nitrodihydroretene was reduced with tin and hydrochloric acid and the resulting amino compound was acetylated. The resulting acetylamino-9,10-dihydroretene was nitrated, giving a faint yellow mononitro derivative in almost quantitative yield. The acetyl

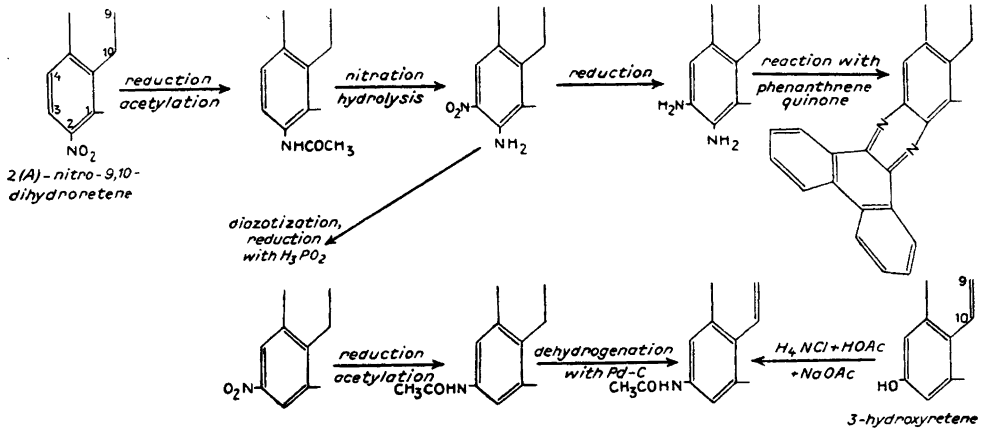

Fig. 1. Reactions showing that the A-position is adjacent to the 3-position.

group was removed and the nitro group reduced, thus producing a diamino-9,10-dihydroretene. This substance was dissolved in hot glacial acetic acid and a hot solution of phenanthraquinone in the same solvent was added. Immediatly a thick, straw-yellow precipitate of the quinoxaline was formed, thus showing that the two amino groups are situated in ortho position to one another (Fig. 1). (If retene quinone is used instead of phenanthraquinone the quinoxaline obtained is not homogeneous but a mixture of the two possible isomers, which can be separated from one another only with difficulty.)

It has thus been proved that on nitration of acet amino-9,10-dihydroretene, the nitro group enters the position ortho to the acet amino group. This nitro derivative was hydrolysed with hydrochloric acid in boiling ethanol, the corresponding ortho nitroamine being obtained as red crystals (the hydrochloride was not formed). The amine was diazotized in dilute acetic acid and the resulting diazonium salt reduced with hypophosphorous acid. A mononitro-9,10-dihydroretene was thus obtained as pale yellow crystals, which on oxidation with chromic acid in glacial acetic acid gave a nitroretene quinone. The nitrodihydroretene was reduced with tin and hydrochloric acid and the resulting amine acetylated with acetic anhydride, giving a monoacetylamino compound. Dehydrogenation by means of palladium-charcoal gave 3-acetylaminoretene m. p. 240—241° C, which was hydrolysed to 3-aminoretene, m. p. 139—140° C.

Thus it is clear that the hydroxyl group in A-hydroxyretene must be situated adjacent to the 3-position, *i. e.* in the 2- or the 4-position (Fig. 1).

The position of the methyl group in 4-methylretene is established by the syntheses of Haworth *et al.*[6] and on account of this, we have been able to decide

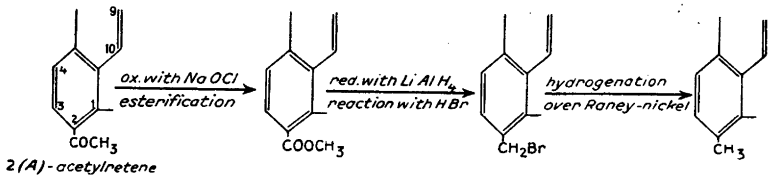

Fig. 2. Reactions leading to A-methylretene, not identical with 4-methylretene.

between 2- and 4-hydroxyretene as follows. A-Acetylretene, prepared by the method of Hakon Lund [7], has been oxidized to the corresponding carboxylic acid and the corresponding methyl ester reduced with lithium aluminum hydride in ether, giving A-hydroxymethylretene in good yield. This substance was treated with hydrobromic acid, giving A-bromomethylretene, which was hydrogenated over Raney nickel at a moderate hydrogen pressure, producing A-methylretene (Fig. 2). This substance is not identical with 4-methylretene as is shown in Table 1 and thus the hydroxyl group in A-hydroxyretene must be situated in the 2-position.

Table 1. Comparison of 2 (A)- and 4-methylretene.

Substance	M. p.	Picrate m. p.	Styphnate m. p.
2 (A)-Methylretene	122.5—123.5°	139.0—139.5°	158—159°
4-Methylretene	60.5— 61.5°	114.5—115°	142—143°

EXPERIMENTAL

2-Acetylamino-3-nitro-9,10-dihydroretene. To 0.38 g of 2-acetylaminodihydroretene, suspended in 7 ml of glacial acetic acid was added a mixture of 2 ml of concentrated nitric acid and 3 ml of glacial acetic acid. The temperature rose from 21° to 25° C and a clear, yellow solution was obtained. After 30 min. excess of water was added and the precipitate formed (0.42 g) was filtered off, dried and recrystallized from toluene, forming pale yellow crystals (0.31 g) of m. p. 228—230° C.

$C_{20}H_{22}O_3N_2$ (338.2)	Calcd.	C 71.0	H 6.56	N 8.28
	Found	» 71.3	» 6.44	» 8.22

2-Amino-3-nitro-9,10-dihydroretene was obtained by boiling a solution of 1.1 g of the above acetyl compound in 15 ml of propanol and 3 ml of concentrated hydrochloric acid for twenty four hours. On cooling, bright-red crystals of the free amine were obtai-

ned, 0.75 g, m. p. 142—143° C. The slow rate of hydrolysis of the acetyl compound is probably due to the presence of substituents in both ortho positions.

$C_{18}H_{20}O_2N_2$ (296.2)	Calcd.	C 72.9	H 6.82	N 9.45
	Found	» 72.9	» 6.63	» 9.37

2,3-Diamino-9,10-dihydroretene. 0.65 g of the 2-amino-3-nitro compound was dissolved in 20 ml of ethanol. 2 g of zinc dust and 2—3 ml of concentrated hydrochloric acid were added and the solution was boiled for 15 hours during which time the red solution gradually turned lighter and was finally almost colourless. The mixture was cooled, water was added, and the resulting colourless precipitate, collected, dried and recrystallized from 35 ml of ethanol, giving 0.41 g of white plates, m. p. 195—197° C.

$C_{18}H_{22}N_2$ (266.2)	Calcd.	C 81.1	H 8.34	N 10.5
	Found	» 81.1	» 8.28	» 10.6

Quinoxalines. To 0.02 g of the diamine in 2 ml of hot glacial acetic acid was added 0.02 g of retene quinone in 2 ml of hot glacial acetic acid. A straw-yellow precipitate (0.35 g) was formed immediately but its melting point was not sharp and the product was probably a mixture of the two possible isomers, as the analysis gave the expected result.

$C_{36}H_{34}N_2$ (494.3)	Calcd.	C 87.4	H 6.94	N 5.66
	Found	» 87.2	» 6.81	» 5.72

When the reaction was repeated with phenanthraquinone, a straw yellow precipitate was obtained.

This was extremely insoluble, but had a sharp melting point, 258—259° C. On account of the symmetry of phenanthraquinone only one isomer can be formed.

$C_{32}H_{26}N_2$ (438.2)	Calcd.	C 87.6	H 5.98	N 6.39
	Found	» 88.0	» 5.77	» 6.31

3-Nitro-9,10-dihydroretene. 2-Amino-3-nitrodihydroretene (0.4 g) was suspended in 30 ml of glacial acetic acid and 9 ml of dilute sulfuric acid (1 : 3) was added. The mixture was cooled to 4° C and 0.4 g of sodium nitrite in 5 ml of water was added with stirring. A clear solution was formed. One hour later 15 ml of 30 % hypophosphorous acid was added and the solution was left in an ice-box for two days, then neutralised with sodium hydroxide and extracted with ether. The ether solution was dried with anhydrous sodium sulphate and evaporated, giving a dark oil which was diluted with hexane and filtered through aluminium oxide (Brockmann). The hexane solution was evaporated giving 0.15 g of pale yellow crystals. Recrystallisation from 5 ml of ethanol gave the pure compound, melting at 88—89° C.

$C_{18}H_{19}O_2N$ (281.1)	Calcd.	C 76.8	H 6.81	N 4.98
	Found	» 76.4	» 6.66	» 5.02

3-Nitroretene quinone. 0.3 g of chromic acid was added to 0.1 g of 3-nitro-dihydroretene in 2 ml of glacial acetic acid and the mixture was heated at 90° C for half an hour,

then cooled and diluted with water. The yellow solid precipitated (0.06 g) was recrystallized from 4 ml of acetic acid, giving orange needles (0.038 g), which did not melt below 280° C.

$C_{18}H_{15}O_4N$ (309.1)	Calcd.	C 69.9	H 4.89
	Found	» 70.1	» 4.76

3-Amino-9,10-dihydroretene. To 0.19 g of 3-nitrodihydroretene in 25 ml of ethanol was added 2 ml of concentrated hydrochloric acid and ca 2 g of granulated tin. The mixture was boiled for half an hour by which time it was almost colourless, then water was added and the precipitate (0.17 g) filtered off and dried. This hydrochloride was treated with sodium hydroxide and extracted with ether. The ether was evaporated, and the residue 0.14 g, taken up in 30 ml of benzene. The solution was treated with charcoal, concentrated to 10 ml, and left in an ice-box overnight, giving 0.046 g of transparent plates, m. p. 111—112° C.

$C_{18}H_{21}N$ (251.2)	Calcd.	N 5.57
	Found	» 5.54

3-Acetylamino-9,10-dihydroretene, was obtained by acetylation of the above amine with acetic anhydride. Recrystallization from ethanol gave colourless crystals, melting at 228—229° C.

$C_{20}H_{23}ON$ (293.2)	Calcd.	C 81.9	H 7.91
	Found	» 81.6	» 7.82

This acetylamino compound (0.020 g) was heated with palladiumcharcoal at 225—235° C for one hour during which time 1.2 ml of hydrogen was evolved. The reaction mixture was extracted with 5 ml of hot toluene, which on cooling deposited 0.011 g of a white substance melting at 240—241° C either alone or mixed with an authentic sample of 3-acetylaminoretene (m. p. 240—241° C). Hydrolysis of the acetyl compound gave the free amine, m. p. 139—140° C.

Methyl ester of retene-2-carboxylic acid. 7.0 g of retene-2-carboxylic acid, prepared as described by H. Lund [7] was boiled in 150 ml of methanol containing 10.5 g of concentrated sulfuric acid for 24 hours. After cooling and filtration, 6.6 g of the methyl ester was obtained, m. p. 174—175° C. Further recrystallization from ethanol raised the m. p. to 175—176° C.

$C_{20}H_{20}O_2$ (292.2)	Calcd.	C 82.2	H 6.90
	Found	» 82.4	» 6.82

The picrate, prepared from the components in ethanol formed yellow needles, m. p. 114—115° C. It is stable in solution only in the presence of an excess of picric acid.

$C_{26}H_{23}O_9N_3$ (521.2)	Calcd.	C 59.9	H 4.45
	Found	» 59.4	» 4.31

2-Hydroxymethylretene. 5.0 g of the methyl ester of retene-2-carboxylic acid was dissolved in 350 ml of dry ether, a solution of 1.0 g of lithium aluminum hydride in 600 ml of dry ether added, the mixture boiled gently for 30 min. Then cooled and treated with 600 ml of water added in small amounts. The ether solution was separated, dried with anhydrous sodium sulfate and evaporated to dryness, giving 3.9 g of product m. p. 166.5—168° C. This was recrystallized from 50 ml of ethanol and gave 2.8 g of pure material as transparent plates, m. p. 168.5—169.5° C.

$C_{19}H_{20}O$ (264.2)	Calcd.	C 86.3	H 7.63
	Found	» 86.7	» 7.79

The picrate, was obtained from the components in ethanol solution as yellow needles, m. p. 138—139° C.

$C_{25}H_{23}O_8N_3$ (493.2)	Calcd.	C 60.8	H 4.70
	Found	» 60.4	» 4.54

2-Acetoxymethylretene, was prepared from the corresponding hydroxymethyl compound and acetic anhydride. It formed white plates, m. p. 136.5—137.5° C.

$C_{21}H_{22}O_2$ (306.2)	Calcd.	C 82.3	H 7.24
	Found	» 82.0	» 7.11

2-Bromomethylretene. 1.5 g of 2-hydroxymethylretene was dissolved in 120 ml of glacial acetic acid and gaseous hydrobromic acid was passed into the solution for twenty minutes. The reaction mixture was then allowed to stand for six hours at 15° C. After filtration, washing and drying, the pure product obtained (1.52 g) melted at 175—176° C.

$C_{19}H_{19}Br$ (327.1)	Calcd.	C 69.7	H 5.85	Br 24.4
	Found	» 70.1	» 5.92	» 24.5

This product has also been obtained by passing gaseous hydrobromic acid into a solution of the carbinol in benzene, or by treating a boiling solution of the carbinol in carbon tetrachloride with phosphorous tribromide.

2-Ethoxymethylretene, was obtained by boiling the bromomethyl compound with ethanol for twenty minutes. It formed transparent plates, m. p. 89—90° C.

$C_{21}H_{24}O$ (292.2)	Calcd.	C 86.2	H 8.28
	Found	» 85.5	» 8.11

2-Methoxymethylretene, was prepared similarly by boiling the bromomethyl compound with methanol. M. p. 112.5—113.5° C.

$C_{20}H_{22}O$ (278.2)	Calcd.	C 86.2	H 7.98
	Found	» 85.8	» 8.05

2-Phenylaminomethylretene. 2-Bromomethylretene (0.1 g) was heated with 0.5 g of aniline at 150° C for some minutes. After cooling, dilute hydrochloric acid was added and the precipitate filtered off, washed and dried. Recrystallization from ethanol gave 0.065 g of white plates, m. p. 192—193° C.

$C_{25}H_{25}N$ (339.2)	Calcd.	C 88.4	H 7.43	N 4.13
	Found	» 87.5	» 7.28	» 4.08

2-Methylretene. 1.00 g of the 2-bromomethyl compound was dissolved in 15 ml of dioxan. Raney nickel (0.5 g) was added to the solution and hydrogenation was carried out for one hour at 70 atmos. and 80° C. After cooling, filtration, and evaporation to dryness 0.71 g (94 %) of crude methylretene was obtained, m. p. 119—121° C. Recrystallization from 40 ml of ethanol gave 0.37 g of white plates, melting at 123—124° C. From the mother liquor a further 0.17 g of the pure compound was obtained.

$C_{19}H_{20}$ (248.2)	Calcd.	C 91.9	H 8.12
	Found	» 91.1	» 8.07

The picrate, was obtained from 0.1 g of 2-methylretene and 0.1 g of picric acid in 5 ml of ethanol, as orange plates, m. p. 139—139.5° C.

$C_{25}H_{23}O_7N_3$ (477.2)	Calcd.	C 62.9	H 4.86	N 8.80
	Found	» 62.6	» 4.76	» 8.85

The styphnate, prepared from the components in ethanol solution formed orange needles, m. p. 158—159° C.

$C_{25}H_{23}O_8N_3$ (493.2)	Calcd.	C 60.8	H 4.70
	Found	» 60.8	» 4.63

SUMMARY

A chemical proof of the position of the hydroxyl group in A-hydroxyretene has been given as follows. Nitration of 9,10-dihydroretene gave a mononitro derivative with the nitro group in the A-position; this compound was reduced to the amine and acetylated. Nitration of this acetylamino compound gave a mononitro derivative, which by hydrolysis and reduction was converted into a diamino dihydroretene. This compound reacted with phenanthraquinone to form a quinoxaline. Thus the two amino groups are situated in adjacent positions. The above acetylamino nitro compound was hydrolysed and the amino group replaced by hydrogen via the diazonium salt. The nitrodihydroretene thus obtained was reduced to the amine, acetylated and dehydrogenated to the corresponding retene derivative. This was identical with 3-acetyl aminoretene and the A-position is thus the 2- or the 4-position.

A-acetylretene was oxidized to the corresponding acid, and the methyl ester reduced with lithium aluminum hydride to the carbinol. This was treated with hydrobromic acid, giving A-bromomethylretene, which, on hydrogenation at moderate pressure over Raney nickel, gave A-methylretene. This product was not identical with 4-methylretene, the structure of which has been fully established by the synthesis of Haworth *et al.*[6]. Thus it has been shown that the A-position is the 2-position.

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Enzymatic Hydrolysis and Synthesis of Benzohydroxamic Acid

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The participation of hydroxylamine in the nitrogen metabolism has been difficult to interpret chiefly because no enzymes have been known to act, *e. g.* in the formation of oximes via reactions between hydroxylamine and oxo-compounds. True, the spontaneous reaction of certain α-keto acids and aldehyde acids with hydroxylamine is so rapid that hydroxylamine, formed in the organisms at the natural concentrations of these acids, may be continuously removed without any catalysts via oxime formation. Of all the examined α-oxo-acids, glyoxylic, pyruvic, oxaloacetic, and ketoglutaric acids, the oximes of which correspond as reduction products to glycine, α-alanine, aspartic and glutamic acids, react according to Virtanen and Alfthan [1] most readily with hydroxylamine in the given order. In the synthesis of amino acids *in vivo* one would expect, however, a certain guidance of reactions by specific enzymes, and hence, a control of chemical reactions.

The first information found in the literature of the participation of hydroxylamine in an enzymatic reaction is the observation of Jacobsohn and Soares [2] on the linkage of hydroxylamine with the double bond of fumaric acid by the effect of aspartase or an analogous enzyme. We have, however, in this laboratory failed to confirm this reaction. Speck [3] and Elliott [4] proved convincingly two years ago that in the enzymatic synthesis of glutamine from glutamic acid and ammonia, the latter is replacable by hydroxylamine whereby hydroxamic acid is formed:

$$\text{Glutamate} + NH_3(NH_2OH) \rightarrow \text{glutamine (glutamohydroxamic acid)} \qquad (1)$$

Waelsch *et al.*[5, 6], Grossowicz *et al.*[7] found recently in certain microorganisms an enzyme, which causes the following reaction:

$$\text{Glutamine (asparagine)} + NH_2OH \rightarrow \text{glutamohydroxamic (aspartohydroxamic) acid} + NH_3 \qquad (2)$$

The experiments with $N^{15}H_3$ showed that the same enzyme extract also catalyzes the exchange of the amide group of glutamine and asparagine with ammonia. Reaction (1) requires utilization of adenosinetriphosphate [3,4] as an external source of energy, reaction (2) proceeds without utilization of energy-rich phosphate bonds [5–7]. Mg^{++} ions are necessary for the reaction (1) which is strongly depressed by sodium fluoride in low concentrations. Neither of these has any influence on the reaction (2).

Stumpf and Loomis [8,9] reported having found in pumpkin seedlings an enzyme (glutamyltransphorase), which labilizes the γ-amide group of glutamine and thus catalyzes the formation of glutamohydroxamic acid from glutamine and hydroxylamine. This enzyme is according to Stumpf (personal communication) very widely spread in the plant kingdom, *e. g.* it is liberally found in the root nodules of lupin and clover as well as in *Azotobacter agilis*. Contrary to the finding of Waelsch *et al.* ATP or ADP is indispensable to the activity of the enzyme of Stumpf and Loomis. Moreover, this enzyme does not act when asparagine replaces glutamine, although a raw preparation of pumpkin seedlings causes a reaction between hydroxylamine and asparagine. Manganese and phosphate or arsenate belong to the complete enzyme system of Stumpf and Loomis, fluoride causes complete inhibition.

We have been interested in finding out whether the activity of the enzyme system catalyzing the above reactions is restricted in glutamic acid, glutamine and asparagine or whether the activity is of more general nature. Therefore, we have examined the enzymatic synthesis and hydrolysis of benzohydroxamic acid and benzamide and report our results in the following. Speck has found a number of carboxyl acids ineffective in the reaction (1) and Grossowicz *et al.* some amides (eg. benzamide) in the reaction (2).

The possible significance of the active amide group of glutamine at the amination reactions has already earlier attracted attention [10, 11] and it might not be impossible that analogously the $>$ NOH-group could be transferred from hydroxamic acid. The formation of hydroxamic acids might also have another function, viz. the reduction of hydroxylamine to ammonia. This would require a reducing system for the $>$ NOH group of hydroxamic acids. If such a reducing system were found in the cells, this explanation would be the most plausible one for the function of hydroxamic acids in the nitrogen metabolism.

EXPERIMENTAL

The substrates and enzyme preparations

Benzamide was prepared according to Fischer [12] from ammonium carbonate and benzoyl chloride; m. p. 126°.

Benzohydroxamic acid was prepared according to Renfrow and Hauser [13] from hydroxylamine and ethylbenzoate. The m.p. of our preparation was 123°.

Yeast autolysate was prepared chiefly according to Grassmann and Mayr [14]. 100 g of baker's yeast was mixed with 10 ml of toluene for an hour whereby the yeast was liquefied. Water was added up to 100 ml and the suspension was centrifuged. The residue was diluted with water to 100 ml, allowed to stand for 15—20 h and centrifuged. The solution thus obtained (yeast-autolysate) decomposes benzamide and benzohydroxamic acid. The baker's yeast was a product of Rajamäki factories (Hyvinkää). The experiments were carried out in summer 1950. As later at the end of the same year the yeast from the same factory was autolyzed, the autolysate was inactive. The factory informed having changed the yeast strain in the meanwhile. The activity of the different yeast strains was not examined in detail, it was merely observed that the laboratory transfer of *Torula utilis* did not produce an active autolysate. Nor could such one be prepared from the brewer's yeast.

Sheep-liver extract. 500 g of liver from a just slaughtered sheep was rapidly cut into pieces, ground in a meat grinder and rubbed with sand. 750 ml of water were added, kept in an ice-cupboard for an hour, and centrifuged. The supernatant liquid was used in the experiments either immediately or after dialysis. Bray *et al.*[15] have employed a similar method when preparing an extract from the liver and kidneys of hen for examination of the hydrolysis of amides.

Analytical methods

Hydroxamic acid was quantitatively determined according to Lipmann and Tuttle [16]. Benzohydroxamic acid served as a standard also for glutamohydroxamic acid since a suitable standard for that was unavailable. The intensity of the colour was determined by a Klett-Summerson photometer using filter S 54. In the latest experiments a slightly modified method of Stumpf (personal communication) was used with filter S 50.

Ammonia was determined according to Pucher.

Hydroxylamine was determined according to the method of Blom as modified by Csáky [17].

Phosphorus was determined according to Berenblum and Chain [18].

Benzoic acid was identified with a paperchromatographic method. Filter paper Whatman no. 1 was used and as a solvent butylalcohol with a drop of ethylamine. After 4 h the paper was dried and sprayed with bromchresolgreen. A distinct blue spot was detectable at about 5 cm distance from the initial drop. The spot was not fluorescent in the ultraviolet light.

In another method the paper was treated at first with 0.1 % fluorescein solution. A drop of benzoic acid solution produces a spot on the paper, which in the ultraviolet light is dark and not fluorescent. Benzohydroxamic acid and benzamide produce a spot which is light and fluorescent in the ultraviolet light. A mixture of benzohydroxamic acid and benzoic acid gives a spot which is similar to that given by benzoic acid.

Experiments and results

Hydrolysis

Experiments were made with both enzyme preparations in order to find out whether yeast autolysate and liver extract, which according to different authors, *e. g.* Gonnermann [19] (liver) Geddes and Hunter [20] (yeast), Grassmann and Mayr [14] (yeast), and Bray *et al.*[15] (liver) hydrolyze different amides, are able to split off hydroxylamine from benzohydroxamic acid.

A. *Experiment with yeast autolysate.* Benzohydroxamic acid (BHA) was dissolved in a phosphate buffer with pH 7.3 (0.002 M BHA-solution).

I 25 ml of BHA-solution + 5 ml of yeast autolysate
II 25 ml of BHA-solution + 5 ml of boiled yeast autolysate
III 25 ml of buffer solution + 5 ml of yeast autolysate
IV 25 ml of BHA-solution + 5 ml of water.

B. *Experiment with liver extract.* Benzohydroxamic acid was dissolved in

I. phosphate buffer with pH 7.3 (0.002 M BHA-solution).
II. glycine-NaOH buffer with pH 7.4 (0.002 M BHA-solution).
a. 25 ml of BHA-solution + 5 ml of liver extract
b. 25 ml of BHA-solution + 5 ml of boiled liver extract
c. 25 ml of buffer solution + 5 ml of liver extract.

Samples of 1 ml were taken at definite intervals from each experiment. E-values from the BHA-determinations are presented in Table 1.

Table 1. E-values from the BHA-determinations.

A. Yeast autolysate					B. Liver extract						
Time h	I	II	III	IV	Time h	Ia	Ib	Ic	IIa	IIb	IIc
0	128	130	11	143	0	148	142	15	165	155	16
3	78	130	11	143	2	110	140	7	135	154	12
					4	91	142	5	121	154	5
					7	69	139	6	116	154	5
19	0	130	11	143	17	60	141	5	103	154	5

The yeast autolysate (expt. A) hydrolyzed in 3 h 39 % of benzohydroxamic acid and in 19 h the hydrolysis was complete; the liver extract (expt. B) hydrolyzed in 2 h 25.6 % and in 17 ½ h 59.5 % of hydroxamic acid in the phosphate solution and in the glycine solution respectively 18.2 % and 37.6 %.

The hydrolysis of benzamide with the same enzyme preparations is illustrated by the following experiments.

A. *Experiment with yeast autolysate.* Benzamide (BA) was dissolved in a phosphate buffer with pH 7.3 (0.0174 M BHA-solution).

I 25 ml of BA-solution + 5 ml of yeast autolysate
II 25 ml of BA-solution + 5 ml of boiled yeast autolysate
III 25 ml of buffer solution + 5 ml of yeast autolysate
IV 25 ml of BA-solution + 5 ml of water

B. *Experiment with liver extract.* Benzamide (BA) was dissolved in

I. phosphate buffer with pH 7.3 (0.0174 mol BA-solution)
II. glycine-phosphate buffer with pH 7.4 (0.0174 M BA-solution)
a. 25 ml of BA-solution + 5 ml of liver extract
b. 25 ml of BA-solution + 5 ml of boiled liver extract
c. 25 ml of buffer solution + 5 ml of liver extract.

Samples of 5 ml were used for NH_3-determinations. NH_3 found in the solutions, is to be seen in Table 2.

Table 2. NH_3 found in the solutions, mg.

A. Yeast autolysate					B. Liver extract						
Time h	I	II	III	IV	Time h	Ia	Ib	Ic	IIa	IIb	IIc
0	0.204	0.204	0.202	0.030	0	0.048	0.039	0.046	0.048	0.041	0.041
4	—	—	—	—	4	0.105	0.043	0.046	0.076	0.043	0.043
17 ½	0.549	0.197	0.172	0.034	17 ½	0.156	—	—	0.117	—	—

Within 17 ½ h the yeast autolysate hydrolyzed 34 % of benzamide, the liver extract 10.6 % in the phosphate solution and 6.8 % in the glycine solution.

The velocity of hydrolysis of BHA and BA cannot be compared on the basis of the above experiments since the concentrations of BHA and BA were different. In a later experiment series, in which liver extract was used for an enzyme preparation with 0.0174 mol BHA- and BA-solutions, it was noted that the hydrolysis of benzamide was considerably slower than that of benzohydroxamic acid (Fig. 1).

The liver extract used in the experiment was 2 weeks old (stored in an ice cupboard, with toluene) and it was much less active than the fresh liver extract used in the previous experiments.

Another similar experiment with a more active liver extract gave a similar result (Fig. 2).

The dependence of hydrolysis of BHA on pH was only roughly examined. At the highest pH used (7.4) the velocity with yeast autolysate was greatest, and at pH 6.0 abt. 2/3 of it. In acetate-buffer of pH 5.4 no hydrolysis occurred.

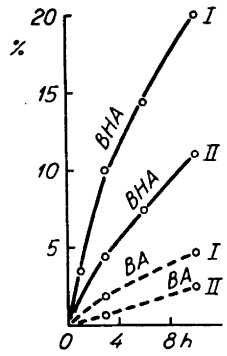

Fig. 1. Hydrolysis of benzohydroxamic acid (BHA, 0.0174M) and benzamide (BA, 0.0174M) with liver extract stored for 2 weeks in an ice-cupboard.

I in phosphate buffer (pH 7.3)
II in NaOH-glycine buffer (pH 7.3)

Borate buffer inactivated the enzyme concerned as appears from Table 3.

Table 3.

I 25 ml of phosphate solution (pH 7.3) with benzohydroxamic acid (0.002 mol) + 5 ml of yeast autolysate
II 25 ml of borate solution (pH 7.3) with benzohydroxamic acid (0.002 mol) + 5 ml of yeast autolysate

Time h	E-values I	II
0	142	144
12	52	144

Dialysis inactivates the yeast autolysate in a very high degree or practically completely. An $MgSO_4$-addition activated considerably the dialyzed autolysate in one experiment, in others again no activation was observed. The liquid outside the dialysis bag had no activating effect when evaporated to a small volume.

The dialysis of the liver extract did not cause any appreciable inactivation when BHA served as a substrate (Fig. 3).

It was proved by the following experiment that benzohydroxamic acid was really hydrolyzed to benzoic acid and hydroxylamine in the above experiments. The system: liver extract + benzohydroxamic acid (0.0174 M, pH 7.3) was examined qualitatively after 20 h in respect to benzoic acid and hydroxylamine. A drop of the solution formed a dark, non-fluorescent spot on a paper treated with 0.1 % fluorescein solution, thus benzoic acid had been formed. Determination of hydroxylamine showed that hydroxylamine, too, had been formed.

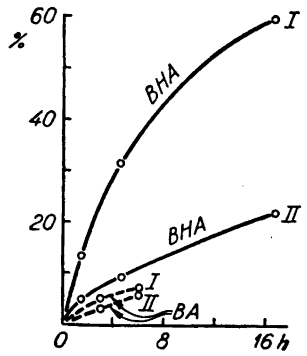

Fig. 2. As in Fig. 1, but with fresh liver extract.

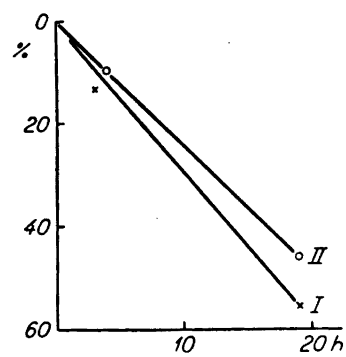

Fig. 3. Hydrolysis of BHA with undialyzed (I) and dialyzed (II) liver extract.

Synthesis

The first directive experiments were made with yeast autolysate (Experiment 8).

Experiment 8

I 122 mg of benzoic acid + 33 mg of NH_2OH in 25 ml of phosphate solution with pH 7.3 + 5 ml of yeast autolysate
II 366 mg of benzoic acid + 99 mg of NH_2OH in 25 ml of phosphate solution with pH 7.3 + 5 ml of yeast autolysate
III 430 mg of benzoic acid + 116 mgNH_2OH in 25 ml of phosphate solution with pH 7.3 + 5 ml of yeast autolysate
1 ml of reaction solution was used for each determination.

E-values from the BHA-determinations

Time h	I	II	III
0	3	0	8
2 ½	14	13	35
4 ½	20	11	37
6	29	15	37

The results suggest that the raw yeast autolysate synthesizes benzohydroxamic acid from benzoic acid and hydroxylamine.

In later experiments liver extract was used for an enzyme preparation (Experiment 13).

Experiment 13

0.5 ml of liver extract
0.1 ml of ATP-solution (0.1 μ M = 5.07 mg/10 ml)
0.1 ml of glutamine (10 μ M = 146.2 mg/10 ml)
0.1 ml of arsenate (10 μ M = 424.1 mg/10 ml)
0.5 ml of 0.1 M malic acid buffer (pH 6.4)
0.1 ml of manganous sulphate solution (0.1 μ M = 1.51 mg/10 ml)
0.1 ml of hydroxylamine solution (10 μ M)

were pipetted in a test tube. The mixture was kept at 35° C for 30 min, after which 1 m l of 10 % trichloracetic acid and 0.5 ml of 5 % ferric chloride were added. The protein precipitate was filtered. Filter S 50 was used in the photometric determinations of hydroxamic acid. Results:

	E
1. The basal solution	85
2. The basal solution, no glutamine	0
3. The basal solution, with boiled liver extract	0
4. The basal solution, benzamide instead of glutamine	37
5. The basal solution, benzoic acid instead of glutamine	35

The results reveal that the reactions

$$\text{glutamine} + NH_2OH \rightleftharpoons \text{glutamohydroxamic acid} + NH_3 \quad (1)$$
$$\text{benzamide} + NH_2OH \rightleftharpoons \text{benzohydroxamic acid} + NH_3 \quad (2)$$
$$\text{benzoic acid} + NH_2OH \rightleftharpoons \text{benzohydroxamic acid} + H_2O \quad (3)$$

have taken place. When the liver extract was boiled, no reaction occurred.

Because the synthesis from benzamide or benzoic acid is weak — in experiment 13 the E-values 37 and 35 correspond to about 20 γ BHA or about 1.5 % of the maximum synthesis — the experiment 14 was carried out on a larger scale and the BHA formed was extracted with ether from the reaction mixture. In this way BHA was strongly concentrated.

Experiment 14.

5 ml of liver extract
1 ml of ATP-solution (0.1 μM)
1 ml of benzamide (10 μM)
1 ml of arsenate (10 μM)
5 ml of 0.1 mol malic acid buffer (pH 6.4)
1 ml of manganous sulphate solution (0.1 μM)
1 ml of hydroxylamine solution, neutr. (10 μM)

After 30 min at 35° C, 2 ml of 50 % trichloracetic acid were added, the reaction mixture was centrifuged, its pH was adjusted to 7.2 with NaOH, and it was extracted with ether in a percolator. After evaporation of ether the extract was diluted with water to 2.2 ml. To the clear solution was added 0.5 ml of 5 % solution of ferric chloride and 1 ml of 10 % trichloracetic acid which brought the pH of the solution to about 2. An intense

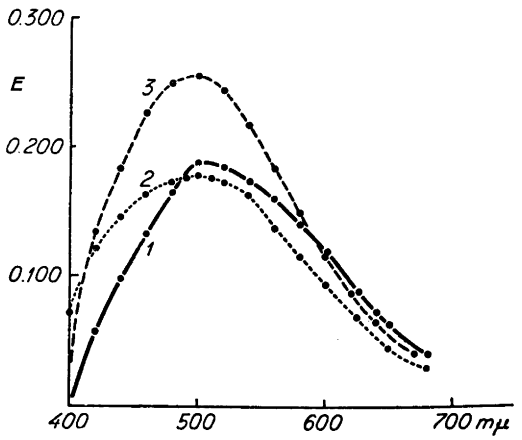

Fig. 4. Absorption spectrum of

1 synth. benzohydroxamic acid (m.p. 123° C)
2 ether extract in exp. 14 (benzamide + NH_2OH with liver extract)
3 basal solution in exp. 13 (glutamine + NH_2OH with liver extract).

red colour appeared and the absorption spectrum of the solution was determined between 400 and 700 mμ with a Beckman spectrophotometer. A parallel experiment was made with the same extract but without benzamide. The absorption of this solution did not show any maximum at 500 mμ, but rose sharply from 450 mμ to 400 mμ. It was subtracted from the absorption values given by the experiment proper.

On the basis of the absorption curve obtained (Fig. 4) the enzymatic synthesis of BHA is apparent.

In the following experiments the effect of ATP on the synthesis was examined. Since the undialyzed liver extract may contain ATP, the fact that the synthesis occurs without an addition of ATP does not prove that the reaction is independent of ATP. The results show, however, to what extent an addition of ATP may promote the synthesis and thus also the significance of ATP to the synthesis. The basal solution was the same as in experiment 13, likewise the experimental time and the method of analysis.

Experiment 15.

	E	Activation by ATP, %
1. The basal solution as in expt. 13	105	36
2. The basal solution, no ATP	77	
3. The basal solution, glutamic acid instead of glutamine	36	140

4. The basal solution, glutamic acid instead of glutamine, no ATP	15	
5. The basal solution, benzamide instead of glutamine	21	10
6. The basal solution, benzamide instead of glutamine, no ATP	19	
7. The basal solution, benzoic acid instead of glutamine	30	50
8. The basal solution, benzoic acid instead of glutamine, no ATP	20	

Experiment 16.

The basal solution was otherwise the same as in expt. 13 except that the liver extract was *dialyzed* for 24 h. The experimental arrangement was the same as in expt. 15.

	E	Activation by ATP, %
1. The basal solution	44	2
2. The basal solution, no ATP	43	
3. The basal solution, glutamic acid instead of glutamine	28	100
4. The basal solution, glutamic acid instead of glutamine, no ATP	14	
5. The basal solution, benzamide instead of glutamine	27	12
6. The basal solution, benzamide instead of glutamine, no ATP	24	
7. The basal solution, benzoic acid instead of glutamine	33	57
8. The basal solution, benzoic acid instead of glutamine, no ATP	21	

Fig. 5 illustrates the activation caused by ATP in experiment 16.

An addition of ATP has in all experiments greatly activated the synthesis of glutamohydroxamic acid from glutamic acid, hence, the indispensability of ATP for the synthesis is obvious, as the investigations of Speck and Elliott had revealed. The same addition had no activation on the synthesis of glutamohydroxamic acid from glutamine with dialyzed liver extract. This is in agreement with the results of Waelsch *et al.*

The activation of the synthesis of benzohydroxamic acid by the addition of ATP was distinct from benzoic acid, but slight from benzamide. It is therefore probable that the activation of the synthesis of hydroxamic acids through ATP is noticeably independent of the nature of the acids or amides,

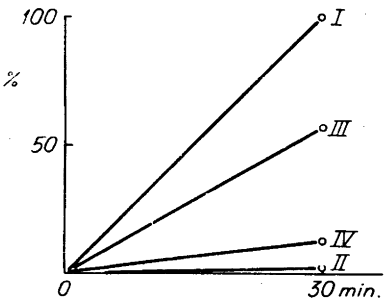

Fig. 5. Activation of the enzymatic synthesis of glutamo- and benzohydroxamic acids with dialyzed liver extract.

I Activation of the synthesis of glutamohydroxamic acid from glutamic acid.
II Activation of the synthesis of glutamohydroxamic acid from glutamine.
III Activation of the synthesis of benzohydroxamic acid from benzoic acid.
IV Activation of the synthesis of benzohydroxamic acid from benzamide.

which take part in the synthesis. The possibility that benzamide were hydrolyzed before the synthesis of benzohydroxamic acid must, however, be considered because ATP has shown a slightly activating effect when benzamide was used for the substrate.

SUMMARY

Hydrolysis of benzamide and benzohydroxamic acid was examined both with yeast autolysate and sheep-liver extract. Hydroxamic acid was hydrolyzed much more rapidly than amide. Benzoic acid and hydroxylamine were qualitatively identified as reaction products of the hydrolysis of benzohydroxamic acid.

No hydrolysis took place in borate buffer. In phosphate buffer the hydrolysis was more rapid than in glycine-NaOH buffer.

The synthesis of benzohydroxamic acid from hydroxylamine and benzoic acid or from benzamide was examined in detail with liver extract. The synthesis took place from both of them. ATP activated distinctly the synthesis from benzoic acid but only slightly that from benzamide.

The results presented in this paper show that the function of the enzymes, which catalyze the synthesis of hydroxamic acids, is not limited to glutamic and aspartic acids or their amides. The specificity of these enzymes is still obscure.

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Investigations in Serum Copper

IV. Effect of Different Anions on the Enzymatic Activity of Coeruloplasmin

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In the previous paper [1] of this series we have presented evidence for the assumption that coeruloplasmin is an oxidase with copper in its active group. It has many properties in common with laccases of plant origin.

When we compared the enzymatic activity per atom of copper in serum and in purified preparations of coeruloplasmin in presence of paraphenylene diamine (ppd) in the same buffers and at the same pH, we found that pure preparations showed less than half the activity of serum. In tracing the cause of this difference, we have made some observations which are of interest for the understanding of the kinetics of the oxidation of ppd by this enzyme. This paper will deal with some observations made in the course of these studies.

To explain the discrepancy between the experiments with crude and pure coeruloplasmin, two explanations may be thought of. Either the isolation procedure has a detrimental effect on coeruloplasmin, or there is present in serum a supplementary substance necessary to promote the full activity of the enzyme. In order to find out which might be the case, pure coeruloplasmin was at first dialyzed against serum. Such dialysis increased the catalytic activity of the pure preparation to the same level as that of serum. Later, different dialyzable components of serum were tested, and the active substance of serum could be identified as the Cl ion.

The experiments described in this paper have been made in order to find out under what conditions and in what way the Cl ion and other anions affect the catalytical activity of coeruloplasmin.

EXPERIMENTAL

Coeruloplasmin has been prepared according to a method published in an earlier paper [2]. The preparations used had a copper content of about 0.35 per cent.

Copper was determined with sodium diethyl dithiocarbamate after wet ashing, protein with the biuret method.

Paraphenylene diamine (ppd) c.p. (Coleman and Cell Co.) was used.

The enzymatic activity was determined with the Warburg technique, temperature 37° C. The consumption of oxygen per unit time during the period of the most rapid oxidation has been used as measure for the velocity of the oxidation. We have thus not used the initial reaction velocity in cases where there has been an induction period before the rapid oxygen consumption starts (discussion see below).

pH was measured with glass electrodes.

Influence of Cl ions on the oxidation of ppd in the presence of coeruloplasmin at different pH:s

In order to study the effect of Cl ions on the oxidation of ppd in the presence of coeruloplasmin at different pH:s the following experiments were performed. Ppd was acidified with HCl to the pH wanted. The saltfree enzyme solution was treated in the same way. Warburg experiments were performed, in which NaCl was added to this system in varying amounts. The buffering capacity in these systems is naturally poor. During the oxidation of ppd there is a slow shift in pH towards the acid side. By making pH measurements we have convinced ourselves that the shift in pH during the initial phase is very small. It is therefore possible to compare the initial reaction velocities in these experiments.

At pH 7.5 we found that the amount of Cl ions added in order to neutralize the solutions was suboptimal, by adding NaCl, higher reaction velocities could be reached. Even at pH 6.8 this was the case. At pH 6, however, the oxidation velocity could not be increased by adding NaCl. Fig. 1 shows this effect at three different pH:s. From these experiments it can be concluded that the Cl ion accelerates the oxidation of ppd by coeruloplasmin.

From Fig. 1 it can also be seen that *there is an optimal concentration of Cl ions which varies with pH*. If the Cl ion concentration is increased over this optimum, an increasing inhibition results. If an experiment is performed at pH 5.5, the amount of HCl necessary to acidify the substrate is so big that the optimal Cl ion concentration at this pH is considerably surpassed. The addition of even a small amount of NaCl therefore causes an inhibition. From Fig. 1 *it is also evident that the inhibitory effect of Cl ions is dependent on pH.*

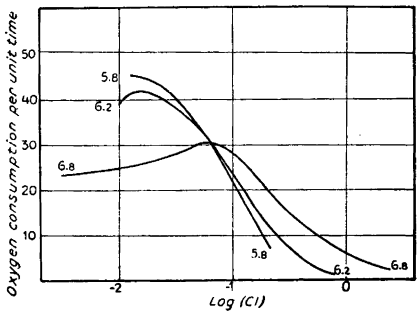

Fig. 1. Influence of chloride ions on the oxidation of ppd in the presence of coeruloplasmin at different pH:s (5.8, 6.2, and 6.8). Ppd was acidified with HCl to the pH wanted. NaCl was added in varying amounts. Abscissa: Logarithm of the chloride concentration (M). Ordinate: Initial reaction velocity (oxygen consumption per unit time). Substrate concentration: 1.8 · 10⁻². Enzyme concentration: About 1 · 10⁻⁶ M. Volume: 2 ml.

An effect similar to that of the Cl ion, *i.e.* acceleration in small concentrations, inhibition when the concentration was increased, has also been found for the following ions: Br^-, NO_3^-, CH_3COO^-, $HCOO^-$, SCN^-. With iodine the results are complicated, probably because this ion is oxidized in the system. F^- has not been investigated in detail, but preliminary experiments indicate that this ion has no accelerating effect.

Fig. 2 shows the acceleratory and inhibitory effects of NO_3^-, $HCOO^-$, SCN^-, and Cl^- ions at pH 6.8 and 5.8 respectively. At pH 6.8 the optimal effect of NO_3^-, $HCOO^-$, and Cl^- ions occurs at approximately the same concentrations, whereas the optimal effect of SCN^- is reached at a lower concentration. The inhibitory effects occur in the following order: $SCN^- > Cl^- > NO_3^- > HCOO^-$. Both

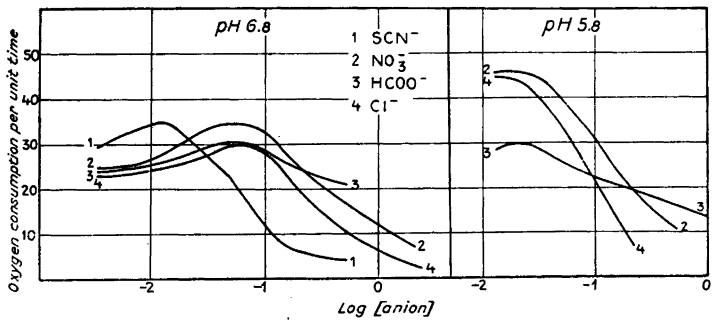

Fig. 2. Influence of different monovalent anions on the oxidation of ppd in the presence of coeruloplasmin at pH 6.8 and 5.8. Ppd was acidified with nitric, formic, or hydrochloric acid. The corresponding sodium salts were added in varying amounts. In the experiments with the rhodanide ion the acidification was performed with hydrochloric acid, and the chloride concentration has not been included in the anion concentration in this case. Abscissa: Logarithm of anion concentration. Ordinate: Initial reaction velocity. Substrate concentration: 1.8 · 10⁻². Enzyme concentration: About 1 · 10⁻⁶ M. Volume: 2 ml.

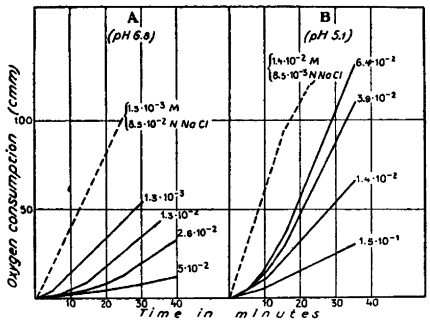

Fig. 3. Influence of phosphate ions on the oxidation of ppd in the presence of coeruloplasmin at pH 6.8 and 5.1. Ppd was acidified with H_3PO_4, and varying amounts of phosphate buffer were added. Abscissa: Time. Ordinate: Oxygen consumption. The broken lines show the reaction when an optimal concentration of NaCl was added to the ppd solution, which had only been adjusted to the pH wanted with H_3PO_4. The figures at the end of each line tell us the molarity of phosphate in each experiment. Substrate concentration: $2.2 \cdot 10^{-2}$. Enzyme concentration: About $2.4 \cdot 10^{-6}$ M. Volume: 2 ml.

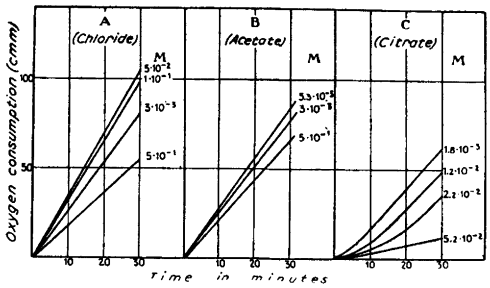

Fig. 4. Influence of chloride, acetate, and citrate ions on the oxidation of ppd in the presence of coeruloplasmin. Abscissa: Time. Ordinate: Oxygen consumption. Ppd was acidified in series A with HCl, in B with HAc, and in C with citric acid. Varying concentrations of the corresponding sodium salts (buffers) of the same pH (6.8) were added. The total anion concentration in M is given at the end of each line. Substrate concentration: $2.2 \cdot 10^{-2}$. Enzyme concentration: About $1.6 \cdot 10^{-6}$ M. Volume: 2 ml.

the accelerating and the inhibitory effects are clearly dependent on pH. At a lower pH, smaller amounts of ions are needed to produce these effects.

The investigation of the anions of polyvalent acids is more complicated, as these acids exist as different anions at different pH:s. The divalent phosphate ion (HPO_4^{--}) has been studied at pH 6.8. No accelerating effect was found, only an increasing inhibition. This inhibition differs in type from the one caused by large amounts of Cl ions (Br^-, CH_3COO^- a.s.o.). The inhibition is in the case of divalent phosphate, most pronounced during the initial phase of the reaction, and so gives rise to an induction period. Fig. 3 A.

The effect of monovalent phosphate ($H_2PO_4^-$) can not be tested in detail, as this has to be done at an acid pH where a comparatively large amount of phosphoric acid must be added in order to correct the pH of the ppd solution. An experiment performed at pH 5.1 is recorded in Fig. 3 B. As in the case of chloride, increasing concentrations of $H_2PO_4^-$ results in increasing reaction velocities, until an optimal concentration of $H_2PO_4^-$ has been reached. If the optimal concentration is surpassed, the reaction velocity decreases. The

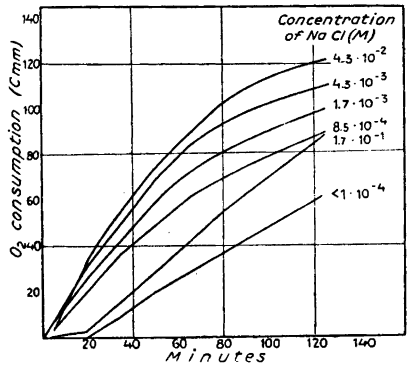

Fig. 5. Influence of chloride ions on the oxidation of ppd in phosphate buffer (M/60) in the presence of coeruloplasmin at pH 6.2. Abscissa: Minutes. Ordinate: Oxygen consumption. The lowest chloride concentration is given as less than $1 \cdot 10^{-4}$. In this case no sodium chloride has been added, but small amounts of chloride ions deriving from substrate, buffer, and enzyme solution can not be excluded. Substrate concentration: $4.6 \cdot 10^{-3}$. Enzyme concentration: About $1.5 \cdot 10^{-6}$ M. Volume: 2 ml.

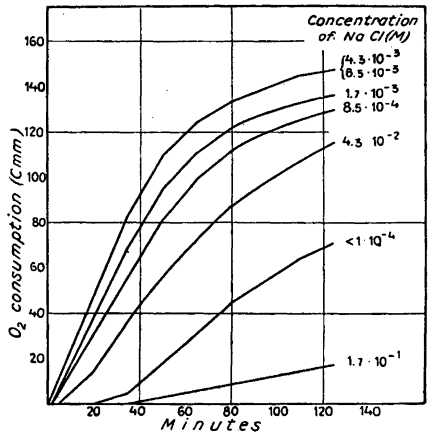

Fig. 6. Influence of chloride ions on the oxidation of ppd in citrate buffer (M/20) in the presence of coeruloplasmin at pH 5.4 Abscissa: Minutes. Ordinate: Oxygen consumption. The lowest chloride concentration is given as less than $1 \cdot 10^{-4}$. Substrate concentration $4.6 \cdot 10^{-3}$. Enzyme concentration: About $15. \cdot 10^{-6}$ M. Volume: 2 ml.

inhibitory effect of high $H_2PO_4^-$ concentrations are less pronounced than in the case of chloride. The concentration of this ion needed for optimal activity is greater than for Cl^-.

Some experiments were performed at pH 6.8 with minimal amounts of different acids. The acids used were hydrochloric acid, sulphuric acid, phosphoric acid, oxalic acid, nitric acid, acetic acid, and citric acid. In all cases fairly good activities were reached, best with nitric acid and hydrochloric acid. The smallest activity was noted with oxalic acid. It amounted to about 50 per cent of the activity measured with nitric acid. No clear induction period could be noted in these experiments. If the concentration of chloride, nitrate, or acetate ions in such experiments is increased within certain limits, the result is an increasing reaction velocity. If these ions are present in still higher concentrations, an inhibition is noted, but no induction period appears. If on the other hand the ions of citric, phosphoric, sulphuric, and oxalic acid are increased, the result is always a lower reaction velocity. In this case an

induction period becomes apparent. The length of this induction period is a function of the ion concentration. Some typical experiments illustrating these phenomena are recorded in Figs. 3 A and 4 (phosphate /3 A/, chloride /4 A/, acetate /4 B/, and citrate /4 C/).

The promotion of an induction period is, however, not only typical of polyvalent anions. A similar effect has, for instance, been noted with hippuric acid.

Effects of the combination of two different anions on the enzymatic activity of coeruloplasmin

If coeruloplasmin is inhibited by an anion as, for instance, the divalent phosphate ion, this inhibition can be broken by adding a small amount of one of the accelerating anions such as Cl^-, Br^-, CH_3COO^-, or NO_3^-. If such ions are added in sufficient amounts, the induction period disappears (Figs. 5 and 6). This effect can only be explained by a competition between the accelerating and the inhibiting ions. Fig. 7 shows clearly the competition between chloride and citrate ions.

Determination of pH optimum for the oxidation of ppd by coeruloplasmin

Before we knew that different anions have specific effects on the oxidation of ppd by coeruloplasmin, we tried to determine the pH optimum of the enzyme (Fig. 8). For this determination phosphate buffers were used between pH 6 and 7.5. Buffer concentration in the final mixture 1/60 *M*. Acetate and citrate buffers were used between pH 4 and 6 (final concentration in both cases 1/20 *M*).

As induction periods of different magnitude occurred in these experiments, the activities were computed from the consumption of oxygen during the most rapid phase of the reaction. In some cases where the buffer capacity was insufficient, there was some drift in pH towards the acid side during the experiments. When this has been the case, determination of pH has been made twice, at the beginning and at the of the experiment. In Fig. 8 this has been marked by combining two points with a straight line. This figure shows clearly that different buffers have different effects on the oxidation regardless of pH. The greatest velocity was observed in acetate buffers between pH 5 and 6. In this region citrate obviously gave an inhibition. A sharp decline in the catalytical effect was observed in changing from acetate to phosphate buffer at pH 6, indicating that phosphate, too, has an inhibitory effect.

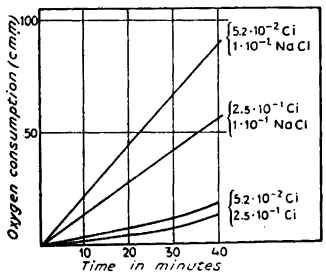

Fig. 7. Experiments showing the competitive effects of chloride and citrate ions on the oxidation of ppd in the presence of coeruloplasmin at pH 6.8. Abscissa: Time. Ordinate: Oxygen consumption. Substrate concentration: $2.2 \cdot 10^{-6}$. Enzyme concentration: About $1 \cdot 10^{-2}$ M. Volume 2 ml.

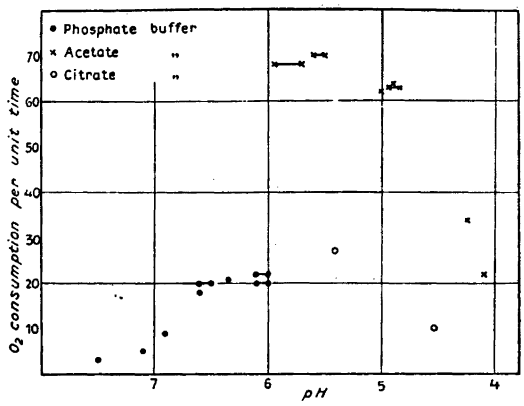

Fig. 8. Oxidation velocity of ppd in the presence of coeruloplasmin in different buffers at different pH:s. Abscissa: pH. Ordinate: Oxygen consumption per unit time. Concentrations of buffer: Phosphate M/60, acetate M/20, and citrate M/20. Substrate concentration: $4.6 \cdot 10^{-3}$. Enzyme concentration: About $1.5 \cdot 10^{-6}$ M. Volume: 2 ml.

By adding NaCl in optimal concentrations to the buffers at each pH, another pH activity curve can be constructed. This has been done and the results are shown in Fig. 9. The pronounced accelerating effect given by Cl ions in phosphate and citrate buffers is very clear when Figs. 8 and 9 are compared. This curve comes very near to the ideal pH activity curve. Between pH 4 and 5 and 6.5 and 8 the real optimal activities are a little better than is suggested by the figures. In the first-mentioned region, the amount of acetate ions used gives some inhibition. In the second region, the amount of Cl ions necessary to break the phosphate inhibition is great enough to cause some Cl ion inhibition. The errors are, however, relatively small.

Do the anions investigated act by combining with coeruloplasmin or with ppd?

As ppd is a weak base, it occurs in neutral and slightly acid solutions partly uncharged and partly as a monovalent cation (ppd H^+). The possibility must be kept in mind that the anions might act by combining with ppd H^+ and not with the enzyme. The experimental attack on this problem presented

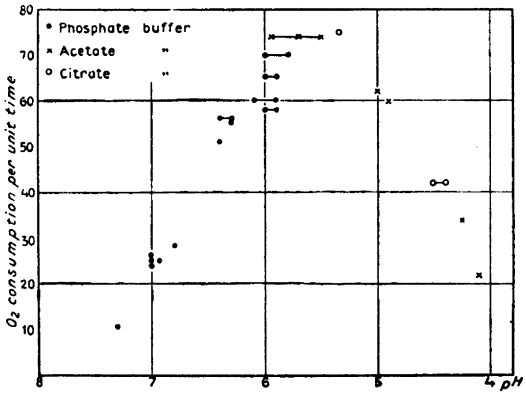

Fig. 9. Oxidation velocity of ppd in the presence of coeruloplasmin in different buffers and with optimal chloride concentration at different pH:s. Concentration of buffer: Phosphate M/60, acetate M/20, and citrate M/20. Abscissa: pH. Ordinate: Oxygen consumption per unit time. Substrate concentration: $4.6 \cdot 10^{-3}$. Enzyme concentration: About $1.5 \cdot 10^{-6}$ M. Volume: 2 ml.

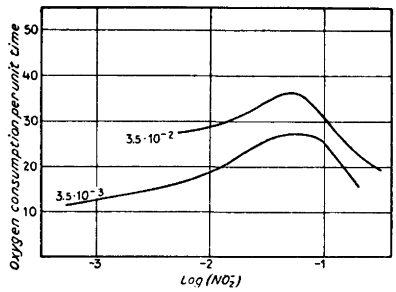

Fig. 10. Influence of nitrate ions on the oxidation velocity of ppd in the presence of coeruloplasmin at different substrate concentrations ($3.5 \cdot 10^{-2}$ and $3.5 \cdot 10^{-3}$). Ppd was acidified with nitric acid in both cases to pH 6.8. Varying amounts of sodium nitrate of the same pH were added. Abscissa: Logarithm of nitrate concentration (M). Ordinate: Oxygen consumption per unit time. Enzyme concentration: About $1 \cdot 10^{-6}$ M. Volume: 2 ml.

some practical difficulties. After some unsuccessful attempts, the problem was attacked in the following way.

1. Keeping the enzyme concentration constant, two series of Warburg experiments were performed at pH 6.8. In the first series, the concentration of ppd was kept at $3.5 \cdot 10^{-2}$ *M*, and in the second series, at $3.5 \cdot 10^{-3}$ *M*. In each series the concentration of the anion chosen (NO_3^-) was varied over a broad interval. The results of these experiments are presented in Fig. 10. From this figure it is evident that optimal reaction velocity is reached at the same concentration of NO_3^- ions in both series. That this is the case seems to us to indicate clearly that the nitrate ions act by combining with the enzyme and not with the substrate. (The differences in absolute activity in both series are due to the fact that the substrate is suboptimal in the second series [1]).

2. The effect of chloride ions on the oxidation of ppd and catechol was compared at pH 6.9. Catechol was chosen as it differs from ppd in being practically undissociated at this pH. These experiments show that the chloride ion has both accelerating and inhibiting effects also in the coeruloplasmin-

catechol system. The chloride concentration at optimal activity was the same in both systems.

These experiments therefore support the assumption that monovalent anions act on the enzyme and not on the substrate.

SUMMARY AND CONCLUSIONS

We have in this paper presented evidence for the occurrence of three different anion effects on the oxidation of ppd in the presence of coeruloplasmin.

Experiments have been performed which show that these anion effects are due to an interaction of enzyme and anion.

Firstly, many monovalent anions as for instance the rhodanide, the nitrate, the chloride, the bromide, the formiate, and the acetate ions, have an accelerating effect on this enzymatic process. Secondly, these same ions, when present in higher concentrations, show an inhibitory effect. Both these effects increase with decreasing pH. Thirdly, some polyvalent ions such as phosphate, citrate, sulphate, and oxalate ions, show an inhibitory effect, which differs from the effect of monovalent ions in being most pronounced in the initial stages of the reaction, and, thus, in causing an induction period. The inhibition and induction caused by polyvalent ions can be eliminated by adding monovalent ions in suitable concentrations.

It is impossible, at the present state of our knowledge, to give a full explanation of the different salt effects here described. We propose, however, to advance a working hypothesis which seems to fit the experimental facts hitherto collected.

Let us start with the inhibition caused by monovalent ions. With reference to the inhibitory effect, the ions investigated by us can probably be grouped in the following order: $SCN^- > Cl^- > NO_3^- > HCOO^- > CH_3COO^-$.

There is some difficulty in interpreting exactly the curves showing this inhibition owing to the fact that the inhibitory and the accelerating effects show different degrees of overlapping. It is therefore possible that further investigations might show that smaller alterations in the order will be necessary.

This arrangement of ions evidently shows similarities to the Hoffman series and to the series published by Klotz and Urquhart [3] and Scatchard and Black [4] relating to the binding of different anions to serum albumin. It seems probable, therefore, that these anions inhibit when they are bound to some cationic groups on the surface of the enzyme molecule. It has been proposed that the inhibitory effect of the anions in the Hoffman series on

different enzymes are due to an aggregation of the protein molecules. With coeruloplasmin this can not be the case. Unpublished experiments made by K. Pedersen at the Institute of Physical Chemistry, Upsala, have not revealed any differences in the sedimentation constant of coeruloplasmin when the concentration of NaCl in the solution was varied over a broad interval.

Let us then proceed to the accelerating effect of these same ions. Here it is still more difficult to group the different ions according to their affinity. The optimal effect is reached with practically the same concentrations of Cl^-, $HCOO^-$, NO_3^-, and CH_3COO^- ions, whereas SCN^- ions seem to reach this activity at a lower concentration. If this effect is also due to a binding between the anion and cationic groups in the enzyme, the cationic groups responsible for the acceleration must have a higher affinity for all the anions investigated than those responsible for the inhibition. It should be kept in mind that Scatchard *et al.* [5,6] have interpreted their finding as regards the binding of Cl^- and SCN^- ions to serum albumin by assuming two sets of groups with widely differing affinities for these anions.

What we assume is that two types of cationic groups exist on the enzyme surface. One of these types has a high affinity for different anions. When these groups are blocked by monovalent anions, the result is generally an increased enzymatic activity. The other type of cationic group has a lower affinity for anions. Blocking of these groups leads to inhibition.

How can now the observations on the specific inhibition of the enzyme by polyvalent anions be made to fit into this hypothesis? As there is evidently a competition between these ions and the monovalent anions, they, too, are probably attached to the cationic groups with high affinity for anions. Contrary to the monovalent ions they must in some way interfere with the activity of the enzyme. Experiments showing whether this is due to aggregation have not yet been performed.

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A Punched Card Method for Radial Distribution Calculations in X-Ray and Electron Diffraction Studies

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The numerical calculations in structure investigations by X-ray or electron diffraction procedures may conveniently be facilitated by punched card methods. Different systems have been developed for this purpose in connection with X-ray investigations of crystals [1–3] and electron diffraction studies of gases [4]. The present paper deals with the numerical calculations involved in the sector method of electron diffraction, and in X-ray investigations of liquids and amorphous solids.

In the methods used in our laboratories [5,6], the structure investigations are carried out using the following functions:

$$M(r) = \int Y(s)\sin sr\, ds \quad \text{and} \quad N(r) = \int Y(s)\cos sr\, ds \tag{1}$$

$Y(s)$ is obtained experimentally, $M(r)$ gives information about the interatomic distances to be obtained, and $N(r)$ may be used for the final structure interpretation and the estimation of accuracy [7].

For the numerical calculation equations (1) are put in the form

$$M(r_m) = \sum_n Y(s_n) \sin s_n r_m \text{ and } N(r_m) = \sum_n Y(s_n) \cos s_n r_m \tag{2}$$

If the values of $Y(s_n)$ are measured at equidistant s values, $s_1, s_2, \ldots\ldots s_n$, the values of $M(r_m)$ and $N(r_m)$ can be obtained for equidistant r values, $r_1, r_2, \ldots\ldots r_m$. The value most frequently used here is $s_n = \frac{1}{4}\, n$ (Å^{-1}), n being equal to 1, 2, 3, ... 90, and $r_m = {}^{8\pi}/_{360}\, m$ (Å), m being equal to 1, 2, 3, ... 90. Then all the angles $s_n r_m$ will be given in whole degrees, expressed by ${}^{2\pi}/_{360}\, nm$.

EQUIPMENT AND FILE

For the evaluation of equation (2) by punched-card machines, the following two or three standard I. B. M. units are used — a "collator" (type 077) and a "numerical accounting machine with progressive totals" (type 416). The latter may be replaced by an "alphabetical accounting machine" (type 405) with a "summary punch" for making progressive totals. Eleven usual selectors, two digit selectors, and sixteen counters are used in the tabulator. For the units (416) and (405) one semi-fixed plugboard can be used for the different operations mentioned below.

For calculating $M(r)$ we use a sine-file consisting of 9 000 cards which will be described below. The calculation of $N(r)$ is carried out using a corresponding file where the sin sr values are replaced by cos sr values.

The 9 000 cards of the sine-file are split up in 10 R groups, called R_1, R_2, ... R_{10}. The 900 cards of each R group are divided in 90 s groups, corresponding to s_1, s_2, s_3, ... s_{90}. Each s group contains 10 cards called α_0, α_1, α_2 ..... α_9. The first two of these, α_0 and α_1 are sign-cards; the rest, α_2 to α_9 are sine-cards. Each of these corresponds to different Y (s_n) sin $s_n r_m$ values. Each α number represents a constant Y value according to the binary system (Table 1).

Table 1.

α:	α_2	α_3	α_4	α_5	α_6	α_7	α_8	α_9
Y	1	2	4	8	16	32	64	128

A given, arbitrary Y value is expressed by a certain selection of α cards. For example, $Y = -69$ is expressed by α_1 and the sum of the cards α_8, α_4 and α_2.

Table

Columns:	1—2	3—4	5	6—8	9—10	11—12
Information punched in the sign-cards:	R	s	α		+ or − for $Y(s)$ $(0<r<2\pi)$	+ or − for $Y(s)$ $(2\pi<r<4\pi)$
Information punched in the sine-cards:	R	s	α	$Y(s)$		

Each of the 9 00 cards belonging to a certain R group is characterized by a certain s value having 90 possible values, and by a certain α-value (10 possibilities). In each sine-card the values of $Y(s_n)$ sin $s_n r_m$ for 9 different r values are punched. The group R_1 corresponds to $r_1, r_2, \ldots\ldots r_9$, the group R_2 to $r_{10}, r_{11}, \ldots\ldots r_{18}$ and so on, the last group R_9 corresponding to $r_{82}, r_{83}, \ldots\ldots r_{90}$.

The 80 columns of a sine-card are used in the following way. Columns 18 to 71 contain the numerical values of the nine Y sin sr values, and the columns 72—80 the corresponding signs. Columns 1 and 2 give the R value, and columns 3 and 4 the s values column 5 gives the α-number, and 6—8 the Y value. In the sine-cards, columns 9 to 17 are not used.

In the sign-cards columns 1 to 5 are used for indication of the R, s, and α values as in the sine-cards. Columns 9 and 10 give the sign for the Y function and columns 11 to 13 are used for special purposes discussed below. A scheme of the information punched in the cards is given in Table 2.

THE WORKING SCHEME

The evaluation of equations (1) on the basis of given Y (s_n) values, is performed as a simple addition of numbers punched in a certain selection of cards from the main file. The addition is first carried out for the 9 r values of the R_1 cards, then for the R_2 cards and so on. The working scheme for a complete calculation will therefore be:

1) Cards corresponding to R_1 are selected by hand from the main file. For each s value one has to pick out a certain number of α cards corresponding to the Y (s) value in question. The hand-sorting is simplified by the α-values which are punched in column 5.
2) Corresponding selections for $R_2, R_3, \ldots\ldots R_{10}$ are performed by use of the collator. The machine picks out the cards by comparing s and α values of the main file with those of the R_1 cards.

2.

13	18—23	24—29	.....	66—71	72—80
termination					
	Y sin sr_i	Y sin $sr_i + 1$	.....	Y sin $sr_i + 8$	sign for sin sr_i—sin $sr_i + 8$

3) The selected cards are run through the accounting machine and the results are tabulated or punched in new cards by the summary punch. In the tabulator the signs for Y (s) and sin sr are combined in the right way by selectors.
4) All the cards are rearranged by the collator, and the card file is ready for the next calculation.

The mean time of a calculation 1) to 4) has been found to be about 1 ½ hours. Using an electrical desk machine the same calculation usually requires more than 25 hours, or 3—4 working days.

ADDITIONAL ARRANGEMENTS

Besides the requirements strictly necessary for the evaluation of equation (2), our system includes some additional features which make the application more effective. These extra features include facilities 1) for checking purposes, 2) for intermediate terminations of the series, and 3) for extension to higher s and r values.

1. Checking

To make sure that the right cards have passed through the machine, a direct summation of the Y (s) values are carried out parallel to the 9 ordinary summations of a given R series. As the same Y (s) values repeat for every new R value, this verification is easily performed by comparison with the corresponding Y (s) summations directly based on the experimental data. To detect failures in the individual counters, a special check-summation is carried out in the tabulator before and after the calculation.

2. Intermediate terminations

Very often smaller parts of the series are wanted, say from s_1 to s_i, i being some arbitrary number. These values are obtained by a special use of column 13 of the sign cards. A certain number, say 1, is punched in the cards following those s values at which the terminations are wanted, and the tabulator (416) is adjusted to print the corresponding progressive totals. The same thing can be achieved with a tabulator (405) and summary punch. The twelve places of column 11 give twelve possibilities for successive series of terminations and combinations of these twelve sequences give still more possibilities.

3. Extension of the s and r values

The card system has been extended to cover s values higher than s_{90} ($= 22.5$ Å^{-1}). This is easily done because of the symmetry of all $\sin s_n r_m$ values with respect to s_{90}. The same symmetry makes it possible to perform a cosine-summation directly from the sine-cards, for all even values of m.

The card system is symmetrical also with respect to r_{90} ($= 2\pi$ Å). One may therefore use the same cards for calculations beyond r_{90} by a change of sign for odd values of n. Columns 11 and 12 of the sign cards are used for these alternative signs of the $Y(s)$ functions.

One may of course use an arbitrary equidistant sequence of s, given by $s_n = kn$. Then the system described will correspond to the r values, $r_m = \frac{2\pi m}{360\, k}$.

We wish to express our sincere gratitude to the Oslo Office of the International Business Machines Corporation, which has made available the machines used in this work.

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Nitrobenzene Oxidation of Compounds of the Lignan Type *

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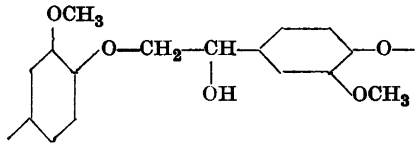
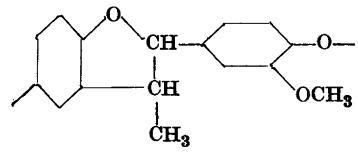
The theory that lignin may be formed in Nature by a process involving the dehydrogenative coupling of coniferyl alcohol or related substances [1,2] appears to have gained wide acceptance by lignin chemists, and Freudenberg has recently shown that the enzymatic dehydrogenation of coniferyl alcohol gives rise to products which are, in many respects, similar to lignin [3].

Consequently the study of simple compounds which may be produced by different modes of union of two molecules of coniferyl alcohol or related compounds has assumed appreciable importance, from the point of view of general lignin chemistry. Dehydrogenative coupling of two molecules of a substance such as coniferyl alcohol can conceivably take place in a number of ways, and previous publications from this laboratory [4,5] have described studies on compounds of the types I and II.



I



II

A further possible type of coupling involves the formation of a C — C bond uniting the carbon atoms in the β-positions of the side chains, thus giving rise to products such as III[6,7] containing the 2,3-dimethyl-1,4-diphenylbutane skeleton.

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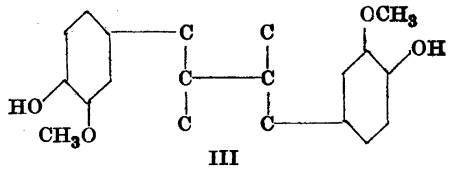
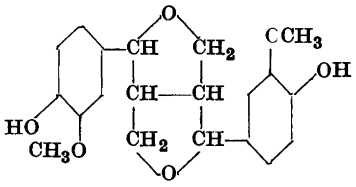
* Part XV in the series "Constitution of resin phenols and their biogenetic relations". Part XIV. *Acta Chem. Scand.* **4** (1950) 391.



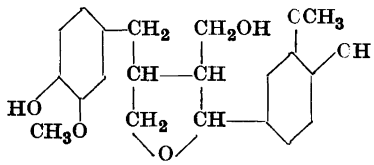
III

This carbon skeleton occurs in Nature in the class of compounds known as lignans [8] — a group of substances whose relationship to lignin has been discussed by several authors [9, 10, 11]. Recently Richtzenhain [12] obtained metahemipinic acid by the oxidation of acid-treated methylated lignin with potassium permanganate. The fact that only lignin which had received prior acid treatment yielded metahemipinic acid was considered [13] to indicate that lignin contains structures of the type III which under the influence of acid undergo rearrangement to products of the type of VIII or IX. However, it would appear that Richtzenhain's results are capable of alternative interpretations.

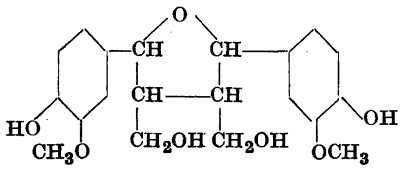
The lignans pinoresinol (IV), lariciresinol (V), olivil (VI), and matairesinol (VII) may be considered to form one sub-group, conidendrin (VIII) and isoolivil (IX) falling into a special cathegory by virtue of the fact that they contain an additional C — C linkage.



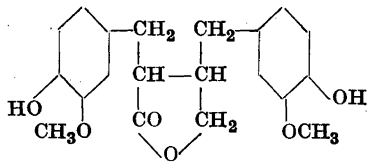
IV



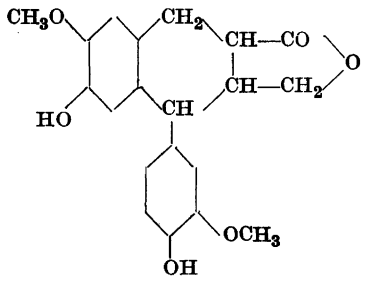
V



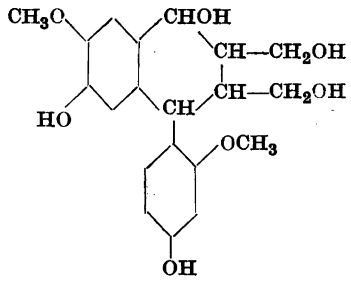
VI



VII



VIII



IX

The present paper deals mainly with the oxidation of IV—IX by means of nitrobenzene and alkali — experiments similar to those carried out by Erdtman [11] who obtained vanillin in 24 % yield by the oxidation of pinoresinol.

Compounds IV—IX have been oxidized under the standard conditions used in our earlier experiments [5] — conditions (reaction temp. 180°; time 2 hours; 60 ml of 2 *N* sodium hydroxide and 8 ml of nitrobenzene per g of substance) which are approximately optimal for most substances of this general type (Table 1).

In addition, a study was made of the effect of varying the alkali concentration and time of heating, on the oxidation of pinoresinol and its sulphonation product [14] (Table 2).

The yields are calculated on the basis of the theoretically possible figures, and corrected for the amount of unchanged material recovered.

Pinoresinol and olivil were also heated with alkali alone at 180° for 2 hours. Pinoresinol was remarkably stable and about 60 % of the starting material

Table 1. Oxidation of IV—IX under standard conditions.

Compound	Vanillin %	Vanillic acid %	Total yield %	Unchanged material %
Pinoresinol (IV)	31	9	40	19
Lariciresinol (V)	63	5	68	0
Olivil (VI)	83	3	86	0
Matairesinol (VII)	15	2	17	0
Conidendrin (VIII)	1	—	—	—
Isoolivil (IX)	3	—	—	—

Table 2. Oxidation of pinoresinol under varying conditions.

Compound	Time hours	Alkali-conc eq/l	Vanillin %	Vanillic acid %	Total yield %	Unchanged material %
Pinoresinol	2	2	31	9	40	19
»	2	3	31	—	—	—
»	2	4	31	8	39	16
»	3	2	33	11	44	6
Barium salt of pinoresinol sulphonic acid	2	2	27	—	—	—
»	2	4	26	—	—	—
»	3	2	31	12	43	0

could be recovered; olivil, on the other hand, was completely destroyed and a dark coloured, highly insoluble condensation product was obtained in about 70 % yield.

DISCUSSION

In a previous communication [5] it was shown that for compounds of type I and II, provided the side chain contains several oxygen atoms or a double bond, the major factor determining the yield of vanillin is the arrangement of the carbon skeleton. For compounds of type III, however, this does not appear to be so.

Pinoresinol (IV) yields less than half the amount of vanillin obtained from olivil (VI), although both compounds contain the same carbon skeleton and differ only in the arrangement of the hydroxyl and ether groups. This is surprising in view of the fact that lariciresinol (V) yields almost as much vanillin as does olivil.

The occurrence of free primary hydroxyl groups may be of importance, as olivil contains two, lariciresinol one, and pinoresinol no such groups. Sulphonation of pinoresinol, however, does not seem to affect the vanillin yield appreciably. It seems that no simple theory will account for all the observed results and possibly the general stereochemical configuration of the molecules is of considerable importance.

The results obtained with matairesinol (VII) on the other hand are readily explained by the fact that both α-carbon atoms are unsubstituted. The smal l vanillin yields obtained from conidendrin (VIII) and isoolivil (IX) are completely in accordance with earlier findings [5], showing that a carbon atom attached to an aromatic nucleus is not split off under the conditions used.

In the same paper it was pointed out that the oxidation studies reported there might make it possible to estimate the relative amounts of "open" and "condensed" coniferyl elements in lignin. The results of the present investigation, however, indicate that, if lignin does really contain significant amounts of groupings of the type III, an estimation of this kind would be more complicated than was at first expected.

EXPERIMENTAL

The oxidation experiments and the isolation and estimation of the products formed were carried out essentially as described in the previous report [5]. Unchanged pinoresinol was isolated as the potassium salt and identified as the diacetate.

SUMMARY

The oxidation, by means of nitrobenzene and alkali, of a number of compounds belonging to the lignan group has been studied. The results indicate

that, in this series, the arrangement of hydroxyl and ether groups has a great influence on the yield of vanillin.

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Extinktiometrische Untersuchungen über die Geschwindigkeit der Isomerisation von festem 1,6-Dinitrito- in 1,6-Dinitrodiäthylendiaminkobalt(III)-nitrat

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Von instabilen festen Dinitritokobalt(III)-verbindungen sind in zwei früheren Arbeiten das 1,6-Dinitritodipyridindiamminkobalt-(III)-nitrat[1] und das 1,2-Dinitritodiäthylendiaminkobalt(III)-nitrat[2] mit Rücksicht auf ihre Isomerisationsgeschwindigkeit vom Verfasser untersucht worden. Es war der Zweck dieser Arbeit, das kinetische Studium auf das Stereoisomere der letztgenannten Verbindung, d.h. auf das 1,6-Dinitritodiäthylendiaminkobalt(III)-nitrat zu erweitern, und somit den Einfluss einer verschiedenartigen, gegenseitigen Stellung der Nitritogruppen auf die Geschwindigkeitsverhältnisse bei sonst unverändertem Bau des Komplexions zu untersuchen.

Folgender Synthesegang wurde zur Herstellung des festen 1,6-Dinitritodiäthylendiaminkobalt(III)-nitrats benutzt.

$$CoSO_4 + en + H_2O + O_2 \rightarrow [(H_2O)_2\,Co\,[(HO)_2\,Co\,en_2]_2]\,(SO_4)_2,\,7H_2O \rightarrow$$

$$\left[\begin{matrix}(1)\ H_2O\\(2)\ H_2O\end{matrix}\ Co\ en_2\right] Br_3,\ 2\ H_2O \rightarrow \left[\begin{matrix}(1)\ HO\\(2)\ H_2O\end{matrix}\ Co\ en_2\right] Br_2,\ 1\ H_2O \rightarrow$$

$$\left[\begin{matrix}(1)\ HO\\(6)\ H_2O\end{matrix}\ Co\ en_2\right] Br_2 \rightarrow \left[\begin{matrix}(1)\ ONO\\(6)\ ONO\end{matrix}\ Co\ en_2\right] Br,\ 1\ H_2O \rightarrow$$

$$\left[\begin{matrix}(1)\ ONO\\(6)\ ONO\end{matrix}\ Co\ en_2\right] NO_3$$

Durch eine äthylendiaminhaltige wässerige Lösung von Kobaltsulfat wurde während mehrerer Stunden bei etwa 50° C Luft gesaugt. (Werner und Jantsch[3]; Adell[2].) Die dabei abgeschiedene Verbindung $[(H_2O)_2\,Co\,[(HO)_2\,Co\,en_2]_2\,](SO_4)_2,\,7H_2O$ gab, wenn man

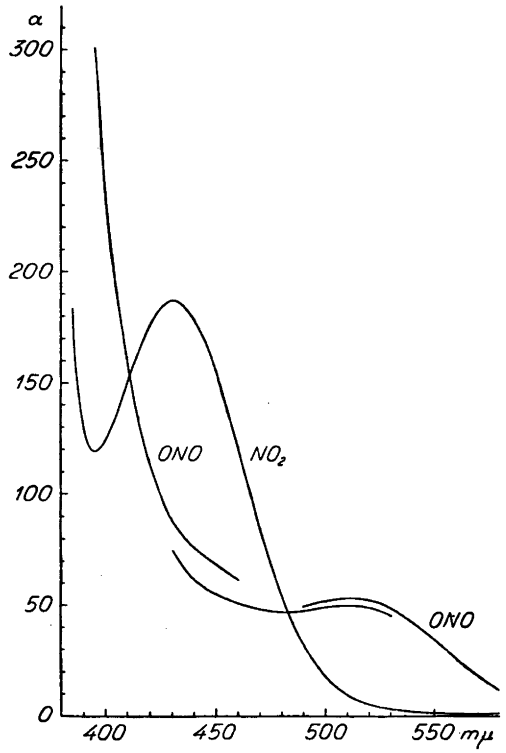


Fig. 1. Der molare, dekadische Extinktionskoeffizient α von 1,6-Dinitrito- und 1,6-Dinitrodiäthylendiaminkobalt (III)-nitrat als Funktion der Wellenlänge λ (in mμ).

sie mit konzentrierter Bromwasserstoffsäure verrieb, 1,2-Diaquodiäthylendiaminkobalt-(III)-bromid (Werner [4]), aus dessen wässeriger Lösung sich das 1,2-Hydroxoaquodiäthylendiaminkobalt(III)-bromid mit Pyridin ausfällen liess (Werner [5]). Dies Salz konnte durch Kochen mit Kalilauge und danach folgende, in der Kälte durchgeführte Neutralisation mit Bromwasserstoffsäure in das isomere 1,6-Hydroxoaquodiäthylendiaminkobalt(III)-bromid übergeführt werden (Werner [6]). Seine wässerige Lösung gab, mit konzentrierter Essigsäure und festem Natriumnitrit versetzt, das 1,6-Dinitritodiäthylendiaminkobalt(III)-bromid (Werner [7]), aus dessen Lösung das entsprechende Nitrat durch Fällung mit festem Ammoniumnitrat dargestellt wurde (Werner [8]). Das Endprodukt wurde schliesslich zur Reinigung mit Ammoniumnitrat umgefällt.

Das für die Messungen nötige 1,6-Dinitrodiäthylendiaminkobalt(III)-nitrat, wurde aus reinstem Dinitritonitrat durch etwa 17-stündige Erwärmung auf 60° C und nachherige Umfällung mit festem Ammoniumnitrat dargestellt.

Die verwendeten Präparate der beiden Isomeren zeigten bei gravimetrischer Bestimmung (vgl. Adell und Thölin [9]) einen Kobaltgehalt, der dem theoretischen (17,69 %) immer sehr nahe kam.

Ganz wie in der vorigen Arbeit [2] wurde für wässerige Lösungen von Dinitronitrat die Extinktionskurve im Wellenlängegebiete $385 \leqq \lambda \leqq 580$ mμ und für Lösungen von neu hergestelltem Dinitritonitrat eine annähernde

Extinktionskurve bei $395 \leqq \lambda \leqq 580$ mμ bestimmt. Für die beiden Salze wurde der Kobaltgehalt 17,71 bzw. 17,71 % gefunden. Es wurde auch kontrolliert, dass das Dinitronitrat nach noch einer Umfällung innerhalb der Grenzen der Messfehler eine unveränderte Extinktionskurve gab. In Fig. 1 ist der molare dekadische Extinktionskoeffizient α als Funktion von λ wiedergegeben. Man findet für die Dinitroverbindung ein Absorptionsminimum bei $\lambda = 395$ mμ und ein Maximum bei $\lambda = 430$ mμ. Das Dinitritosalz hat ein flaches Minimum für $\lambda = 480$ mμ und ein ebenso flaches Maximum für $\lambda = 515$ mμ. Kuroya und Tsuchida[10] finden für die erste Absorptionsbande des Dinitronitrats die Frequenz $\nu_{Max} = 69{,}9 \cdot 10^{13}$ sec^{-1}, der Wellenlänge $\lambda_{Max.} = 429$ mμ entsprechend. Aus ihrer Extinktionskurve kann man weiter für das angrenzende Minimum $\nu_{Min.}$ auf $75{,}4 \cdot 10^{13}$ sec^{-1} und $\lambda_{Min.}$ somit auf 398 mμ schätzen. Die Übereinstimmung mit den Daten dieser Arbeit ist also gut. Es ist auch zu bemerken, dass der Übergang vom Dinitrosalz zum Dinitritosalz wie bei den analogen *cis*-Verbindungen (vgl. Adell[2]) eine bedeutende Verschiebung des Maximums und Minimums gegen grössere Wellenlängen bewirkt. Die mit dem Übergang verknüpfte Erniedrigung von $\alpha_{Max.}$ ist hier noch ausgeprägter als bei den *cis*-Salzen. Tsuchida[11] schreibt die langwelligste Absorptionsbande komplexer Verbindungen Elektronensprüngen in der ungesättigten Übergangsschale des Zentralions zu, und die Verschiebung dieser Bande bei Substitution eines Liganden durch einen anderen wird durch den grösseren oder kleineren Einfluss des elektrischen Feldes des Liganden auf die erwähnten Elektronensprünge erklärt. Empirisch findet er folgende Reihe der Liganden, nach abnehmendem hypsochromem Effekt geordnet

$$NO_2,\ NH_3,\ ONO,\ H_2O,\ NCS,\ NO_3,\ SO_4 \text{ u. s. w.}$$

Als einzige Beispiele zur Klarlegung der gegenseitigen Lage der Nitro- und Nitritogruppen in dieser Reihe werden die Verbindungen $[Co(NH_3)_5NO_2]Cl_2$ und $[Co(NH_3)_5ONO]Cl_2$ angezogen. Die in der vorigen[2] und der vorliegenden Arbeit für 1,2- bzw. 1,6-Dinitro- und Dinitritodiäthylendiaminkobalt(III)-nitrat bestimmten Extinktionskurven bestätigen nun die Annahme, dass die Nitrogruppe eine hypsochromere Wirkung als die Nitritogruppe auf die fragliche Absorptionsbande ausübt.

Bei den mit einem Beckman-Quarz-Spektrophotometer extinktiometrisch durchgeführten kinetischen Messungen war die Wellenlänge des verwendeten Lichtes $\lambda = 510$ mμ, und die Versuchstemperaturen des festen Salzes 20,0 °, 30,0 ° und 40,0 ° C. Die Probelösungen hatten immer die Zusammensetzung

$$0{,}004\ M \text{ Komplexsalz} + 0{,}01\ M\ HOCOCH_3 + 0{,}01\ M\ NaOCOCH_3,$$

und deren Schichtdicke war 3,00 cm.

Betreffs der Einzelheiten der Messmethode wie der Extrapolation der Extinktion der Versuchslösung zum Auflösungsaugenblicke des Salzes sei auf zwei vorhergehende Arbeiten [1, 2] des Verfassers hingewiesen.

In den Tabellen 1—8 sind die primären Versuchsdaten unter den Überschriften t und $(E_V)_{gef.}$ zusammengestellt. Die Zeit t wird vom Auflösungszeitpunkt der ersten Probe jeder Reihe als Nullpunkt gerechnet. E_V gibt den zum Auflösungszeitpunkt der fraglichen Probe extrapolierten Extinktionswert der Versuchslösung an.

Tabelle 1. Versuchsreihe Nr 78a. Temperatur 20,0° C.

Formeln: a) $E_V - 0{,}111 = 0{,}470 \cdot 10^{-0{,}00155 \cdot t} - 0{,}024 \cdot 10^{-0{,}00442 \cdot t}$
b) $E_V - 0{,}111 = 10^{-0{,}00250 \cdot t} \cdot (0{,}441 + 0{,}00136 \cdot t)$

t(Stdn)	$(E_V)_{gef.}$	$(E'_V)_{ber.}$	$(E''_V)_{ber.}$	t(Stdn)	$(E_V)_{gef.}$	$(E'_V)_{ber.}$	$(E''_V)_{ber.}$
0,00	0,556	0,557	0,552	77,93	0,456	0,456	0,460
2,55	0,551	0,553	0,549	93,38	0,443	0,439	0,443
6,29	0,545	0,548	0,545	126,01	0,410	0,404	0,407
20,63	0,526	0,528	0,527	165,44	0,369	0,367	0,368
26,38	0,519	0,521	0,521	189,63	0,349	0,346	0,346
31,33	0,515	0,514	0,515	213,49	0,327	0,328	0,325
44,99	0,495	0,496	0,498	263,56	0,288	0,293	0,286
54,21	0,486	0,485	0,488				

Tabelle 2. Versuchsreihe Nr 81a. Temperatur 20,0°C.

Formeln: a) $E_V - 0{,}113 = 0{,}743 \cdot 10^{-0{,}00160 \cdot t} - 0{,}165 \cdot 10^{-0{,}00427 \cdot t}$
b) $E_V - 0{,}113 = 10^{-0{,}00230 \cdot t} \cdot (0{,}570 + 0{,}00200 \cdot t)$

t(Stdn)	$(E_V)_{gef.}$	$(E'_V)_{ber.}$	$(E''_V)_{ber.}$	t(Stdn)	$(E_V)_{gef.}$	$(E'_V)_{ber.}$	$(E''_V)_{ber.}$
0,00	0,685	0,691	0,683	206,22	0,443	0,439	0,442
14,58	0,668	0,674	0,667	231,08	0,418	0,413	0,416
38,48	0,636	0,645	0,640	255,06	0,393	0,390	0,392
62,47	0,613	0,614	0,612	281,61	0,369	0,366	0,368
86,72	0,591	0,582	0,582	304,75	0,351	0,347	0,347
111,98	0,558	0,550	0,551	326,23	0,331	0,330	0,330
134,97	0,515	0,521	0,524	350,41	0,312	0,312	0,311
159,03	0,489	0,492	0,495	375,42	0,294	0,295	0,293
183,23	0,457	0,464	0,467	457,95	0,242	0,249	0,244

Tabelle 3. Versuchsreihe Nr 64a. Temperatur 30,0° C.

Formeln: a) $E_V - 0{,}105 = 0{,}622 \cdot 10^{-0{,}00769 \cdot t} - 0{,}118 \cdot 10^{-0{,}0316 \cdot t}$
b) $E_V - 0{,}105 = 10^{-0{,}0105 \cdot t} \cdot (0{,}500 + 0{,}00754 \cdot t)$

t(Stdn)	$(E_V)_{gef.}$	$(E'_V)_{ber.}$	$(E''_V)_{ber.}$	t(Stdn)	$(E_V)_{gef.}$	$(E'_V)_{ber.}$	$(E''_V)_{ber.}$
0,00	0,609	0,609	0,605	49,53	0,361	0,362	0,369
0,508	0,605	0,608	0,603	54,98	0,355	0,338	0,347
1,41	0,604	0,605	0,599	68,72	0,310	0,288	0,298
2,92	0,596	0,600	0,591	78,63	0,278	0,259	0,268
7,44	0,571	0,582	0,569	102,37	0,207	0,206	0,212
19,50	0,499	0,517	0,509	123,57	0,170	0,175	0,177
25,85	0,473	0,481	0,477	140,53	0,153	0,157	0,157
30,47	0,451	0,456	0,454	211,84	0,120	0,120	0,118
43,73	0,385	0,387	0,393				

Tabelle 4. Versuchsreihe Nr 77a. Temperatur 30,0° C.

Formeln: a) $E_V - 0{,}110 = 0{,}626 \cdot 10^{-0{,}00789 \cdot t} - 0{,}135 \cdot 10^{-0{,}0270 \cdot t}$
b) $E_V - 0{,}110 = 10^{-0{,}0118 \cdot t} \cdot (0{,}499 + 0{,}00919 \cdot t)$

t(Stdn)	$(E_V)_{gef.}$	$(E'_V)_{ber.}$	$(E''_V)_{ber.}$	t(Stdn)	$(E_V)_{gef.}$	$(E'_V)_{ber.}$	$(E''_V)_{ber.}$
0,00	0,609	0,601	0,609	44,28	0,376	0,381	0,382
0,93	0,605	0,598	0,605	50,08	0,357	0,356	0,356
3,13	0,593	0,590	0,595	53,62	0,338	0,342	0,341
5,67	0,583	0,580	0,582	56,45	0,328	0,331	0,330
8,85	0,568	0,565	0,566	68,20	0,293	0,289	0,287
20,33	0,497	0,505	0,505	71,30	0,278	0,280	0,276
26,38	0,472	0,471	0,472	73,62	0,275	0,273	0,269
29,55	0,452	0,455	0,455	140,10	0,151	0,159	0,150

Tabelle 5. Versuchsreihe Nr 82a. Temperatur 30° C.

Formeln: a) $E_V - 0{,}111 = 0{,}870 \cdot 10^{-0{,}00880 \cdot t} - 0{,}322 \cdot 10^{-0{,}0232 \cdot t}$
b) $E_V - 0{,}111 = 10^{-0{,}0120 \cdot t} \cdot (0{,}550 + 0{,}0128 \cdot t)$

t(Stdn)	$(E_V)_{gef.}$	$(E'_V)_{ber.}$	$(E''_V)_{ber.}$	t(Stdn)	$(E_V)_{gef.}$	$(E'_V)_{ber.}$	$(E''_V)_{ber.}$
0,00	0,677	0,659	0,661	68,88	0,332	0,318	0,324
2,72	0,653	0,656	0,654	74,68	0,306	0,297	0,302
7,52	0,628	0,643	0,636	80,63	0,289	0,277	0,281
20,63	0,555	0,577	0,571	94,02	0,248	0,238	0,241
24,07	0,541	0,556	0,552	104,87	0,218	0,214	0,215
26,57	0,527	0,541	0,538	116,97	0,193	0,192	0,192
32,63	0,497	0,504	0,504	128,72	0,172	0,176	0,174
44,62	0,440	0,434	0,438	141,02	0,159	0,161	0,159
51,12	0,407	0,399	0,404	165,30	0,136	0,142	0,139
56,62	0,386	0,372	0,378				

Tabelle 6. Versuchsreihe Nr 76a. Temperatur 40,0° C.

Formeln: a) $E_V - 0{,}111 = 0{,}720 \cdot 10^{-0{,}0311 \cdot t} - 0{,}234 \cdot 10^{-0{,}0533 \cdot t}$
b) $E_V - 0{,}111 = 10^{-0{,}040 \cdot t} \cdot (0{,}486 + 0{,}0247 \cdot t)$

t(Stdn)	$(E_V)_{gef.}$	$(E'_V)_{ber.}$	$(E''_V)_{ber.}$	t(Stdn)	$(E_V)_{gef.}$	$(E'_V)_{ber.}$	$(E''_V)_{ber.}$
0,00	0,597	0,597	0,597	26,05	0,211	0,213	0,214
1,00	0,582	0,574	0,577	28,00	0,201	0,200	0,200
1,80	0,563	0,556	0,560	30,05	0,188	0,189	0,188
4,33	0,506	0,502	0,509	33,00	0,174	0,175	0,173
6,40	0,463	0,460	0,468	44,00	0,121	0,141	0,138
8,97	0,420	0,412	0,421	50,05	0,119	0,131	0,127
22,75	0,242	0,238	0,240	54,67	0,116	0,125	0,123

Tabelle 7. Versuchsreihe Nr 79a. Temperatur 40,0° C.

Formeln: a) $E_V - 0{,}110 = 0{,}725 \cdot 10^{-0{,}0350 \cdot t} - 0{,}225 \cdot 10^{-0{,}647 \cdot t}$
b) $E_V - 0{,}110 = 10^{-0{,}050 \cdot t} \cdot (0.492 + 0{,}0412 \cdot t)$

t(Stdn)	$(E_V)_{gef.}$	$(E'_V)_{ber.}$	$(E''_V)_{ber.}$	t(Stdn)	$(E_V)_{gef.}$	$(E'_V)_{ber.}$	$(E''_V)_{ber.}$
0,00	0,602	0,610	0,602	22,75	0,214	0,218	0,214
1,00	0,585	0,585	0,585	24,93	0,201	0,202	0,196
1,97	0,571	0,561	0,567	27,82	0,170	0,183	0,177
4,43	0,509	0,501	0,515	30,24	0,165	0,171	0,163
6,53	0,463	0,453	0,469	33,95	0,148	0,156	0,148
9,53	0,401	0,392	0,405	44,98	0,125	0,129	0,123
21,07	0,236	0,233	0,230	50,42	0,118	0,122	0,118
21,45	0,232	0,229	0,226				

Tabelle 8. Versuchsreihe Nr 80a. Temperatur 40,0° C.

Formeln: a) $E_V - 0{,}112 = 1{,}015 \cdot 10^{-0{,}0365 \cdot t} - 0{,}450 \cdot 10^{-0{,}0580 \cdot t}$
b) $E_V - 0{,}112 = 10^{-0{,}045 \cdot t} \cdot (0{,}555 + 0{,}0351 \cdot t)$

t(Stdn)	$(E_V)_{gef.}$	$(E'_V)_{ber.}$	$(E''_V)_{ber.}$	t(Stdn)	$(E_V)_{gef.}$	$(E'_V)_{ber.}$	$(E''_V)_{ber.}$
0,00	0,667	0,677	0,667	23,33	0,241	0,235	0,235
2,00	0,615	0,626	0,620	24,33	0,228	0,226	0,225
4,42	0,567	0,563	0,561	26,13	0,206	0,211	0,210
5,77	0,528	0,529	0,529	28,50	0,196	0,195	0,193
7,02	0,498	0,499	0,499	34,03	0,162	0,165	0,164
9,67	0,449	0,439	0,440	44,75	0,132	0,135	0,133
20,90	0,262	0,260	0,260	51,02	0,123	0,125	0,124
21,97	0,256	0,248	0,248	53,17	0,122	0,123	0,122

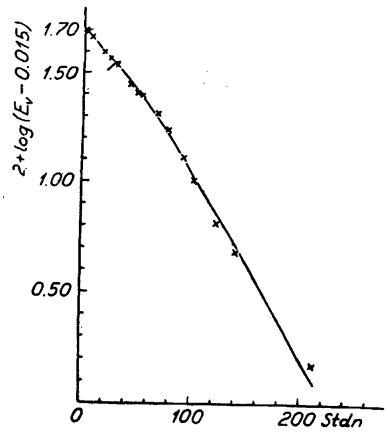


Fig. 2. Versuchsreihe Nr. 64a. (30° C.) Die Veränderung von log ($E_V - E_G$) mit der Zeit t (in Stunden).

Die Extinktion E_G der Lösung

0,004 *M* Dinitronitrat + 0,01 *M* $HOCOCH_3$ + 0,01 *M* $NaOCOCH_3$

der sich die E_V-Werte einer Versuchsreihe mit wachsendem Alter des festen Dinitritosalzes immer mehr näherten, wurde mit dem in jeder Versuchsreihe benutzten, zu 60 ° C über Nacht erhitzten und dann umgefällten Salzpräparate bestimmt. Es wurde auch mehrmals kontrolliert, dass noch eine Umfällung des ursprünglichen Dinitritonitrats eine nur sehr kleine und innerhalb der Messfehler liegende Änderung in E_G bewirkte. Die E_G-Werte der verschiedenen Versuchsreihen stimmten unter sich gut überein.

Des Überblickes halber wurden wie in den beiden früheren Arbeiten [1,2] für jede Reihe die Werte von log (E_V—E_G) gegen *t* graphisch repräsentiert.

Es zeigte sich, dass die resultierenden Kurven von dem in Fig. 2 wiedergegebenen Typ waren. Sie sind gegen die Abszissenachse schwach konkave Linien und unterscheiden sich also mit Rücksicht auf ihre Form wesentlich von den für *trans*- $[(ONO)_2Co\ py_2(NH_3)_2]NO_3$ und für *cis*- $[(ONO)_2Co\ en_2]NO_3$ gefundenen Kurven (vgl. Adell [1, S. 17; 2 S. 61], die gegen die Abszissenachse konvex sind und mit wachsendem *t* ziemlich schnell in Geraden übergehen.

Es entstand nun die Frage, ob die durch die Kurven repräsentierten, empirisch gefundenen Zusammenhänge zwischen E_V und *t* mit der Annahme einer zweistufigen Isomerisation nach dem Schema

$$trans\text{-}\left[(ONO)_2Co\ en_2\right]^+ \rightarrow trans\text{-}\left[\begin{matrix}ONO\\O_2N\end{matrix}\ Co\ en_2\right]^+ \rightarrow trans\text{-}\left[(O_2N)_2Co\ en_2\right]^+$$

wie bei den früher untersuchten Dinitritoverbindungen [1,2] könnten erklärt werden. Falls die Teilumwandlungen Reaktionen erster Ordnung mit *verschiedenen* Geschwindigkeitskonstanten wären, dann würde sich E_V—E_G durch eine Gleichung der Form

$$E_V—E_G = A \cdot 10^{-K_1 t} + B \cdot 10^{-K_2 \cdot t} \qquad (1)$$

schreiben lassen, wo K_1 und K_2 die Geschwindigkeitskonstanten der ersten und zweiten Teilisomerisation sind. A und B sind u.a. von dem Alter des verwendeten Salzpräparates abhängig und deshalb für jede Versuchsreihe individuelle Konstanten. (Vgl. Adell [1, S. 14—16]).

Wenn die obige Annahme richtig sei, wäre es zu erwarten, dass die log (E_V—E_G)-Kurven für grosse t-Werte immer mehr in Geraden übergehen würden. Das kommt auch bei den längeren empirischen Kurven zum Vorschein, tritt aber bei so grossen t-Werten ein, dass der Einfluss der Versuchsfehler auf die entsprechenden Ordinatwerte allzu gross ist, um eine einigermassen zuverlässige graphische Bestimmung der kleineren Geschwindigkeitskonstante zu erlauben.

Deshalb wurde die zweite der in dem vorigen Aufsatz [1] diskutierten Methoden zum weiteren Studium des Zusammenhanges zwischen (E_V—E_G) und t verwendet. Die Werte von (E_V—E_G) waren in einem Diagramm graphisch gegen t einzuzeichnen und die bestmögliche ausgleichende Kurve durch die resultierenden Punkte zu ziehen. Die den Abszisswerten t_1 ; $t_1 + \tau$; $t_1 + 2\tau$; $t_1 + 3\tau$ ..... $t_1 + n\tau$ zugehörigen Ordinatewerte a_1, a_2, a_3 ..... a_{n+1} wurden abgelesen (t_1 ist ein willkürlicher Anfangszeitpunkt und τ ein beliebiges, konstantes Intervall, das hier bei 20 °, 30 ° und 40 ° C gleich 20 ; 5 und 2 Stunden gesetzt wurde). Wenn die erwähnte Annahme stichhaltig sei, würde für drei konsekutive a-Werte a_n, a_{n+1} und a_{n+2} der Zusammenhang

$$\frac{a_{n+2}}{a_n} = (10^{-K_1\tau} + 10^{-K_2\tau}) \cdot \frac{a_{n+1}}{a_n} - 10^{-K_1\tau} \cdot 10^{-K_2\tau} \qquad (2)$$

gelten. (Vgl. Adell [1, S. 17—19]). Das bedeutet, dass eine Reihe von empirischen Quotienten $\frac{a_{n+2}}{a_n}$ als Ordinaten gegen die zugehörigen $\frac{a_{n+1}}{a_n}$ -Werte graphisch eingezeichnet, Punkte geben sollen, die auf oder in der Nähe einer Gerade liegen. Das war auch mit ziemlich guter Annäherung der Fall.

Die Gleichung der bestmöglichen durch die empirischen Punkte zu ziehenden Gerade wurde nach der Methode der kleinsten Quadrate ermittelt. Aus

dem Winkelkoeffizient k und der Ordinate im Nullpunkt l dieser Gerade liessen sich für K_1 und K_2 mittels der Gleichungen

$$10^{-K_1\tau} + 10^{-K_2\tau} = k \tag{3}$$

$$10^{-K_1\tau} \cdot 10^{-K_2\tau} = -l \tag{4}$$

zwei Wertepaare $K_1 = \lambda_1$; $K_2 = \lambda_2$ bzw. $K_1 = \lambda_2$; $K_2 = \lambda_1$ berechnen. (Es sei $\lambda_1 > \lambda_2$)

Die Konstanten C und D der Gleichung

$$E_V - E_G = C \cdot 10^{-\lambda_1 t} + D \cdot 10^{-\lambda_2 t} \tag{5}$$

konnten weiter aus zusammengehörigen empirischen (E_V—E_G)- und t-Werten auf folgende Weise bestimmt werden. Nach Überführung von (5) in die Form

$$(E_V - E_G) \cdot 10^{\lambda_1 t} = C + D \cdot 10^{(\lambda_1 - \lambda_2)t} \tag{6}$$

gaben die Werte des linken Gliedes von (6) als Ordinaten mit $10^{(\lambda_1 - \lambda_2)t}$ als Abszissen graphisch Punkte, die im allgemeinen gut um eine neue Gerade gruppiert waren. D liess sich dann gleich dem Winkelkoeffizient dieser Gerade und C gleich ihrer Ordinate im Nullpunkt setzen. C war immer negativ, B positiv.

Man hat nun die empirische Gleichung (5) mit der theoretischen (1) zusammenzustellen und die Frage zu entscheiden, ob K_1 und K_2 mit λ_1 und λ_2 wie A und B mit C und D oder K_1 und K_2 mit λ_2 und λ_1 bzw. A und B mit D und C zu identifizieren sind. Der theoretische Quotient $\frac{A}{B}$, der annäherungsweise aus der Gleichung

$$\frac{A}{B} \simeq 1 - 2 \cdot \frac{K_2}{K_1} \tag{7}$$

berechnet werden kann, gibt die Antwort. (Vgl. Adell [2, Gleichung (8)]). In Tabelle 9 sind die gefundenen Werte von C/D mit den nach (7) ermittelten von A'/B' und B''/A'' zusammengestellt. Durch Indices' und '' wird der erste, bzw. zweite der beiden obigen Identifizierungsfälle angegeben.

Da C/D durchgehend viel besser mit B''/A'' als mit A'/B' übereinstimmt, muss man schliessen, dass K_1 in allen Versuchsreihen kleiner als K_2 ist und dass die erste Teilisomerisation also langsamer als die zweite verläuft. Die Differenzen zwischen den empirischen C/D-Werten und den theoretischen B''/A''-Werten

Tabelle 9. Empirische Werte von C/D und nach (7) berechnete Werte von A'/B' bzw. B''/A''.

Versuchsreihe	Temp.	$(C/D)_{gef.}$	$(A'/B')_{ber.}$	$(B''/A'')_{ber.}$
78a	20° C	− 0,051	+ 0,30	− 0,21
81a	20° C	− 0,22	+ 0,25	− 0,23
64a	30° C	− 0,19	+ 0,51	− 0,14
77a	30° C	− 0,22	+ 0,42	− 0,17
82a	30° C	− 0,37	+ 0,24	− 0,23
76a	40° C	− 0,33	− 0,17	− 0,41
79a	40° C	− 0,31	− 0,16	− 0,37
80a	40° C	− 0,44	− 0,26	− 0,46

sind hier viel kleiner als zwischen den entsprechenden Daten für das in der vorigen Arbeit [2] untersuchte isomere *cis*-Dinitritonitrat. Das hängt sicherlich damit zusammen, dass wegen der vielmals kleineren Geschwindigkeitskonstante der ersten Isomerisationsstufe beim *trans*-Salz die bei der Herleitung der approximativ gültigen Gleichung (7) gemachte Vernachlässigung der Konzentration des Nitronitritonitrats beim Zeitpunkte $t = 0$ hier mehr berechtigt ist als beim *cis*-Salz. (Vgl. Adell [2]).

Tabelle 10. Übersicht über die Geschwindigkeitskonstanten K_1 und K_2 (in h^{-1}, auf dekadische Logarithmen bezogen) für die erste und zweite Isomerisationsstufe.

Versuchsreihe Nr	Tabelle Nr	Temp.	K_1	K_2	A	B
78a	1	20,0° C	0,00155	0,00442	0,470	− 0,024
81a	2	20,0° C	0,00160	0,00427	0,743	− 0,165
	Mittelwerte:		$K_1 = 0{,}00158 \pm 0{,}00002$	$K_2 = 0{,}00435 \pm 0{,}00005$		
64a	3	30,0° C	0,00769	0,0316	0,622	− 0,118
77a	4	30,0° C	0,00789	0,0270	0,626	− 0,135
82a	5	30,0° C	0,00880	0,0232	0,870	− 0,322
	Mittelwerte:		$K_1 = 0{,}0081 \pm 0{,}0003$	$K_2 = 0{,}027 \pm 0{,}002$		
76a	6	40,0° C	0,0311	0,0533	0,720	− 0,234
79a	7	40,0° C	0,0350	0,0647	0,725	− 0,225
80a	8	40,0° C	0,0365	0,0580	1,015	− 0,450
	Mittelwerte:		$K_1 = 0{,}034 \pm 0{,}002$	$K_2 = 0{,}059 \pm 0{,}003$		

Die aus den obigen Bestimmungsmethoden resultierenden Werte der Konstanten K_1, K_2, A und B sind in der Tabelle 10 zusammengestellt, und im Haupte jeder der Tabellen 1—8 ist die zu der fraglichen Versuchsreihe gehörige Formel vom Typ (1) angegeben. Nach dieser Formel sind die in den Kolumnen (3) und (7) der erwähnten Tabellen unter $(E'_V)_{ber.}$ verzeichneten Werte von E_V ermittelt. Die Übereinstimmung mit den gefundenen E_V-Werten (im Mittel innerhalb 1,5 %) muss als zufriedenstellend betrachtet werden.

Wenn man in einem Diagramm die drei Mittelwerte von log K_1 gegen $1/T$ wiedergibt und durch die sich ergebenden Punkte die bestmögliche Gerade zieht, findet man für diese die Gleichung

$$\log K_1 = 18{,}119 - \frac{6131}{T} \tag{8}$$

Für log K_2 erhält man in analoger Weise

$$\log K_2 = 15{,}481 - \frac{5211}{T} \tag{9}$$

Daraus ergibt sich für die erste Isomerisationsstufe die Aktionskonstante $\alpha' = 1{,}32 \cdot 10^{18}\ h^{-1}$ und die Aktivierungsenergie $q' = 28{,}04$ kcal; für die zweite die Aktionskonstante $\alpha'' = 3{,}03 \cdot 10^{15}\ h^{-1}$ und die Aktivierungsenergie $q'' = 23{,}83$ kcal.

Wenn die log $(E_V—E_G)$-t-Kurve die Form einer gegen die t-Achse schwach konkaven Linie hat, wie sie sich für das hier aktuelle *trans*-$[(ONO)_2\,Co\,en_2]NO_3$ ergibt, so ist es nicht ohne weiteres klar, dass die zwei Teilisomerisationen sich mit v e r s c h i e d e n e n Geschwindigkeitskonstanten vollziehen müssen. Im Gegenteil scheint es ganz notwendig zu prüfen, ob die Annahme g l e i c h e r Geschwindigkeitskonstanten den gefundenen Zusammenhang zwischen $(E_V—E_G)$ und t nicht ebenso gut oder besser erklären könne.

Falls man in dem grundlegenden, theoretischen Ausdruck

$$E_V—E_G = ad\ (\alpha_1 — \alpha_2 \cdot \frac{K_1}{K_1 — K_2} + \alpha_3 \cdot \frac{K_2}{K_1 — K_2}) \cdot 10^{-K_1 t} + (a \cdot \frac{K_1}{K_1 — K_2} + b) \cdot \\ \cdot d \cdot (\alpha_2 — \alpha_3) \cdot 10^{-K_2 t} \tag{10}$$

(vgl. Adell[1], Gleichung (14)), wo d gleich der Schichtdicke der Versuchslösung, α_1, α_2 und α_3 gleich den molaren Extinktionskoeffizienten der Dinitrito-, der Nitronitrito- und der Dinitroverbindungen, wie a und b gleich den Konzentrationen der beiden ersteren Formen beim Zeitpunkt $t = 0$ sind, K_1 und K_2 beide den Wert K annehmen lässt, so geht (10) in die folgende Gleichung über:

$$E_V — E_G = d \cdot 10^{-Kt} \cdot [a\,(\alpha_1 — \alpha_3) + b\,(\alpha_2 — \alpha_3) + \frac{a \cdot (\alpha_2 — \alpha_3) \cdot K}{\log e} \cdot t] \tag{11}$$

(Bei Gleichsetzung von K_1 und K_2, wird (10) im rechten Glied anfangs einen Ausdruck von der Form $\frac{0}{0}$ enthalten, dessen wahrer Wert aber in bekannter Weise bestimmt werden kann.)

Gleichung (11) kann natürlich kürzer als

$$E_V - E_G = [A' + B' \cdot t] \cdot 10^{-Kt} = f \tag{12}$$

geschrieben werden. A' und B' sind dabei für jede Versuchsreihe individuelle Konstanten, da ihre Werte u.a. von den Anfangskonzentrationen a und b abhängen. Wenn dieser Zusammenhang zwischen f und t gültig sei und wenn man die zu den Zeitpunkten $t_1 + n\tau$; $t_1 + (n+1)\tau$ und $t_1 + (n+2)\tau$ gehörigen f-Werte f_n ; f_{n+1} und f_{n+2} schreibe, dann müsste man folgende Relationen haben

$$f_n \cdot 10^{K[t_1 + n\tau]} = A' + B' \cdot [t_1 + n\tau] \tag{13}$$

$$f_{n+1} \cdot 10^{K[t_1 + (n+1)\tau]} = A' + B' \cdot [t_1 + (n+1)\tau] \tag{14}$$

$$f_{n+2} \cdot 10^{K[t_1 + (n+2)\tau]} = A' + B' \cdot [t_1 + (n+2)\tau] \tag{15}$$

(t_1 ist der Anfangszeitpunkt und τ ein konstantes Intervall, beide willkürlich wählbar). Aus der Tatsache, dass das rechte Glied von (14) das arithmetrische Medium derjenigen von (13) und (15) ist, ergibt sich weiter:

$$2\, f_{n+1} \cdot 10^{K \cdot [t_1 + (n+1)\tau]} = f_n \cdot 10^{K \cdot [t_1 + n\tau]} + f_{n+2} \cdot 10^{K[t_1 + (n+2)\tau]} \tag{16 a}$$

$$\text{oder } f_n \cdot 10^{-2K\tau} - 2 f_{n+1} \cdot 10^{-K\tau} + f_{n+2} = 0 \tag{16 b}$$

$$\text{bzw. } 10^{-K\tau} = \frac{f_{n+1} \pm \sqrt{f_{n+1}^2 - f_n \cdot f_{n+2}}}{f_n} \tag{16 c}$$

Aus drei mit konstantem Zeitintervall bestimmten Werten von $f = E_V - E_G$ würde man also die Geschwindigkeitskonstante K berechnen können. Weiter lässt sich Gleichung (12) folgenderweise umformen:

$$F = (E_V - E_G) \cdot 10^{Kt} = A' + B' \cdot t \tag{17}$$

d.h. die mit dem so ermittelten K aus zusammengehörigen Werten von $(E_V - E_G)$ und t bestimmten F-Werte müssten, gegen t eingezeichnet, graphisch eine Gerade geben.

Die experimentellen Werte von $f = E_V - E_G$ einer Versuchsreihe wurden deshalb in einem Diagram gegen t repräsentiert und durch die sich so ergebenden Punkte die bestmögliche ausgleichende Kurve gezogen. Auf dieser Kurve liessen sich mit konstantem Zeitintervall τ die f-Werte vom Nullpunkt an ablesen. (Für die Versuchsreihen 78 a und 81 a wurde τ gleich 20 Stunden; für 64 a gleich 10, für 77 a und 82 a gleich 5 wie für 76 a; 79 a und 80 a gleich 2 Stunden gewählt.) Aus je drei konsekutiven f-Werten: $f_0\ f_1\ f_2$; $f_1\ f_2\ f_3$; $f_2\ f_3\ f_4$ u.s.w. wurde dann mit Hilfe von Gleichung (16 c) und mit Minuszeichen vor dem Wurzelausdruck ein Wert von $10^{-K\tau}$ berechnet. Innerhalb jeder Reihe waren diese Werte ziemlich gut konstant, wenn auch die Messfehler einen bedeutenden Einfluss ausübten. (Der Unterschied zwischen den Extremwerten von $10^{-K\tau}$ konnte 10—15 % betragen.) Der Mittelwert dieser $10^{-K\tau}$ -Werte gab in einigen Fällen (wie in den Versuchsreihen 64 a und 76 a) eine Geschwindigkeitskonstante K, die ihrerseits in (17) eingesetzt, F-Werte berechnen liess, welche in einem t-Diagramm durch um eine Gerade gut gruppierten Punkte repräsentiert waren. In den übrigen Fällen konnte man ohne Schwierigkeit innerhalb der Grenzen von $10^{-K\tau}$ einen Wert dieser Grösse finden, dem eine Konstante K entsprach, die aus (17) einen linearen Zusammenhang zwischen F und t gab. Dieser $10^{-K\tau}$-Wert war immer nur wenig vom Mittelwert entfernt. Aus dem F-t-Diagramm liessen sich schliesslich die Konstanten A' und B' der fraglichen Reihe bestimmen. Beide hatten durchgehend positive Werte.

Wenn man bei Durchführung der obigen Rechnungen in (16c) das Pluszeichen vor dem Wurzelausdruck benutzt, so geben die Gruppen von je drei konsekutiven f-Werten unwahrscheinlich kleine K-Werte, die z. B. bei 20° C etwa 8-mal kleiner als die mit Minuszeichen resultierenden sind und die, wenn man sie zur Ermittlung von A' und B' in (17) einsetzt, negative B'-Werte erfordern würden. Wie ein Vergleich von (17) mit dem theoretischen Ausdruck (11) zeigt, wäre dies nur möglich, wenn α_2 kleiner als α_3 sei. Für eine neu bereitete Lösung ist E_V aber vielmals grösser als E_G. α_1 muss also auch erheblich grösser als α_3 sein. Es scheint somit sehr unwahrscheinlich, dass α_2, der molare Extinktionskoeffizient der Nitronitritoform nicht zwischen denjenigen der Dinitrito- und Dinitroformen liege, sondern noch kleiner als α_3 sei. Deshalb wurde in (16c) das Pluszeichen verworfen.

Die nach dem obigen Verfahren bestimmten Werte der Konstanten K, A' und B' sind in der Tabelle 11 gesammelt, und die zu den Versuchsreihen gehörigen Formeln vom Typ (12) finden sich unter b) im Haupte der Tabellen 1 bis 8. Aus diesen Formeln sind die als $(E''_V)_{ber.}$ bezeichneten Werte von E_V berechnet. Sie stimmen mit den gefundenen E_V-Werten nicht unbedeutend besser als die unter der Annahme verschiedener Geschwindigkeitskonstanten erhaltenen $(E'_V)_{ber.}$-Werte überein. (Mittlere Abweichung bzw. 1,0 und 1,5 %).

Tabelle 11. Übersicht über die Geschwindigkeitskonstanten K (in h^{-1}, auf dekadische Logarithmen bezogen und unter der Annahme $K_1 = K_2 = K$ berechnet).

Versuchsreihe Nr	Tabelle Nr	Temp.	K	A'	B'
78a	1	20,0° C	0,00250	0,441	0,00136
81a	2	20,0° C	0,00230	0,570	0,00200
		Mittelwert: K =	0,0024	± 0,0001	
64a	3	30,0° C	0,0105	0,500	0,00754
77a	4	30,0° C	0,0118	0,499	0,00919
82a	5	30,0° C	0,0120	0,550	0,0128
		Mittelwert: K =	0,0114	± 0,0005	
76a	6	40,0° C	0,040	0,486	0,0247
79a	7	40,0° C	0,050	0,492	0,0412
80a	8	40,0° C	0,045	0,555	0,0351
		Mittelwert: K =	0,045	± 0,003	

Der Zusammenhang zwischen den mittleren K-Werten und der absoluten Versuchstemperatur T lässt sich innerhalb der Grenzen der Messfehler durch die Formel

$$\log K = 17{,}324 - \frac{5845}{T} \tag{18}$$

darstellen, was einer Aktionskonstante $2{,}11 \cdot 10^{17}\ h^{-1}$ und einer Aktivierungsenergie 26,73 kcal entspricht.

Zum Schluss sind in Tabelle 12 die kinetischen Daten aller vom Verfasser untersuchten festen komplexen Mono- und Dinitritokobalt(III)-nitrate zusammengestellt.

Die Isomerisation vom Kation *trans*- $[(ONO)_2Co\ en_2]^+$ lässt sich räumlich durch die Formeln

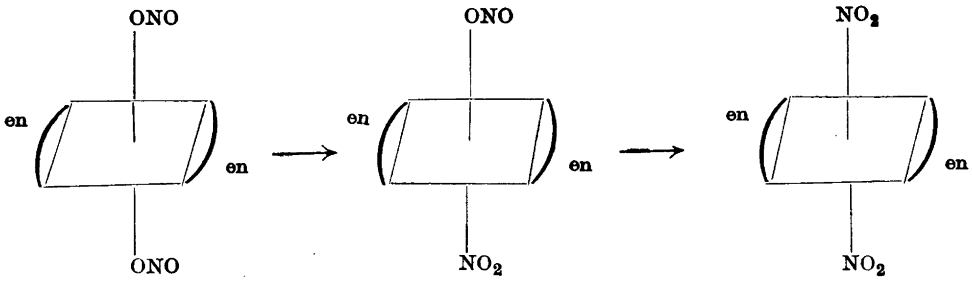


Tabelle 12. Die kinetischen Daten der vom Verfasser untersuchten komplexen, festen Mono- und Dinitritokobalt(III)-nitrate. (Die Geschwindigkeitskonstanten auf dekadische Logarithmen bezogen; die Zeit dabei in Stunden ausgedrückt.)

Komplexsalz	20° C		30° C		40° C	
	K_1	K_2	K_1	K_2	K_1	K_2
$[ONO \cdot Co \cdot (NH_3)_5](NO_3)_2$ [9]	0,016	—	0,051	—	0,192	—
trans-$[(ONO)_2 \cdot Co \cdot py_2(NH_3)_2]NO_3$ [1]	0,012	0,000254	0,033	0,00089	0,105	0,0039
cis-$[(ONO)_2Co\ en_2]NO_3$ [2]	0,0195	0,0025	0,049	0,0093	0,162	0,039
trans-$[(ONO)_2Co\ en_2]NO_3$	0,00158	0,00435	0,0081	0,027	0,034	0,059
trans-$[(ONO)_2Co\ en_2]NO_3$	0,0024	0,0024	0,0114	0,0114	0,045	0,045

Komplexsalz	$K_1 : K_2$			q'	q''	α'	α''
	20° C	30° C	40° C	kcal	kcal	sec^{-1}	sec^{-1}
$[ONO \cdot Co \cdot (NH_3)_5](NO_3)_2$	—	—	—	21,89	—	$9,5 \cdot 10^{10}$	—
trans-$[(ONO)_2 \cdot Co\ py_2(NH_3)_2]NO_3$	47,2	37,1	26,9	19,89	24,70	$2,2 \cdot 10^{9}$	$1,8 \cdot 10^{11}$
cis-$[(ONO)_2Co\ en_2]NO_3$	7,8	5,3	4,2	19,30	25,05	$1,3 \cdot 10^{9}$	$3,2 \cdot 10^{12}$
trans-$[(ONO)_2Co\ en_2]NO_3$	0,36	0,33	0,58	28,04	23,83	$3,7 \cdot 10^{14}$	$8,4 \cdot 10^{11}$
trans-$[(ONO)_2Co\ en_2]NO_3$	1	1	1	26,73	26,73	$5,9 \cdot 10^{13}$	$5,9 \cdot 10^{13}$

veranschaulichen. Unter der Voraussetzung, dass die beiden Nitritogruppen ganz gleichartig sind und dass ihre Umwandlung in derselben Weise vorgeht, ist es rein statistisch zu erwarten, dass K_1 gleich 2 K_2 sei.

Wie in der nächst vorhergehenden Arbeit [2] könnte man ansatzweise die Geschwindigkeitskonstanten K_1 und K_2 in folgender Form schreiben:

$$K_1 = 2\,k \cdot (f_{ONO})_a \cdot (f_{en})^2_{kk} \tag{19}$$

$$K_2 = k \cdot (f_{NO_2})_a \cdot (f_{en})^2_{kk} \tag{20}$$

wo k ein Proportionalitätsfaktor ist wie $(f_{ONO})_a$, $(f_{NO_2})_a$ und $(f_{en})_{kk}$ Koeffizienten sind, durch die der Einfluss einer Nitrito- bzw. einer Nitrogruppe in Axialstellung und einer Äthylendiamingruppe in Kanten-Kantenstellung im Verhältnis zu der sich umlagernden Nitritogruppe ausgedrückt wird. Es scheint billig anzunehmen, dass $(f_{en})_{kk}$ denselben Wert in beiden Isomerisationsstufen habe. Dann ergibt sich

$$\frac{K_1}{2\ K_2} = \frac{(f_{ONO})_a}{(f_{NO_2})_a} \tag{21}$$

Wenn man von der Annahme verschiedener K_1- und K_2-Werte ausgeht, wird $K_1/2\ K_2 \simeq 0{,}2$. Für K_1 gleich K_2 wird $K_1/2\ K_2$ ja 0,5. Welche dieser beiden Voraussetzungen die richtigere ist, lässt sich zur Zeit kaum entscheiden. Zwar gibt die letztere eine Formel, die die gefundenen (E_V—E_G)-Werte entschieden besser wiedergibt als die aus der ersteren hervorgehende. Andrerseits scheint aber kein hinreichender theoretischer Grund zu der Annahme vorzuliegen, K_1 sei exakt gleich K_2. Um das Problem zu lösen, muss man die Versuchstechnik noch weiter treiben und dadurch den Einfluss der Messfehler auf die berechneten Werte der Geschwindigkeitskonstanten beschränken. Jedenfalls kann man es als festgestellt betrachten, dass für das *trans*- $[(ONO)_2\ Co\ en_2]^+$--Ion $K_1/2\ K_2 \leqq 0{,}5$, während dieselbe Grösse für das entsprechende *cis*-Ion rund 2,9 und für das *trans*- $[(ONO)_2\ Co\ py_2\ (NH_3)_2]^+$-Ion ungefähr gleich 18,5 ist.

Für die beiden *trans*-Verbindungen zeigt $K_1/2\ K_2$ also auffallend grosse Abweichungen von dem statistischen Wert 1. Der verschiedenartige Einfluss einer Nitrito- und einer Nitrogruppe in Axialstellung zur der sich verwandelnden Nitritogruppe macht sich somit bei ihnen besonders stark geltend. Die Abweichungen sind ja aber entgegengesetzt gerichtet. Die grossen $K_1/2\ K_2$-Werte bei dem *trans*- $[(ONO)_2\ Co\ py_2(NH_3)_2]^+$-Ion werden weiter von sehr kleinen K_2-Werten bedingt, während die kleinen $K_1/2$ K_2-Werte bei dem *trans* $[(ONO)_2Co\ en_2]^+$-Ion von unerwartet niedrigen K_1-Werten verursacht sind. Der Unterschied der beiden Fälle kommt auch dadurch zum Vorschein, dass die kleinen K_2-Werte des ersten *trans*-Ions ihren Grund in einer kleinen Aktionskonstante haben, die kleinen K_1-Werte des zweiten hauptsächlich von einer auffallend grossen Aktivierungsenergie abhängen.

Es liegt nahe, die auffallende Sonderstellung der *trans*-Ionen oben anderen Eigenschaften komplexer Verbindungen zur Seite zu stellen, die man durch die Annahme zu erklären versucht hat, Liganden in *trans*-Position stehen in besonderer Beziehung zu einander. Cox und Preston [12] haben z. B. mit Hilfe von Röntgenstrahlen die Symmetrieeigenschaften der komplexen Ionen oder Moleküle fester Platin- uud Palladiumverbindungen untersucht und aus ihren Resultaten geschlossen, dass die Valenzen der Metallatome nicht alle kristallographisch äquivalent sind sondern in *trans*-Paare unter sich äquivalenter Valenzen zu zerlegen sind. (Vgl. auch Drew, Pinkard, Wardlaw und Cox [13]). Das Problem einer speziellen *trans*-Wirkung ist weiter von mehreren japanischen Forschern erörtert worden. H. Sueda [14] fand beim Studium der zweiten im Wellenlängegebiete 320—350 mμ liegenden Absorptionsbande der Lösung einiger komplexen Nitroamminkobalt(III)-verbindungen u. a., dass *cis*-$[Co(NH_3)_4(NO_2)_2]Cl$ sein Absorptionsmaximum bei fast derselben Wellenlänge ($\lambda \simeq 325$ mμ) wie $[Co(NH_3)_5NO_2]Cl_2$ habe und dass der molare Extinktionskoeffizient der Dinitroverbindung rund doppelt so gross wie derjenige der Mononitroverbindung sei. Für *trans*-$[Co(NH_3)_4(NO_2)_2]Cl$ war das Maximum aber erheblich nach grösseren Wellenlängen verschoben ($\lambda_{Max.} = 347$ mμ) und noch höher als beim *cis*-Chlorid. Das brachte ihn auf den Gedanken, die gegenseitige Wirkung *cis*-

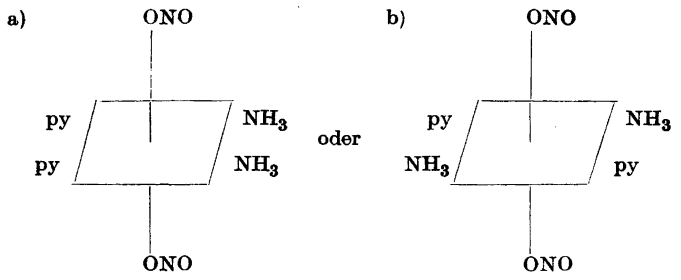
koordinierter Gruppen sei sehr gering und könnte vernachlässigt worden. Bei *trans*-gestellten Liganden müsste sie aber bedeutend sein. Sueda konnte sogar mit ziemlich guter Annäherung die Extinktionskoeffizienten mehrerer komplexen Aquochloroamminkobalt(III)- und -chrom(III)-ionen additiv aus konstanten Extinktionswerten berechnen, die er den drei *trans*-gestellten Ligandpaaren jedes Ions zuschrieb.

In den zwanziger und dreissiger Jahren hatten russische Forscher, vor allem Tschernaieff und Grünberg beim Studium komplexer Verbindungen von Platin, Palladium, Rhodium und Iridium empirisch gefunden, dass die Reaktionsfähigkeit, in erster Linie die Ersatzleichtigkeit, eines Ligands von der Natur seines *trans*-Ligands in höchstem Grade abhängig sei und dass ein Ligand, der in *trans*-Stellung zu einem Anion stehe, mehr labil erscheine als einer, dessen *trans*-Partner ein Neutralmolekül sei. Es wurde weiter von Grünberg[18] und anderen gezeigt, dass die Intensität der von einem Anion herrührenden *trans*-Wirkung mit seiner Polarisierbarkeit stetig zunimmt. Grünberg[19] meinte nun, diese *trans*-Wirkung sei in erster Hand von der Polarisierbarkeit des Zentralions wie von derjenigen der Liganden abhängig, und hat einige Berechnungen zur Schätzung deren Grössenordnung ausgeführt. Von seinem Standpunkte aus konnte er auch die Dissoziationsverhältnisse der beiden Säuren *cis*- und *trans*- $[Pt(H_2O)_2(NH_3)_2]^{2+}$ qualitativ erklären. (Vgl. K. A. Jensen[20].)

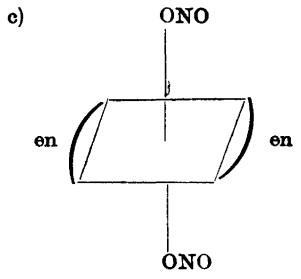
Diese *trans*-Wirkung wird schliesslich von Basolo[17] durch die Absorptionsspektren in Methanol-Wasser-Lösung der *cis-trans*-Paare von $[Co(NH_3)_4(NO_2)_2]$ Cl, $[Co\ en_2(NO_2)_2]NO_3$ und $[Co\ en_2Cl_2]$ Cl illustriert. Man findet, dass die zweite Absorptionsbande, deren Lage, wie Tsuchida[11] gezeigt hat, als ein qualitatives Mass der Stabilität des Komplexions betrachtet werden kann, für die *trans*- immer bei grösserer Wellenlänge als für die *cis*-Verbindung liegt. Die erste soll also die labilere sein.

Man muss somit die *trans*-Wirkung als experimentell gut belegt, wenn auch nicht theoretisch hinreichend klargemacht betrachten. Mit ihr lässt sich das Ergebnis dieser Arbeit gut vereinen, dass sich die Verschiedenheit einer Nitrito- und einer Nitrogruppe mit Rücksicht auf ihre geschwindigkeitsbeeinflussende Wirkung viel kräftiger geltend macht, wenn sie *trans*- als wenn sie *cis*-gestellt zur labilen Nitritogruppe sind. Warum $(f_{ONO})_a/(f_{NO_2})_a = K_1/2\,K_2$ für das erste *trans*-Ion, $[Co(ONO)_2py_2(NH_3)_2]^+$, viel grösser, für das zweite, $[Co(ONO)_2\ en_2]^+$ erheblich kleiner als eins wird, ist bedeutend schwerer zu verstehen.

In diesem Zusammenhange sei an den räumlichen Bau der beiden Ionenarten erinnert. Es ist noch eine offene Frage, ob das erste Ion die Konfiguration



hat. Für das zweite Ion muss man die Formel



als sichergestellt betrachten. Der sehr grosse Unterschied der Ionen mit Rücksicht auf den Quotienten $(f_{ONO})_a/(f_{NO_2})_a$ macht aber die Formel a) plausibler als b), denn mit der ersteren Konfiguration würde das Ion nur zwei Symmetrieebenen haben und wäre dem zweiten Ion, das drei Symmetrieebenen besitzt, unähnlicher sein, als wenn die Formel b) gültig sei, wo drei Symmetrieebenen ebenso vorhanden sind. Es ist jedenfalls nicht undenkbar, dass die einseitige Beeinflussung zweier Pyridin- bzw. zweier Ammoniakmoleküle auf das Zentralion eine Hauptbedingung des erwähnten Unterschiedes sein kann.

ZUSAMMENFASSUNG

1. Bei 20° C und für $385 \leqq \lambda \leqq 580$ mμ wird die Extinktionskurve wässeriger Lösungen von $\left[\begin{matrix}(1)\ O_2N \\ (6)\ O_2N\end{matrix}\ Co\ en_2\right] NO_3$ mit einem Beckman Quartz-Spektrophotometer bestimmt. Sie zeigt ein Absorptionsminimum bei $\lambda = 395$ mμ und ein Maximum bei $\lambda = 430$ mμ.

2. Lösungen von dem neu hergestellten, entsprechenden Dinitritonitrat gibt eine Extinktionskurve, die bei $\lambda = 480$ mμ ein flaches Minimum und bei $\lambda = 515$ mμ ein ebenso flaches Maximum hat. Die Kurve kann wegen der Isomerisation des Salzes in festem Zustande und vor allem in der Lösung nur als eine annähernde Extinktionskurve der Dinitritoverbindung betrachtet werden. Der Übergang vom Dinitrosalz zum Dinitritosalz bewirkt somit wie bei den analogen *cis*-Verbindungen [2] eine erhebliche Verschiebung des Maximums und Minimums gegen grössere Wellenlängen. Das Maximum ist weiter für das Dinitritonitrat bedeutend niedriger als für das Dinitronitrat.

3. Es wird der Isomerisation von festem $\left[\begin{matrix}(1)\ ONO \\ (6)\ ONO\end{matrix}\ Co\ en_2\right] NO_3$ bei 20,0°;

30,0° und 40,0° C in derselben Weise wie in zwei vorhergehenden Untersuchungen[1, 2] gefolgt. Die Wellenlänge des benutzten Lichtes ist 510 mμ, und die Probelösungen haben immer die Zusammensetzung

$$0{,}004\ M \text{ Komplexsalz} + 0{,}01\ M\ HOCOCH_3 + 0{,}01\ M\ NaOCOCH_3$$

Deren Schichtdicke ist 3,00 cm.

4. Es wird gefunden, dass die Variation der Extinktion E_V der erwähnten Versuchslösung, zum Auflösungszeitpunkte der festen Probe extrapoliert, mit dem Alter t des über P_2O_5 verwarten festen Salzes durch die Annahme einer zweistufigen, als zwei konsekutive Reaktionen erster Ordnung verlaufenden Isomerisation:

$$\left[\begin{matrix}ONO\\ONO\end{matrix}\ Co\ en_2\right] NO_3 \rightarrow \left[\begin{matrix}O_2N\\ONO\end{matrix}\ Co\ en_2\right] NO_3 \rightarrow \left[\begin{matrix}O_2N\\O_2N\end{matrix}\ Co\ en_2\right] NO_3$$

gut erklärt werden kann.

5. Mit wachsendem t (von dem Auflösungsaugenblicke der ersten Probe gerechnet) nähert sich E_V immer einem Grenzwert E_G, der gleich der Extinktion einer Versuchslösung mit reinem Dinitronitrat als Komplexsalz ist.

6. Es zeigt sich, dass $E_V - E_G$ sowohl in der Form

$$\text{a)}\quad E_V - E_G = C \cdot 10^{-\lambda_1 t} + D \cdot 10^{-\lambda_2 t}$$

wie

$$\text{b)}\quad E_V - E_G = (C' + D'\ t) \cdot 10^{-\lambda t}$$

geschrieben werden kann. ($\lambda_1 > \lambda_2$).

7. Die Konstanten C, D, λ_1 und λ_2 lassen sich nach einer früher angegebenen Methode aus den Versuchsdaten bestimmen. (Vgl. Adell[1, S. 17–19]). Zur Ermittlung von C', D' und λ aus dem empirischen Material wird ein neues Verfahren ausgearbeitet. Die Formeln vom Typ b) können die gefundenen E_V-Werte besser wiedergeben als die vom Typ a) (Mittlere Abweichung 1,0 bzw. 1,5 %).

8. Die a)-Formeln können durch die Annahme zweier Teilisomerisationen mit verschiedenen Geschwindigkeitskonstanten K_1 und K_2 erklärt werden. Eine nähere Erörterung zeigt, dass K_1 gleich λ_2 und K_2 gleich λ_1 sein muss. Die erste Teilisomerisation soll also nach diesen Formeln langsamer als die zweite verlaufen.

9. Die b)-Formeln fordern gleiche Geschwindigkeitskonstanten K der beiden Teilisomerisationen. K wird gleich λ.

10. Die gefundenen mittleren Werte von K_1 sind bei 20°, 30° und 40° C 0,00158; 0,0081 und 0,034. Für K_2 resultiert entsprechend bei denselben

Temperaturen 0,00435; 0,027 und 0,059. (Dekadische Logarithmen; Zeit in Stunden gerechnet.) Die zugehörigen Werte der Aktionskonstante und Aktivierungsenergie sind $a' = 3{,}7 \cdot 10^{14}$ sec^{-1} und $q' = 28{,}04$ kcal, wie $a'' = 8{,}4 \cdot 10^{11}$ sec^{-1} und $q'' = 23{,}83$ kcal.

11. Wenn man gleiche Geschwindigkeitskonstanten K der Teilisomerisationen voraussetzt, erhält man für K bei 20°, 30° und 40° C im Mittel die Werte 0,0024; 0,0114 und 0,045. Daraus ergibt sich $a = 5{,}9.\ 10^{13}$ sec^{-1} und $q = 26{,}73$ kcal.

12. Die b)-Formeln geben zwar die empirischen E_V-Werte besser wieder als die a)-Formeln. Es scheint aber noch kein hinreichender, theoretischer Grund vorzuliegen, K_1 exakt gleich K_2 zu setzen.

13. Ein Vergleich mit den Resultaten der beiden vorigen Arbeiten [1,2] über die Isomerisationsgeschwindigkeit fester Dinitritokobalt(III)-nitrate zeigt, dass für die *trans*-Ionen

$$\left[\begin{matrix}(1)\ ONO\\(6)\ ONO\end{matrix}\ Co\ py_2(NH_3)_2\right]^+ \text{ und } \left[\begin{matrix}(1)\ ONO\\(6)\ ONO\end{matrix}\ Co\ en_2\right]^+$$

der Quotient $\dfrac{K_1}{2\,K_2}$ etwa 18,5 und 0,2 (bzw. 0,5) wird und also von dem theoretischen Wert 1 in hohem Grade abweicht. Für das *cis*-Ion $\left[\begin{matrix}(1)\ ONO\\(2)\ ONO\end{matrix}\ Co\ en_2\right]^+$ hat $K_1/2\,K_2$ den mittleren Wert 2,9.

14. Die Sonderstellung der *trans*-Verbindungen wird aus dem Gesichtspunkte einer starken gegenseitigen Beeinflussung *trans*gestellter Liganden durch die chemischen Bindungen hindurch erörtert.

Statens Naturvetenskapliga Forskningsråd hat mir Geldmittel zu der hier vorliegenden Untersuchung bewilligt. Dafür spreche ich meinen herzlichsten Dank aus. Ebenso bin ich dem Institutionschef, Professor Dr S. Bodforss für sein die Arbeit förderndes Interesse grossen Dank schuldig. Mit meinem Freunde, Lektor Torsten Nilson habe ich wertvolle Diskussionen der theoretischen Probleme dieser Arbeit gehabt. Ich bin ihm dafür dankbar. Meinen Assistenten Boo Frejrud, Irene Petersson und Brita Linderot verdanke ich schliesslich eine sehr gute Mitarbeit.

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Short Communications

Different Nitrogen Fractions in Normal and Low-Nitrogen Cells of Microorganisms. II. Changes in the Free Amino Acids of Low-Nitrogen *Torulopsis utilis* Yeast during Nitrogen Enrichment by Various Nitrogen Sources

Preliminary communication

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The enrichment experiments reported earlier [1] have been continued with other nitrogen sources. Low-nitrogen *Torulopsis utilis* yeast (N appr. 4 % of dry weight) was prepared from normal *Torulopsis utilis* yeast (N appr. 8 % of dry weight) by driving, in a Kluyver-flask, a strong current of air through a sugar nutrient solution (without combined nitrogen) in which the normal yeast was suspended [2].

The enrichment experiments were made as follows: The washed low-nitrogen yeast was suspended in water in a Kluyver-flask and a strong current of air was driven through the suspension. A 0-sample was taken, whereupon one of the following nitrogen compounds: ammonium sulphate, sodium nitrate, glutamic acid, aspartic acid, glycine, alanine, or asparagine, was added in concentrated water solution to make the initial molarity of the added nitrogen 0.03–0.2. Samples were taken 5, 15, 30 min and 1, 2, and 4 h after the addition of the nitrogen compound.

Each sample was rapidly chilled and washed, and an aliquot was extracted with 70 % ethanol. The ninhydrin-positive compounds of the ethanol extracts were determined semiquantitatively by paper-chromatography using the spot dilution technique [3]. The chromatograms were made by the "continuous development technique" [4] using 1-dimensional runs with *tert.* amyl alcohol for leucine, *iso*-leucine, phenylalanine, methionine, valine, and tyrosine (see ref. 4), and 2-dimensional runs (see Fig. 1) for the other amino acids.

From other aliquots, measured simultaneously, dry weight, total-N, N soluble in hot (10 min extraction at 100° C) and cold (1 h at 0° C) 8.3 % trichloroacetic acid, total nucleic acids (by a slight modification of the method of Di Carlo *et al.* [5]), and desoxyribonucleic acid (Dische-method) were determined and microscopic preparations made. Protein-N and ribonucleic acid were calculated as differences.

The 70 % ethanol extract of low-nitrogen Torulopsis utilis contains:
richly: glutamic acid and alanine,
considerably: arginine, lysine, unknown no. 26 (probably a peptide), aspartic acid, glutathione, glycine, serine, threonine, unknown no. 30 (probably a difficultly hydrolysable peptide), valine, tyrosine, unknown no. 43 (probably a peptide) and unknown no. 41 (unhydrolysable basic compound),
slightly: leucine, isoleucine, phenylalanine, glutamine, asparagine, proline, and methionine.

Histidine, cystine, hydroxyproline, ethanolamine phosphoric acid esther, sarcosine, taurine, γ-aminobutyric acid, α-amino-*n*-butyric acid, α-amino-*iso*butyric

acid and β-alanine have not been present in identifiable amounts.

In hydrolysis (20 % HCl 24h 108° C) glutamine, glutathione, asparagine, and the unknown compounds nos. 26 and 43 disappear completely and compound no. 30 partly and considerable amounts of amino acids, mainly glutamic acid, glycine, alanine, and unknown 41 are set free. In addition an unknown 42 appears.

The presence of *peptides* containing mainly glutamic acid, glycine and alanine, but in smaller amounts other amino acids, has been further confirmed by cutting strips (corresponding to compounds 26 and 30) out from a set of chromatograms, eluting them with water, and examining the concentrated eluate paperchromatographically before and after hydrolysis.

The 70 % ethanol extract of normal Torulopsis utilis was studied for comparison. In general, it contains the same free amino acids as the low-nitrogen yeast but in considerably higher concentrations, arginine, glutamine and alanine being (relatively) most enriched.

The most remarkable changes in the concentrations of the free amino acids in the *enrichment experiments* were the following ones:

I. *Ammonium sulphate:* Ammonium nitrogen was rapidly assimilated and a very powerful formation of glutamine (but not asparagine) rapidly (in less than 5 min) started. Glutamic acid and alanine, too, were rapidly enriched. The unknown no. 30 disappeared completely (in one experiment) or partly in 5 min. Later (after about 1—2 hours) a slow and continuous enrichment of other amino acids, especially of the basic ones, took place.

II. *Sodium nitrate* was only very slowly assimilated and no change in the free amino acids took place within the first 30 min. In 1,2 and especially 4-hours' samples an enrichment of glutamine, alanine and glutamic acid and probably glutathione was to be noticed.

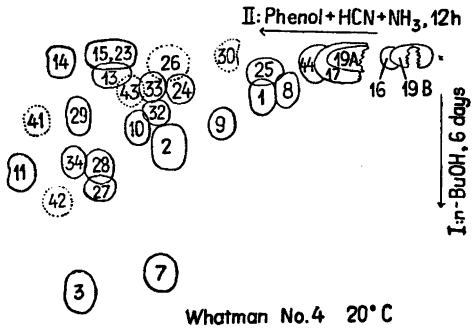

Fig. 1. 1 = Gly, 2 = ala, 3 = val, 7 = tyr, 8 = ser, 9 = thr, 10 = hypro, 11 = pro, 13 = his, 14 = arg, 15 = lys, 16 = asp, 17 = glu, 19 = glutathione, 23 = orn, 24 = glutamine, 25 = asparagine, 27 = α-amino-n-butyric acid, 28 = α-amino-iso-butyric acid, 29 = γ-aminobutyric acid, 32 = β-alanine, 33 = sitr, 34 = sarc, 35 = tau, 44 = phosphorylaminoethanol.

III. *Aspartic acid* was rapidly assimilated but not much enriched in the yeast cells, since it was rapidly metabolised further. Instead, glutamic acid, glutamine, and alanine were strongly enriched. In the 4 hours' sample asparagine was enriched.

IV. *Glutamic acid* was rapidly assimilated and metabolised. At first (5 to 30 min) mainly glutamine and alanine, but later (1 to 2 hours) other amino acids were enriched. In the 4 hours' sample asparagine was enriched.

V. *Glycine* was rapidly assimilated — in 15 min a large amount of glycine was enriched in the cells — but the only change in the other amino acids was at first the disappearance of the compound no. 30. Not until after 2 hours was a formation of glutamine, alanine and glutamic acid noticeable.

VI. *Alanine* was rapidly assimilated but only after about 30 min did a more considerable enrichment of glutamine start.

VII. *Asparagine* was the most potential of the nitrogen sources studied. It was rapidly assimilated and (in addition to

asparagine) a rapid enrichment of glutamine, glutamic acid, aspartic acid, and probably glutathione immediately started. A distinct but less intense enrichment of the other amino acids started rapidly, too.

Ribonucleic acid was rapidly enriched with all the other nitrogen sources, expect sodium nitrate, during the first and second hour, but no greater change took place in the *desoxyribonucleic acid.*

Roine [2] has earlier studied in this laboratory with other methods changes in the free amino acids during the enrichment of low-nitrogen *Torulopsis* with ammonium nitrogen being able to show enrichment of amides — glutamine and asparagine — and glutamic acid and alanine. His results have now been confirmed paperchromatographically.

By the sensitive paperchromatographic method many other free amino acids have now been shown to be present and other changes have been observed.

Virtanen *et al.* [6] have studied enrichment with NO_3-nitrogen finding that amino acid and amide content of the cells rises hereby much less than with ammonium nitrogen, which is in accordance with the results in this paper.

The rapid enrichment of *alanine* in these experiments must be emphasized as well as the great activity of *asparagine* as a nitrogen source, and the presence of *peptides*, which in their amino acid composition resemble more the free amino acid fraction of the yeast than the protein of the yeast.

Full details of this work are being published elsewhere.

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Notiz über die Reaktion zwischen Trialkyl-amino-silanen und Schwefelwasserstoff

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Eaborn [1] hat gezeigt, dass Trimethyl-jod-silan und Silbersulfid mit einander unter Bildung von Hexamethyl-thiodisiloxan reagieren. In entsprechender Weise entstand Hexaäthyl-thiodisiloxan aus Triäthyl-jod-silan. Im folgenden werden wir eine Methode angeben, nach der Hexaalkylthiodisiloxane ohne Verwendung der schwierig zugänglichen Trialkyl-jod-silane dargestellt werden können.

Gasförmiges Schwefelwasserstoff wurde während einigen Stunden in Triäthyl-amino-silan eingeleitet. Bei der Destillation des Reaktionsgemisches wurde *teils* Triäthyl-thiosilanol vom Sdp. 158°, *teils* Hexaäthyl-thiodisiloxan vom Sdp. 128° (7 mm) erhalten. 163 g Triäthylamino-silan ergaben in dieser Weise 34,5 g Triäthyl-thiosilanol und 95,2 g Hexaäthyl-thiodisiloxan. Weiter wurden aus 89,5 g Tri-*n*-propyl-amino-silan und Schwefelwasserstoff 18,4 g Tri-*n*-propyl-thiosilanol vom Sdp. 83—84° (7 mm) und 54,3 g Hexa-*n*-propyl-thiodisiloxan vom Sdp. 168° (7 mm) erhalten.

Versuche mit Trimethyl-amino-silan konnten nicht ausgeführt werden, weil dieses Silan noch nicht dargestellt worden ist. Man hat immer statt dessen Hexamethyl-disilazin erhalten. Dieses reagierte sehr schlecht mit Schwefelwasserstoff. Dagegen ergab Trimethyl-N-phenyl-amino-silan mit Schwefelwasserstoff Trimethyl-thiosilanol vom Sdp. 75—76°. In einem Versuch betrug aber die Ausbeute nur 14 %.

Über die obigen Versuche und einige andere Umsetzungen zwischen Verbindungen $R_3Si \cdot N\begin{smallmatrix}R'\\R''\end{smallmatrix}$ (wo R Alkylgruppen und

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R′ und R″ Wasserstoffatome oder Alkyl-, Aryl- oder Aralkylgruppen sind) und Schwefelwasserstoff oder Merkaptanen und die dabei erhaltenen Produkte werden wir später eingehend berichten.

1. Eaborn, C., *Nature* **165** (1950) 685; *J. Chem. Soc.* (1950) 3077.

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Precipitation of Phosphate in the Gomori Test

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In the histochemical test for localization of alkaline phosphatase described by Gomori [1] and by Takamatsu [2] thin tissue slices are incubated in solutions containing glycerophosphate and calcium ions. Calcium phosphate is supposed to be precipitated at the sites of enzyme action. But calcium phosphate (in its least soluble form, *viz.* that of hydroxyapatite $Ca_5(PO_4)_3OH$) has a strong tendency to form supersaturated solutions as shown by the experiments recorded in Table 1 where $p(Ca) = -\log c_{Ca^{++}}$, $p(HPO_4) = -\log c_{HPO_4^{--}}$ and p(HAP) is the negative logarithm of

$$\frac{c^5_{Ca^{++}}\, c^3_{PO_4^{---}}}{c_{H^+}\, c_{OH^-}} \left(\frac{c_{HPO_4^{--}}}{c_{H^+}\, c_{PO_4^{---}}}\right)^3 = \frac{c^5_{Ca^{++}}\, c^3_{HPO_4^{--}}}{c^4_{H^+}}$$

$$p(HAP) = 5\,p(Ca) + 3\,p(HPO_4) - 4pH$$

In the four last experiments a precipitate was formed, in expt. 2 only a slight turbidity appeared, and in expt. 1 none at all. From these and from a few other experiments in which the ratio Ca^{++}/HPO_4^{--} was varied from 1 to 4 it is concluded that the tendency for hydroxyapatite to crystallize *spontaneously* is negligible when p(HAP) > − 13.3 whereas the value of p(HAP) corresponding to solubility equilibrium is about +1. Hence there is a possibility that in the Gomori test calcium phosphate will precipitate, not at the sites of enzyme action where the concentration of phosphate is highest, but at places where there are pre-formed crystal nuclei or cell structure elements particularly favorable for adsorption, complex formation or the like.

Table 1. Formation of precipitate in a solution (A) containing sodium diethylbarbiturate (0.023 M), sodium glycerophosphate (0.017 M) and the concentrations of calcium chloride and disodium phosphate given below. pH 9.4, temp. 37° C.

p(Ca)=p(HPO_4)	p(HAP)	log t_{prec}
3.08	− 12.96	—
3.00	− 13.60	3.30
2.93	− 14.16	1.74
2.90	− 14.40	1.30
2.87	− 14.64	1.00
2.85	− 14.80	0.70

In a bifurcated vessel 5 ml solution A containing calcium chloride was rapidly mixed at 37° C with 5 ml A containing disodium phosphate to give the above solutions. A strong ray of light was thrown through the solution. t_{prec} is the time in seconds when a faint but definite Tyndall beam was observed.

According to Reis [3] the phosphatase activity at pH 9 of human tissues may be put at 1 μg phosphorus per hour per mg wet weight on an average. Per cell of radius 10 μ it corresponds to $\gamma = 4 \cdot 10^{-17}$ moles per sec. In the pre-treatment of sections in the Gomori technique there are severe losses of phosphatase activity but we take $4 \cdot 10^{-17}$ as an upper limit for γ in a treated cell of this size. The turnover of alkaline phosphatase, *i.e.*, the number of substrate molecules converted per second

R′ und R″ Wasserstoffatome oder Alkyl-, Aryl- oder Aralkylgruppen sind) und Schwefelwasserstoff oder Merkaptanen und die dabei erhaltenen Produkte werden wir später eingehend berichten.

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$$\frac{c^5_{Ca^{++}}\, c^3_{PO_4^{---}}}{c_{H^+}\, c_{OH^-}} \left(\frac{c_{HPO_4^{--}}}{c_{H^+}\, c_{PO_4^{---}}}\right)^3 = \frac{c^5_{Ca^{++}}\, c^3_{HPO_4^{--}}}{c^4_{H^+}}$$

$$p(HAP) = 5\,p(Ca) + 3\,p(HPO_4) - 4pH$$

In the four last experiments a precipitate was formed, in expt. 2 only a slight turbidity appeared, and in expt. 1 none at all. From these and from a few other experiments in which the ratio Ca^{++}/HPO_4^{--} was varied from 1 to 4 it is concluded that the tendency for hydroxyapatite to crystallize *spontaneously* is negligible when p(HAP) > − 13.3 whereas the value of p(HAP) corresponding to solubility equilibrium is about +1. Hence there is a possibility that in the Gomori test calcium phosphate will precipitate, not at the sites of enzyme action where the concentration of phosphate is highest, but at places where there are pre-formed crystal nuclei or cell structure elements particularly favorable for adsorption, complex formation or the like.

Table 1. Formation of precipitate in a solution (A) containing sodium diethylbarbiturate (0.023 M), sodium glycerophosphate (0.017 M) and the concentrations of calcium chloride and disodium phosphate given below. pH 9.4, temp. 37° C.

p(Ca)=p(HPO_4)	p(HAP)	log t_{prec}
3.08	− 12.96	—
3.00	− 13.60	3.30
2.93	− 14.16	1.74
2.90	− 14.40	1.30
2.87	− 14.64	1.00
2.85	− 14.80	0.70

In a bifurcated vessel 5 ml solution A containing calcium chloride was rapidly mixed at 37° C with 5 ml A containing disodium phosphate to give the above solutions. A strong ray of light was thrown through the solution. t_{prec} is the time in seconds when a faint but definite Tyndall beam was observed.

According to Reis [3] the phosphatase activity at pH 9 of human tissues may be put at 1 μg phosphorus per hour per mg wet weight on an average. Per cell of radius 10 μ it corresponds to $\gamma = 4 \cdot 10^{-17}$ moles per sec. In the pre-treatment of sections in the Gomori technique there are severe losses of phosphatase activity but we take $4 \cdot 10^{-17}$ as an upper limit for γ in a treated cell of this size. The turnover of alkaline phosphatase, *i.e.*, the number of substrate molecules converted per second

per enzyme molecule, is unknown, but we adopt the value 1000 which is very high compared with those found for other hydrolases. On this basis we find that the γ assumed corresponds to the action of $4 \cdot 10^{-17} \cdot 6 \cdot 10^{23}/1000 = 2.4 \cdot 10^4$ enzyme molecules/cell.

We assume that these molecules are packed in spherical sites of uniform size and postulate that each molecule occupies a space of $\frac{4}{3} \pi \cdot 10^{-18}$ cm^3. If the "sites" are evenly distributed throughout the cell each one of them may be regarded as being surrounded by an approximately spherical space containing no enzyme activity. When the enzymatic reaction goes on at a uniform rate in all sites the phosphate concentration will rise in accordance with a pattern which is the same in all spheres so there is no net transfer of phosphate from one sphere to another before precipitation sets in. Hence each sphere may be treated as if it were bordered by an impermeable membrane and it is possible to calculate the rate of diffusion of phosphate from the sites after a short time needed for the establishment of a quasi-steady state. The result of such a calculation is shown in Table 2 where $p(HPO_4)_{crit}$ is the negative logarithm of the concentration of secondary phosphate ion needed to bring p(HAP) to the critical value for spontaneous precipitation, p(Ca) and pH = 9.4 being given. ν is the number of sites per cell, t_{crit} the number of seconds required for the maximum concentration of phosphate to reach the critical value $(c'_o)_{crit}$. $(c'_R)_{crit}$ is the value at this time of the minimum concentration and F_R the factor by which $(c'_R)_{crit}$ exceeds the value corresponding to the solubility product. t'_s is the time required for reaching the quasi-steady state.

Table 2.

$c_{Ca++} \cdot 10^3$	$p(HPO_4)_{crit}$	ν	t_{crit}(sec)	$(c'_o)_{crit} \cdot 10^5$	$(c'_R)_{crit} \cdot 10^5$	F_R
8.4	4.63	$2.4 \cdot 10^4$	2.45	2.34	2.34	60000
»	»	10^4	2.45	2.34	2.34	
»	»	10^3	2.44	2.34	2.33	
»	»	10^2	2.41	2.34	2.30	
»	»	10	2.27	2.34	2.17	
»	»	1	1.61	2.34	1.54	39000
50	5.93	$2.4 \cdot 10^4$	0.122	0.118	0.117	59000
»	»	10^4	0.121	0.118	0.116	
»	»	10^3	0.115	0.118	0.110	
»	»	10^2	0.084	0.118	0.080	41000
»	»	10	$< t'_s$	—	—	
»	»	1	$< t'_s$	—	—	

The figures in Table 2 are only valid for a thin tissue section covered with a 10 μ thick layer of substrate solution. However, if the thickness of this layer is increased, all the values except t_{crit} will remain constant while t_{crit} will increase proportional to the thickness. It is seen that before conditions for spontaneous precipitation at the sites have become favorable there is abundant possibility for crystallization around stray crystal nuclei in the cytoplasmic material, for adsorption onto suitable cell structure elements *etc.*

In the foregoing we have assumed that if the macroscopic conditions for spontaneous precipitation are present then a sufficiently large number of crystal nuclei will be formed to ensure a defined distribu-

tion of solid particles within microscopic dimensions. But in a study on crystallization of calcium fluoride, Tovborg Jensen[4] found that a comparatively small number of solid particles (10^8 per cm^3) appear, seemingly formed from an approximately equal number of crystal nuclei by capture of ions from the solution and not by coalescence of a much larger number of nuclei. In the case of calcium fluoride there would not be one solid particle available per cell of radius $R = 10^{-3}$ cm ($10^8 \frac{4}{3} \pi R^3 = 0.4$) and even if in the case of calcium phosphate the number of nuclei per cm^3 exceeded 10^8 by several powers of ten it would be hard to imagine that finer cell structures could come out by well defined local precipitation.

The question therefore arises whether spontaneous precipitation ever occurs in the Gomori system. It seems more likely that precipitation is always induced either by pre-formed crystal nuclei or by other structure elements so that the deposits obtained are determined not only by the localization of enzymic sites but also by the localization of "precipitation centers". Recent experiments [5] confirm this view.

A more detailed treatment of the problem including aspects such as the distribution of phosphate within the sites themselves and the possibility of wandering of primary deposits of calcium phosphate toward sources of phosphate ions so as to give an increasingly correct picture, will appear in the *Acta Medica Scandinavica*.

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Aromatic Seleno- and Telluropolythionic Compounds

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This note gives a preliminary report on three new types of benzene- and *p*-toluenesulphonyl derivatives, *viz.*, selenium disulphinates, $Se(SO_2R)_2$, and thiosulphonates of divalent selenium and tellurium, $Se(S_2O_2R)_2$ and $Te(S_2O_2R)_2$. The corresponding methanethiosulphonates have been described recently [1], whereas benzene- and *p*-toluenesulphinates and -thiosulphonates of divalent sulphur, $S(SO_2R)_2$ and $S(S_2O_2R)_2$, are of older date [2, 3].

The selenium disulphinates were prepared from finely powdered, dry sodium benzene- or *p*-toluenesulphinate, suspended in dry benzene or ether, and selenium tetrachloride:

$$SeCl_4 + 3\ RSO_2Na = Se(SO_2R)_2 + RSO_2Cl + 3\ NaCl$$

The compounds also result, in equally smooth reactions, if selenium oxychloride, $SeOCl_2$, is used instead of selenium tetrachloride. Furthermore, they occur as products, beside triselenium disulphinates, when diselenium dichloride reacts with the sodium sulphinates suspended in dry ether [4].

The thiosulphonates of divalent selenium were got from diselenium dichloride and dry, powdered potassium benzene- or *p*-toluenethiosulphonate suspended in dry ether, and the thiosulphonates of divalent tellurium were obtained from the same salts, suspended in dry, ethanol-free chloroform, and tellurium tetrachloride:

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tion of solid particles within microscopic dimensions. But in a study on crystallization of calcium fluoride, Tovborg Jensen[4] found that a comparatively small number of solid particles (10^8 per cm^3) appear, seemingly formed from an approximately equal number of crystal nuclei by capture of ions from the solution and not by coalescence of a much larger number of nuclei. In the case of calcium fluoride there would not be one solid particle available per cell of radius $R = 10^{-3}$ cm ($10^8 \frac{4}{3} \pi R^3 = 0.4$) and even if in the case of calcium phosphate the number of nuclei per cm^3 exceeded 10^8 by several powers of ten it would be hard to imagine that finer cell structures could come out by well defined local precipitation.

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$$Se_2Cl_2 + 2\ RSO_2SK = Se(S_2O_2R)_2 + Se + 2\ KCl$$

$TeCl_4 + 4\ RSO_2SK =$
$Te(S_2O_2R)_2 + S_2(SO_2R)_2 + 4\ KCl$

The thiosulphonates of divalent selenium are pale greenish, and so are the selenium disulphinates, whereas the thiosulphonates of divalent tellurium are yellow. When kept in a dry, neutral atmosphere, shielded from light, the crystals remain unchanged for months.

In the following, the symbols Bs and Ts denote the benzenesulphonyl and *p*-toluenesulphonyl group, respectively.

Compound	M.p., °C (preheated bath, dec.)	% Se or Te Calc.	Found
$SeBs_2$	132–3	21.85	21.94
$SeTs_2$	121–2	20.29	20.16
$Se(SBs)_2$	152–4	19.00	19.04
$Se(STs)_2$	Ca. 200	17.41	17.53
$Te(SBs)_2$	Ca. 170	26.91	26.73
$Te(STs)_2$	Ca. 215	25.41	25.28

The crystals of the selenium disulphinates, $SeBs_2$ and $SeTs_2$, are monoclinic prismatic, and are isomorphous with those of the corresponding sulphur derivatives, the crystal structure of which was described resently by Mathieson and Robertson [5].

The unit cells and space group of the benzene- and *p*-toluenethiosulphonates of divalent sulphur, $S(SBs)_2$ and $S(STs)_2$, have been reported by Dawson, Mathieson and Robertson [6]. The crystals are tetragonal trapezohedral. The crystals of the following four compounds are isomorphous with those of the sulphur thiosulphonates: Selenium di(benzenethiosulphonate), selenium and tellurium di(*p*-toluenethiosulphonate), and triselenium di(*p*-toluenesulphinate), *i.e.*, $Se(SBs)_2$, $Se(STs)_2$, $Te(STs)_2$, and Se_3Ts_2. Tellurium di(benzenethiosulphonate), $Te(SBs)_2$, is orthorhombic, whereas triselenium di(benzenesulphinate), Se_3Bs_2, is triclinic. Work on the crystal structure of the *p*-toluene compounds, $Se(STs)_2$, $Te(STs)_2$ and Se_3Ts_2, is in progress in this Institute.

1. Foss, O. *Acta Chem. Scand.* **5** (1951) 115.
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5. Mathieson, A. McL., and Robertson, J. M. *J. Chem. Soc.* (1949) 724.
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Explosions in the Preparation of Diethylaminoethyl Dinitrate

JØRGEN FAKSTORP and JYTTE CHRISTIANSEN

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In the preparation of diethylaminoethyl dinitrate, $O_2NO \cdot CH_2 \cdot CH_2 \cdot N(C_2H_5)_2$, HNO^3 according to the directions given by Barbière [1], we have experienced several very violent explosions.

Hence we feel compelled to call attention to the potential danger of this compound.

The procedure as described by Barbière (*l.c.*) calls for addition of 23.4 g of diethylaminoethanol to 75.6 g of fuming nitric acid under stirring in an ice bath and evaporating excess nitric acid *in vacuo* being careful not to let the bath temperature exceed 40° C.

When most of the nitric acid had evaporated our mixture invariably exploded. This explosion could not be prevented by nitrating in the presence of urea.

The following alternative procedure is suggested: After addition of the reagents,

$TeCl_4 + 4\ RSO_2SK =$
$Te(S_2O_2R)_2 + S_2(SO_2R)_2 + 4\ KCl$

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The following alternative procedure is suggested: After addition of the reagents,

which should take at least two hours, the reaction mixture is diluted with 200 ml of methanol and precipitated with 2 l of ether.

After a night in the icebox the solvents are decanted from the oily precipitate, which is washed with ether and dissolved in 50 ml of anhydrous ethanol. This solution is evaporated to half its volume *in vacuo* keeping a bath temperature of no more than 30° C. Upon cooling, and addition of a small amount of ether, a crystalline precipitate starts to settle, which is removed, washed with ether and dried over conc. sulphuric acid and paraffin.

The prisms melted at 60° C (Barbière finds 63° C). Yield 22 g = 50 % of the theoretical.

We have prepared other similar compounds without difficulty according to Barbière's directions.

1. Barbière, J. *Bull. Soc. Chim.* [5] **11** (1944 473.

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The Structure of Pauly's Dibromodiethyl Ketone

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A dibromo-diethyl ketone with the bromine atoms in the α-position of the keto group has been prepared by Pauly [1], and he assumed the substance to be symmetrically substituted. No structure proof is given, however, and he made his conclusions from the attributed similarity to 1,3-dichloroacetone.

When a ketone is brominated, it is well known that the first bromine atom will substitute at the α-carbon atom and the second one, if possible, in most cases at the same α-position.[2] Acetone and methyl ethyl ketone are thus brominated according to the given rule, and it is surprising to find that diethyl ketone gives a symmetrical dibromide. This substance was needed in connection with other work, and a structure proof and an examination of the dibromide of diethyl ketone was therefore performed.

The usual way to get evidence about the structure of a dibromo substituted ketone is to hydrolyze the substance.[2] The dibromide was thus hydrolyzed in an alkaline medium, and from the products acetylpropionyl could be isolated. The hydrolysis is very rapid and has been performed with potassium hydroxide in cooled alcohol as well as with potassium carbonate in water solution. Acetylpropionyl was easily identified by the dioxime and the orange-coloured nickel precipitate, formed from the dioxime, a well known reagent of aliphatic α-diketones. Duroquinone was also obtained from acetylpropionyl and dilute alkali hydroxide,[3] and if a mixture of the dibromide and potassium carbonate in water solution is refluxed, until the dibromide is dissolved, duroquinone can be isolated from the reaction products. A similar investigation has once been performed by Faworsky on α,α-dichloropropyl methyl ketone. [4]

From the results it is obvious that the dibromide must be unsymmetrically substituted, as the hydrolysis gives an α-diketone and not α,α′- dioxi-diethyl ketone. The author is conscious, however, of the possibility of a rearrangement of the dibromo ketone from α,α′- to α, α-substitution during the hydrolysis, but it must be emphasized that such an isomerisation is not very probable in an alkaline medium and not found in analogous reactions; the Faworsky rearrangement [2] only results in other products of hydrolysis and is specially studied for α,α′-dibromo ketones. *The dibromide is thus α, α-dibromo-diethyl ketone.*

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The substance is found to oxidize the iodide ion in a water solution of potassium iodide to iodine on heating, and on the other hand it reduces Fehling's solution and Tollen's reagent. However, these two reductions might be caused by acetylpropionyl, formed to some extent in a rapid hydrolysis of the dibromide, as both the reagents are alkaline. The aliphatic α-diketones diacetyl and acetylpropionyl reduce namely Fehling's solution easily. An oxidation of the dibromide with potassium permanganate gives propionic acid and acetic acid.

Experimental. α,α-Dibromo-diethyl ketone. 320 g (2 moles) of bromine was added dropwise to 86 g (1 mole) of diethyl ketone in the presence of red phosphorus. Nitrogen gas was bubbled through the solution in order to remove hydrogen bromide. After half the bromine was added, the mixture was warmed to 80° and maintained at that temperature for one hour after the addition. The product was washed with water and some 5 % sodium carbonate and distilled with benzene, then fractionated *in vacuo*. B. p. 75—77°/ 10 mm. Yield: 180 g (74 %). The density as well as the boiling point is in agreement with the values found by Pauly. [1]

$C_5H_8OBr_2$ (243.94) Calc. Br 65.52
Found » 65.56

The hydrolysis and some derivatives. 24.4 g (0.1 mole) of the dibromide of diethyl ketone was dropped into an ice-cooled solution of 16.8 g (0.3 moles) of potassium hydroxide in 80 ml of alcohol. The mixture was stirred and allowed to stand overnight. Potassium bromide was separated, and the solution neutralized with concentrated hydrochloric acid and then distilled in order to remove the alcohol and some water. The residue was dissolved in ether, dried over anhydrous potassium carbonate and, after removal of the ether by distillation, fractionated and a middle fraction, b. p. 109—112°/ 752 mm, taken. The liquid was dark-yellow coloured. Yield: 5.9 g (59 %).

A qualitative test showed the substance to be an α-diketone, and the dioxime could be prepared from the liquid and hydroxylamine hydrochloride in alcoholic solution in the presence of sodium acetate, according to the general process. M. p. 172—173°. From an alcoholic solution of the dioxime and a water solution of nickel chloride the typical nickel salt could be obtained. M. p. 276—280°. — When a water solution of the α-diketone was treated with hot dilute sodium hydroxide for a few minutes a product, crystallizing in yiellow needles with the melting point, 108—110°, identified as duroquinone, was obtained.[3]

The three above-mentioned derivatives give full evidence for the identification of the hydrolysis product as acetylpropionyl.

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A Synthesis of *dl*-2-Methyl-5,7-dihydroxyflavanone

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5,7-Dihydroxyflavanone (dihydrochrysin) has been synthesised from phloroglucinol and cinnamoyl chloride by the Friedel-Crafts method [1]. Under the same conditions phloroglucinol and β-methylcinnamoyl chloride gave a mixture of two colourless substances. The main product, m. p. 154—156° *, had the composition $C_{16}H_{14}O_4$, gave a yellow colour when reduced with magnesium and hydrochloric acid and a reddish brown colour with ferric chloride, and thus must be the expected *dl*-2-methyl-5,7-dihydroxyflavanone (I). On the paper chromatogram, its R_F value is about 0.55 (benzene-ligroin solvent [2], and the substance gives a red spot with diazotised benzidine. The R_F value of 5,7-dihydroxyflavanone in the same solvent is 0.44, so that, as expected, the in-

* All melting points uncorrected.

The substance is found to oxidize the iodide ion in a water solution of potassium iodide to iodine on heating, and on the other hand it reduces Fehling's solution and Tollen's reagent. However, these two reductions might be caused by acetylpropionyl, formed to some extent in a rapid hydrolysis of the dibromide, as both the reagents are alkaline. The aliphatic α-diketones diacetyl and acetylpropionyl reduce namely Fehling's solution easily. An oxidation of the dibromide with potassium permanganate gives propionic acid and acetic acid.

Experimental. α,α-Dibromo-diethyl ketone. 320 g (2 moles) of bromine was added dropwise to 86 g (1 mole) of diethyl ketone in the presence of red phosphorus. Nitrogen gas was bubbled through the solution in order to remove hydrogen bromide. After half the bromine was added, the mixture was warmed to 80° and maintained at that temperature for one hour after the addition. The product was washed with water and some 5 % sodium carbonate and distilled with benzene, then fractionated *in vacuo*. B. p. 75—77°/ 10 mm. Yield: 180 g (74 %). The density as well as the boiling point is in agreement with the values found by Pauly. [1]

$C_5H_8OBr_2$ (243.94) Calc. Br 65.52
Found » 65.56

The hydrolysis and some derivatives. 24.4 g (0.1 mole) of the dibromide of diethyl ketone was dropped into an ice-cooled solution of 16.8 g (0.3 moles) of potassium hydroxide in 80 ml of alcohol. The mixture was stirred and allowed to stand overnight. Potassium bromide was separated, and the solution neutralized with concentrated hydrochloric acid and then distilled in order to remove the alcohol and some water. The residue was dissolved in ether, dried over anhydrous potassium carbonate and, after removal of the ether by distillation, fractionated and a middle fraction, b. p. 109—112°/ 752 mm, taken. The liquid was dark-yellow coloured. Yield: 5.9 g (59 %).

A qualitative test showed the substance to be an α-diketone, and the dioxime could be prepared from the liquid and hydroxylamine hydrochloride in alcoholic solution in the presence of sodium acetate, according to the general process. M. p. 172—173°. From an alcoholic solution of the dioxime and a water solution of nickel chloride the typical nickel salt could be obtained. M. p. 276—280°. — When a water solution of the α-diketone was treated with hot dilute sodium hydroxide for a few minutes a product, crystallizing in yiellow needles with the melting point, 108—110°, identified as duroquinone, was obtained.[3]

The three above-mentioned derivatives give full evidence for the identification of the hydrolysis product as acetylpropionyl.

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A Synthesis of *dl*-2-Methyl-5,7-dihydroxyflavanone

GÖSTA LINDSTEDT and
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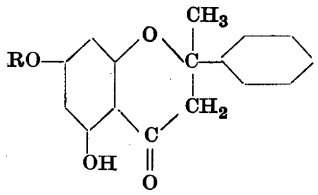
5,7-Dihydroxyflavanone (dihydrochrysin) has been synthesised from phloroglucinol and cinnamoyl chloride by the Friedel-Crafts method [1]. Under the same conditions phloroglucinol and β-methylcinnamoyl chloride gave a mixture of two colourless substances. The main product, m. p. 154—156° *, had the composition $C_{16}H_{14}O_4$, gave a yellow colour when reduced with magnesium and hydrochloric acid and a reddish brown colour with ferric chloride, and thus must be the expected *dl*-2-methyl-5,7-dihydroxyflavanone (I). On the paper chromatogram, its R_F value is about 0.55 (benzene-ligroin solvent [2], and the substance gives a red spot with diazotised benzidine. The R_F value of 5,7-dihydroxyflavanone in the same solvent is 0.44, so that, as expected, the in-

* All melting points uncorrected.

troduction of the C-methyl group causes an increase in R_F.



I: R = H
II: R = CH_3

Methylation of 2-methyl-5,7-dihydroxyflavanone with diazomethane yields a monomethyl ether, m. p. 92—93°. Since the hydroxyl group in the 7-position is always much more readily methylated than that in the 5-position, this compound must be 2-methyl-5-hydroxy-7-methoxyflavanone (II).

A second product formed during the synthesis of 2-methyl-5,7-dihydroxyflavanone was isolated from the mother liquors. It melted at 233—234° and had the same composition, $C_{16}H_{14}O_4$, and is thus isomeric with the main product. It gave no colour reactions either with magnesium-hydrochloric acid or with ferric chloride. The R_F value was very low (0.05), and diazotised benzidine gave a reddish-brown colour. Methylation with diazomethane did not lead to any crystalline product. A possible structure for this compound would be phloroglucinol mono-β-methylcinnamate: a chalkone structure is less probable owing to the absence of colour.

The structure (I) was once considered for stropopinin, a flavanone from the heartwood of *Pinus strobus* L.[3] However, stropopinin has recently been proved to be 6- or 8-methyl-5,7-dihydroxyflavanone [4].

Experimental. A suspension of phloroglucinol (4.8 g) in nitrobenzene (45 ml) was mixed with a solution of β-methylcinnamoyl chloride (6.7 g) in nitrobenzene (50 ml). The mixture was cooled in ice water, and a solution of aluminium chloride (7.1 g) and a few drops of thionyl chloride in nitrobenzene (120 ml) was added in small portions. After two days, the mixture was heated to 50—60° for one hour and then poured into a mixture of ice (100 g) and conc. hydrochloric acid (10 ml). The nitrobenzene was removed by steam distillation, and the remaining resinous product extracted three times with boiling water. The aqueous extracts were cooled and extracted with ether, and the ether solution dried over anhydrous sodium sulphate, decolourised by filtration through aluminium oxide and concentrated to a yellow oil, which soon crystallised. Recrystallisation from 50 % acetic acid gave colourless crystals, m. p. 153—154° (1.7 g). Further recrystallisation raised the m. p. to 154—156°. Colour reactions: magnesium-hydrochloric acid, yellow; ferric chloride, reddish-brown; diazotised benzidine, red.

$C_{16}H_{14}O_4$ (270.3)	Calc.	C	71.1	H 5.22
	Found	»	70.8	» 5.13

The monomethyl ether was obtained by treatment of the flavanone with diazomethane in ether solution. After recrystallisation from methanol, it formed colourless crystals, m. p. 92—93°. Colour reactions: Magnesium-hydrochloric acid, pale yellow; ferric chloride, reddish brown.

$C_{17}H_{16}O_4$ (284.3)	Calc.	OCH_3	10.9
	Found	»	10.8

The acetic acid mother liquors from the recrystallisation of 2-methyl-5,7-dihydroxyflavanone were diluted by water, and the precipitate thus formed recrystallised from toluene and then from methanol, yielding 0.3 g of colourless crystals, m. p. 233—234°. Colour reactions: Magnesium-hydrochloric acid, nil; ferric chloride, nil; diazotised benzidine, reddish brown.

$C_{16}H_{14}O_4$ (270.3)	Calc.	C	71.1	H 5.22
	Found	»	71.2	» 5.36

This work has been financially supported by *Fonden för Skoglig Forskning.*

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The Acyl Exchange in Acidolysis of Diethyl Acylmalonates with Organic Acids

ARNE BRÄNDSTRÖM

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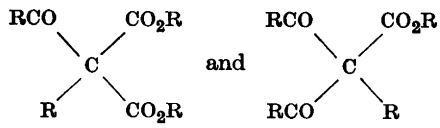
The present author has recently shown that diethyl acylmalonates are very valuable substances in the synthesis of β-ketoesters [1]. The diethyl acylmalonates were acidolysed with the organic acid containing the *same* acyl group thus giving excellent yields of the corresponding ethyl acylacetate according to the reaction:

$$RCOCH(CO_2C_2H_5)_2 + RCO_2H \longrightarrow RCOCH_2CO_2C_2H_5 + RCO_2C_2H_5 + CO_2$$

However, if the acid and the diethyl acylmalonate have *different* acyl groups a mixture of the two possible β-ketoesters, corresponding to the two different acyl groups is obtained. This indicates that the acyl group in the diethyl acylmalonate is not hold so firmly by the rest of the molecule.

A similar mobility of the acyl group has been observed by Dickmann and Wittmann [2] who studied the catalytic cleavage of compounds of the types:



with an alcoholic solution of sodium ethoxide. This reaction was found to proceed easily even when cold.

As the mobility of the acyl group is of great interest from both theoretical and preparative points of view a preliminary result of the investigation will be given here.

Equimolecular amounts of diethyl acetylmalonate and an organic acid were heated, using a little magnesium oxide and copper acetate as catalyst [1], for 5—8 hours, and the resulting product was fractionated in vacuo through a 30 cm Widmer column. In this way *ethyl* n-*butyrylacetate* b. p. 84—88°/10 mm (yield 38 %) and *ethyl* iso*butyrylacetate* b. p. 78—82°/10 mm (yield 35 %) were obtained. The actual amounts of these β-ketoesters in the mixtures are probably still higher, since there were losses in the isolation of the products. It seems to me very probable that this exchange is the result of a "redistribution reaction" [3] and that the yields hence would be 50 %.

From preparative point of view this method of preparing β-ketoesters has the advantage that the corresponding diethyl acylmalonate is not required as a starting material.

The reaction will be studied further to see if the acyl group exchange will be effected by other reagents, and if the reaction might be forced in a desired direction to give high yields of β-ketoesters.

1. Brändström, A. *Acta Chem. Scand.* **5** (1951).
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3. Gilman, H. *Organic Chemistry* **II** (1943) 1807.

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The Acyl Exchange in Acidolysis of Diethyl Acylmalonates with Organic Acids

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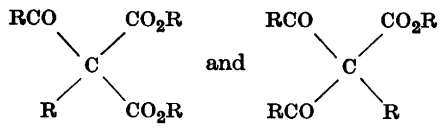

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The Solubilization of Long-Chain Alcohols in Association Colloid Solutions below the Critical Concentration for Micelle Formation

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It is possible to follow with the microscope the solubilization of the in water sparingly soluble *n*-decanol by aqueous solutions of sodium laurate. A compact layer of a double-refracting liquid-crystalline substance is seen to form and grow on the surface of each drop of decanol. A similar phenomenon is observed even when the concentration of the sodium laurate solution is below the critical concentration for micelle formation, C.M.C., of sodium laurate, *e. g.* at 40° C down to approximately 0.006 *M*.

The turbidities of laurate solutions containing a constant mole-fraction of *n*-decanol vary with the laurate concentration as shown by the nephelometric curves given in Fig. 1. Two turbidity ranges may be distinguished. The turbidity range at low concentrations terminates with an abrupt upper limit, at 40° C at a laurate concentration of 0.006—0.007 *M*. In ths other turbidity range, the turbidity ie much weaker; its upper limit or its maximum is near the critical concentration, 0.024—0.030 *M*.

The turbidity in the first range is due to emulsified decanol. In the second range the substance causing the turbidity is of a different nature. The substance is evidently highly dispersed and is difficult to observe with the microscope; at higher decanol concentrations double-refracting spheres of a liquid-crystalline substance are noted, at least above the C.M.C. Above the C.M.C. the precipitated substance goes into solution when the temperature is raised. The turbidity observed

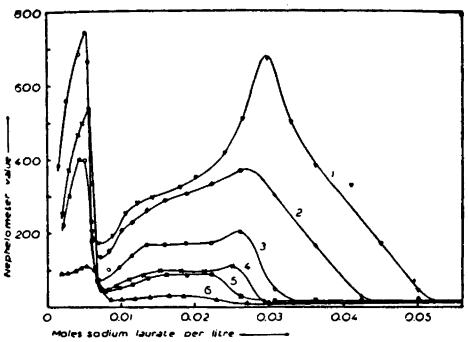

Fig. 1. The turbidities of sodium laurate solutions containing n-decanol.

1. 21 mole per cent decanol.
2. 15.7 » » » »
3. 10.5 » » » »
4. 7.35 » » » »
5. 5.25 » » » »
6. 3.20 » » » »

between the C.M.C. and the lower limit of the turbidity range is very little affected by a change in temperature. The upper limit of the first turbidity range shifts upwards rapidly with increasing temperature and the phenomenon is reversible.

These experiments thus show that the interaction between decanol and laurate which above the C.M.C. leads to a solution of the alcohol in the micelles continues at laurate concentrations as low as 0.006 *M*, *i. e.* down to the so-called limiting concentration (limiting association concentration, L.A.C.) of sodium laurate [1-2]. Between the C.M.C. and L.A.C., the interaction leads to the formation of a poorly soluble substance which separates as a fine suspension.

The equivalent conductivity of sodium laurate below the L.A.C. is not affected by the addition of decanol. Above the L.A.C., however, the equivalent conductivity is altered considerably by the addition. When approximately 250 moles per cent have been added, the equivalent conductivity has lowered to approximately the value at the L.A.C. in the absence of

the alcohol. This indicates that the interaction between decanol and laurate extends down to the L.A.C., but no further.

The interaction results in an increased solubility of the decanol. Beginning at the L.A.C., the solubility increases at first slowly, but attains already at the critical concentration a value that is many times higher than at the L.A.C. Above the C.M.C. the solubility of decanol rises rapidly with increase in the laurate concentration. The solubilization of decanol thus begins already at the L.A.C.

A similar interaction is noted between decanol and the other fatty acid soaps. In all the cases studied this was observed to take place below the C.M.C., down to the L.A.C. of the soaps. Similar interactions take place in the case of *n*-octanol and *n*-hexanol.

Analogous observations have previously been described for another group of polar-nonpolar compounds, the fatty acids. Under the microscope the reaction between lauric acid and laurate solutions can be followed at temperatures above the melting point of the acid. In this case a liquid-crystalline acid laurate is formed. The reaction continues down to the limiting concentration of sodium laurate, 0.006 *M* [4–6]. Two turbidity ranges are observed in laurate solutions containing lauric acid [2–5, 7], one immediately below the L.A.C. in which the turbidity is due to free lauric acid and the other between the L.A.C. and C.M.C. in which range acid laurate is responsible for the turbidity. Above the C.M.C., fairly large quantities of lauric acid are dissolved, colloid acid laurate being formed [2, 3]. The equivalent conductivity also varies in a manner similar to that described above [1, 2].

Interaction with the formation of acid salt takes place between other fatty acid soaps and the corresponding fatty acids down to the L.A.C. of the soaps [1, 2]. This interaction has been considered evidence for the belief that association in soap solutions begins already at the limiting concentration [1, 2, 8, 9] and not at the critical concentration, as it has been previously assumed, and as it is still frequently suggested. The solubilization of alcohols beginning at the L.A.C. furnishes additional support for this assumption.

At 40° C decanol is solubilized by sodium myristyl sulphate solutions in a manner analogous to that described above. This takes place down to a myristyl sulphate concentration of 0.0003 *M*; the C.M.C. of the sample of myristyl sulphate was 0.0012 *M*. In hexadecyltrimethylammonium bromide solutions, the solubilization of decanol extends down to a concentration of 0.0004 *M* (C.M.C. : 0.001 *M*). This phenomenon thus seems to be quite general, and indicates that in the case of the association colloids of the paraffin chain type, premicelle formation begins to take place at a limiting association concentration which is below the C.M.C.

Harkins and co-workers [10–12] have shown it to be probable that the solubilization of polar-nonpolar compounds occurs through the incorporation of the latter in the palisade layers of the micelles. This process they have termed penetration. The difference between the solubilization of hydrocarbons and that of alcohols, fatty acids, *etc.*, is clearly shown by our experiments. When the substances of the former type are added in excess, the unchanged hydrocarbon separates, sometimes in the form of an emulsion; when polar-nonpolar substances are added in excess, these substances separate along with the association colloid until the concentration of the latter is reduced to a value close to the L.A.C. The incorporation of the molecules of the polar-nonpolar substances in the micelles makes the latter less hydrophilic, and thus leads to a decrease in the solubility of the micellar substance.

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Chemical Nature of the Thiobarbituric Acid Test for the Oxidation of Unsaturated Fatty Acids

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Thiobarbituric acid was used by Kohn and Liversedge[1] as a reagent for a compound formed by aerobic incubation of tissue suspensions. It was found by Bernheim *et al.*[2] that the colors obtained upon addition of thiobarbituric acid to incubated tissues are due to a product of oxidation of unsaturated fatty acids, especially linolenic acid. Subsequently, Wilbur *et. al.*[3] studied further thiobarbituric acid as a reagent for oxidized fatty acids. They found that the strongest colors were produced with oxidized linolenic acid, weaker with oxidized linoleic and arachidonic acids. Furthermore, while oxidized fatty acids gave orange-red colors, various sugars and aliphatic aldehydes gave yellow colors, and glyoxylic acid a pink color.

As stated by Wilbur *et al.* the chemical reaction underlying the thiobarbituric acid reaction has not been completely elucidated. However, a comparison of thiobarbituric acid with phloroglucinol with respect to chemical properties will probably contribute to its explanation. The two compounds show some similarity with respect to chemical structure, including an analogous tautomerism and both are used as sensitive reagents for the quantitative determination of aldehydes, especially furfural. Furthermore, phloroglucinol is used as a reagent for the detection of oxidative changes of fats in the well-known Kreis-test[4]. The question therefore arises whether thiobarbituric acid is a reagent for the presence of the same chemical structures.

In order to investigate this question we have compared the two reactions using a number of fatty acids and other products of various origin. The thiobarbituric acid reaction was carried out as described by Wilbur *et al.* In order to facilitate the comparison of the results, the Kreis-test was carried out in a one phase-system using the modification of Pool *et al.*[5]. Finally, the peroxide values of the substances were determined by Hartmann and Glavind's colorimetric method[6].

Some representative results are given in Table 1. The colors were measured on the Beckman spectrophotometer at the respective wave-lengths. For presentation in the table they are calculated as the extinctions obtained in the three methods by the same amount of substance in the same final volume of 10 ml using a 1 cm cuvette.

It can be seen from the table that a minute amount of purified methyl oleate hydroperoxide gave a strong color in the thiobarbituric acid reaction. Such a strong color cannot be explained by the presence in the oleate of small amounts of linolenate. This observation does not agree with the statement by Wilbur *et al.* that thiobarbituric acid is a reagent for oxidized linolenic acid alone.

A study of the table further shows that the thiobarbituric acid reaction and the Kreis-test follow each other rather closely,

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Table 1. Intensity of colors produced by different methods for assay of oxidative changes in fats.

Method	Thiobarbituric acid reaction	Kreis-test	Peroxide method of Hartmann and Glavind
Wave length	532 mμ	550 mμ	520 mμ
Substance	Extinction		
Lard, 400 mg	0.175	0.325	0.73
Fatty acids from cod liver oil, 1.3 mg*	0.165	0.35	1.65
Methyl oleate hydroperoxide**, 2.5 mg	0.10	0.36	7.3
Epihydrin aldehyde***	0.64	0.60	0.89
Benzoyl peroxide, mg	0.00	0.00	2.5

* Highly unsaturated fraction precipitated at −75° C from acetone solution after more satured acid had been removed at higher temperatures. Stored for 7 years at room temperature.

** Prepared from purified oleic acid by the method of Swift *et al.*[7]. Peroxide value 5 000 milliequivalents per 1 000 gramms.

*** To 330 mg freshly distilled acrolein dissolved in saturated KCl was added 1 ml 30 % H_2O_2. Since all reactions were to be carried out in a non-aqueous medium, the mixture was extracted with 40 ml $CHCl_3$, and 2.5 ml of the extracts was used in each trial.

while there is no such parallelism with Hartmann and Glavind's method for the determination of peroxides. Benzoyl peroxide reacts quantitavely with leucodichlorophenolindophenol in the latter reaction but produces no color in the Kreis- or thiobarbituric acid tests. Purified methyl oleate hydroperoxide produces rather weak colors in the thiobarbituric acid as well as the Kreis-test as compared with the colors produced with leucodichlorophenolindophenol. On the other hand, strong colors are produced in the two tests in comparison with Hartmann and Glavind's method by such substances as oxidized fatty acids and lard. Especially intense colors are produced with epihydrin aldehyde which, according to Powick [8], is the carrier of the Kreis-test.

These observations seem to justify the conclusion that while Hartmann and Glavind's method is a specific reaction for peroxide groups, the thiobarbituric reaction like the Kreis-test is indicative of more complex structures formed in the course of the peroxidation process, and which, in accordance with Powick's explanation, are split off in the form of epihydrin aldehyde by the treatment with strong acids used in both methods.

1. Kohn, H. I., and Liversedge, M. *J. Pharm. Exptl. Therap.* **82** (1944) 292.
2. Bernheim, F., Bernheim, M. L. C., and Wilbur, K. M. *J. Biol. Chem.* **174** (1948) 257.
3. Wilbur, K. M., Bernheim, F., and Shapiro, O. W. *Arch Biochem.* **24** (1949) 305.
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Paper Cromatography on Borate-Impregnated Paper

CARL AXEL WACHTMEISTER

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Paper impregnated with phosphate buffers has been employed with advantage for the separation of organic acids[1,2]. As emphasized by Levi in a review[3] it should also be possible to utilise paper treated in various other ways, and in connection with work on lichen acids capable of forming complexes with boric acid it seemed desirable to investigate the possible use of borate buffers.

A number of phenols, phenolic aldehydes and phenolic acids as well as some sugars were studied. With every substance or group of isomeric substances parallel

Table 1. Intensity of colors produced by different methods for assay of oxidative changes in fats.

Method	Thiobarbituric acid reaction	Kreis-test	Peroxide method of Hartmann and Glavind
Wave length	532 mμ	550 mμ	520 mμ
Substance	Extinction		
Lard, 400 mg	0.175	0.325	0.73
Fatty acids from cod liver oil, 1.3 mg*	0.165	0.35	1.65
Methyl oleate hydroperoxide**, 2.5 mg	0.10	0.36	7.3
Epihydrin aldehyde***	0.64	0.60	0.89
Benzoyl peroxide, mg	0.00	0.00	2.5

* Highly unsaturated fraction precipitated at −75° C from acetone solution after more satured acid had been removed at higher temperatures. Stored for 7 years at room temperature.

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*** To 330 mg freshly distilled acrolein dissolved in saturated KCl was added 1 ml 30 % H_2O_2. Since all reactions were to be carried out in a non-aqueous medium, the mixture was extracted with 40 ml $CHCl_3$, and 2.5 ml of the extracts was used in each trial.

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A number of phenols, phenolic aldehydes and phenolic acids as well as some sugars were studied. With every substance or group of isomeric substances parallel

chromatograms were run, using paper impregnated with a sodium borate buffer and, for comparison, a sodium phosphate buffer of approximately the same pH, 8.7. The buffers were 0.1 *M* with respect to boric or phosphoric acid. Spots of phenolic compounds were detected by spraying the chromatograms with a solution of bis-diazotised benzidine [3] or in some cases by examination of the strips in ultra-violet light. The sugar chromatograms were developed with the aniline hydrogen phtalate reagent.

On the addition of sodium borate the distribution between water and an organic solvent of substances capable of forming strongly acidic complexes with boric acid (*cf.* Böeseken [4]) should be displaced in favour of the aqueous phase. Accordingly, such substances would be expected to show decreased R_F values on borate-impregnated paper as compared with the values on unimpregnated or phosphate-impregnated paper.

The experiments (Table 1) proved the decrease in R_F values to be considerable in the case of phenols and phenolic aldehydes containing two adjacent hydroxyl groups and less but still distinct in the case of saligenin. On the other hand, compounds with isolated hydroxyl groups as well as *o*-hydroxyaldehydes moved at approximately the same rate on phosphate- and borate-impregnated paper. Isomeric substances often do not separate well on chromatograms of the conventional type, but as shown in Table 1, borate-impregnated paper may facilitate the separation if one of the isomers is reactive towards boric acid.

Phenolic acids are known to form complexes with boric acid if they contain two adjacent hydroxyl groups or a hydroxyl group in ortho-position to a free carboxyl group. The results obtained (Table 2) are in accordance with these facts, although there are small differences in the R_f values of the unreactive acids also, probably due to the sensitivity of the R_f values to small changes in pH.

Table 1. R_F values for phenols and phenolic aldehydes.

Substance	R_F values on paper impregnated with borate	phosphate
a. n-Butanol-water		
Pyrocatechol	0.40	0.88
Resorcinol	0.91	0.96
Hydroquinone	0.91	0.94
Pyrogallol	0.16	0.78
Phloroglucinol	0.78	0.80
3-Methoxypyrocatechol	0.45	0.92
4- »	0.46	0.95
Methoxyhydroquinone	0.87	0.90
3,4-Dihydroxybenzaldehyde	0.28	0.84
2,4- »	0.92	0.91
b. Benzene-water		
Saligenin	0.07	0.31
p-Orsellinaldehyde (Atranol)	0.48	0.52

Table 2. R_F values for phenolic acids (n-Butanol-water).

Substance	R_F values on paper impregnated with borate	phosphate
o-Hydroxybenzoic acid	0.35	0.60
m- » »	0.22	0.27
p- » »	0.21	0.27
3,4-Dihydroxybenzoic acid	0.02	0.06
2,4- » »	0.17	0.38
3,5- » »	0.13	0.18
4-Methoxy-2,6-cresotic acid (Everninic acid)	0.42	0.70

Table 3. R_F values for hexoses (n-Butanol-etanol-water 4,1,5).

Substance	R_F values on paper impregnated with borate	phosphate
Glucose	0.04	0.09
Fructose	0.02	0.09
Sorbose	0.02	0.08

Experiments with a few hexoses (Table 3) indicate the usefulnes of the method also in sugar chemistry. The sugars investigated move more slowly on phosphate-impregnated than on untreated paper, but their sequence is not changed. On borate-impregnated paper, however, a god separation of *e. g.* glucose and sorbose can

be achieved, although these two sugars appear to move at the same rate in the systems ordinarily used.

Generally, the preparation of parallel chromatograms on borate- and phosphate-impregnated paper affords a simple micro-method for the detection of borate reactive groupings. Apart from the analytical use in separating isomers, the method may be of value for the determination of the relative positions of hydroxyl groups in complex natural products. Thus it will sometimes be possible to localize the position of methoxyl- or acyl-groups.

1. Levi, A. A., in *Partition chromatography (Biochemical Society Symposia No. 3)* Cambridge (1949).
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Hemicellulose Extracted from Wood Holocellulose Swollen in Liquid Ammonia

K. J. BJÖRKQVIST and L. JÖRGENSEN

Research Laboratory, Billeruds AB, Säffle, Sweden

The chemistry of the so-called hemicellulose portion of the woods is still far from being clarified [1,2]. This is partly due to the difficulties encountered in the extraction of the hemicellulose portion without changing it chemically. The preparation and the use of holocellulose as a starting material in the isolation of hemicelluloses was a large step forwards, but still rather strong alkaline solution had to be applied in the isolation of the various fractions.

It is well known [3] that liquid ammonia will swell cellulosic fibers, and after evaporation of the ammonia, change the native cellulose structure (Cellulose I) into the hydrate structure (Cellulose II). The swelling of wood in liquid ammonia to render the lignin portion more accessible was first applied by Yan [4]. Later Purves and Neubauer [5] used this swelling on maple wood. They investigated the carbohydrates in the water soluble portion. A deesterification was noted at the conditions applied, pressure and room temperature. Bishop and Adams [6] recently described the isolation of hemicellulose fractions from wheat straw holocellulose swollen in liquid ammonia at the boiling temperature of ammonia (— 33° C). Noteworthy, was the pronounced increase in the water soluble portion (an increase from 3 to 20.2 %) from the unswollen to the swollen material. It is believed that the

Table 1. Yield of hemicelluloses extracted from ammonia-swollen wood.

Fraction	Birch *Betula verrucosa*	Spruce *Picea excelsa*
Hot water	14.4 %	7.1 %
1 % sodium carbonate	2.3 »	2.8 »
2 » » hydroxide	19.1 »	7.3 »
5 » » »	7.9 »	9.9 »
Residue	52.3 »	69.0 »
Total recovery	96.0 %	96.1 %

Table 2. The pentosan and uronic acid content of birch and spruce holocellulose and extracted hemicellulose fractions (expressed in %).

Fraction	Pentosan birch	Pentosan spruce	Uronic acids birch	Uronic acids spruce
Holocellulose	28.0	7.8	6.0	5.1
Hot water	62.7	25.3	12.8	16.6
1 % sodium carbonate	50.4	47.3	17.9	20.3
2 » sodium hydroxide	40.5	40.8	4.9	8.0
5 » sodium hydroxide	52.3	13.8	7.1	7.5
Residue	6.4	1.4	0.14	0.01

be achieved, although these two sugars appear to move at the same rate in the systems ordinarily used.

Generally, the preparation of parallel chromatograms on borate- and phosphate-impregnated paper affords a simple micro-method for the detection of borate reactive groupings. Apart from the analytical use in separating isomers, the method may be of value for the determination of the relative positions of hydroxyl groups in complex natural products. Thus it will sometimes be possible to localize the position of methoxyl- or acyl-groups.

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Residue	6.4	1.4	0.14	0.01

Table 3. Constituent sugars in birch and spruce holocellulose and extracted hemicellulose fractions (relative amounts expressed in %).

Fraction	Galactose		Glucose		Mannose		Arabinose		Xylose	
	b.	spr.	b.	spr.	b.	spr.	b.	spr.	b.	spr.
Holocellulose	0	3.0	65.5	70.5	2.7	17.9	2.3	1.0	29.5	8.0
Hot water	0	5	5	12	tr.	45	tr.	17	95	21
1 % sodium carbonate	0	5	5	15	tr.	24	tr.	22	95	34
2 » » hydroxide	0	tr.	5	20	tr.	30	tr.	20	95	30
5 » » »	0	tr.	10	27	5	50	tr.	5	85	18
Residue	0	0	83	90	4	10	0	0	13	tr.

deesterification may be avoided at this low temperature during the swelling.

We have applied a similar technique to holocellulose from birch and spruce wood. The holocelluloses were prepared from ethanol-benzene extracted wood (12—20 mesh) according to Wise [7]. The sample to be extracted was dried under high vacuum (0.1 mm) for 36 hours over phosphoric anhydride at 20° C. The evacuated flask with the sample was then cooled to — 70° C in dry ice-acetone mixture and filled with liquid ammonia from a cylinder. The flask was then allowed to stand at room temperature for 30—40 hours. The ammonia evaporated slowly and the last traces thereof were removed at high vacuum at 35° C. The swollen holocellulose was then successively extracted with water (70—75° C), 1 % sodium carbonate, 2 % sodium hydroxide and 5 % sodium hydroxide. The solid-solvent ratio was approximately 1 : 10 and three extractions of three hours each were required for exhaustive extraction. The fractions were precipitated with acetone and separated by centrifuging. The yields are given in Table 1. Table 2 gives the pentosan and uronic acid content of the fractions. All calculations are based on absolute dry holocellulose.

No marked drop in DP of the cellulose could be observed during the swelling and extraction of the holocellulose as measured by the viscosities of corresponding cellulose nitrates in acetone. The constituent sugars were determined after hydrolysis by a paper partition chromatographic technique similar to that of Jermyn and Isherwood [8]. The results are recorded in Table 3, analytical data should only be regarded as approximative.

These results differ very highly from results we obtained by direct alkaline extraction of unswollen wood hemicellulose. Water will dissolve only traces thereof and the total recovery is 85 %, apparently because the hemicelluloses are degraded and changed by strong alkali. It should be mentioned that all the hemicellulose fractions obtained from liquid ammonia treated holocelluloses are, in contrast to those from unswollen extracted holocelluloses, completely soluble in warm water. This will facilitate further investigations of them.

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Received September 20, 1951.

IInd. International Congress of Biochemistry

The Second International Congress of Biochemistry is to be held in Paris from the 21st to the 27th of July 1952.

The programme has not yet been definitely decided on. Nevertheless the Congress Committee has drawn up a plan for the study of biochemical question of current interest during seven symposia:

1. *Biochemistry of steroids*
2. *Biochemistry of haematopoiesis*
3. *Biogenesis of the proteins*
4. *Tricarboxylic acid cycles*
5. *Bacterial metabolism*
6. *Mecanism of action of the antibiotics*
7. *Protein hormones and hormones derived from proteins.*

Communications dealing with other biochemical problems will be grouped together in homogeneous sections.

Four General Lectures will be given by well known scientists.

Authors should send the titles of their communications before the 1st of March 1952 and a summary of less than 200 words before the 1st of April 1952 to the General Secretary: Professor J. E. Courtois, 4 Avenue de l'Observatoire, Paris VI.

In order to receive the programmes and bulletins of adhesion, please write to the Secretary of the Congress, same address.

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On the Structure of Hyaluronic Acid

GUNNAR BLIX

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In a case of mesothelioma about 20 g of pure hyaluronic acid were isolated from the highly viscous pleural and peritoneal fluid [1]. Advantage was taken of the relatively great amount of hyaluronic acid thus obtained to attempt to elucidate the structure of this substance, using the same principles as those applied by Meyer *et al.*[2] in their investigation of the structure of chondroitin sulfuric acid. When the present work had proceeded for some time, investigations on the same subject were communicated, first by Jeanloz [3], in a preliminary note, and somewhat later, briefly, by K. H. Meyer and Fellig [4].

Based on reasons which, owing to the rather short form of the communication, are somewhat difficult to judge, Jeanloz suggests that the acetylglucosamine and glucuronic acid residues of the hyaluronic acid are mutually joined by 1,3-linkages. According to Meyer and Fellig periodic acid at 0° C and pH 4.7 attack only the end groups of the hyaluronic acid molecule. The same was found to be true also with the fully methylated and subsequently methanolysed substance. Assuming the hyaluronic acid to be a linear polymer, composed of alternating residues of acetylglucosamine and glucuronic acid, both being present in the pyranose form, the results mentioned would indicate that the basic units of the chain are bound by glucuronido-4-glucosamine and glucosaminido-3-glucuronic acid linkages. Recently Kaye and Stacey [5] reported the isolation of a dimethyl ether of methyl glucopyruronide methyl ester from the methylated product of a somewhat degraded hyaluronic acid, thus giving definite evidence of the pyranose form of glucuronic acid in that substance. On the basis of their analyses of the methylated hydrolysis products of hyaluronic acid they reject the idea that the hyaluronic acid consists of linearly linked disaccharidic units and suggest that the N-acetyl amino sugar groups form a chitin-like "core" to which is attached a complicated polyglucuronic acid structure. (The report is a short abstract of a paper read to the Biochemical Society in London.)

ISOLATION, COMPOSITION AND MOLECULAR WEIGHT OF THE HYALURONIC ACID INVESTIGATED

The isolation of the preparation used has earlier been described in detail [1]. The proteins of the tumor fluid were degraded by papain and mostly removed by dialysis. Protein residues were removed by the Sevag technique. The native tumor fluid was very viscous. As not infrequently happens with the procedure employed, the viscosity of the hyaluronic acid solution decreased markedly during the isolation, the pure product showing a relative viscosity of only 1.76 at +25 ° and pH 7.0 in 0.05 *M* phosphate and 0.05 *M* NaCl.

The reduction power as measured according to the method of Willstaetter and Schudel as modified by Linderström-Lang and Holter [6] indicated a mean molecular weight of about 28 000.

The analysis of the pure product gave the following results.

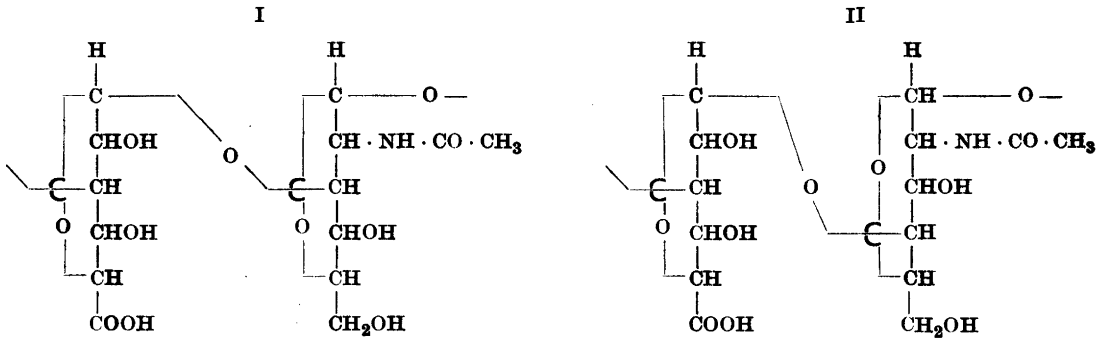
	%	Calculated for the Na-salt of hyaluronic acid %
Glucosamine	45.1	44.6
Glucuronic acid	47.5	48.4
Acetyl	9.8	10.7
Nitrogen	3.39	3.49
Sulfuric acid	0	0
Optical rotation (end value)	− 77.9° ± 1°	

Glucosamine was determined with the method of Elson and Morgan as modified by Blix [7], glucuronic acid according to Burkhart, Baur and Link [8] and acetyl according to Friedrich and Rapoport [9]. — The values obtained showed the product to be very pure. A negative ninhydrin test indicated the absence of proteins and protein split products.

THE STRUCTURE OF HYALURONIC ACID

According to the chemical analyses the hyaluronic acid is composed of an equal number of acetylglucosamine and glucuronic acid residues. Studies of double refraction of flow and viscosity (Blix and Snellman [10]) of the native substance have shown that it has a long chain structure. As a working hypothesis it therefore may be reasonably assumed that the molecule of the hyaluronic acid is composed of glucosidically linked repeating units of acetylglucosamine and glucuronic acid. Of the monosaccharides the glucuronic acid is with

certainty, the glucosamine, probably, present in the pyranose form. The negative optical rotation indicates that at least one of the glucosidic linkages is of the β type. Based on the mean molecular weight found for the product studied, its molecules should contain about 70 disaccharide units. On the basis of these facts and assumptions, 9 different structures are conceivable. Of these only those two given diagrammatically below should be possible, if — as found by Meyer and Fellig — periodic acid attacks only end groups, leaving the interior of the chain intact.



After permethylation and *methanolysis* no periodic acid should be consumed with structure II. On the other hand in case of structure I the substance should consume one mole of periodic acid per mole of the dimer. In the latter case NH_3 should also be liberated. The fully methylated product should, after *hydrolysis*, in both cases consume two molecules periodic acid per period.

1. Oxidation of the unmethylated substance

Some experiments are represented in Fig. 1. One molecule periodic acid is consumed per approximately 10 disaccharide units. Identical results were obtained at $+20°$ and $+10°$, but the secondary reactions proceeded at a lower rate with the lower temperature. Determinations were also made on two pure specimens of hyaluronic acid, isolated from navel cord, the one a highly polymerised product, and on a tumor fluid preparation which had been kept for 36 h at $+20°$ in 0.1 *N* NaOH.

The consumption of periodic acid was about the same in all these cases and definitely higher than that of a specimen of chondroitin sulfuric acid also tried. The oxidation value found for the hyaluronic acid is consistent with the view that the substance is in the main constituted by disaccharide units

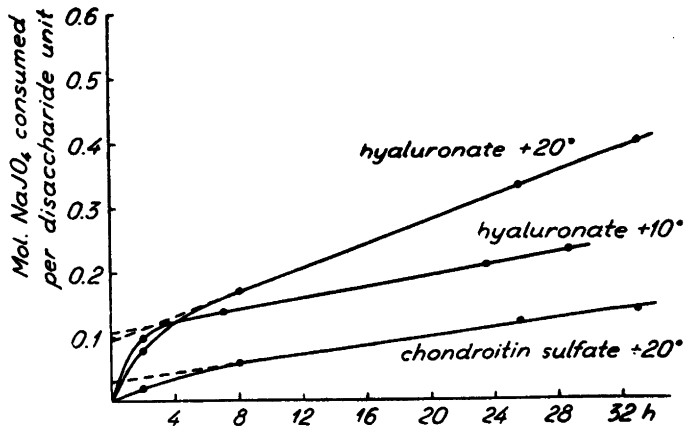


Fig. 1. Oxidation of unmethylated substances.

of structure I or II. However, the consumption of periodic acid is probably greater than can be due to the oxidation of the end groups of a straight chain composed of 70 disaccharide units. Two explanations may be suggested: 1. In addition to the main chain the molecule may have a few short side chains with nonreducing end groups. 2. In addition to a great number of units of structure I or II, the molecular chain may contain a small number of units with some other kind of linkages, for example 1,6 linkages.*)

2. Oxidation of the methylated and methanolysed substance

Several experiments were conducted of which one is given in Fig. 2. The consumption of periodic acid was found to be about 0.5 molecule per disaccharide unit. This result is most simply interpreted by the assumption that the hyaluronic acid molecule is composed of an equal number of disaccharide units of structure I and II. In agreement with this assumption one half or somewhat less of one molecule of ammonia per disaccharide unit was found to be liberated on the oxidation.

* As the determination of the molecular weight with the aid of the reduction power can be regarded only as a rather approximate method, the moderate excess oxidation found might possibly at least partly be due to the use of a too high value of the molecular weight.

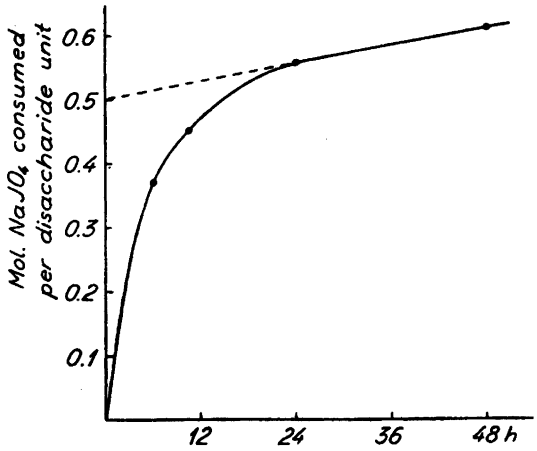


Fig. 2. Oxidation of methylated and methanolysed hyaluronic acid.

3. Oxidation of the methylated and hydrolysed substance

The results of the experiments given above might, to some extent, be checked by experiments on the methylated and hydrolysed product. With structure I or II two molecules of periodic acid should be consumed per disaccharide unit, whereas with most of the other structures three molecules should be consumed. Ammonia should be liberated in both cases. Actually, the hydrolysis showed a biphasic course. At +55°—+60° in 4 *N* HCl reducing values (obtained by the method of Linderström-Lang and Holter), corresponding to one reducing group per disaccharide, were reached in about 12 h, whereafter the reduction power increased only very slowly, reaching a constant value in 5—6 days. The reducing value arrived at after that time amounted to only about 80 % of that calculated for 2 reducing groups per dimer, the deficit most probably being due to secondary reactions involving the C_1 group of the glucuronic acid. After hydrolysis for 8 days the oxygen consumption found was about 2.0 atoms per dimer (Fig. 3) and the oxidation liberated about 0.8 molecule ammonia on the same basis.

Considering especially the secondary reaction in which the liberated ammonia is likely to be involved, these results agree fairly well with those of the preceding experiments and thus give some further support of the conclusions drawn

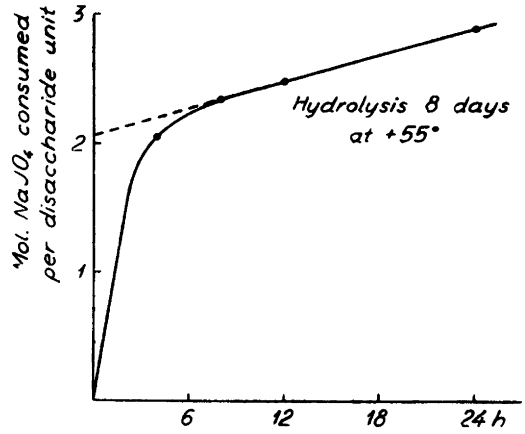


Fig. 3. Oxidation of methylated and hydrolysed hyaluronic acid.

from them. (The possibility of using the determination of the formed formic acid for further evidence was tried, but owing to a technical misfortune the results received are unreliable).

COMMENT

The observations made are consistent with the idea that the molecule of the hyaluronic acid investigated is in the main constituted of linearly linked dimers of N-acetyl-glucosamine and hyaluronic acid and indicate that the glucosamine residue is glucosidically linked to the 3rd carbon atom of the glucuronic acid residue. The results further make it probable that one half of the glucuronic acid residues are linked to the 3rd and the other half to the 4th C-atom of the glucosamine part. If this is true, these two kinds of linkages may either be present alternateingly in the same molecular chain or, less probably, may belong to two different kinds of molecules, present in equal amounts. The studies on hyaluronic acid from navel cord by Meyer and Fellig indicated presence exclusively of glucuronido-4-glucosamine linkages. Possibly the structure may vary in hyaluronic acid of different origin. It should also be emphasized that in the present work, contrary to Meyer's findings, a consumption of periodic acid of the native product was found which could not be ascribed purely to the end groups of a straight chain with glucuronido-4-(or -3-)-glucosamine and glucosaminido-3-glucuronic acid linkages, provided that

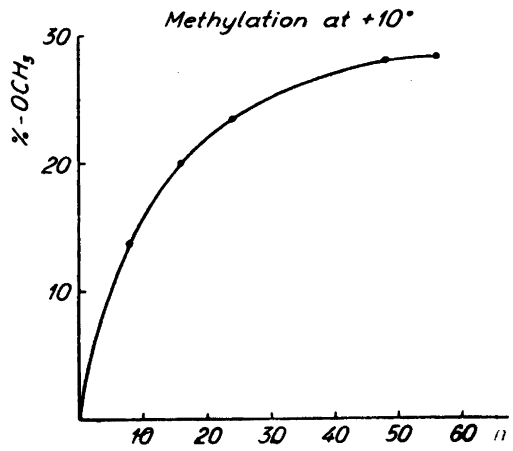


Fig. 4. Methylation of hyaluronic acid.

the molecular weight deduced from the reducing power is approximately correct. The oxidizability of the native substance was in the present work found to be the same for three different preparations of hyaluronic acid, two of which were isolated from navel cord, and was distinctly lower for a specimen of chondroitin sulfuric acid tried. This result agrees with that of Jorpes, Werner and Åberg [11], who in this regard found a distinct difference between the two substances.

The oxidizability of the native substance might indicate the presence of a few short side chains per molecule but may also be otherwise explained. Many unsuccessful attempts to get X-ray diffraction pictures of hyaluronic acid might perhaps be taken as an additional evidence of an irregular structure of the substance. Experiments with periodic acid can give valuable guidance in studies of the structure of hyaluronic acid and related substance, but they obviously do not suffice for a definite settlement of the structural problems of these compounds. However, the application of the classical methods for structural studies within the carbohydrate domain, seem to be connected with unusual difficulties in the case of hyaluronic acid and related substances.

EXPERIMENTAL

Methylation

Meyer *et al.* methylated chondroitin sulfate with the aid of dimethyl sulfate and obtained a fully methylated product on operating at +5° or +20°, whereas the methylation became incomplete at +40° and +60°, probably due to disturbing secondary reac-

tions. In a preliminary experiment on chondroitin sulfate, the methylation did not become quite complete at +20°. The methylation of the hyaluronic acid was therefore carried out at +10°. A quantity of 8 g was dissolved in 160 ml 1 % NaOH. Using an apparatus similar to that of Meyer, Wertheim and Bernfeld [12] and following the directions given by Meyer, Odier and Siegrist [2], 40 ml of dimethyl sulfate and 51.2 ml 30 % NaOH were added during 8 h. The solution was then precipitated by addition of 3.5 liters of alcohol, the mixture centrifuged, washed with alcohol and ether and dried. Of this product 0.3 g were dissolved in water and dialysed against 0.5 % sodium acetate until free from sulfate. The solution was then precipitated with alcohol, washed with alcohol and ether and dried *in vacuo*, at first at room temperature, and then at +70° over P_2O_5. Some moisture was very tenaciously held by the methylated products and constant weight was usually attained only after drying for several days at the elevated temperature. The preparation thus obtained was used for the methoxyl determination, carried out according to Vieböck and Brecher [13]. The main portion was again dissolved in 1 % NaOH (200 ml) and methylated in the same way as before. After 6 methylations 3.5 g of a methylated, quite white product were obtained, which contained 28.1 % methoxyl; calculated for the Na-salt of the tetramethylated disaccharide unit, 27.1 %. For the precipitation of the highly methylated products ether had to be added. The course of the methylation is shown in Fig. 4.

Periodate oxidation

The oxidations were performed principally in the same way as given by Meyer, Odier and Siegrist [2].

A suitable quantity of the substance to be oxidized was dissolved in a small amount of water. In most instances 12 ml 2 *M* acetate buffer of pH 4.3 and 6 ml *M* sodium periodate were then added and the solution filled up to 60 ml with water. After standing, as a rule at +18°—+20° for suitable lengths of time, 10 ml portions were taken for titration. 2 ml of a 7.5 % KI solution and 4 ml 2 % HCl were added and the solution then titrated with 0.1 *M* $Na_2S_2O_3$, using a 5 ml burette allowing a reading of 0.01 ml.

The liberated ammonia was distilled after addition of magnesia.

Methanolysis and hydrolysis

Meyer, Odier and Siegrist [2] used 7.3 % HCl and reflux for 44 h. On boiling methanol containing 7.3 % HCl with reflux at ordinary pressure the greater part of the HCl escapes in a short time. In the present work the same results were obtained when the substance was boiled under reflux for 40 h in 7 % HCl as when this procedure was repeated once with the solution evaporated to dryness in vacuo after the first treatment. The outcome was also the same on heating with 7 % HCl in a closed tube for 6 or 12 h. (The formation of risky amounts of methyl chloride forbids the use of longer heating times in closed tubes.) In the experiment given in Fig. 2 the substance was boiled under reflux twice with (initially) 7 % methanolic HCl for 40 h.

The hydrolyses were carried out in closed glass bulbs at +55° or +60° in 4 *N* HCl. Small losses of fluid through inevitable evaporation were replaced by addition of water before samples were taken for analyses.

SUMMARY

Hyaluronic acid obtained from the tumor fluid in a case of mesothelioma was submitted to structural analysis with the aid of periodate oxidation of the native and the methylated product. The results found are consistent with the view that the molecule of the hyaluronic acid is in the main constituted of linearly linked dimers of N-acetylglucosamine and hyaluronic acid, and indicate that in this case the glucosamine residues were glucosidically linked to the 3rd carbon atom of the glucuronic acid residue. They further made probable the interpretation that half of the glucuronic acid residues were linked to the 3rd and the other half to the 4th C-atom of the glucosamine part.

Added in proof: The full report of the work of Meyer and Fellig [4] has now been published in *Helv. Chim. Acta* **34** (1951) 939 and that of Kaye and Stacey [5] in *Biochem. J.* **48** (1951) 249.

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The Stability of the Micelles in Bile Acid Salt Solutions of Different Acidities

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Recent research [1-6, 17] has proved that micelle formation occurs in aqueous solutions of many of the bile acid salts (sodium cholate, desoxycholate, glycocholate, taurocholate). This micelle formation begins at relatively low critical concentrations and progresses slowly at first with increasing salt concentration [6, 17]. Upon addition of a strong acid to the solutions of these salts, the bile acids are liberated sooner or later. The possibilities for the preservation of the micelles formed by bile acid anions or the formation of micelles by the undissociated bile acid molecules or by both anions and acid molecules together are determined primarily by the strengths and solubilities of the bile acids. In respect of these latter properties, wide variations are noted [7-11]. By following potentiometrically the variation of the hydrogen ion concentration on addition of hydrochloric acid and by taking note of the other changes occurring in the solutions we have attempted to obtain knowledge of the pH ranges in which micelles are able to exist and in which micelles are no longer stable.

The pH values of the solutions were measured using a glass electrode and Radiometer valve potentiometer, Type PHM 3. Figs. 1 and 2 show typical titration curves for sodium desoxycholate, sodium cholate, sodium glycocholate and sodium taurocholate. In the first two cases the liberated bile acids separated as crystals from the solutions after a relatively small addition of hydrochloric acid, in the case of sodium glycocholate a fairly large amount of hydrochloric acid was necessary, whereas with sodium taurocholate no separation occurred. This is in accordance with the acid strengths and solubilities of these acids [10, 7], the former increasing and the latter decreasing in the order mentioned. Josephson has pointed out that some of the bile acids

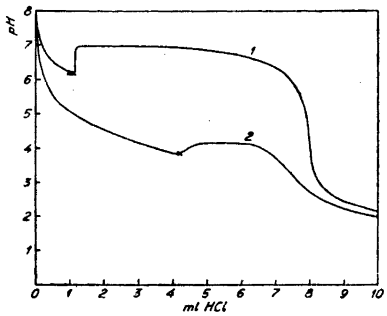

Fig. 1. Potentiometric titration of sodium cholate and sodium glycocholate with 0.1 N hydrochloric acid.

1. 20 ml 0.04 M Sodium cholate.
2. 20 ml 0.04 M Sodium glycocholate.

△ *The solution becomes turbid.*
× *Crystals begin to precipitate.*

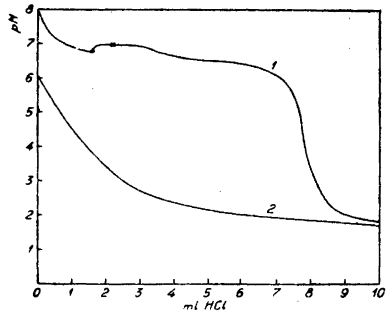

Fig. 2. Potentiometric titration of sodium desoxycholate and sodium taurocholate with 0.05 N hydrochloric acid.

1. 20 ml 0.02 M Sodium desoxycholate.
2. 10 ml 0.1 M Sodium taurocholate.

△ *The solution becomes turbid.*
× *Crystals begin to precipitate.*

(cholic, desoxycholic, glycocholic acid) easily form supersaturated, sometimes colloidal solutions. In our experiments this tendency was appreciable. When the bile acid salt concentration is above the critical concentration for micelle formation the liberated bile acid obviously is solubilized in the micelles, but also in more diluted salt solutions the acids are not always precipitated immediately.

From the titration curves it is seen that desoxycholate and cholate micelles are not generally able to exist in solutions whose pH values are below 6.5—6. In cholate solutions which temporarily were supersaturated with cholic acid, however, we have measured pH values down to 5. — Glycocholate-glycocholic acid micelles are stable down to pH 4.5—4.0 and taurocholate-taurocholic acid micelles still at pH 2—1.

The bile acid salt micelles are good solubilizers of various hydrocarbons [5, 6, 12, 13, 17]. The different stabilities of the micelle systems in media of different acidities become evident as a variation in the solubilizing power at pH values below 7. This is shown by experiments with bile acid salt solutions containing the strongly fluorescent polycyclic hydrocarbon 3,4-benzpyrene solubilized in the micelles. In a 0.1 *M* sodium cholate solution the fluorescence remains unaltered on adding hydrochloric acid even after cholic acid has begun to precipitate (fluorescence measured in the filtered solution). A continued further addition of hydrochloric acid resulted in a lowering of the concentra-

tion of the micelles and a precipitation of increasing amounts of the benzpyrene along with the cholic acid. Only after the pH had fallen below 6 did the fluorescence begin to decrease more rapidly and it disappeared altogether when the concentration of undecomposed cholate decreased below the critical concentration. In a similar experiment in which the solution studied was a 0.05 *M* sodium glycocholate solution containing benzpyrene, the fluorescence remained constant to pH 4.4. At this pH the glycocholic acid began to precipitate and this resulted in a gradual lowering of the fluorescence intensity (of the filtered solution). The fluorescence disappeared altogether at about pH 3.5 when the greater part of the salt had been decomposed. — In taurocholate solutions (0.1 and 0.05 *M*) the fluorescence of benzpyrene remained unchanged even after three times the equivalent amount of hydrochloric acid had been added and the pH had fallen below 1.5.

Bile is an association colloid solution in which the micelle-forming substances are various bile acid and fatty acid salts (in human bile primarily taurocholate, glycocholate, oleate), probably also cholesterol and lecithin. When hydrochloric acid is added to fresh ox bile, a titration curve with two jumps (Fig. 3, 1) is obtained and precipitation occurs at a pH below 4.5. As the bile becomes older, it becomes more alkaline and the titration curve passes through higher pH values, without, however, changing its form (Fig. 3, 2). The fluorescence of benzpyrene solubilized in bile did not disappear on the addition of hydrochloric acid, even when the pH attained the value 1.

These results indicate that the micelle systems in solutions of certain bile acid salts and in bile itself are stable at the low pH values which prevail in the stomach and consequently that even in such media they are still able to solubilize fat-soluble substances. This is the case at least with the taurocholate-taurocholic acid micelles. At the higher pH values which may sometimes prevail in the stomach in conditions of low acidity, the same may also apply to the micelle system glycocholate-glycocholic acid. When bile regurgigates into the stomach, an association colloid solution enters the stomach, which, thus, in spite of the acidity, is able to solubilize and keep solubilized fat-soluble substances such as the carcinogenic polycyclic hydrocarbons.

After our previous studies had shown that carcinogenic hydrocarbons solubilized in aqueous solutions of different association colloids easily penetrate into the skin of mice[14], the question arose whether these aqueous solutions are also able to assist the penetration of carcinogenic hydrocarbons into the walls of the glandular stomach through the protecting mucin layer. We continued our studies with the working hypothesis that such a penetration should be possible if the micelles are stable in the acid media of the stomach [15]. The experiments described above show that as far as the stability of the micelles

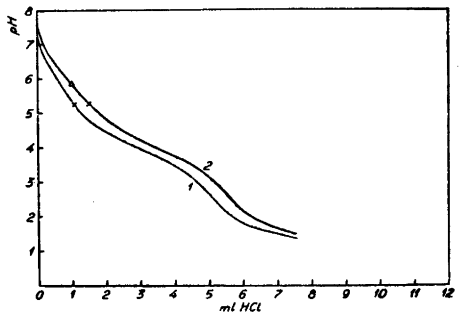

Fig. 3. Potentiometric titration of ox bile (25 ml) with 1 N hydrochloric acid.

1. Fresh ox bile.
2. Four hours' old ox bile.

is concerned certain bile acid salts and bile itself should be able to assist the penetration of carcinogenic hydrocarbons. Experiments which have been conducted later with animals have confirmed that such a penetration can be effected with solutions of these substances [16].

SUMMARY

A potentiometric titration study has been conducted to determine the pH ranges at which micelles are stable in aqueous solutions of the bile acid salts. In solutions of desoxycholate and cholate, micelles are generally not able to exist below pH 6.5—6.0, but in glycocholate solutions micelles are present down to pH 4.5—4.0 and in the taurocholate solutions even to pH 2—1, primarily owing to the greater strengths and solubilities of glycocholic and taurocholic acids.

Our studies of the ability of these solutions to solubilize the polycyclic hydrocarbon 3,4-benzpyrene confirm these findings.

Ox bile also contains micelles which are able to solubilize benzpyrene, even at pH 1.

The results have a bearing on the possible significance of the bile for the development of gastric cancer via chemical carcinogenesis.

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Antibiotic Substances from the Heart Wood of *Thuja plicata* D. Don. VII *. A Partial Synthesis of Thujic Acid

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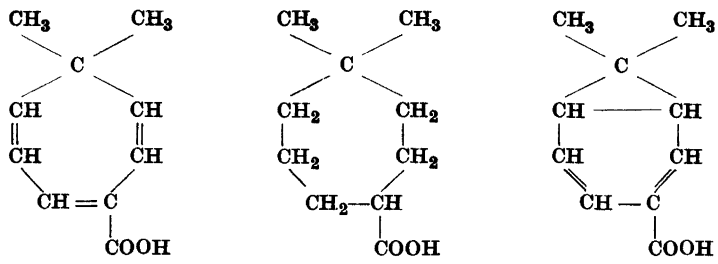
In a previous paper in this series the structure (I) was proposed for thujic acid [1]. This suggestion was based on the hydrogenation of thujic acid to hexahydrothujic acid and degradation of the latter to known compounds. While the formulation of hexahydrothujic acid as 4,4-dimethyl*cyclo*heptane carboxylic acid (II) appears to be well founded, the deduction of structure (I) for thujic acid was based on the fact that this was the only way in which three double bonds could be placed in the ring skeleton of hexahydrothujic acid. The presence of three double bonds in the molecule of thujic acid was inferred from the ready uptake of three molecules of hydrogen on catalytic hydrogenation [1,2].

Later experiments on the hydrogenation of thujic acid, however, led to evidence, which appeared to throw some doubts on the validity of this assumption. When a less active platinum catalyst was used, a marked decrease in the hydrogenation rate was observed after an initial uptake of two molecules of hydrogen. This effect was even more marked when a palladium-charcoal catalyst was used, the uptake of hydrogen ceasing altogether when two molecules had been absorbed. These observations could be interpreted on the assumption that thujic acid contains a *cyclo*propane ring as in (III); the slower uptake of the third molecule of hydrogen would then correspond to the rupture of this ring.

Although from a biogenetic point of view this formulation appears very attractive, being closely related to the carenes, there are several objections to it. The most serious of these are: 1) The *cyclo*propane ring in the carenes cannot be hydrogenated under conditions similar to those used in the hydro-

* Part VI. Gripenberg, J. *Acta Chem. Scand.* 3 (1949) 1137



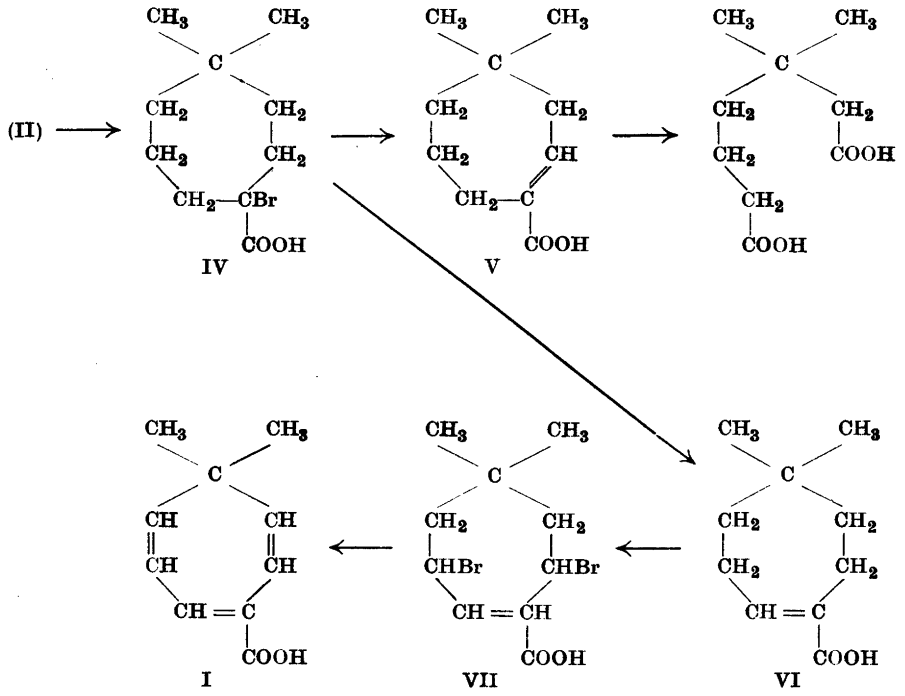
I II III

genation of thujic acid [3], and even if such a ring fission were to occur in the latter case, it would be expected to give rise to either 3- or 4-*iso*propyl*cyclo*hexane carboxylic acid rather than 4,4-dimethyl*cyclo*heptane carboxylic acid, or at least to a mixture of these compounds [4]. The hydrogenation product is, however, definitely quite homogeneous. 2) Permanganate oxidation of thujic acid gives only dimethylmalonic acid [1]; no caronic acid, the product which would be expected from a substance of structure (III), is formed. The possibility that caronic acid can be further oxidised to dimethylmalonic acid can be ruled out since Baeyer [5] has observed that caronic acid is definitely stable towards permanganate.

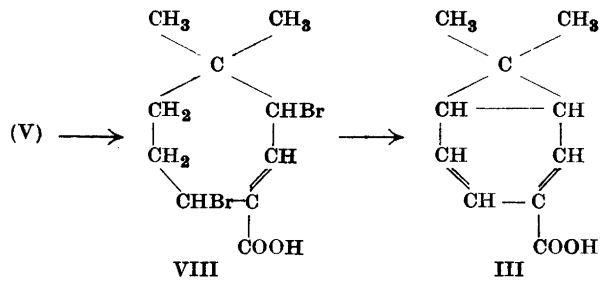
In view of this conflicting evidence, some confirmation of the structure of thujic acid appeared to be highly necessary, and a logical way to achieve this seemed to be to synthesise the acid itself. A natural intermediate in such a synthesis is hexahydrothujic acid (II). While the synthesis of this acid was expected to present little difficulty, its conversion into thujic acid was more uncertain owing to its low stability and the ease with which it rearranges into cumic acid. The second part of the synthesis was therefore carried out first and will be described in this paper. Experiments on the synthesis of hexahydrothujic acid are in progress, and it is hoped to report the completion of the total synthesis of thujic acid in the near future.

The synthesis of thujic acid from hexhydrothujic acid was carried out according to the following scheme.

Hexahydrothujic acid (II) was converted into α-bromohexahydrothujic acid (IV) by the Hell-Volhard-Zelinsky-method. Hydrogen bromide was removed from (IV) by heating with diethylaniline, which was expected to give a mixture of the two unsaturated acids 4,4-dimethyl*cyclo*hepta-1-ene carboxylic acid (V) and 4,4-dimethyl*cyclo*hepta-6-ene carboxylic acid (VI). The acid (VI), after bromination with N-bromosuccinimide to give (VII) and subsequent removal of two molecules of hydrogen bromide, should give the compound (I).



From the acid (V) it should, on the other hand, be possible to obtain a substance of formula (III) in the following way:



This formation of a *cyclo*propane ring on removal of hydrogen bromide would be analogous to the formation of carone from dihydrocarvone hydrobromide [6].

It must however be borne in mind that the bromination with N-bromosuccinimide can be accompanied by an allylic rearrangement [7], whereby the dibromo-acid (VII) could be formed from (V) and the dibromo-acid (VIII) from (VI).

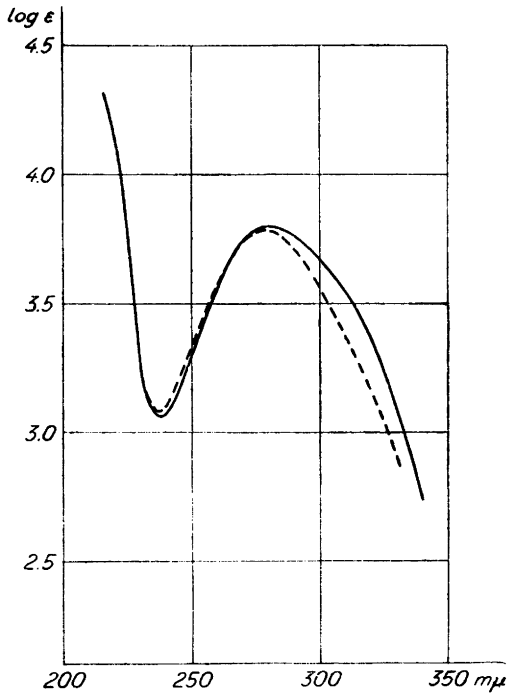


Fig. 1. U. V.-absorption spectra of natural thujic acid (————) and synthetic thujic acid (— — — —) both in ethanol.

Unfortunately it proved to be impossible to separate completely the two acids obtained upon dehydrobromination of bromohexahydrothujic acid. When the mixture of acids was fractionally crystallised a small amount of an apparently pure compound of m.p. 83—85 ° was obtained as the least soluble fraction, which on ozonisation gave β,β-dimethylpimelic acid, thus proving that it was the acid (V). From the more soluble fractions only preparations with very indefinite melting-points between 30 ° and 50 ° could be obtained; analysis of one of these preparations agreed well with the theoretical values for (V) and (VI) thus showing that it was a mixture of these acids. In view of this failure to obtain the acid (VI) in a pure state, the following reaction was carried out on the crude product, in spite of the uncertainty this introduces into the synthesis.

Treatment with two molecules of N-bromosuccinimide gave a very viscous oil, which without further purification was heated with diethylaniline. The product obtained consisted of a steam-volatile component accompanied by relatively large amounts of non-volatile products. The steam-volatile product was a colourless oil, which rapidly solidified. After several recrystallisations from light petroleum a small amount of a substance with m.p. 83—85 ° was

obtained, and a mixture of this with thujic acid of m.p. 86—88° melted at 84—86°. The difficulty encountered in its purification was not unexpected, since thujic acid obtained from *Thuja plicata* has to be recrystallised very many times before it can be obtained in a pure state. The absorption spectrum of the synthetic product was very similar to, although not quite identical with, the spectrum of authentic thujic acid, as is seen from Fig 1.

Definite evidence of the identity of the synthetic product with thujic acid was obtained by the preparation of its *p*-bromophenacyl ester, which was easy to purify and had the same m.p. as the *p*-bromophenacyl ester prepared from authentic thujic acid and did not depress the melting point of the latter on admixture.

Although the transformation of hexahydrothujic acid into thujic acid has thus been realised it does not differentiate between the structure (I) and (III) for thujic acid, for reasons given above.

A definite proof that thujic acid has the structure (I) was however obtained by a study of the product of the hydrogenation of thujic acid using a palladium-charcoal catalyst. As already mentioned, this led to the uptake of two molecules of hydrogen. From the reaction product there could be isolated a tetrahydrothujic acid which proved to be identical with the acid (V) described above. This fact was established by a mixed melting point determination and by the identity of their absorption spectra with a maximum at 223 mμ ($\log \varepsilon = 4.0$). It would actually have been expected to obtain in this hydrogenation the acid (VI) formed by addition of two molecules of hydrogen to the double bonds more remote from the carboxyl group. The formation of the acid (V) must be assumed to proceed by addition of one molecule of hydrogen to the double bond in 5,6-position followed by addition of a second molecule of hydrogen to the ends of the remaining conjugated doublebond system. In view of the difficulty earlier encountered in obtaining the acid (VI) in a pure state, no search for it was made, but it is quite possible that small amounts of it can occur in the mother liquors of (V). The amount of (VI) produced in the hydrogenation must, however, be very small as judged from the ease with which the acid (V) was obtained in a pure state.

The structure of the acid (V) is established by its mode of formation, its behaviour on ozonisation and its u.v.-absorption spectrum, which definitely show the presence of a double bond in conjugation to the carboxyl group. Thus the failure of thujic acid to take up more than two molecules of hydrogen upon hydrogenation with a palladium-charcoal catalyst is due to the resistance of the α, β-unsaturated linkage under the conditions employed, and not to the presence of a *cyclo*propane ring in the molecule.

EXPERIMENTAL

α-Bromohexahydrothujic acid

Hexahydrothujic acid (7 g), obtained by catalytic hydrogenation of thujic acid, was treated with bromine (13 g) in the presence of red phosphorus (0.45 g). After all the bromine had been added, the mixture was heated on a water-bath for one hour, then poured into water and allowed to stand for two days. The solid precipitate was collected and recrystallised from dilute acetic acid. Yield 8.9 g (87 %). For analysis it was further recrystallised from dilute acetic acid and finally sublimed *in vacuo*. M. p. 106°.

$C_{10}H_{17}O_2Br$	Calc.	C	48.2	H	6.9
	Found	»	48.2	»	7.0

Dehydrobromination of α-bromohexahydrothujic acid

α-Bromohexahydrothujic acid (8 g) and diethylaniline (20 g) were heated on an oil bath for one hour at 180°. On cooling diethylaniline hydrobromide crystallised out. The reaction mixture was poured into water, ether was added and the solution made alkaline with sodium carbonate. The ether layer was then separated and extracted with additional portions of sodium carbonate. Acidification of the combined sodium carbonate extracts gave a somewhat sticky precipitate, which was recrystallised from 85 % formic acid. Yield 4.2 g (77 %). On further recrystallisation of this product from formic acid a small amount (0.04 g) of 4,4-dimethylcyclohepta-1-ene carboxylic acid, m. p. 83—85°, was obtained.

$C_{10}H_{16}O_2$	Calc.	C	71.4	H	9.6
	Found	»	71.3	»	9.7

Fractional crystallisation of the mother liquors failed to give any pure substance, except some additional amounts of the same acid. All the more soluble fractions showed very indefinite melting points between 40—50°. A fraction of m. p. 35—40° gave the following analysis:

$C_{10}H_{16}O_2$	Calc.	C	71.4	H	9.6
	Found	»	71.0	»	9.7

Ozonisation of 4,4-dimethylcyclohepta-1-ene carboxylic acid

The acid (0.1 g) was dissolved in chloroform and ozonised at 0°. The chloroform was then evaporated *in vacuo* and the ozonide warmed with water on a water-bath until a clear solution was obtained. Steam was passed in, in order to remove any volatile products, and the solution was then evaporated to dryness giving an oil which subsequently crystallised. Recrystallisation from light petroleum-ether furnished crystals of m. p. 101—102°. No depression was observed on admixture with authentic β,β-dimethylpimelic acid of m. p. 103—104°.

Thujic acid

The acid obtained by dehydrobromination of bromohexahydrothujic acid (m. p. (40—50°) (1.5 g) was dissolved in carbon tetrachloride (100 ml); N-bromosuccinimide (3.2 g) was added and the mixture refluxed for 8 hours. During the heating a yellow colour developed, which, however, had nearly disappeared when heating was discontinued. The solvent was then distilled off on a boiling water bath, the last traces being removed *in vacuo*. The very viscous oil thus obtained was dissolved in diethylaniline (10 ml) and heated at 180° for one hour. The mixture was then treated in the same way as described above for the dehydrobromination of bromohexahydrothujic acid. The acid liberated upon acidification was taken up in ether and steam distilled; the distillate contained an oil which rapidly solidified and was collected by filtration. Yield 0.31 g (20 %). The crude product was purified by recrystallisation from light petroleum, giving crystals of m. p. 82—84°.

$C_{10}H_{12}O_2$	Calc.	C	73.1	H	7.4
	Found	»	72.0	»	7.3

The p-bromophenacyl ester was prepared from the crude acid and recrystallised from methanol. Glistening leaflets of m. p. 105—106° were obtained.

$C_{18}H_{17}O_3Br$	Calc.	C	59.8	H	4.8
	Found	»	59.0	»	4.7

The p-bromophenacyl ester prepared from authentic thujic acid had m. p. 106°.

$C_{18}H_{17}O_3Br$	Calc.	C	59.8	H	4.8	Br	22.1
	Found	»	60.0	»	5.2	»	22.2

Admixture of these two bromophenacyl esters showed m. p. 105—106°.

Hydrogenation of thujic acid with palladium catalyst

Thujic acid (1 g) was hydrogenated in alcoholic solution with a palladium-charcoal catalyst. The total uptake of hydrogen was 280 ml (calc. for two molecules, 274 ml). The catalyst was then filtered off, the alcohol removed *in vacuo*, and the residue taken up in ether and extracted with sodium carbonate. Acidification of the extract gave an oil that solidified. After recrystallisation, first from dilute methanol and then from light petroleum, it had m. p. 82—84°, not depressed on admixture with 4,4-dimethylcyclohepta-1-ene carboxylic acid.

SUMMARY

A partial synthesis of thujic acid has been achieved by conversion of hexahydrothujic acid into thujic acid. Definite evidence has been obtained that thujic acid is 4,4-dimethyl*cyclo*hepta-2,5,7-triene carboxylic acid.

The analyses were carried out by J. Hukki and K. Salo.

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The Crystal Structure of $(NH_4)_2Cr_2O_7$

With a Discussion of the Relation between Bond Number and Interatomic Distances

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Very little is known on the crystal chemistry of the dichromates and the polychromates. The symmetry and the cell dimensions have been determined for $K_2Cr_2O_7$ and $(NH_4)_2Cr_2O_7$ by Gossner and Mussgnug [1]. $K_2Cr_2O_7$ is triclinic with $a = 7.50$ kX, $b = 7.38$ kX, $c = 13.40$ kX, $\alpha = 82.0°$, $\beta = 96.26°$, $\gamma = 90.85°$, and $(NH_4)_2Cr_2O_7$ is monoclinic with $a = 7.78$ kX, $b = 7.54$ kX, $c = 13.27$ kX, and $\beta = 93.7°$. The space group for $(NH_4)_2Cr_2O_7$ is C_{2h}^3 or C_{2h}^6 according to Gossner and Mussgnug. There are four formula units in the unit cell.

It is our intention to study the crystal chemistry of the dichromates and polychromates as part of an investigation of the chromium oxides. The present paper reports a determination of the structure of $(NH_4)_2Cr_2O_7$.

UNIT CELL AND SPACE GROUP

A determination of the unit cell from powder photographs (CrK radiation, $\lambda_\alpha = 2.2909$ Å) gave the following cell dimensions, agreeing well with Gossner's and Mussgnug's values:

$$a = 13.26 \pm 0.01 \text{ Å}, \quad b = 7.54 \pm 0.02 \text{ Å}, \quad c = 7.74 \pm 0.02 \text{ Å}, \quad \beta = 93.2°$$

Weissenberg photographs (Cu-K radiation) of the reflexions $hk0$, $h0l$, $h1l$, $h2l$, $0kl$, $1kl$, and $2kl$ showed that the space group is C_{2h}^6—$C2/c$.

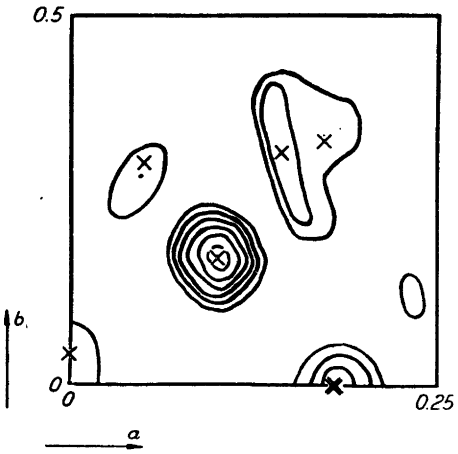

Fig. 1. $(NH_4)_2Cr_2O_7$. *Projection of the electron density on (001).*

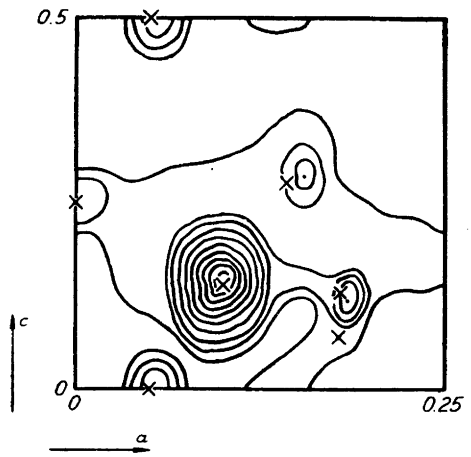

Fig. 2. $(NH_4)_2Cr_2O_7$. *Projection of the electron density on (010). The positions of the atoms from the trial and error process are marked with asterisks, the maxima in the Fourier projections are marked with points.*

DETERMINATION OF THE STRUCTURE

Approximate values of the x and y parameters of the Cr atoms were derived from a Patterson projection in (001). These parameter values rendered it possible to guess the signs of most of the reflexions $hk0$. A two dimensional Fourier synthesis of these reflexions showed maxima belonging to the oxygen atoms also. A trial and error process, followed by a new Fourier synthesis, gave the final values of the x and y parameters. A similar procedure for the $h0l$ reflexions gave the z parameters for the atoms. The final Fourier projections in (001) and (010) are shown in Figs. 1 and 2. Table 1 gives a comparison between observed and calculated F values.

In the calculations of the F values, scattering factors for O^{2-} and Cr^{6+} have been used. The values for Cr^{6+} were calculated from the expression $f_{Cr^{6+}} = f_{Cr} - \frac{6}{4}\,(f_{Ti} - f_{Ti^{4+}}) \cdot f_{NH_4^+}$ was assumed to be equal to $f_{O^{2-}}$.

The following parameter values were obtained:

		x	y	z
8 Cr	in 8(f):	0.100 ± 0.002	0.175 ± 0.003	0.139 ± 0.003
8 O_1	in 8(f):	0.050 ± 0.005	0.300 ± 0.010	0.000 ± 0.010
8 O_2	in 8(f):	0.144 ± 0.005	0.311 ± 0.010	0.278 ± 0.010
8 O_3	in 8(f):	0.178 ± 0.005	0.000 ± 0.010	0.070 ± 0.010
4 O_4	in 4(e):	0	0.042 ± 0.010	0.250 ± 0.010
8 NH_4	in 8(f):	0.172 ± 0.005	0.333 ± 0.010	0.667 ± 0.010

The errors are derived from the trial and error calculations.

Table 1. Weissenberg Photographs of $(NH_4)_2Cr_2O_7$. Comparison between F_{obs} and F_{calc}. Cu—K Radiation.

hkl	F_{obs}	F_{calc}	*hkl*	F_{obs}	F_{calc}
200	30	37	150	30	22
400	140	— 140	350	30	— 23
600	25	12	550	50	— 45
800	30	— 10	750	—	— 22
1000	80	54	950	55	61
1200	55	47	1150	50	79
1400	65	— 69	1350	30	— 21
1600	30	— 40			
			060	120	147
110	50	68	260	—	2
310	—	— 11	460	110	— 86
510	—	— 12	660	55	— 16
710	—	— 9	860	—	— 3
910	60	53	1060	50	53
1110	90	65	1260	—	39
1310	—	— 11			
1510	30	— 26	170	20	31
			370	—	— 13
020	100	— 117	570	20	— 6
220	30	— 45	770	—	0
420	75	91	970	—	— 11
620	45	72	1170	—	— 1
820	—	— 14			
1020	—	— 2	080	80	— 82
1220	—	11	280	20	— 34
1420	25	25	480	30	50
1620	35	46	680	30	42
			880	—	— 27
130	—	13			
330	20	— 40	190	65	— 41
530	90	158	390	—	— 21
730	35	62	590	60	72
930	105	— 127			
1130	70	— 55	1602	70	80
1330	45	25	1402	40	— 69
1530	85	57	1202	105	— 123
			1002	30	15
040	—	2	802	30	3
240	20	0	602	50	6
440	30	33	402	—	42
640	50	39	202	130	— 163
840	30	— 19	002	35	— 41
1040	30	— 20	20$\bar{2}$	130	199
1240	—	— 3	40$\bar{2}$	55	56
1440	—	12	60$\bar{2}$	80	— 68

hkl	F_{obs}	F_{calc}
$80\bar{2}$	130	− 134
$100\bar{2}$	30	− 63
$120\bar{2}$	80	70
$140\bar{2}$	—	27
$160\bar{2}$	—	− 48
1404	70	83
1204	30	− 11
1004	75	− 98
804	85	− 51
604	40	54
404	120	123
204	30	32
004	60	− 51
$20\bar{4}$	30	3
$40\bar{4}$	50	54
$60\bar{4}$	110	83
$80\bar{4}$	—	26
$100\bar{4}$	110	− 92
$120\bar{4}$	40	− 66
$140\bar{4}$	40	56
1206	30	39
1006	—	26
806	30	− 70
606	80	− 101
406	—	2
206	85	103
006	85	48
$20\bar{6}$	75	− 57
$40\bar{6}$	100	− 75
$60\bar{6}$	—	− 4
$80\bar{6}$	60	37
$100\bar{6}$	—	1
$120\bar{6}$	50	− 40
608	—	− 22
408	25	− 40
208	25	− 12
008	75	59
$20\bar{8}$	100	114
$40\bar{8}$	—	− 34
$60\bar{8}$	70	− 94
021	70	− 101
022	30	27
023	60	− 60
024	35	32
025	90	96
026	45	− 73
027	—	26
028	35	− 51
029	50	− 59
041	35	58
042	20	33
043	65	82
044	70	43
045	110	− 112
046	30	− 41
047	—	− 12
048	—	− 18
049	30	56
061	—	− 15
062	—	− 18
063	20	− 14
064	70	− 65
065	—	− 1
066	40	36
067	—	7
081	50	− 52
082	—	− 34
083	—	− 12
084	—	− 27
085	30	41

DESCRIPTION OF THE STRUCTURE

The following interatomic distances are obtained:

$Cr-O_1 = 1.55 \pm 0.1$ Å
$Cr-O_2 = 1.57 \pm 0.1$ Å
$Cr-O_3 = 1.78 \pm 0.1$ Å
$Cr-O_4 = 1.91 \pm 0.05$ Å

$NH_4-3\ O_1 = 3.13$, 3.16, and 3.42 Å ($\pm$ 0.2 Å)
$NH_4-3\ O_2 = 2.68$, 2.85, and 3.02 Å
$NH_4-3\ O_3 = 2.51$, 3.04, and 3.04 Å

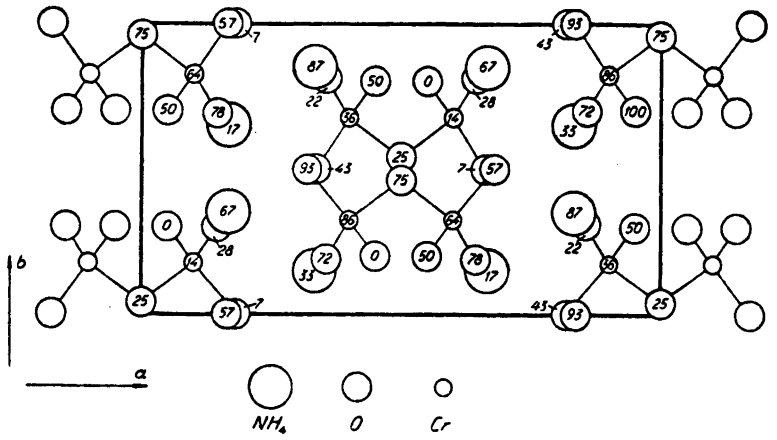

Fig. 3. $(NH_4)_2Cr_2O_7$. Projection of the structure on (001). The figures denote the heights of the atoms in fractions of c. Superimposed oxygen atoms are symmetrically displaced.

The shortest O—O distances are:

$O_1-O_2 = 2.43$ Å,	$O_1-O_3 = 2.86$ Å,	$O_2-O_3 = 2.89$ Å,
$O_1-O_4 = 2.85$ »	$O_3-O_4 = 2.82$ »	$O_2-O_4 = 2.79$ »

The structure is shown in Fig. 3. The chromium atoms are surrounded by four oxygen atoms, situated in the corners of a distorted tetrahedron. Two tetrahedra share one corner so that a $Cr_2O_7^{2-}$ group is formed. The Cr-O_4-Cr angle is 115° and the Cr—Cr distance in the bridge 3.2 Å. The Cr—Cr distances between different $Cr_2O_7^{2-}$ groups are much longer, being 4.8 Å. In CrO_3, the distance Cr—Cr between adjacent atoms in the same chain is 3.3 Å and the angle Cr—O—Cr = 136° [2].

The most stable arrangement of a $Cr_2O_7^{2-}$ group, consisting of two joined tetrahedra, would seem to imply equilinear bonds from the two Cr atoms to the shared oxygen atom. Actually these two bonds form an angle of 115°. A satisfactory explanation of this strong distortion of the dichromate group has not been found. It may be due to the formation of strong N—H—O bonds, but the accuracy of the NH_4—O (or rather N—O) distances is so low that it is not possible to decide whether this explanation is likely to be the real one. If this is the case, these bent $Cr_2O_7^{2-}$ groups ought not to exist in dichromates of the alkali metals. An attempt to solve the structure of another dichromate will be made.

Previous investigations on chromates and CrO_2Cl_2 have shown, that for an oxygen atom bonded to only one Cr atom the distance Cr—O is close to

1.60 Å (see table in ref. 2 p. 1 140). As will be seen the distances Cr—O_1 and Cr—O_2 found agree with this figure. However, the distance Cr—O_3 is considerably longer. No reasonable explanation for this effect has been found, but a considerable part of the difference may be due to errors in the parameters.

For oxygen atoms bonded to two Cr atoms, Cr—O distances of 1.79 $\pm$ 0.05 Å were found in CrO_3[2]. Again in $(NH_4)_2Cr_2O_7$, the corresponding distances are considerably longer (Cr—O_4), being 1.91 $\pm$ 0.10 Å. However, this difference in distances may also be due to errors in parameters.

ESTIMATION OF THE EFFECT OF BOND NUMBER ON INTERATOMIC DISTANCES

In a recent paper, Pauling [3] suggested a formula relating interatomic distances and coordination number in metals and alloys:

$r(1) - r(n) = 0.300 \log n$
n = bond number
$r(1)$ = radius for bond number 1.
$r(n)$ = radius for bond number n.

A similar logarithmic relation may also hold for the bonds (dominatingly) covalent in character in other compounds. In V_2O_5 there are oxygen atoms bonded to one, two, and three V atoms with the following distances [4]:

d_1 = O—V = 1.54 $\pm$ 0.06; d_2 = O—2 V = 1.77 $\pm$ 0.03; d_3 = O—3 V = 1.93 $\pm$ 0.05

d_3 is a mean value of three distances 1.88, 1.88 and 2.02 Å (see below). The errors denote maximal errors derived from a trial and error process and from two dimensional Fourier projections, so that the distances are probably rather more accurate then the errors would indicate.

Assuming a similar equation to Pauling's to be valid, we can calculate one of these distances, knowing two of them.

$$\Delta_{1,2} = -d_1 + d_2 = 2\ K \log 2 \qquad \Delta_{1,3} = -d_1 + d_3 = 2\ K \log 3$$

$$\frac{\Delta_{1,2}}{\Delta_{1,3}} = \frac{\log 2}{\log 3}$$

From $\Delta_{1,2} = -1.54 + 1.77 = 0.23$, we calculate $\Delta_{1,3} = 0.36$ Å, which is close to the observed value 0.39 Å. The value of the constant K in the equation used:

$$r_V(n_1) + r_0(n_1) - r_V(n_2) + r_0(n_2) = 2\ K \log \frac{n_2}{n_1}$$

is calculated from the observed values of $\Delta_{1,2}$ and $\Delta_{1,3}$ and found to be 0.38 and 0.41 respectively, with a mean value of 0.39. As was seen, the corresponding value of the constant in Pauling's equation was 0.300.

Using the value 0.39 for the constant we can calculate the bond numbers of the four longer O—V bonds, with the bond numbers 2 for O—V_1 = 1.54 and 1 for O—2 V = 1.77 Å. For the vanadium atoms we find

		n	
V—O = 1.54 Å	(one distance)	2.0	(assumed value)
V—O = 1.77 Å	(» »)	1.0	» »
V—O = 1.88 Å	(two »)	0.73	(calculated value)
V—O = 2.02 Å	(one »)	0.50	» »
V—O = 2.81 Å	(» »)	0.05	» »

Thus for the V atoms $\sum n = 2.0 + 1.0 + 2 \cdot 0.73 + 0.50 = 4.96$ close to the expected value of 5. It is of interest to note that the sixth bond is much weaker than the other five, and it seems appropriate to call the V atoms five-coordinated, as was done in a previous paper [4].

The only Cr—O distance in CrO_3 known with reasonable accuracy is that to the shared oxygen atoms in the chain, which is 1.79 ± 0.05 Å [2]. From the distance Cr—O in CrO_2F_2 with bond number 2, we calculate this distance to be 1.57 + 0.78 log 2 = 1.80 Å, thus almost exactly the same as the observed value. The other Cr—O distances in CrO_3 are almost equally long, being 1.81 Å (expected value 1.57 Å), but their accuracy is very low, as is evident from the structure determination.

The mean Cr—O distance in chromates such as K_2CrO_4 and $CuCrO_4$ is calculated to be 1.57 + 0.78 log $\frac{4}{3}$ = 1.67 Å. This is actually the value found by Brandt in $CuCrO_4$ [5] and within the accuracy of the value in $CaCrO_4$ (1.64 Å) [6]. The distance Cr—O in K_2CrO_4 [7] and Na_2CrO_4 [8] is somewhat lower, 1.60 Å, but the limits of errors may include the calculated value.

The individual bond numbers of the Cr—O_1, Cr—O_2, and Cr—O_3 bonds in $(NH_4)_2Cr_2O_7$ are difficult to evaluate because of the large errors in the NH_4—O distances. However an expected mean value for the length of the three bonds is 1.57 + 0.78 log $\frac{6}{5}$ = 1.63 Å. The observed Cr—O distances are rather inaccurate (± 0.1 Å) but their mean value, which may be more accurate than the three individual bonds, is actually 1.63 Å, the same as the calculated value. For the fourth Cr—O bond in $(NH_4)_2Cr_2O_7$, a value of 1.57 + 0.78 log 2 = 1.80 Å is calculated but the observed value 1.91 ± 0.05 Å indicates that this distance may be somewhat longer. A more accurate determination of the interatomic distances would be of great interest.

SUMMARY

The crystal structure of $(NH_4)_2Cr_2O_7$ has been determined. The cell dimensions are:

$$a = 13.26 \pm 0.01 \text{ Å}, \quad b = 7.54 \pm 0.02 \text{ Å}, \quad c = 7.74 \pm 0.02 \text{ Å}, \quad \beta = 93.2°$$

The space group is $C_{2h}^6 - C2/c$ with the atoms in the positions:

		x	y	z
8 Cr	in 8(*f*):	0.100 ± 0.002	0.175 ± 0.003	0.139 ± 0.003
8 O_1	in 8(*f*):	0.050 ± 0.005	0.300 ± 0.010	0.000 ± 0.010
8 O_2	in 8(*f*):	0.144 ± 0.005	0.311 ± 0.010	0.278 ± 0.010
8 O_3	in 8(*f*):	0.178 ± 0.005	0.000 ± 0.010	0.070 ± 0.010
4 O_4	in 4(*e*):	0	0.042 ± 0.010	0.250
8 NH_4	in 8(*f*):	0.172 ± 0.005	0.333 ± 0.010	0.667 ± 0.010

The $Cr_2O_7^{2-}$ group consists of two tetrahedra sharing one corner. The two bonds in the Cr—O—Cr bridge form an angle of 115°.

The relation between bond numbers and interatomic distances in V_2O_5, CrO_3, and $(NH_4)_2Cr_2O_7$ are discussed.

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A Colorimetric Method for the Determination of Monosaccharides in Organic Solvents for Use in Partition Chromatography

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It is necessary, when using partition chromatography for the separation of substances according to the flowing chromatogram technique, to have some simple identification reaction. It should preferably be directly applicable to the collected fractions and be suitable for mass analyses. Moore and Stein [1] used the ninhydrin reaction for the determination of amino acids quantitatively. For the identification of purine bases Edman, Hammarsten, Löw and Reichard [2] made use of the light absorption at 260 mμ. Both methods can be applied directly to the mobile phase in partition chromatography.

No simple method applicable to sugars has hitherto been described. Bell [3] was the first to separate sugars by means of partition chromatography on a column. He separated methylated monosaccharides on a column of silica gel. Hough, Jones and Wadman [4] later separated unsubstituted sugars on a column of pulverised cellulose. The last-mentioned authors applied the flowing chromatogram technique and made qualitative identification of the sugars by means of paper chromatography.

The usual reduction methods with more or less concentrated salt solutions cannot be used for the determination of sugars in organic solvents. In this case the solvent must at first be removed by evaporation under reduced pressure.

Colorimetric methods which involve the use of strong mineral acids cannot be used either, because the organic solvents are decomposed by the strong acid and give rise to dark-coloured compounds.

The methods using *e.g.* picric acid, dinitro-salicylic acid or o-dinitrobenzene, could possibly be used in the organic solvents.

The picric acid method was found to be applicable only to sugar concentrations greater than 100 μg/ml. The o-dinitro-benzene reaction is very sensitive. It was possible to detect glucose in concentrations as low as approximately 1 μg/ml. The dark-blue colour formed was, however, too unstable.

Aniline phthalate or oxalate has been recommended by Partridge [5] for the qualitative identification of sugars in paper chromatography. Hough, Jones and Wadman [6] have recently studied the salts of a number of bases such as aniline, dimethylaniline, naphthylamine and *p*-anisidine with organic acids such as acetic acid, trichloracetic acid and oxalic acid with regard to their use as spraying reagents in paper chromatography. They found that these salts on heating reacted with aldohexoses and aldopentoses with the formation of coloured compounds. Aldohexoses gave a green to a greenish-brown colour, depending on the sugar and the base used, whereas aldopentoses gave a reddish colour. With these reagents, ketohexoses gave no colour. The authors recommended — as did Forsyth [7] and Partridge [8] — naphthoresorcinol and hydrocloric acid or trichloracetic acid for the identification of ketohexoses. Aniline salts were also found to give coloured compounds with methylated sugars.

It should therefore be possible to use aniline in combination with organic acids for quantitative determinations as well.

Whilst the writer was working on the method described in the following Blass, Macheboeuf and Núñez [9] reported a method for the quantitative determination of sugars. They used Partridge's aniline phthalate reagent. They did not, however, obtain any colour directly till after the reagent was added and the solvent evaporated. After evaporation to dryness the coloured reaction product was taken up in methanol for colorimetry.

On a study of the ability of a number of bases to form colour in combination with various organic acids, the present writer found aniline trichloracetate in a strong solution of trichloracetic acid to give the best results. Colour was formed with aldopentoses and aldohexoses but not with fructose. In the case of both pentoses and hexoses, the colour formed showed the strongest light absorption at 370 mμ. A similar colour with the same absorption maximum was obtained with furfural. The reaction for both categories of sugars and for furfural was found to follow Lambert-Beer's law within the 5—500 μg/ml range. The method was therefore suitable for the quantitative determination of sugars in organic solvents.

EXPERIMENTAL

Reagent: 32 ml of an 8.5 *N* aqueous solution of CCl_3COOH are mixed with approximately 50 ml of cold absolute alcohol in a flask placed in an ice-water bath and 2 ml of aniline are added. After dilution to 100 ml with cold absolute alcohol, the reagent is ready for use.

If alcohol of room temperature is used in preparing the reagent, or if this takes place without chilling, higher blanks are obtained. If the reagent is stored in a refrigerator at approximately 0° C for 3—4 hours, the blank remains constant. After this time a faint yellow colour appears in the reagent and the blank rises simultaneously. The trichloracetic acid to be used for the preparation of the 8.5 *N* solution is kept airtight in a closed bottle in the cold. The 8.5 *N* aqueous solution of trichloracetic acid remains stable for a week.

Sugar solutions: Stock solutions are made in organic solvents saturated with water and containing 1 or 2 mg/ml of the aldopentoses or the aldohexoses. Suitable dilutions can then be prepared from these stock solutions.

Method: 1 ml of the solvent used containing between 5 and 300 μg sugar per ml is pipetted into thin-walled test tubes, 15.4—16 mm by 120 mm, standing in a cooling bath. 1 $\pm$ 0.02 ml of the reagent is added to each test tube. After shaking for 3—5 minutes, the test tubes are placed in a specially constructed stand, provided with a cooling arrangement (see below). The openings of the tubes are covered with aluminium caps and the stand placed in a strongly boiling water bath for 15 $\pm$ 0.5 minutes. After boiling, it is cooled for 5 minutes in a water bath (running tap water or an ice-water bath) and 2 $\pm$ 0.1 ml of 95 per cent alcohol added to each test tube. Reading off takes place in 1 cm cuvettes at 370 mμ in a Beckman spectrophotometer (model B) or a similar apparatus.

At least three blanks are boiled at the same time. The blank consists of 1 ml of solvent saturated with water and 1 ml of the reagent. In chromatography, fractions that do not contain sugar should be used for the blanks. The blanks are compared with a reference solution of the same composition as the blank but that has not been heated in a water bath. The sample is read off against the blank which corresponds most closely to the mean value of the three blanks read off against the reference solution. The blank must not give a higher optical density than 0.070 in a 1 cm cuvette.

Calculations: A series of standard solutions is boiled at the same time as the sample. The series should contain 3 blanks and 3 samples of each of the following solutions: 10, 20, 50, 100 and 200 μg/ml of the sugar to be determined dissolved in the solvent used, saturated with water. On the basis of the optical density of these standard solutions, a standard curve is drawn from which the sugar concentration in the sample is read off directly. When less accuracy is required, it is unnecessary to boil the standard solutions simultaneously each time.

Apparatus: Because the volume of the reagent affects the intensity of the colour (see below), the volume must be kept constant during boiling. It was therefore necessary to construct a stand for the test tubes, provided with a cooling arrangement. It was constructed in such a way, that during boiling, the lower one fourth of each test tube was standing in the boiling water, the middle was rinsed by the cooling water and the upper one fourth was above the surface of the cooling water, the openings of the test tubes being covered with an aluminium cap. The construction of the stand and the water bath is shown in Fig. 1. The level of the boiling water is kept just above the level of the liquid in the test tubes. The water bath is heated by means of five 12 mm Bunsen burners.

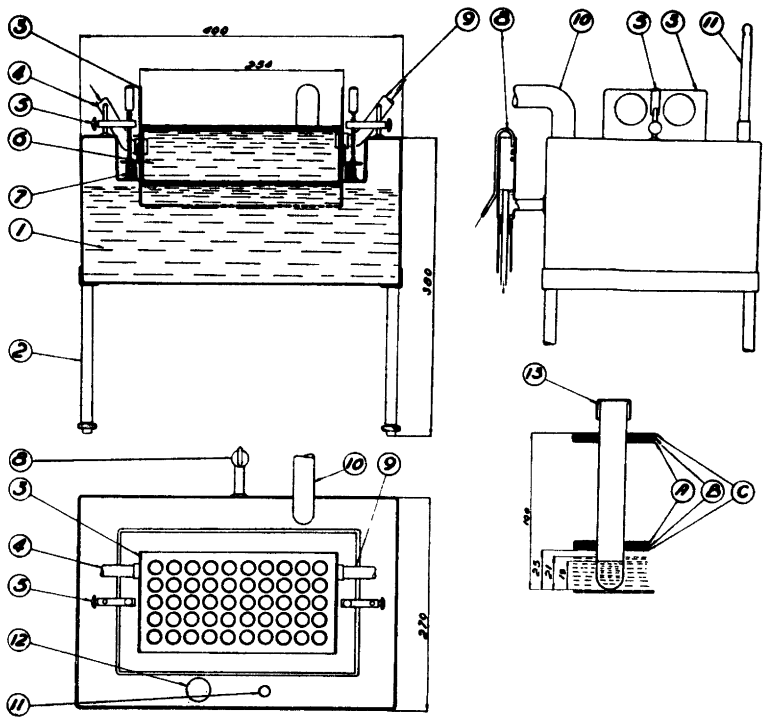

Fig. 1. Test tube stand and water bath.

The stand and the water bath are made of aluminium, thickness 2 mm.

1. *Water bath with boiling water*
2. *Iron stand supporting water bath*
3. *Test tube stand*
4. *Inlet for cooling water*
5. *Screws and clamps for keeping stand in place*
6. *Cooling water in the test tube stand*
7. *Rubber packing*
8. *Water level regulator for water bath*
9. *Outlet for cooling water*
10. *Steam outlet*
11. *Thermometer*
12. *Window for controling of the boiling*
13. *Aluminium cap covering the test tubes*

In order to obtain good tightening of the holes in the stand supporting the test tubes an arrangement shown in detail in the figure was made. A is an aluminium plate with holes 18 mm in diameter (test tube diam. 15.4—16 mm) for the test tubes. B is a rubber plate with holes 14 mm in diameter and C is a plate similar to A.

Factors affecting the formation of colour

1. *Concentration of aniline*: An increase in the concentration of aniline increases the intensity of the colour. That of the blank is increased simultaneously. 2 ml of aniline per 100 ml of reagent resulted in the greatest difference between the blank and the sample, the colour due only to the solvent — *i.e.*, the blank — then being the smallest fraction of the total colour formed.

2. *Concentration of trichloracetic acid*: The concentration of trichloracetic acid has a corresponding effect as that of aniline on the formation of colour, although to a lesser degree.

3. *The degree of purity of the aniline and trichloracetic acid:* The aniline used for preparation of the reagent should be practically colourless. Compared with a somewhat less pure compound, very highly purified aniline causes no improvement. Strongly coloured aniline, on the contrary, gives a high blank. Aniline (A. R.) is distilled *in vacuo* once or twice. Despite repeated vacuum distillations, individual lots of the preparation can nevertheless continue to give high blanks. In such cases, purification according to Keyes and Hildebrand [10] has been found to be satisfactory. The method consists of dissolving the aniline in hydrochloric acid and subsequent removal of the traces of nitro compounds by means of steam distillation. The solution is made alkaline and the aniline distilled with steam. It is then dried over potassium hydroxide and fractionated *in vacuo*.

The trichloracetic acid should be of the highest degree of purity. The optical density of the blank which should not exceed 0.070 in a 1 cm cuvette, is the best gauge of the degree of purity of the aniline and the trichloracetic acid. In the experiments reported here, the trichloracetic acid was obtained in 100 grms lots from A. B. LKB Produkter. It was stored in firmly closed glass vessels in a refrigerator.

4. *Water content*: An increase in the water content of the reagent gives a lower colour intensity and reduces the stability of the reagent. The water content of the reagent is regulated by the quantity of trichloracetic acid solution used and its concentration. An 8.5 N aqueous solution of trichloracetic acid was found to be most suitable.

5. *Volume of the reagent and of the solution*: An increase of $\pm$ 5 per cent in the volume of the sugar solution added causes an inappreciable change in the intensity of the colour formed. The volume of the reagent, on the contrary, has a considerable effect. The reagent should therefore be pipetted off with an accuracy of $\pm$ 0.02 ml.

6. *Duration of boiling and temperature of the water bath:* It is necessary to have a vigorously boiling water bath. If boiling is prolonged, the intensity

of the colour is increased, that of the blank also increasing. Optimal conditions for colorimetry were chosen for the same reason as those given in the case of the concentration of aniline, trichloracetic acid and water.

7. *Effect of dilution after boiling:* If the samples, after boiling, were diluted with different volumes of 95 per cent alcohol, butanol or the reagent the colour did not follow Lambert-Beer's law. The dilution with 2 ml of 95 per cent alcohol after boiling must therefore be made accurately (2 $\pm$ 0.1 ml). If the optical density of a sample is too high for being read off accurately from the apparatus, dilution must take place with a mixture of one part of the solvent in question saturated with water, one part of the reagent and two parts of 95 per cent alcohol. The figure for the sugar concentration obtained from the curve must then be corrected for the dilution.

8. *Influence of amino acids*: When solutions containing 50 μg/ml of glucose and 500 μg/ml of any one of the common amino acids were analysed there was no difference in colour intensity compared with that of solutions containing no amino acid except for cystein. This amino acid caused a detectable depression of colour intensity the amino acid concentration being as low as 50 μg/ml.

Stability of the colour: The change in the intensity of the colour at various times after boiling is shown in Table 1.

Table 1.

Glucose	Optical density after		
μg/ml	15 min.	1 h	24 h
0	0.050	0.050	0.050
10	0.055	0.050	0.055
50	0.260	0.260	0.260
100	0.530	0.515	0.520
200	1.035	1.010	1.010

In these cases, the blank was read off against the reference solution and the other solutions against the blank. Between the readings the solutions were kept in closed test tubes at room temperature.

Absorption spectrum: The light absorption for wave lengths between 320 and 600 mμ is shown in Fig. 2 for hexoses (glucose) and in Fig. 3 for pentoses (ribose). The blank and the standard solutions with 50 and 100 μg/ml were read off against the reference solution, the standard solutions then being read off against the blank. From 355 mμ up to 600 mμ the light absorption of the blank was low and almost constant. Both pentoses and hexoses showed a maximum at 370 mμ.

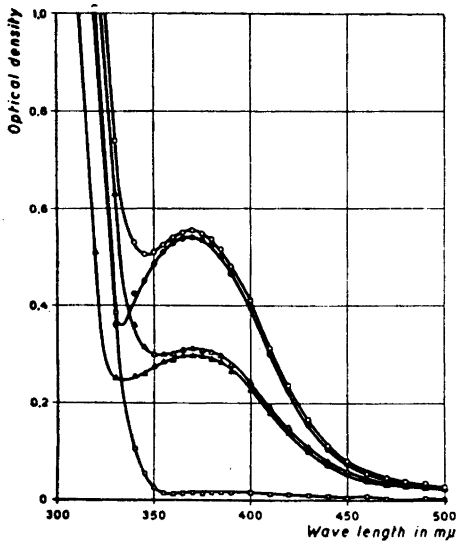

Fig. 2. Absorption spectrum for glucose.

—□—□—□— *Blank read against reference solution*
—△—△—△— *50 μg/ml read against reference solution*
—○—○—○— *100 μg/ml read against reference solution*
—▲—▲—▲— *50 μg/ml read against blank*
—●—●—●— *100 μg/ml read against blank*

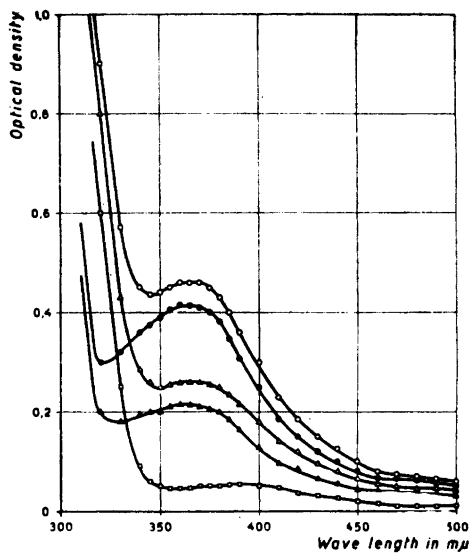

Fig. 3. Absorption spectrum for ribose.

—□—□—□— *Blank read against reference solution*
—△—△—△— *50 μg/ml read against reference solution*
—○—○—○— *100 μg/ml read against reference solution*
—▲—▲—▲— *50 μg/ml read against blank*
—●—●—●— *100 μg/ml read against blank*

Correlation between the sugar concentration and the intensity of colour: The standard curves for glucose and ribose are shown in Fig. 4 and Fig. 5 respectively. It is seen that there is a satisfactory correlation between the colour formed and the sugar concentration in the whole range covered by the curve.

Accuracy: In order to determine the accuracy of the method the following experiment was made.

Three blanks and 10 samples of each of the following solutions: 10, 20, 50 and 100 μg/ml dissolved in butanol saturated with water were boiled simultaneously. A curve was plotted through the means found for the different concentrations from which the sugar content of each individual sample was read off. The mean and the standard deviation of a single determination (σ) were calculated for each solution. The result is shown in Table 2.

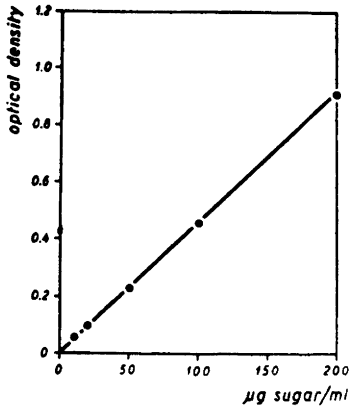

Fig. 4. Standard absorption curve for glucose dissolved in butanol.

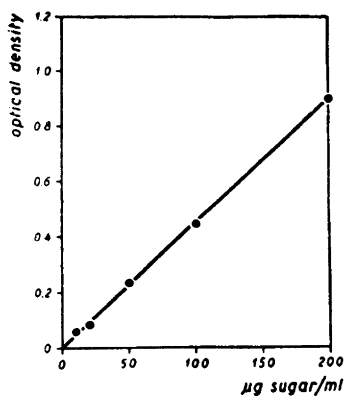

Fig. 5. Standard absorption curve for ribose dissolved in butanol.

Table 2.

Sugar concentration in µg/ml		Standard deviation (σ)	
Added	Found mean	µg	Per cent of the mean
10	10.1	1.9	19
20	18.75 (!)	1.25	6.7
50	49.5	1.20	2.4
100	100.7	2.65	2.7

Applicability to different solvents: The method was applied to mixtures of butanol and ethanol and of butanol and propanol. High blanks were obtained in ethylene glycol and in ethyl acetate.

SUMMARY

A method is described for the determination of monosaccharides in organic solvents to be used in partition chromatography. It is based on a reaction between aniline salts and sugar

The method can be used for aldopentoses, methyl pentoses and aldohexoses in concentrations between 10 and 300 µg/ml.

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Brauchbarkeit und Anwendungsbereich der Papierelektrophorese für Serumproteine

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Nachdem es Haugaard und Kroner [1], Wieland [2] und neuerdings auch Biserte [3] gelungen war, Aminosäuren- und Peptidgemische in der Papierelektrophorese aufzutrennen, gingen Durrum [4], Turba und Enenkel [5] und Cremer und Tiselius [6] dazu über, die Methode auf Eiweisskörper auszudehnen. Es gelang in verschiedenen Versuchsanordnungen Serumproteine ausreichend aufzutrennen. Die Methoden unterscheiden sich im färberischen Nachweis der Eiweisskörper und vor allem in dem Vorgehen, das Papier während des Versuches vor Austrocknung zu schützen. Während Durrum und Turba und Enenkel das Papier zur Elektrophorese in eine feuchte Kammer [7] einschliessen, versenken Cremer und Tiselius den mit Glasplatten bedeckten Papierstreifen in ein Chlorbenzolbad. Das Chlorbenzol schliesst das Papier gegen die Aussenluft ab und ist in der Lage, die beim Stromdurchfluss erzeugte Wärme aufzufangen. Da die Abdunstung von Pufferlösung zu Flüssigkeitsverschiebungen im Papierstreifen und eine Erwärmung des Streifens zur Veränderung seiner Leitfähigkeit führen kann, erscheint diese Massnahme angebracht.

Cremer und Tiselius konnten durch Ausführung der Papierelektrophorese in einem Chlorbenzolbad verschiedene Seren einwandfrei auftrennen und durch kolorimetrische Auswertung der Bromphenolblaufärbung der Proteinflecke Konzentrationsgefällekurven der Seren erzielen, die weitgehend den bekannten Elektrophoresekurven bei optischer Registrierung ähneln. Indessen birgt Cremers Apparatur eine Unsicherheit in der Stromführung in sich. Zur Überleitung des Stromes von der Elektrodenflüssigkeit auf das Filterpapier werden angefeuchtete Papierrollen benützt. Diese können den Kontakt mit

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dem Filterpapierstreifen in wechselnder Breite eingehen. So kann die Leitfähigkeit dieses Kontaktes von Versuch zu Versuch variieren, was Schwankungen des für die Auftrennung im Filterpapierstreifen wirksamen Spannungsgefälles zur Folge hat. Einerseits leidet die Auftrennung darunter und andererseits sind verschiedene Untersuchungen schlecht miteinander vergleichbar. Um stets gleiches Spannungsgefälle im Papierstreifen zu haben, wurde deshalb die Elektrodenanordnung vereinfacht und ein direkter Kontakt zwischen der Elektrodenflüssigkeit und dem Filterpapierstreifen hergestellt. Die Änderung ermöglicht weiterhin eine gleichzeitige Analyse mehrerer Seren in einem Untersuchungsgang.

Mit der modifizierten Apparatur wurden Analysen der verschiedensten Seren durchgeführt. Die Ergebnisse mehrerer Parallelbestimmungen und einiger Kontrollbestimmungen mit der freien Elektrophorese nach Tiselius sind tabellarisch zusammengestellt. Im Allgemeinen gestattet die Methode auch bei stark pathologisch veränderten Seren einen guten Überblick über die Zusammensetzung der Seren. In einigen Fällen, wo die freie Elektrophorese wegen Trübung oder starker Färbung der Seren optisch nicht messbar war, konnte mit der Papierelektrophorese noch eine gute Auftrennung registriert werden.

VERSUCHSANORDNUNG

Sollen drei verschiedene Proben gleichzeitig untersucht werden, so trägt man auf jeden der drei 40 cm langen und 40 mm breiten Streifen des Filterpapiers Munktell No. 20 (fünffache Dicke gegenüber dem handelsüblichen) am Startpunkt, der 10 cm vom kathodischen Ende durch ein Bleistiftkreuz markiert wurde, 2 mg Gesamteiweiss auf. Man wird bei normalem Gesamteiweissgehalt des Serums am Besten 0,025 ml auftropfen. Dialyse des Serums ist nicht erforderlich. Kurz nach dem Auftropfen werden die Papierstreifen durch eine 0,05 mol. Veronallösung (102 g Natriumdiaethylbarbituricum auf 10 l destilliertes Wasser) gezogen und auf Filterpapier gelegt. Nachdem die überschüssige Veronallösung abgesaugt ist, werden die Streifen einzeln zwischen 25 × 4,5 cm grosse Glasplatten gelegt. Dabei sollen die markierten Startpunkte genau übereinander zu liegen kommen. Die Platten werden fest aneinander gepresst, damit die zwischen Platten und Papierstreifen befindliche Luft verschwindet, und schliesslich durch mehrere Stahlklammern verschiebungssicher fixiert. Die beiderseits das Plattenpaket überragenden Enden der Papierstreifen werden jetzt auf dieselbe Länge zugeschnitten, so dass etwa 2—3 cm beiderseits aus dem Paket herausragen. Man legt dann das Paket in das Chlorbenzolbad ein. Die überstehenden Papierenden kommen dabei über Glasstopfen zu liegen, so dass sie etwa 5 mm über das Niveau des Chlorbenzols

herausragen. Nun wird auf der Anoden- und Kathodenseite je ein am einen Ast trichterförmig ausgeweiteter Heber mit seinem dünnen Ende in die Veronallösung des Elektrodengefässes und mit dem ausgeweiteten Ende über das hochragende Ende der Papierstreifen in das Chlorbenzolbad versenkt. Unter die trichterförmige Ausweitung des im Chlorbenzol versenkten Heberastes werden mit der Pipette vorsichtig etwa 15 ml Veronallösung gegeben. Wegen ihrer geringeren spezifischen Dichte schichtet sich die Lösung über das Chlorbenzol und lagert sich um das Papierstreifenende an.

Saugt man nun aus dem Hebersystem durch den oben angebrachten Hahn die Luft ab, so steigt die Flüssigkeit unter der Heberglocke und aus dem Elektrodengefäss hoch und findet an der Spitze des Hebers luftfreien Kontakt. Da im Chlorbenzolbad und im Elektrodengefäss vor Einsetzen des Hebers gleiches Flüssigkeitsniveau hergestellt wurde, fliesst im Heber keine Flüssigkeit nach. Wird in das Elektrodengefäss mehr Flüssigkeit nachgegeben, so verdrängt diese das Chlorbenzol am Papierstreifenende. Man gibt deshalb zur Herstellung eines stabilen Kontaktes soviel Veronallösung in das Elektrodengefäss nach, dass die Papierenden in ihrer ganzen Breite von der Elektrodenflüssigkeit umspült sind. Die Leitfähigkeit des ganzen Systems ist dann immer gleich gross. Der Gesamtwiderstand zwischen beiden Elektroden beträgt bei drei Streifen 73 000 Ω (190 Volt bei 2,6 mA).

Die Trennung des Kontaktes bei Beendigung des Versuches nach 12—24 Stunden ist einfach. Es wird aus dem Elektrodengefäss soviel Lösung abgesaugt, dass die Grenze Chlorbenzol-Veronallösung im Heber bis unter den Hahn ansteigt. Wird dieser geöffnet, so fällt gut abgetrennt das Chlorbenzol in die Wanne und die Veronallösung in das Elektrodengefäss zurück. Die Streifen werden nach vorsichtigem Absaugen des sich zwischen den Glasplatten ausserhalb der Papierstreifen ansammelnden Chlorbenzols aus dem Platten paket herausgenommen und sorgfältig in der Hitze getrocknet. Abbildung 1 gibt einen Überblick über die Versuchsanordnung. Während die Elektrodenbechergläser beliebig gross gewählt werden können (Inhalt etwa 500 ml), soll das für die Heber verwendete Glasrohr mindestens 12 mm weit und für jeden Heber nicht länger als 18 cm sein. Bei diesen Massen ist der Spannungsverlust durch die beiden Elektrodensysteme etwa die Hälfte der angelegten Spannung. Das Spannungsgefälle ist ausreichend hoch für eine rasche Wanderung der Komponenten mit guter Auftrennung.

In den zahlreichen Versuchen war die von Cremer verwendete Bromphenolblaufärbung zuverlässig. Nach Trocknung der Streifen bei etwa 100° C werden sie auf 5 Minuten in eine 1 %ige Lösung von Bromphenolblau in 96 %igem *a*ethanol gelegt. Das *a*ethanol ist mit $HgCl_2$ gesättigt (32,2 g $HgCl_2$ auf 100 ml *a*ethanol) und fixiert dadurch im gleichen Gang mit der

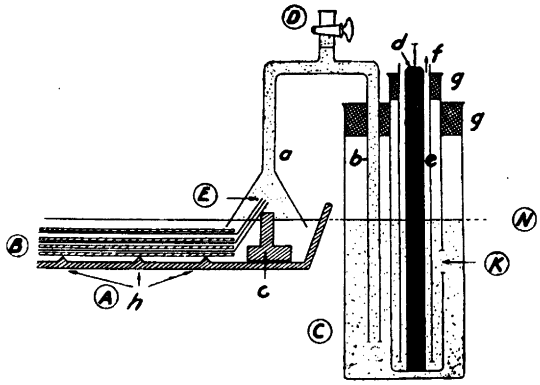

Abb. 1. Schema der Versuchsanordnung. (Zur Vereinfachung wird nur eine Elektrodenseite im Aufriss gegeben.)

A. Glaswanne, gefüllt mit Chlorbenzol (40 × 15 cm).
B. Plattenpaket, bestehend aus 4 Glasplatten und 3 dazwischenliegenden Papierstreifen.
C. Becherglas mit Pufferlösung und Elektrodengefäss.
D. Heber mit Hahn eingetaucht in C und A.
E. Kontaktstelle zwischen der Elektrodenflüssigkeit und den Papierstreifen.
N. Gleiches Flüssigkeitsniveau des Chlorbenzols und der Elektrodenflüssigkeit.

Detail: a Trichterförmiges Ende
b Dünnes Ende des Hebers
c Glasstopfen
d Kohlenelektrode
e Elektrodengefäss mit Öffnung bei K
f Rohr für Gasabzug (↑)
g Durchbohrte Korkstopfen
h Querleisten am Boden der Glaswanne oder eingelegte Glasstückchen zur Hochlagerung des Plattenpakets.

Färbung das angetrocknete Protein auf dem Papierstreifen. Anschliessend werden die proteinfreien Teile des Papiers durch Einlegen des Streifens in 96 %iges Methanol und Äthanol entfärbt. Beide Alkohole enthalten noch 1 % $HgCl_2$. Nach 1/2 bis 1 Stunde ist das Papier bis auf die proteinhaltigen Teile farbfrei. Der Streifen wird nun für wenige Minuten in reines Methanol und Äther überführt und getrocknet. Die selektive Anfärbung der Proteinflecke geht einwandfrei, wenn man in den Endstufen stets frischangesetztes Äthanol mit 1 %igen $HgCl_2$ verwendet und das zum Schluss benützte Methanol- und Ätherbad farbfrei sind.

Die entwickelten Streifen wurden auch kolorimetrisch ausgewertet. Sie werden dazu in 5 mm breite Querstreifen geschnitten. Der Farbstoff jedes Streifchens wird mit 3 ml (bei starkem Farbgehalt mit 6 ml) einer Mischung von 96 %igem Methanol und 10 %iger Na_2CO_3-Lösung ($Na_2CO_3 \cdot 10\ H_2O$)

zu gleichen Teilen extrahiert. Die Farbextinktion der Extraktionsflüssigkeiten wird nach einer Extraktion über 1/2—1 Stunde am Beckman-Spektrophotometer bei einer Wellenlänge von 5 950 Å abgelesen. Trägt man die Extinktionswerte als Ordinate gegen die Abscisse des Abstandes der Streifchen vom Startpunkt auf, so erhält man Konzentrationsgefällekurven für die Serumproteine ähnlich den Kurven der freien Elektrophorese. Da die Papierentfärbung nicht immer in gleich hohem Masse gelingt, empfiehlt es sich, von der kathodischen und anodischen Seite des Papierstreifens je ein proteinfreies Streifchen ebenfalls zu extrahieren. Der Mittelwert beider Streifchen wird als Papierleerwert von den Extinktionswerten der proteinhaltigen Streifchen abgezogen. Erst dadurch bekommt die Kurve eine exakte Basislinie. Da die Bindungsfähigkeit des Bromphenolblaus gegenüber dem Globulin geringer ist als zum Albumin, ermittelte Cremer an künstlichen Albumin-Globulingemischen einen Korrekturfaktor für die Extinktionswerte der Globulinhaltigen Streifchen. Multipliziert man die Extinktionswerte des globulingebundenen Bromphenolblaus mit 1,6 nach Abzug des Papierleerwertes, so kann man proportionale Konzentrationskurven aufstellen.

Mit dieser Technik wurden vergleichende Untersuchungen an Normalseren und pathologischen Seren ausgeführt. Vor allem interessierte es, ob Einzelergebnisse gut reproduzierbar sind und ob der von Cremer ermittelte Faktor 1,6 auch bei Gemischen verschiedenartiger Globuline wie dem Serum für jede Komponente anwendbar ist. So könnte man z. B. annehmen, dass das α- und γ-Globulin verschieden grosse Affinität zum Bromphenolblau haben — ganz abgesehen vom Verhalten pathologischer Globulinkomponenten.

ERGEBNISSE

Schon die einfache Betrachtung der gefärbten Papierstreifen lässt weitgehende Schlüsse über Art und Menge der einzelnen Komponenten des untersuchten Serums zu. Nach einer Versuchsdauer von 12—16 Stunden bei 190 Volt und 2,6 mA für drei Streifen liegen die Komponenten über eine Papierstrecke von 12—16 cm ausgebreitet vor. Das Albumin wandert dabei etwa einen Zentimeter in der Stunde. Die Normalseren trennten sich innerhalb 12 Stunden immer gut in ihre 4 Hauptkomponenten auf. Auch vereinzelt wurde eine Auftrennung des β-Globulins in zwei Untergruppen beobachtet. Bei den pathologischen Seren waren Albumin und pathologisch vermehrte Komponenten stets deutlich erkennbar. Verminderte Globuline erschienen häufiger weniger deutlich auf dem Papier. Besonders bei Nephroseseren trennte sich mitunter das α-Globulin schlecht vom β-Globulin. Bei der häufigen Gesamteiweissverarmung solcher Seren musste man stets mehr Ausgangsmaterial

auf das Papier auftropfen, um die optimale Gesamteiweissmenge von 2 mg am Startpunkt anzureichern. Wurde unter gleichzeitiger Erhöhung der Ausgangsproteinmenge die Versuchsdauer auf 18—24 Stunden verlängert, so war auch in diesen Fällen eine einwandfreie Darstellung sämtlicher Hauptkomponenten möglich. Anfangs fiel bei den Versuchen reichlich Protein am Startpunkt aus. Dies lässt sich jedoch vermeiden, wenn man die Papierstreifen sofort nach dem Auftropfen der Eiweisslösung mit der Veronallösung befeuchtet. Antrocknen kann sonst zur Denaturierung des Eiweisses führen. Die Flecke der gewanderten Proteine sind wegen der während der Wanderung stattfindenden Diffusion stets breiter als ihr Startpunkt. Der γ-Globulinfleck ist im allgemeinen gegenüber den anderen Flecken deutlich verbreitert, was auf die Inhomogenität dieses Globulins schliessen lässt.

Bei 140 mm Wanderungsstrecke des Albumins, also etwa 14 Stunden Versuchsdauer, war die kathodische Ausbreitung von Proteinen meist gering (10—27 mm). Die kathodische Ausbreitung von Eiweisskörpern bei der Papierelektrophorese ist Folge der Elektroosmose und Diffusion, da man eine kathodische Wanderung von Proteinen bei dem hohen pH der verwendeten Veronallösung (pH 9,2—9,3) für unwahrscheinlich halten muss. Stärkere kathodische Ausbreitung von Proteinen ist stets auch mit einer mangelhaften Auftrennung verbunden und weitgehend von der Konzentration der Elektrodenflüssigkeit oder Elektrolytkonzentration der untersuchten Eiweisslösung abhängig. Wurde so zum Beispiel der Eiweissgehalt stark verdünnter Lösungen durch Wasserentzug im Vakuum angereichert, so ergab die gleichzeitige starke Erhöhung ihres Salzgehaltes eine schlechte elektrophoretische Auftrennung mit starker kathodischer Ausbreitung von Proteinen.

Für die Beurteilung pathologischer Seren, die sich manchmal schlecht auftrennen liessen, ist es vorteilhaft, im gleichen Untersuchungsgang ein normales Serum mitlaufen zu lassen. Nach der Färbung kann man dann beide Papierstreifen startpunktgleich aneinanderlegen und mit dem Bild des Normalserums als Schlüssel Abweichungen in der Beweglichkeit und Konzentration der Komponenten des pathologischen Serums direkt ablesen. Der methodische Gleichlauf ist durch die neue Elektrodenanordnung gesichert wie dies die Abbildungen 2 und 3 zeigen. Die Abbildung 2 stellt eine Sammlung von drei pathologischen Seren (α-, β-, γ-Globulinvermehrung) mit den entsprechend gleichgeschalteten Normalseren dar. Bei Abbildung 3 wurden die Extinktionskurven eines Normalserums und eines gleichgeschalteten pathologischen Serums durch Aufeinanderlegen der Startpunkte übereinanderprojiziert. Sämtliche Gipfel beider Seren kommen zur Deckung. Es bestehen lediglich deutliche quantitative Abweichungen des pathologischen Serums vom Normalen.

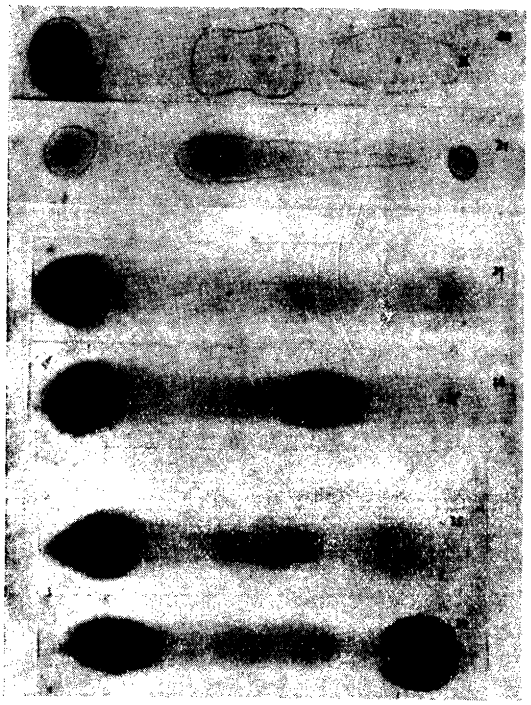

Abb. 2. Originalstreifen der Papierelektrophorese. 21 Nephroseserum, 30 β-Myelom und 34 γ-Myelom. 22, 29 und 35 sind die entsprechenden parallelgelaufenen Normalseren.

Im Weiteren wurden Untersuchungen über die Reproduzierbarkeit der Befunde in der Papierelektrophorese und quantitative Vergleiche zur freien Elektrophorese ausgeführt. Von 15 verschiedenen Seren wurden insgesamt 35 Diagramme angefertigt. Zwei Normalseren wurden dabei viermal und die übrigen Seren in Doppelbestimmungen fraktioniert. Alle Seren wurden auch in der Standardelektrophoreseapparatur nach Tiselius untersucht. Die Tabelle 1 bringt die Ergebnisse der Kurvenauswertungen. Die Extinktionskurven wurden von Bromphenolblaugefärbten Filterpapierstreifen unter Anwendung des Globulinfaktors 1,6 gewonnen. Die Flächen der einzelnen Gipfel wurden nach dem üblichen Kurvenausgleich ausplanimetriert, wobei die Basislinie nach den obigen Angaben festgelegt wurde. Vereinzelt macht es Schwierigkeiten nach der Ablesung der Extinktionswerte den Übergangspunkt vom Albumin zum α-Globulin genau festzulegen. Er ist wesentlich für das Einsetzen des Globulinmultiplikators. Im Allgemeinen enthält das anodische Drittel des gesamten Bromphenolblaugefärbten Proteinbezirks reines Albu-

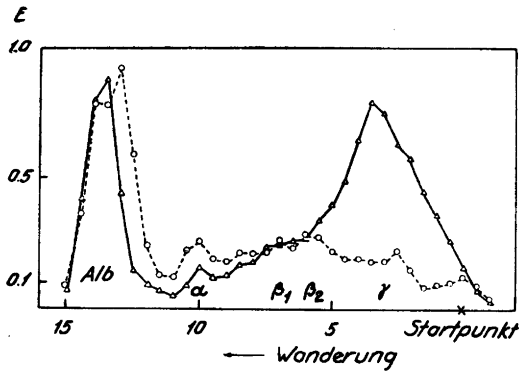

Abb. 3. Vergleich von normalem (—○—○—)und pathologischem (—△—△—) Serum nach Gleichlauf.

Abszisse: Abstand vom Startpunkt. *Ordinate: Extinktionswerte bei 5950 Å.*

min. Man beginnt am besten am tiefsten Punkt der Extinktionskurve unmittelbar nach dem meist steilen Albumingipfel mit der Multiplikation der Globuline und schreitet damit kathodenwärts fort. Erfahrungsgemäss ist dieser Punkt stets der Extinktionswert des 9.—11. von insgesamt 27—33 Streifchen.

DISKUSSION

Vergleicht man an Hand der Tabelle 1 die Ergebnisse der einzelnen Bestimmungen am selben Serum miteinander, so stimmen sie gut überein. Die Auftrennung war bei Normalseren einwandfrei. Bei 2 Seren war in 4 Paralellbestimmungen der mittlere Fehler des Mittelwertes für sämtliche Komponenten bei 10 %. Während die Albuminwerte sehr geringe Streuung zeigen, sind die Schwankungen der relativ kleinen Flächenwerte der Globuline etwas grösser. Auch bei pathologischen Seren ist in sämtlichen Versuchen das Ergebnis in Doppelbestimmungen gut reproduzierbar, allerdings war die Auftrennung mehrmals unvollständig. In solchen Fällen wurde die Zwischenfläche zwischen Albumin und dem nächsten deutlich auszumachenden Globulingipfel als Ganzes ausplanimetriert und das Summenergebnis dieser nicht deutlich unterteilbaren Strecken von Bestimmung zu Bestimmung einander gegenüber gestellt. Die methodische Fehlerbreite war auch hier nicht wesentlich grösser als bei Normalseren.

Stellt man diese methodisch sicheren Werte aus Doppelbestimmungen der Papierelektrophorese den Daten der freien Elektrophorese gegenüber (letzte Spalte der Tabelle 1), so besteht eine zufriedenstellende Überein-

Tabelle 1. Ergebnisse der Auswertung der Papierelektrophoresekurven und Vergleichsdaten der optischen Elektrophorese (in % des Gesamtproteins).

a) *Normalseren*

		Papierelektrophorese					Optische Elektrophorese				
Bez.	Nr	Alb.	α	β	γ	Nr	Alb.	α	β	γ	
G. R.	1	63.1	10.1	9.8	17.0	436					
	2	65.9	10.6	5.9+4.4	13.2						*) Mittlerer Fehler des Mittelwertes
	4	64.4	7.7	9.3	18.6						
	8	66.3	10.1	11.2	12.4						
Mittelwert		64.9	9.6	10.2	15.3						$F_m = \sqrt{\frac{\Sigma (l)}{n (n-1)}}$
F_m*)		±0.7	±0.7	±0.4	±1.5		61.5	8.1	12.1	18.3	
L. P.	14	46	16	22	16	406					
	15	55	9	16	20						
	17	60	12	10	18						
	28	55	11	14+6	14						
									8.9+ 6.1		
Mittelwert		54	12	17	17		60.8	9.5	15.0	14.7	
F_m		±3.0	±1.4	±2.8	±1.3						
S. Ju	49	60	8	11	21	442					
	56	57	8	14	21						
								3.0+ 8.2	10.1+ 4.8		
Mittelwert		58.5	8	12.5	21		50.8	11.2	14.9	23.1	
S. In	46	60	7	14	19	445					
	55	57	10	12	21						
									7.2+ 8.5		
Mittelwert		58.5	8.5	13	20		56.8	10.0	15.7	17.5	
S. G.	45	50	9	9.5+7.5	24	443					
	51	59	8	13	20						
								2.6+ 8.2	10.9+ 7.0		
Mittelwert		54.5	8.5	15	22		49.6	10.8	17.9	21.7	
M. R.	50	60	8	5+10	17	444					
	54	69	5	14	12						
									11.0+8.6		
Mittelwert		64.5	6.5	14.5	14.5		54.0	7.9	19.6	18.5	

b) *Pathologische Seren.*

M. D.	32	43	11	37	9	440					
	39	50	9	35	6						
								3.3+ 5.6			
Mittelwert		46.5	10	36	7.5		52.1	8.9	30.2	8.8	
H. K.	13	26	6	10+6	52	439					
	40	30	6	6+5	53						
								3.9+ 5.6	12.2		
Mittelwert		28	6	8+5.5	52.5		28.2	9.5		50.1	
O. B.	5	17		19	64	437					
	9	14		23	63						
Mittelwert		15.5		21	63.5		20.4	14.5	9.3	55.8	
M. S.	6	27		18	55	433					
	7	35		14	51			6.2+11.0			
Mittelwert		31		16	53		26.1	17.2	56.7		
E. L.	47	42		25	33	441					
	48	48		20	32						
								5.9+ 9.0	9.6+10.0		
Mittelwert		45		22.5	32.5		36.2	14.9	19.6	29.3	

stimmung. Zunächst ergeben die Mittelwerte der Untersuchungen von 6 Normalseren mit der Papierelektrophorese und freien Elektrophorese folgende relative Zusammensetzung.

	Papierelektrophorese:	Freie Elektrophorese:
Albumin	59,1 %	55,6 %
α-Globulin	8,8 %	7,9 %
β-Globulin	13,8 %	15,9 %
γ-Globulin	18,3 %	20,6 %

Die Zusammenstellung zeigt eine gute Übereinstimmung der Normalserumwerte. Bei pathologischen Seren fällt der Vergleich ebenfalls günstig aus (Tab. 1 b.), wenn man auch hier wegen den geringeren Trennungsmöglichkeiten der Papierelektrophorese das oben erwähnte Summenergebnis einer Zusammenfassung der entsprechenden Komponenten bei der freien Elektrophorese gegenübersetzen muss. Bemerkenswert ist bei dieser Gegenüberstellung von Papierelektrophorese und freier Elektrophorese, dass der Korrekturfaktor 1,6 fü alle Globulinanteile normaler Seren gelten kann und die verschiedenen zum Teil erheblich vermehrten Globuline der hier untersuchten pathologischen Seren im Wesentlichen doch gleich grosse Affinität zum Bromphenolblau haben. In der Tabelle der Normalseren fällt auf, dass von insgesamt 6 Seren 5 in der Papierelektrophorese etwas niederere Globulinwerte als in der freien Elektrophorese haben. Möglicherweise liegt der optimale Multiplikatorwert etwas über 1,6. Eine feinere Korrektur des Faktors auf Grund dieser Versuche ist jedoch nicht angebracht, da die methodische Fehlerbreite von 10 % dies nicht zulässt und weiterhin die bei beiden Methoden verwendeten Puffer nicht gleich waren.

Neben den Serumuntersuchungen wurde auch eine Auftrennung von Citratplasma mit der Papierelektrophorese versucht. Doch fällt hierbei das Fibrinogen schon beim Auftropfen am Startpunkt aus. Während die freie Elektrophorese mit optischer Registrierung zur Untersuchung lichtundurchlässiger Proteinlösungen nicht geeignet ist, bestehen für die Papierelektrophorese zur Untersuchung solcher Lösungen wie stark eigengefärbter Zellextrakte und Körperflüssigkeiten geringere Schwierigkeiten. Durch ihren geringen Proteinbedarf ist sie eine wertvolle Ergänzung zu den bisherigen Möglichkeiten elektrophoretischer Auftrennung. Immerhin ist die Papierelektrophorese in der qualitativen Beurteilung von Eiweisskörpern der freien Elektrophorese noch unterlegen. Für Serumeiweisskörper kann der Parallellauf des pathologischen Serums mit einem Normalserum einige Aussagen gestatten, doch wäre eine Charakterisierung der Eiweisskörper durch die Bestimmung ihrer absoluten Wanderungsgeschwindigkeit unter den Ver-

hältnissen der Papierelektrophorese wünschenswert. Diesbezügliche Versuche sind am hiesigen Institut durch Dr. H. G. Kunkel im Gang.

ZUSAMMENFASSUNG

Die Papierelektrophorese nach Cremer und Tiselius wurde nach Vereinfachung der Elektrodenanordnung an Serumproteinen erprobt. Bei Normalseren trennen sich die vier Hauptkomponenten stets einwandfrei ab, während bei pathologischen Seren die Auftrennung geringer sein kann. Es besteht weitgehende Übereinstimmung zwischen den Ergebnissen der Papierelektrophorese und der freien Elektrophorese. Die Abweichungen betragen 10—20 %. Die Affinität des zur Färbung der Proteinflecke verwendeten Bromphenolblaus zum Albumin ist 1,6 mal grösser als zu den Globulinen der untersuchten Seren.

Die Papierelektrophorese ist eine brauchbare Mikromethode zur Untersuchung klarer wie auch stark getrübter oder eigengefärbter Seren bei einem Gesamtproteinbedarf von 2 mg. Indessen fehlt noch eine brauchbare Methode zur Bestimmung der absoluten Beweglichkeit der Komponenten unter den Verhältnissen der Papierelektrophorese.

Es ist mir eine angenehme Pflicht, Herrn Prof. Arne Tiselius und Dr. Kai O. Pedersen für ihr lebhaftes Interesse an dieser Arbeit und wertvolle Anregungen zu danken. Durch ihr grosszügiges Entgegenkommen war es mir möglich, diese Studien im Rahmen eines Stipendiums des »Schwedischen Institutes für kulturellen Austausch» durchzuführen, dem auch an dieser Stelle aufrichtiger Dank abgestattet werden soll.

Diese Arbeit, vom Frühjahr 1950, wurde im Rahmen eines Arbeitsprogrammes über die Entwicklung von Elektrophoresemethoden ausgeführt, das von dem Schwedischen Naturwissenschaftlichen Forschungsrat finanziell unterstützt wird.

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The Use of Starch Chromatography and Ion Exchange Resin for Large Scale Separations of N^{15}-Labeled Amino Acids

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In the past few years several methods have been worked out for the fractionation of amino acids and other organic compounds by means of the chromatographic technique. Since the first separation of N-acetyl-amino acids on silica gel by Martin and Synge [1], the field has been developed by various other workers.

The introduction of starch as the supporting media was made by Elsden and Synge [2, 3], and its usefulness has been demonstrated in the excellent procedures developed by Moore and Stein for the quantitative estimation of amino acids, both in synthetic mixtures and in protein hydrolysates [4-8]. With this technique it is possible to obtain almost all of the ordinary amino acids by two or three different separations. The original procedure was described and used only on a micro scale, for a few milligrams of protein, but it is also applicable to work on a larger scale, separating hydrolysates of 200—500 mg of protein.

The use of ion exchange resins for the separation of amino acids has also been extensively studied by several workers. Stein and Moore [7] describe the use of Dowex 50 and point out that one of its advantages, as compared with starch, is its greater capacity, which allows the separation of gram amounts of amino acids.

In the first part of the present paper a technique is described for the separation, on Dowex 50 ion exchange resin, of large amounts of N^{15}-labeled amino acids obtained from hydrolysates of biologically marked proteins. These labeled amino acids were used as tracers in later isotope experiments.

The second part presents a method of starch chromatography which has been used by us in these isotope experiments for the isolation of the common amino acids of tissue proteins, in amounts large enough to allow determination of the identity and purity of the different amino acids and their contents of N^{15}.

EXPERIMENTAL AND RESULTS

The separation of N^{15}-labeled amino acids on Dowex 50 ion exchange resin

The sources of N^{15}-labeled amino acids were the proteins formed when yeast (*Torulopsis utilis*), in one experiment, and bacteria (*Escherichia coli*), in another, were allowed to grow on a medium in which the only source of nitrogen was an N^{15}-labeled ammonium salt[9,10]. The excess of N^{15} in the protein from *Torulopsis utilis* was about 17 % and in that of *Escherichia coli* 4.2 %. These biologically marked tissues were also used for the isolation of labeled components of nucleic acids. In the procedure of the experiment, the protein was taken as the residue which remained after extraction of the polynucleotides (Hammarsten[11]) and treatment with hot trichloroacetic acid (Schneider[12]).

The protein was hydrolyzed by boiling in 6 *N* HCl on a hot sandbath for 24 hours. Excess hydrochloric acid was removed by repeated evaporations *in vacuo*; the residue was dissolved in distilled water and made up to a suitable volume.

In order to remove insoluble particles from the hydrolysate the sample was kept at —17 ° C. overnight and then thawed; after this treatment even the smallest particles were easily spun down in a centrifuge. The clear, brown supernatant was decanted; the hydrolysate was then ready for further use.

The first treatment of the hydrolysate was the removal of tyrosine by isoelectric precipitation. The hydrolysate was then divided into neutral, acidic and basic amino acids by means of electrodialysis according to the procedure of Sperber[13]. From the acid fraction glutamic acid was isolated as the hydrochloride and aspartic acid was precipitated as the copper salt. Both the basic fraction and the neutral fraction were further fractionated on Dowex 50 ion exchange resin.

The glass column used had a sintered glass plate at the bottom; it was 8 cm in diameter for the first 40 cm of length and 10 cm. in diameter above this. The ion exchanger which is available in very small particle size (250—500 mesh) was stirred up in 4 *N* HCl, poured into this glass column and allowed to settle. Washing with 4 *N* HCl was continued for a week or until about 10 l of acid had run through. The acid concentration was then changed to 1.5 *N*, which caused swelling of the resin and packed it in more tightly. The column was considered ready for use when the effluent had the same normality as the hydrochloric acid applied to the top. The length of the resin column was now 67 cm. The column was used at a temperature of 18 ° C.

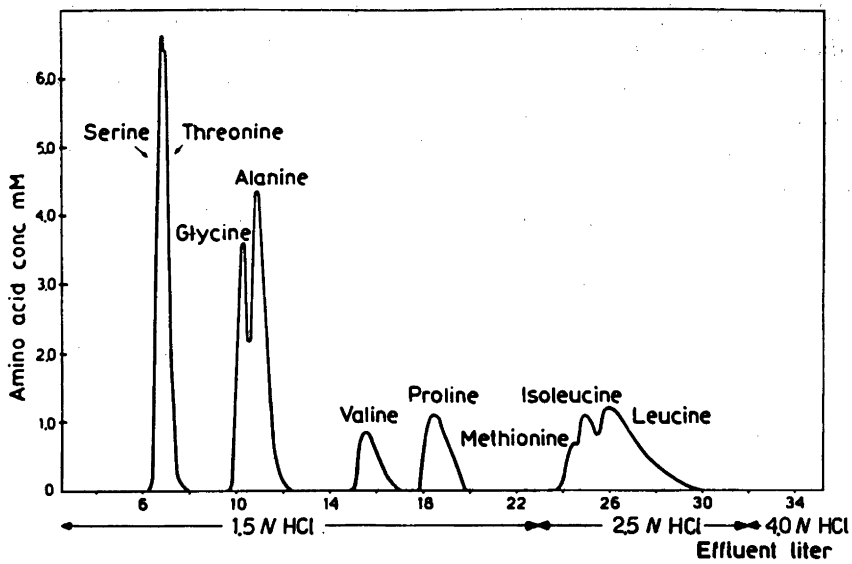

Fig. 1. Separation of neutral amino acids on Dowex 50 ion exchange resin.

The amino acid mixture to be separated was dissolved in 1.5 *N* HCl and pressed into the column with slight air pressure. The walls were rinsed twice with a small amount of hydrochloric acid and the washings likewise pressed in. The column was then connected with a reservoir of 20 l of 1.5 *N* HCl. A slight pressure was applied to maintain a rate of effluent of about 175 ml per hour. Hourly fractions were collected with the aid of a time-controlled fraction collector, specially built at this laboratory for these large volumes. During the separation the hydrochloric acid concentration was first raised to 2.5 *N* and later on to 4 *N* in order to speed up the migration of the more slowly moving amino acids.

To localize the positions of the different amino acids the photometric ninhydrin method of Moore and Stein [14] was applied to 0.05 ml of each fraction. The hydrochloric acid was neutralized with 0.2 ml of sodium hydroxide of a proper concentration, and the color was developed by the addition of 0.5 ml of the ninhydrin solution.

The separation of the neutral amino acids is shown in Fig. 1. In this experiment neutral amino acids equivalent to 1.4 g of nitrogen were applied to the column. It can be seen that the amino acids move out partly or completely separated from each other. The phenylalanine which is not plotted in the curve appeared in the effluent of 4 *N* HCl between 55 and 63 l.

In order to identify the different amino acids and to tell in which fractions two acids overlap each other an aliquot from every fraction was tested on a paper chromatogram and compared with known amino acid standards. A sample of 0.2—0.3 ml was evaporated to dryness on a small glass dish in an oven at 80 ° C. and then a few drops of distilled water was added and the process repeated three times to remove excess hydrochloric acid. The final residue was dissolved in a drop of water and applied to filter paper. These chromatograms were developed in different suitable solvent systems — *s*-collidine-water, *s*-collidine-water with diethylamine in the atmosphere, phenol-water and butanol-benzylalcohol-water (1 : 1 : 0.25). The chromatogram was always heavily overloaded in order to detect even very small traces of another acid.

From this picture of the distribution of the amino acids it was possible to judge which fractions contained a single amino acid, and to select pure fractions for almost all of these neutral amino acids. However, no fraction of methionine could be found which did not contain a trace of isoleucine. No significant amount of cystine could be detected; it is assumed to have been destroyed during the hydrolysis.

Fractions containing the same single amino acid were combined and evaporated to dryness *in vacuo*. The excess hydrochloric acid was removed by repeating the evaporation several times. The amino acids crystallized beautifully from the final residue without further purification. Since they had been isolated as hydrochlorides, the hydrochloric acid was removed with silver carbonate and the amino acids were recrystallized from water. The purity was again checked by means of paper chromatography; all the amino acids were found to be free from other ninhydrin-reacting substances except serine which contained a very slight and previously undetected trace of threonine.

The separation of the basic amino acids on Dowex 50 was similar to that of the neutral acids. The result is shown in Fig. 2. It can be seen that these amino acids separate very well from each other. However, since they emerge in such large volumes of 4 *N* HCl, there is some contamination by impurities from the acid and from the resin. Fractions containing the same amino acid were combined and evaporated as before; the residue was electrodialyzed and the free base isolated at the cathode. No further purification was performed.

With these separations it was possible to isolate the amino acids serine, threonine, glycine, alanine, valine, proline, isoleucine, leucine, phenylalanine, lysine, arginine, histidine and, by other means than Dowex, tyrosine, glutamic acid and aspartic acid. All of these acids were used as N^{15}-labeled tracers in an investigation on interrelationships among different amino acids and between these and the formation of purines and pyrimidines. The isolation of amino

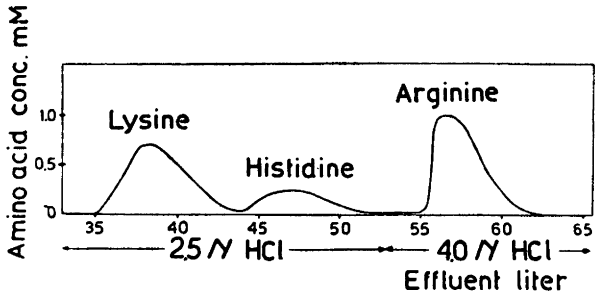

Fig. 2. Separation of basic amino acids on Dowex 50 ion exchange resin.

acids from tissue proteins in this study is described in the following section. Further details and results of these experiments will be presented in other publications.

The separation of N^{15}-labeled amino acids by means of starch chromatography

For this and other investigations it was necessary to isolate the labeled amino acids from different tissue proteins and to determine their content of isotope. For this determination it is desirable to have amounts of each amino acid equivalent to 1—2 mg of amino acid nitrogen, which corresponds to 200—400 mg of protein. For the separation of amino acids on this scale a system of starch cromatography has been employed. This is the same as that used by Moore and Stein but on a scale about 80 times larger.

MATERIALS AND METHODS

Purification of the starch: For these experiments we have used potato starch, either from Morningstar Nicol, Inc.* or a commercial specimen of Swedish potato starch. Before use, the starch was extracted with methyl alcohol in a heated percolator at 55° C for 36 hours. The amount of alcohol necessary for this extraction is about 3—4 l per kg of starch. The material was then dried in a vacuum oven at 55° C for 24 hours.

Purification of the solvent: To commercial samples of n-butyl alcohol and n-propyl alcohol were added 100 ml of concentrated sulfuric acid per 10 l of solvent and the solvent was distilled *in vacuo* in a glass apparatus.

Preparation of the column: The first separation of the protein hydrolysate was performed on a column 8 cm in diameter filled with starch to a height of 30 cm. Subsequent reseparations were made with columns 4 cm in diameter with the same height of starch.

* Purchased from Amend Drug and Chemical Co., New York City.

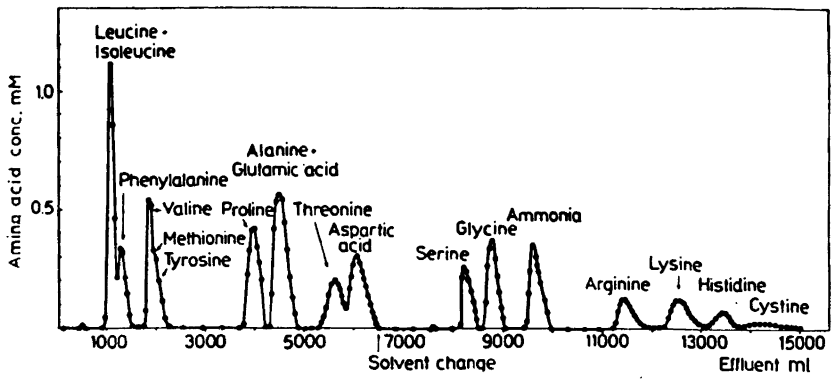

Fig. 3. Separation on starch of liver protein hydrolysate. Column 8 cm in diameter, 30 cm high. Solvents, 1 : 2 : 1 butanol-propanol-0.1 N HCl, followed by 2 :1 propanol-0.5 N HCl.

The extracted starch was stirred up in a mixture of butyl alcohol and water according to the directions of Stein and Moore [5], then poured into the column and allowed to settle under an air pressure of 3—5 cm of mercury. When using starch with larger granules than those of the American sample, it has been found that more uniformly and tightly packed columns could be obtained if the starch was stirred up in butyl alcohol containing half as much water as that originally proposed.

The columns were then washed according to the directions of Moore and Stein [6] to remove impurities which could be subsequently eluted by the developing solvents. When these solvents contained hydrochloric acid, the column was washed for 36 hours with a mixture of 1 : 1 propyl alcohol and 0.5 *N* HCl and then for 24 hours with the developing solvent itself before being used for chromatography. The one column for which no acid solvents were used (butanol-water being the eluting solvent) was treated instead with 8-hydroxy-quinoline.

Hydrolysis of the protein: 250—300 mg of protein were refluxed for 24 hours in a solution of equal parts of concentrated hydrochloric acid and 50 % formic acid. Excess acid was removed by repeated evaporations *in vacuo* and the final residue was made up to a volume of 10 ml. Insoluble particles were centrifuged down and the clear, brown supernatant was decanted. To 9 ml of this solution were added 1 ml of 1 *N* HCl, 20 ml of propyl alcohol and 10 ml of butyl alcohol. The resulting 40-ml mixture was applied to the starch column.

Separation and identification of the amino acids: The chromatograms were developed with different solvent systems, and hourly fractions of the eluate collected in the conventional way. The original separation employed first a solvent mixture of 1 : 2 : 1 butanol—propanol—0.1 *N* HCl, and later a mixture of 2 : 1 propanol—0.5 *N* HCl. Reseparations were made with 0.1 *N* HCl or with the butanol-water mixture as eluting solvent. All chromatograms were run under an air pressure of 7—10 cm of mercury.

The amino acids were localized by colorimetry in the same way as in the Dowex separation. The test was made on 0.2 ml of each fraction. With the 2 : 1 propanol—0.5 *N* HCl and the 0.1 *N* HCl solvents, 0.1 ml of sodium hydroxide of suitable concentration

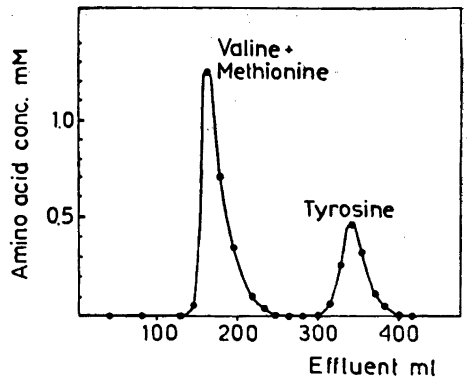

Fig. 4. Reseparation on starch of valine, methionine and tyrosine. Column 4 cm in diameter, 30 cm high. Solvent, 0.1 N HCl.

was added to neutralize the hydrochloric acid, and the color was developed with ninhydrin in the usual way.

The positions of the individual amino acids were verified with standards run separately on identical starch columns. The volume of effluent corresponding to the top of each peak is reproducible, from one run to another, to within a few percent. Throughout the experiment the amino acids isolated were also identified and tested for contaminating amino acids and other ninhydrin-reacting substances by means of paper chromatography. The solvent mixtures used were the same as those mentioned above for the ion exchange procedure.

The result of the first separation is shown in Fig. 3. As mentioned above this separation was begun in a solvent mixture of 1 : 2 : 1 butanol—propanol—0.1 *N* HCl, and continued with a mixture of 2 : 1 propanol—0.5 *N* HCl.

It can be seen that several of the faster-moving amino acids overlap each other. The fraction containing valine, methionine, and tyrosine was reseparated on another column with an eluting medium of 0.1 *N* HCl. Under these conditions tyrosine is retarded and separated from valine and methionine. The separation is illustrated in Fig. 4. The mixed

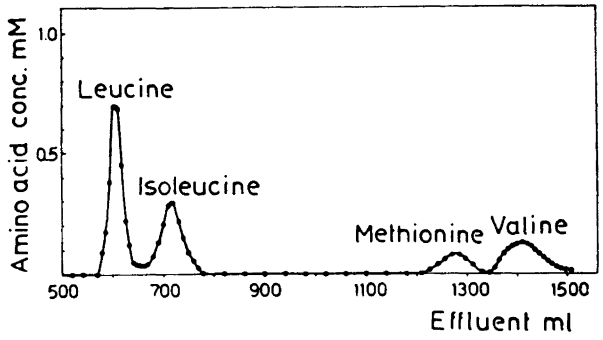

Fig. 5. Reseparation on starch of leucine, isoleucine, methionine and valine. Column 4 cm in diameter, 30 cm high. Solvent, butanol saturated with water.

fraction of leucine and isoleucine from the first separation could be collected free from phenylalanine if some of the fractions in the valley were discarded. (The same was done to obtain pure threonine and aspartic acid.) Even when this was done, sufficient amounts of both fractions could be obtained. The leucine-isoleucine fraction was then added to the valine and methionine from the second chromatogram and the four acids separated on a third column in a butanol-water system as illustrated in Fig. 5. If traces of phenylalanine remained as a contamination of the leucine-isoleucine fraction, they could be detected on the paper chromatogram and removed in the reseparation with butanol-water, where the phenylalanine appears as a peak between leucine and isoleucine.

With these three different separations all the amino acids except alanine and glutamic acid are obtained in essentially pure form. These remaining two were separated by means of electrodialysis on a microscale, according to the directions of Theorell and Åkeson [15].

When the glutamic acid and alanine fraction was tested on a paper chromatogram, the expected amino acids were identified besides four other definite ninhydrin-positive substances. Two of these were present in large amounts and moved very fast in both *s*-collidine and phenol. The other two gave faint spots and had slower travelling rates. None of these materials could be identified with any known amino acid or with any other of various ninhydrin-positive substances tested. It was considered possible that these compounds were formed when the glutamic acid and alanine fractions were evaporated to dryness in the alcohol-acid media before being tested with paper chromatography. The residue left after this evaporation was therefore hydrolyzed in hydrochloric acid, and the resulting solution again tested on a paper chromatogram. In this case only alanine and glutamic acid were found.

In order to isolate these substances, 500 mg of glutamic acid and 500 mg of alanine were refluxed in 500 ml of 1 : 2 : 1 butanol—propanol—0.1 *N* HCl on a hot sandbath for 3 hours. The solution was then evaporated to dryness *in vacuo*, dissolved in 40 ml of the above solvent mixture and applied to a starch column 8 cm in diameter and 30 cm high. Aliquots of the effluent were tested as usual with ninhydrin. The result is shown in Fig. 6.

The first material to emerge was slightly colored and is visible in the curve as a small peak labeled E_0. In addition to this, four different peaks, partly separated from each other, were observed. The two fractions corresponding to the tops of peaks E_1 and E_2 were evaporated to dryness and tested with paper chromatography. They were identical with the two fast-moving components which gave strongly colored spots. Upon hydrolysis both gave rise to no ninhydrin-positive compound except glutamic acid. When glutamic acid was heated in butanol-acid the compound corresponding to peak E_1 was formed and with propanol compound E_2. Acid hydrolysis of the materials from peaks E_3 and E_4 also produced only glutamic acid as amino acid constituent, but no further investigation was made to establish which alcohol takes part in their formation.

Both of the compounds labeled E_1 and E_2 have neutral properties during electrodialysis. The formation of these substances therefore introduces a hydrolysis of the dried glutamic acid-alanine fraction before these amino acids are separated by electrodialysis.

Aspartic acid has been tested in this same way but no substances like those formed from glutamic acid seem to be synthesized.

The only other contaminating ninhydrin-positive material found in these starch chromatograms in any significant amount was an unknown substance in exactly the same region as threonine. Its travelling rate on a paper chromatogram is about the same as that of γ-aminobutyric acid, but the two substances are not the same. All attempts to identify this material with known reference standards failed.

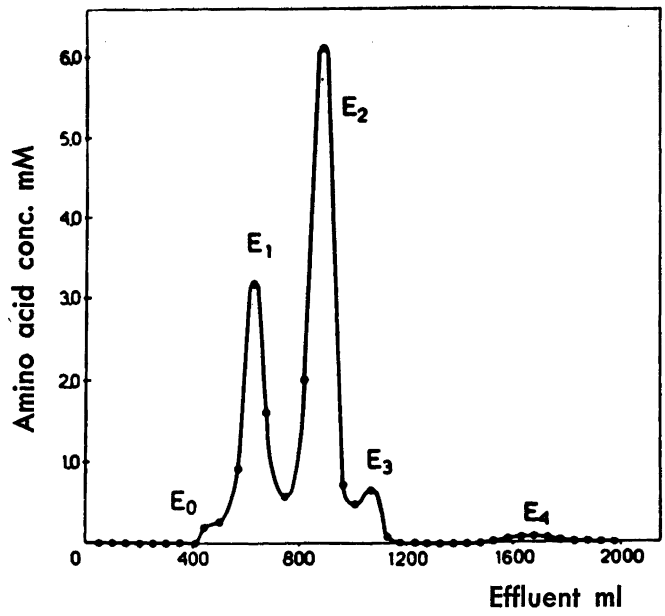

Fig. 6. Beginning of separation on starch of compounds formed by heating glutamic acid and alanine with 1 : 2 : 1 butanol-propanol-0.1 N HCl. Column 8 cm in diameter, 30 cm high. Solvent, 1 : 2 : 1 butanol-propanol-0.1 N HCl.

The substance was isolated free from threonine by a reseparation on Dowex 50. It is strongly retained on this resin and was eluted with the aid of 2.5 *N* HCl. The threonine, which moves fast (see Fig. 1), is well ahead of the unknown compound, which has a travelling rate about like that of arginine.

The substance was found in the neutral fraction when electrodialyzed. It was only partly affected by hydrolysis for 24 hours in boiling 6 *N* HCl but was completely decomposed when heated in 6 *N* HCl in a sealed glass tube at 140° C for 48 hours. The hydrolysis products were separated on a starch column and identified as almost equimolar amounts of lysine and ammonia. The origin of this compound was established in an isotope experiment in which lysine labeled with N^{15} was injected into rats. From the liver proteins both the lysine and the unknown compound were collected. The latter was hydrolyzed as above and lysine and ammonia separated. Isotope determinations on these substances showed an excess of N^{15} in the lysine from protein which was exactly the same as that found in lysine isolated from the unknown compound. On the other hand, although the ammonia from protein contained a significant amount of N^{15}, that from the unknown compound had none.

This indicated that both lysines were derived from the same source; the lysine in the unknown compound must have been that liberated in the hydrolysis of the protein, for both had the same excess of N^{15}, and it is very unlikely that all lysine residues in the protein molecule have the same content of isotope. However, the ammonia in the unknown compound apparently comes from some external source, and it was concluded

that the compound itself was probably formed artificially during the treatment of the protein. When the polynucleotides are extracted from these tissues prior to the analysis of the protein, the materials are in one step treated with hot concentrated urea solution. A control experiment was performed in which a sample of lysine was heated with urea and the products separated on starch. Besides urea and lysine a considerable amount of the unknown compound was found. The urea necessary for the combination with lysine probably becomes attached to the protein in some way during this treatment with urea in the extraction of nucleotides.

Isotope determination and correction for foreign nitrogen: The total amount of nitrogen in each amino acid was determined on an aliquot of the sample by a semi-micro Kjeldahl procedure. The neutralized distillate obtained after titration in this determination was evaporated to a volume of about 1 ml, and the sample was then used for isotope determination. Nitrogen was liberated from the ammonium salt with the aid of alkaline hypobromite, according to the technique of Springson and Rittenberg [16], and the excess of N^{15} estimated in either a 120° or 180° mass spectrometer.

Since each amino acid emerges in rather large volumes of solvent, a significant amount of non-amino nitrogen is derived from the solvent and the starch and is present in each sample. Some ammonia is also picked up by the acid solvents during the separation and the further treatment of the fractions. This foreign nitrogen will introduce a dilution of the isotope present in the amino acid nitrogen and a correction must be made for it. Our procedure for this has been as follows.

The actual amino acid nitrogen present in each case was calculated from the amount of α-amino nitrogen, which was determined on another aliquot of the amino acid sample by means of the Van Slyke procedure, as modified by Kendrick and Hanke [17]. Since about 25 % of the total nitrogen in ammonia is also set free in the Van Slyke estimation, ammonia must be removed prior to this analysis. This was done by distillation *in vacuo* in the presence of a slight excess of alkali. The ratio of the total amount of nitrogen found by the Kjeldahl procedure to the amino acid nitrogen calculated from the Van Slyke determination was taken as a measure of the dilution of the isotopic nitrogen and used as a correction factor. This factor varied from 1.05 to 1.25 for different amino acids and was estimated for each one separately.

In order to determine whether or not the foreign nitrogen contained any isotope, each of the amino acids was put through a Dowex 50 column with hydrochloric acid as the eluting solvent. Almost all of the foreign nitrogen was separated from the amino acid by this procedure and the factor determined by the same technique for the purified amino acids was much lower, ranging from 1.00 to 1.05. When a sample was tested for content of N^{15} both before and after this final purification on Dowex 50 and the isotope excess found in each case corrected by the respective factor, the two values for excess of N^{15} always agreed to within 1 %, indicating that the foreign nitrogen did not contain any appreciable amount of isotope.

The amino acid nitrogen from proline can not be determined by the Van Slyke reaction and isotope figures for this amino acid are therefore uncorrected for foreign nitrogen.

DISCUSSION

In general the procedures used in these separations of amino acids have been similar to those previously employed by Stein and Moore and already

discussed in their original publications. However, it has seemed desirable to modify their techniques in certain respects for this preparative work on a larger scale.

In the separation on Dowex 50 ion exchange resin, one of the chief modifications has been the separation of the protein hydrolysate into acid, neutral and basic amino acids before the fractionation on the ion exchange column. This was done for several reasons. If all the amino acids are put through the column together, aspartic acid, although well separated from glutamic acid, extensively overlaps the neutral amino acids serine and threonine. In addition phenylalanine, the neutral amino acid most strongly retained by the resin, appears as a flat peak in exactly the same place as the basic amino acid, arginine. Also, if electrodialysis is introduced, the original amide nitrogen, which has been converted to ammonia, is aerated away during this procedure, so that the amino acid valine emerges without any contamination of ammonia, which otherwise would appear close in front of it in the eluate. Thus, although the electrodialysis introduces an additional procedure, it enables one to obtain much cleaner separations of several of the amino acids and seemed to be a necessary addition for a fractionation of the amounts [3] of protein hydrolysates we have worked with here.

The amounts of amino acids separated on Dowex in our experiments are approximately 100 to 300 times larger than those of the Stein and Moore separation, and our columns, therefore, have probably been heavily overloaded. This may partly explain our less successful separation of methionine, isoleucine and leucine. However, another explanation may be the fact that this separation has been carried out at a somewhat lower temperature (18° C.) than the other, which was run at 25° C. The temperature effect has been discussed in detail by Moore and Stein [7] and by Partridge and Brimley [18]. However, except for this fractionation, the separation appears to be quite satisfactory, even on this large scale.

Our starch separation has also been carried out on a larger scale than that of Stein and Moore, but this has been accomplished by the use of larger columns, so that the proportions of starch used are approximately the same. The resolving power of these large starch columns is as good as that of the smaller columns described in the original method. The amounts of amino acids should not exceed 500 mg for an 8-cm column if clean-cut separation is to be obtained for the amino acids which emerge close together. The amounts which can easily be accomodated in the reseparations are comparatively larger, and the amounts of leucine, isoleucine, valine, methionine and tyrosine obtained from the separation on an 8-cm column can be cleanly reseparated on a column of half this diameter.

Before the starch columns can be used for the separation of amino acids they must be freed of organic impurities and metal ions. This is performed by allowing alcohol-acid solvents to pass through the column for 36 hours. This washing is enough to extract from the starch the metal ions and the soluble nitrogenous impurities, but there still remain appreciable amounts of fatty material which are later eluted into the amino acid fractions and can be seen as a fatty residue after the sample has been evaporated down to dryness. Washing the column for a longer time with alcohol-acid lowers the resolving power of the column, and it has therefore been found preferable to remove this fatty material by extracting the starch prior to its use for columns with a better fat-extracting solvent, namely methyl alcohol.

Although these washings remove most of the impurities from the starch, there are also impurities in the solvents themselves, so that amino acid fractions isolated from the starch separation contain appreciable amounts of extraneous nitrogen, as shown by the differences between total nitrogen and amino nitrogen determinations. Although it has been demonstrated that this foreign nitrogen does not contain any significant amount of N^{15}, it is undesirable from several standpoints to have to deal with such high correction factors In more recent work, therefore, the reseparation of each amino acid on Dowex 50 has been introduced as a routine procedure. The removal of ammonia prior to the Van Slyke estimation can then be eliminated, since the Dowex columns can be made of proper length to give a satisfactory separation of ammonia from each amino acid.

The solvent system suggested by Stein and Moore [5] for the separation of the first six amino acids — leucine, isoleucine, phenylalanine, methionine, tyrosine and valine — was found to be somewhat unsatisfactory for a separation on this larger scale, in that the separations were not reproducible from one run to another. In addition it was very difficult to free the benzyl alcohol which was available from nitrogenous impurities, and it was extremely time-consuming to redistill the necessary large volumes of this alcohol because of its high boiling point. We have, therefore, used instead a solvent system of acid-propanol-butanol, even though this introduces the necessity of reseparating five of these amino acids.

The reseparation of valine, methionine and tyrosine has been simplified in later work in this laboratory by the use of Dowex 50 instead of starch. Hydrochloric acid is used as eluting solvent and all three amino acids are isolated free from each other in a single run. In a similar manner it is easier to reseparate alanine and glutamic acid on Dowex 50 rather than by use of electrodialysis. This modification is also currently in use in our laboratory. Still another application of ion exchange separation has been in the isolation of

threonine from aspartic acid. If the overlapping of these two amino acids is extensive enough to cause large losses of material, it is better to reseparate them on the anion exchange resin, Dowex A 1. We have found that such combinations of starch chromatography with ion exchange separations are frequently the simplest and most effective way to separate amino acids on this scale.

Throughout these experiments the identification of the different amino acids obtained from both the ion exchange and the starch separations has been made by means of paper chromatography. Nearly all of the solvent systems used for this technique are well known from previous work. The addition of diethylamine to the atmosphere of the *s*-collidine-water system improved the separation between phenylalanine and the two leucines isolated from starch. The mixture of butanol-benzylalcohol-water was used for the identification of the mixed fraction of leucine, isoleucine and methionine from Dowex 50.

Calculations of the quantities of amino acids separated from the proteins have been made both from the light absorption of the color developed with ninhydrin (for an aliquot of each fraction) and from the amino nitrogen determined by Van Slyke on the isolated amino acids. The calculations from the ninhydrin figures were made according to the method of Moore and Stein[14]. A calculation of recovery of nitrogen can be made from these values, although it is only approximate because of error due to overlapping of amino acids in some fractions. By both methods such a calculation indicates the total nitrogen recovered to be 85—95 % of that present in the original hydrolysate. When a synthetic mixture of amino acids has been analyzed the nitrogen has been recovered quantitatively. It appears therefore that there may be some nitrogen present in the trichloracetic-acid-precipitated protein which is not amino acid nitrogen, although the error in the calculations makes exact figures questionable.

The form in which this nitrogen exists is unknown, for this figure for recovery of nitrogen is based not just on amino acids isolated but on all nitrogen-containing materials that we have been able to obtain from the hydrolysate. Thus during the hydrolysis of the protein a dark brown, acid-insoluble, solid material is formed and centrifuged off from the hydrolysate. This material is named acid-insoluble humin. The brown supernatant, when applied to the column, gives rise not only to individual amino acids, but also to a brownish-colored constituent which moves very fast, almost at the solvent front. This is termed acid-soluble humin. These two substances, formed during the acid hydrolysis, contain decomposition products of certain of the amino acids and of carbohydrates. The nitrogen contents of both of these were always

determined and taken into consideration in the calculation of the recovery figure. Both were also tested for content of N^{15}, but no attempt was made to fractionate these humins or to identify their constituents.

Although cystine should be among the neutral amino acids isolated by these techniques, we have never found appreciable quantities of this amino acid in eluates from either type of column. The amounts of methionine found have also been low, actually too low to allow an accurate determination of amino nitrogen or of content of N^{15}. This is thought to be due to partial destruction of cystine during hydrolysis and to a very low content of both of these sulfur amino acids in the liver protein analyzed. Since the use of acid as hydrolyzing agent also destroys all of the tryptophane, it is not surprising that no trace of this amino acid has ever been found. These disadvantages of acid hydrolysis of proteins are well known, but, on the other hand, no method is completely satisfactory, and acid hydrolysis has an advantage in simplicity. It has been considered quite adequate for our purposes in the present experiments.

From the present results it seems highly probable that the four ninhydrin-positive substances found in the glutamic acid-alanine region on the starch chromatograms are different esters of butyl and propyl alcohols with glutamic acid. The two present in greatest quantities, corresponding to peaks E_1 and E_2 in Fig. 6, are most likely the monoesters, since they have neutral properties in electrodialysis. Other kinds of esters of glutamic acid with these alcohols can probably explain the formation of E_3 and E_4. It is quite possible that these substances are the same as those observed by Borsook [19].

In the Dowex separation of amino acids from the protein of *Escherichia coli* two small peaks were observed ahead of the first amino acid, aspartic acid. The constituents of these fractions were both crystalline substances which gave a slight reaction with ninhydrin; their identity has not yet been established. These unknown substances have also been observed by Ehrensvärd [20] in a similar analysis of hydrolysates of *Escherichia coli* protein.

SUMMARY

Methods previously described by Stein and Moore for the separation of amino acids have been satisfactorily applied in the present experiments to preparative work on a larger scale. All of the common amino acids have been isolated from acid hydrolysates of 25—50 grams of protein by means of the cation exchange resin Dowex 50 with hydrochloric acid as eluting solvent. Starch chromatography has been employed to separate the same amino acids from hydrolysates of 250—300 milligrams of protein. Both techniques have

been used to isolate samples of N^{15}-labeled amino acids, and have been found quite satisfactory for the isolation of materials with the degree of purity required for isotope work. The identity of the amino acids isolated has been proved by means of paper chromatography. The existence of several other unknown substances has been revealed by these separations, and the identity of these substances is discussed.

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Metabolic Interrelationships among Amino Acids Studied with Isotopic Nitrogen

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Isotope investigations in this laboratory concerning the formation of proteins and nucleic acid bases in rat liver have been performed with N^{15}-labeled glycine. Experiments were also carried out to test the relationship of other N^{15}-labeled amino acids to purine and pyrimidine synthesis, thus allowing, at the same time, possibilities for a study of interrelationships between the different amino acids involved in protein synthesis. The present paper deals with these different relationships among amino acids, as far as can be judged from a study in which the nitrogen of the amino acids has been labeled with N^{15}. The utilization of amino acid N^{15} for the formation of nucleic acid bases will form the subject of another publication [1].

EXPERIMENTAL

The N^{15}-labeled amino acids used in this investigation were isolated from biologically marked protein by the use of Dowex 50, according to the technique described by Åqvist [2]. The amino acids were administered to adult albino rats fed on a diet of bread, milk, oats and carrots. The animals were allowed to eat *ad libitum* throughout the experiment. In most of the experiments two 200-gram rats were used for each amino acid tested, and the amount of amino acid administered was on the order of 100 mg per 100 g rat weight. The exact amount of each amino acid, together with its content of isotope, is presented with the table in each case. The amino acid was dissolved in 4 – 16 ml of water, depending on the solubility of the substance in question. This volume was divided into four doses, which were injected subcutaneously at two-hour intervals. Two hours after the last injection the animals were killed by a blow on the head and bled. The total period of exposure to isotope was therefore eight hours.

The livers were taken out and blotted to remove blood. They were cut into small pieces into ethyl alcohol, and then ground in a Turmix blendor and dried with alcohol and ether. The dried tissue was fragmented in absolute alcohol by means of mechanical vibration, as described by Hammarsten [3]. The remaining lipid material was extracted by

boiling with 3 : 1 alcohol-ether for two hours and the preparation was then dried again. Nucleic acids were isolated by the method of Hammarsten [3] and the residue was twice treated with hot trichloroacetic acid solution, according to Schneider [4], to insure complete removal of nucleotides. The total remaining material was taken for the liver protein and used for the studies reported in this paper. This liver protein was dried and hydrolyzed, and the individual amino acids isolated by the use of starch chromatography, according to Åqvist [2]. The incorporation of isotope from the injected amino acid into each amino acid of the total liver protein was determined in each experiment by estimating the content of N^{15} by means of a mass spectrometer.

RESULTS AND DISCUSSION

Glutamic acid

N^{15}-labeled glutamic acid was injected in the form of the neutralized hydrochloride. The amount of amino acid injected and its content of N^{15} are indicated with Table 1. The isotope values found in each amino acid isolated from the liver protein are shown in this table, where the figures for excess of N^{15} are expressed as calculated on the basis of 100 % N^{15} in the injected compound. The amino acids isolated are listed in the table in the order in which they emerge from the starch column. The figure for threonine includes that for a lysine compound. The existence of this compound in the threonine fraction has been demonstrated and discussed in previous work [2].

As might be expected, the highest content of N^{15} in the amino acids isolated is found in glutamic acid itself, but the isotope has also been distributed, in somewhat lesser amounts, to almost all the other compounds listed. Of the other amino acids, the ones showing greatest incorporation of N^{15} from injected glutamic acid are alanine and then aspartic acid. The leucines, serine and arginine are next, and the excesses of N^{15} in the remaining compounds are

Table 1. Incorporation of N^{15} from glutamic acid into liver protein amino acids. Two 200-gram rats, 350 mg glutamic acid each, 17.2 % excess N^{15}.

Insoluble humin	Soluble humin	Leuc. + isoleuc.	Leucine	Isoleucine	Phenylalanine	Val., meth., tyr.	Valine + meth.	Valine	Methionine	Tyrosine	Proline	Alanine	Glutamic acid	Threonine	Aspartic acid	Serine	Glycine	Ammonia	Arginine	Lysine	Histidine
.26*	.24	.75	.58		.26		.45	.38	.91	.37	.23	1.39	1.88	.05	.94	.86	.36	.43	.63	.08	.04

* All figures in Tables 1—9 are given as atom per cent excess N^{15} calculated on the basis of 100 % N^{15} in the administered compound.

of a somewhat lower order of magnitude. The amino acids threonine, lysine and histidine show no significant incorporation of N^{15}. The amounts of methionine and cystine were too low to allow any accurate determination of their content of N^{15}. The values for these amino acids have, therefore, been omitted. The isotope contents of the two humin fractions are presented, since the nitrogen in these fractions is derived from the breakdown of certain amino acids during the acid hydrolysis.

It is, of course, an arbitrary choice to decide what excess of N^{15} should be accepted as significant. In this paper absolute values below 0.01 % excess N^{15} are not regarded as significant. It must be kept in mind that the figures presented in the tables are expressed on the basis of 100 % N^{15} in the administered compound and are, therefore, the absolute values multiplied by a factor, which is of different size for the various amino acids. The size of this factor must, therefore, be remembered when considering the significance of the excesses of N^{15} recorded in the tables, and also the significance of the differences between values.

Ammonia

Table 2 shows the figures for the isotope contents of the different amino acids isolated from liver protein after the injection of ammonium citrate labeled with N^{15}. The figures are calculated in the same way as in the glutamic acid experiment.

It can be seen that the utilization of N^{15} from the injected ammonium salt is somewhat less than from glutamic acid, but the general pattern of distribution shows similarities. The highest excess of N^{15} is found in ammonia itself. Next to this is glutamic acid and then alanine and aspartic acid. The figures for the other amino acids are all lower, except for arginine, and it is hard to evaluate differences in these figures. The ratios between the excesses for the different amino acids are even more interesting than the direct values. For

Table 2. Incorporation of N^{15} from ammonium salt into liver protein amino acids. Four 250-gram rats, 130 mg ammonium citrate each, 32.1 % excess N^{15}.

Insoluble humin	Soluble humin	Leuc. + isoleuc.	Leucine	Isoleucine	Phenylalanine	Val., meth., tyr.	Val. + meth.	Valine	Methionine	Tyrosine	Proline	Alanine	Glutamic acid	Threonine	Aspartic acid	Serine	Glycine	Ammonia	Arginine	Lysine	Histidine
.08	.09	.10		.09	.06		.10			.17	.04	.60	.71	.02	.46	.09	.11	1.05	.47	.01	.02

example, from both injected glutamic acid and ammonium salt the incorporation of N^{15} into alanine is about 80 % of that into glutamic acid, and the figure for aspartic acid about 50—60 % of the glutamic acid value.

These similarities in ratios and in pattern of distribution of N^{15} seemed significant, especially when it became apparent, as shown below, that these similarities also appeared in the incorporation of N^{15} from other injected amino acids. Next to the injected amino acid, glutamic acid has always been found to have the greatest excess of N^{15} and the excesses in the other amino acids have usually shown the same general ratio to glutamic acid in each experiment. This suggested the idea that amino acid interconversions, at least with respect to nitrogen, might follow a common pathway under our experimental conditions, and that glutamic acid might occupy a central position in this metabolism. To clarify these relationships, the figures for excesses of N^{15} in the various amino acids were recalculated for each experiment on the basis of the excess in glutamic acid as 1.00. These recalculated values are shown in Table 10. The similarity, in the different experiments, in the ratios between the figures for the various amino acids can be clearly seen from this table. Since the amounts of labeled amino acids available were insufficient to allow the use of large enough numbers of rats to eliminate biological variations, direct figures could not be comparable from one experiment to another, and no attempt was made, therefore, to administer the same molar amount of each amino acid. Recalculating the figures on the same basis, as is done for Table 10, makes it possible to compare the figures from different experiments, even though the amounts of injected amino acid were unequal and the extents to which the amino acids were utilized in metabolism were different.

From Table 10, the hypothesis of a common pathway of amino acid metabolism appears to be justified by the similarity in the ratios between the figures. The few cases in which the values are significantly higher than expected from the general pattern can be explained by direct interconversions between the injected amino acids and these specific compounds, usually interconversions which are already well-known. The similarity of ratios from one experiment to another would, of course, be apparent no matter what amino acid was used as a basis of calculation. However, the predominant incorporation of N^{15} into glutamic acid from all amino acids seems to justify using this amino acid as a basis of calculation and postulating a central position for it in the metabolism. Thus the hypothesis suggests itself that in metabolism the amino acids distribute their nitrogen to other amino acids by way of glutamic acid, except for certain direct conversions.

Even injected ammonia may distribute its nitrogen by first forming the α-amino group of glutamic acid, as indicated by the similarity in the two

experiments in the ratios between glutamic acid and the other amino acids. This does not explain the high N^{15} content in the ammonia isolated from liver protein, but this could be attributed to direct contribution of injected ammonia to the amide nitrogen of dicarboxylic amino acids, which is the source of most of the ammonia from protein. The high figure for N^{15} in arginine from injected ammonia may well be due to the contribution of the urea moiety of the arginine molecule.

Aspartic acid

The solution of aspartic acid was neutralized by the addition of sodium bicarbonate and injected in the usual manner. Table 3 shows the figures for the incorporation of N^{15} from the administered compound into the other amino acids.

Table 3. Incorporation of N^{15} from aspartic acid into liver protein amino acids. Two 200-gram rats, 350 mg aspartic acid each, 8.1 % excess N^{15}.

Insoluble humin	Soluble humin	Leuc. + isoleuc.	Leucine	Isoleucine	Phenylalanine	Val., meth., tyr.	Val. + meth.	Valine	Methionine	Tyrosine	Proline	Alanine	Glutamic acid	Threonine	Aspartic acid	Serine	Glycine	Ammonia	Arginine	Lysine	Histidine
.49		.83	.66	1.51	.36		.52		.18	.51	.39	1.70	2.54	.03	1.36	.55	.52	.79	.82	.21	.34

The most striking thing about the figures in this experiment is that the highest excess of N^{15} is not found in the injected amino acid, but in glutamic acid; both glutamic acid and alanine have higher values than does aspartic acid itself. The ratios between glutamic acid and these other two amino acids are almost the same as in the first two experiments with glutamic acid and ammonia, and in fact follow the general pattern illustrated by Table 10. It would seem that the metabolic activity of aspartic acid is a rapid and complete equilibration in terms of the common pattern of nitrogen distribution.

The other amino acids also bear the same general relation to glutamic acid that is indicated in Table 10. The excess of N^{15} in isoleucine seems high, but it is hard to know whether or not the difference from the general pattern is significant.

A higher excess of N^{15} in glutamic acid than in aspartic acid, from administered aspartic acid, has also been observed by Wu and Rittenberg [5]. However, their suggestion that the metabolic behavior of aspartic acid is comparable

to that of ammonia does not seem to be supported by the present figures. In the experiment with injected ammonia, the figure for amide nitrogen was found to be higher than both those for glutamic acid and aspartic acid, but in the aspartic acid experiment the N^{15} content of the amide nitrogen is lower than either the glutamic acid or the aspartic acid figure, indicating that the path of aspartic acid metabolism can not be only by way of ammonia.

Alanine

The figures for N^{15} in the amino acids isolated after injection of labeled alanine are shown in Table 4. Both this table and Table 10 show that the

Table 4. Incorporation of N^{15} from alanine into liver protein amino acids. Two 200-gram rats, 200 mg alanine each, 17.0 % excess N^{15}.

Insoluble humin	Soluble humin	Leuc. + isoleuc.	Leucine	Isoleucine	Phenylalanine	Val., meth., tyr.	Val. + meth.	Valine	Methionine	Tyrosine	Proline	Alanine	Glutamic acid	Threonine	Aspartic acid	Serine	Glycine	Ammonia	Arginine	Lysine	Histidine
.32	.53		1.07	1.10	.24	.90	.96	.80		.27	.43	2.76	2.13	.01	1.21	.63	.58	.73	.92	.12	.09

distribution of N^{15} from labeled alanine follows the same general pattern already pointed out. As in the other experiments (except that with aspartic acid) the highest excess of N^{15} is found in the injected amino acid itself. However, the figure for the excess in alanine is not much above the usual level for this amino acid in the general pattern, indicating a rather rapid and extensive activity of alanine in nitrogen metabolism. The ratios between the figures for the other amino acids and that for glutamic acid are approximately the same as in the other experiments. The figures for both leucines and for valine are somewhat higher than usual, but perhaps not enough higher to indicate direct conversions.

Proline

As shown in Tables 5 and 10, injected N^{15}-marked proline has distributed its nitrogen to the other amino acids in approximately the same proportion and to about the same extent as have the other administered compounds. Stetten and Schoenheimer [6] have shown that proline forms glutamic acid directly, by opening of the ring. It was of interest, therefore, to observe

Table 5. Incorporation of N^{15} from proline into liver protein amino acids. Two 200-gram rats, 200 mg proline each, 16.7 % excess N^{15}.

Insoluble humin	Soluble humin	Leuc. + isoleuc.	Leucine	Isoleucine	Phenylalanine	Val., meth., tyr.	Val. + meth.	Valine	Methionine	Tyrosine	Proline	Alanine	Glutamic acid	Threonine	Aspartic acid	Serine	Glycine	Ammonia	Arginine	Lysine	Histidine
27	.31		.36	.40	.12	.40	.46	.38		.19	8.09	1.47	1.89	.02	1.15	.37	.24	.45	.95	.13	.05

whether or not the relative level of N^{15} in glutamic acid was higher than in other experiments where such direct transformations are not known to occur. It can be seen that the ratios between glutamic acid and the other amino acids are not altered in this experiment, but agree with the general pattern. This supports the hypothesis that the level of N^{15} in the other amino acids is dependent on that in glutamic acid and that in all experiments the transfer of nitrogen proceeds from the injected amino acid to glutamic acid and from glutamic acid to the other amino acids.

The actual level of N^{15} in glutamic acid from proline is high but not above that in certain other experiments; the transfer of nitrogen from proline to glutamic acid by this direct conversion does not appear to be any more extensive than transfer of nitrogen by deamination from these other amino acids. It is probable, however, that the transfer which does occur here proceeds in this direct fashion, since proline might be expected to be relatively resistant to deamination due to its ring structure. In connection with this, the rather low level of N^{15} in proline in the general pattern indicates a low extent of incorporation of nitrogen into this amino acid, which is also in line with its stable molecular structure. The very high excess of N^{15} remaining in proline in this experiment probably also reflects a limited nitrogen exchange; there may well be a decreased transfer of nitrogen to compounds other than glutamic acid (and the other amino acids) and at the same time there would be a limited uptake of nitrogen to form new proline and therefore less dilution of the isotopic content of the injected acid.

Threonine, serine and glycine

These three amino acids were injected in the usual manner, in the amounts indicated with Table 6. The procedures were the same as in the other experiments, except in the case of glycine. The glycine experiment was part of another investigation, of which the results are reported elsewhere [7,8]. In this

Table 6. Incorporation of N^{15} from threonine, serine and glycine into liver protein amino acids. One 200-gram rat, 230 mg threonine, 17.0 % excess N^{15}. Two 200-gram rats, 200 mg serine each, 17.2 % excess N^{15}. Ten 200-gram rats, 200 mg glycine each, 31.0 % excess N^{15}.

Administered compound	Insoluble humin	Soluble humin	Leuc. + isoleuc.	Leucine	Isoleucine	Phenylalanine	Val., meth., tyr.	Val. + meth.	Valine	Methionine	Tyrosine	Proline	Alanine	Glutamic acid	Threonine	Aspartic acid	Serine	Glycine	Ammonia	Arginine	Lysine	Histidine
Threonine	.27	.30		.04	.12	.09	.17	.19	.24		.04	.08	.30	.34	5.76	.28	1.15	.80	.39	.27	.08	.02
Serine	.32	.19		.16	.13	.12		.17	.11		.31	.11	.62	.71	.74	.47	5.23	2.62	.70	.45	.04	.03
Glycine	.46	.10	.12			.13	.10				.06	.73	.85	.00	.55	3.98	4.53	.71	.52	.01	.07	

experiment the amino acid was administered to a larger group of rats, and from the pooled livers, cell nuclei were separated from cytoplasm. Details of this procedure have been described previously [9]. The liver protein used for the isolation of the labeled amino acids recorded in Table 6 was that from the cytoplasm only. The figures are therefore not really comparable to those in the other amino acid exepriments in this paper, but the cytoplasm protein is probably fairly representative of the whole cell protein.

It can be seen from this table that in all three experiments by far the highest excess of N^{15} is found in the injected amino acid itself. All three have contributed N^{15} to the other amino acids, although in the case of threonine the extent of nitrogen transfer is quite low. All these amino acids show the usual pattern when recalculated over glutamic acid, as can be seen from Table 10. This is true even for glycine, although the protein in this experiment is not exactly the same as in the other experiments. The fact that the ratios (Table 10) in the threonine experiment all seem to be somewhat high may be due to a slightly low figure for glutamic acid, since a rather small error could produce such variations.

It is apparent that the values for serine and glycine are high in all three experiments, much higher than could be expected from the common metabolic pattern over glutamic acid. After the injection of labeled glycine, the serine isolated from the protein contains 88 % as much N^{15} as does the isolated glycine itself. Administration of labeled serine produces glycine with an N^{15} content 50 % as high as that in the isolated serine. These results indicate a direct interconversion of glycine and serine, with the equilibrium favoring serine. Both of these conversions have been previously demonstrated in experiments both with carbon isotopes and with N^{15} [10–14].

In the experiment with marked threonine a high level of N^{15} is found in both serine and glycine, with more in the former. Unfortunately, this experiment is complicated by a slight contamination of the injected threonine with serine. The labeled amino acids injected in all these experiments were isolated by a separation on Dowex 50 ion exchange resin, from which threonine and serine emerge only partly separated from each other. Presumably pure fractions were selected, as indicated by paper chromatography, but in this case slight contamination was detected in a later paper chromatogram [2]. Some highly-labeled serine must, therefore, have been injected together with the threonine, and the high figures for isolated serine must be due partly to this. It is thus rather difficult to draw definite conclusions about the contribution of threonine to either glycine or serine, but certain indications are apparent. If glycine were formed only from serine here, one would expect a glycine to serine ratio of about 0.5, based on the experiment with injected serine. The fact that the glycine to serine ratio is higher than this makes it appear probable that the formation of glycine does not proceed only from injected contaminating serine and that some of the glycine, and in turn serine, is formed by a path which proceeds from threonine to glycine. Such a conversion of threonine to glycine has been reported by Meltzer and Springson [15]. The experiment with injected glycine shows no transfer of N^{15} to threonine, indicating that such a reaction is not reversible.

It can be seen that throughout the experiments in this paper threonine has received no N^{15} from any of the amino acids administered. Except for the experiment with injected threonine itself, the only experiment which shows an appreciable concentration of N^{15} in threonine is the one with administered serine. It does not seem probable that this is due to a conversion of serine to threonine, for in other experiments in which serine is highly labeled, as from injected glycine, there is no significant incorporation of N^{15} into threonine. It is more likely that the explanation is a contamination of the injected serine with highly-labeled threonine, as in the reverse case, although no contamination of serine was detected by paper chromatography at any stage. If the figures found from injected threonine itself can be used as reference, the contamination of threonine in injected serine must have been on the order of 10 %. This extent of contamination seems surprisingly high, even in view of the fact that threonine gives a weak ninhydrin reaction on filter paper, but a direct conversion of serine to threonine seems even less likely.

It can be seen in the tables that the ammonia value is slightly higher than usual in all three experiments. This is probably due to a partial destruction of the serine and threonine during hydrolysis, which has been demonstrated by Rees [16]. This destruction may result in the formation of labeled ammonia,

which would be found in the ammonia isolated, but the decomposition products may also contribute to some extent to the two humins.

In the experiment with injected threonine, the lysine compound found in the isolated threonine fraction was separated from the threonine on Dowex 50. The figure for N^{15} excess in this fraction, therefore, represents undiluted threonine.

Leucine, isoleucine and valine

Table 7 shows the N^{15} contents in the liver protein amino acids after the administration of labeled leucine, isoleucine and valine. Included in Table 10 are the figures recalculated on the basis of the excess in glutamic acid as 1.00.

Table 7. Incorporation of N^{15} from leucine, isoleucine and valine into liver protein amino acids. Two 200-gram rats, 200 mg leucine each, 17.1 % excess N^{15}. One 200-gram rat, 215 mg isoleucine, 17.4 % excess N^{15}. Two 200-gram rats, 200 mg valine each, 17.5 % excess N^{15}.

Administered compound	Insoluble humin	Soluble humin	Leuc. + isoleuc.	Leucine	Isoleucine	Phenylalanine	Val., meth., tyr.	Val. + meth.	Valine	Methionine	Tyrosine	Proline	Alanine	Glutamic acid	Threonine	Aspartic acid	Serine	Glycine	Ammonia	Arginine	Lysine	Histidine
Leucine	.41	.44	2.34	3.07	.76	.09			.36	.19	.21		.76	.92	.02	.47	.22	.23	.29	.33	.00	.04
Isoleucine	.41	.64	1.39	.81	2.39	.18	.64	.74	.37	.94	.29	.27	.54	.68	.01	.34	.21	.20	.20	.24	.10	.08
Valine	.38			1.12	.99	.12	2.06		2.43	.35	.15	.17	.70	.83	.00	.45	.29	.24	.40	.33	.03	.06

As usual, the highest excess of N^{15} is found in the injected amino acid in each case. All three acids have contributed high amounts of N^{15} to glutamic acid and to the other amino acids, for the most part in conformance with the general pattern of ratios. The chief significant variation from this pattern is the transfer of nitrogen among these amino acids themselves. The injection of N^{15}-leucine results in a high incorporation of isotope into isoleucine, labeled isoleucine transfers a very large amount of N^{15} to leucine and injected valine produces high labeling in both the leucines. The excesses in valine from both leucines are only slightly higher than the values that could be expected. These results would seem to indicate a direct interconversion between leucine and isoleucine (with the equilibrium favoring leucine) and a conversion of valine to one or both of them (with an equilibrium strongly in favor of the leucines).

It is interesting that such conversions should appear to exist among amino acids that are known to be essential in the diet. However, it must be remembered that since these results have been obtained with N^{15}-labeled amino acids, they give no information about the extent to which the carbon skeleton takes part in such conversions, and may well indicate a shift of only part of the molecule or of just the amino group. Perhaps some doubts could be raised about these figures because of the possible existence of slight contamination of the leucines with each other. They emerge close together from the Dowex 50 isolation (compare threonine and serine), and although they have been found free of contamination when tested with paper chromatography, traces of the other leucine could perhaps have been present in each one. However, the valine definitely could not have contained any contaminating leucines.

The transfer of N^{15} from isoleucine to methionine is also very high and might indicate some direct conversion. It has already been pointed out that all the methionine values are questionable because the amounts isolated have been too low for accurate analysis. The excess here is less doubtful, however, because the figure for the fraction of valine plus methionine is considerably higher than that for valine alone.

Phenylalanine and tyrosine

N^{15}-labeled tyrosine could not be injected subcutaneously in the desired amount, due to its low solubility in water, and was therefore given as a water suspension by stomach tube. The animals received the tracer in one dose and were then killed twelve hours after the feeding. Phenylalanine was injected in the usual way. The N^{15} excesses in the amino acids isolated from the liver

Table 8. Incorporation of N^{15} from phenylalanine and tyrosine into liver protein amino acids. Two 200-gram rats, 200 mg phenylalanine each, 16.4 % excess N^{15}. Two 200-gram-rats, 130 mg tyrosine each (by stomach tube), 17.6 % excess N^{15}.

Administered compound	Insoluble humin	Soluble humin	Leuc. + isoleuc.	Leucine	Isoleucine	Phenylalanine	Val., meth., tyr.	Val. + meth.	Valine	Methionine	Tyrosine	Proline	Alanine	Glutamic acid	Threonine	Aspartic acid	Serine	Glycine	Ammonia	Arginine	Lysine	Histidine
Phenyl-alanine		.16		.16	.25	4.61	.91		.13	.16	3.41	.09	.84	1.11	.02	.56	.13	.11	.19	.45	.33	.10
Tyrosine	.25	.28		.15	.16	1.48	.80	.17	.13		3.34	.11	.55	.78	.01	.42	.12	.15	.18	.30	.05	.06

protein are shown in Table 8. The ratios of the excesses to that of glutamic acid are given in Table 10.

In both experiments the highest excess of N^{15} is found in the injected amino acid and the isotope has been distributed to the other amino acids in line with the general pattern. The variation in experimental conditions for the administration of tyrosine apparently has not affected this pattern. The only variation can be seen in the figures for these two amino acids themselves. A direct conversion of phenylalanine to tyrosine is indicated by the high figure for tyrosine in the phenylalanine experiment; this has been observed previously by Moss and Schoenheimer [17] in isotope experiments. However, the high excess in phenylalanine after feeding of tyrosine would seem to indicate that this conversion is reversible, in contrast to the results in the feeding experiments of Womack and Rose [18].

In neither experiment is there a significantly high excess of N^{15} in alanine, although a direct relation might be expected. However, since alanine bears such a close relationship to glutamic acid, its content of N^{15} may equilibrate rapidly with that of glutamic acid and may therefore not appear to be significantly higher. This is supported by the results in the experiment with injected alanine.

Arginine, lysine and histidine

The three basic amino acids were each injected in the form of the free base. The amounts used are shown with Table 9. Since the labeled amino acids were isolated from biologically marked proteins, the isotope was distributed throughout the molecule, and the figures are overall excesses of N^{15}. In

Table 9. Incorporation of N^{15} from arginine, lysine and histidine into liver protein amino acids. Two 200-gram rats, 200 mg arginine each, 4.2 % excess N^{15}. Two 200-gram rats, 200 mg lysine each, 4.1 % excess N^{15}. Two 200-gram rats, 200 mg histidine each, 4.1 % excess N^{15}.

Administered compound	Insoluble humin	Soluble humin	Leuc. + isoleuc.	Leucine	Isoleucine	Phenylalanine	Val., meth., tyr.	Val. + meth.	Valine	Methionine	Tyrosine	Proline	Alanine	Glutamic acid	Threonine	Aspartic acid	Serine	Glycine	Ammonia	Arginine	Lysine	Histidine
Arginine	.28	.22	.34			.52	.46	.39			.48	.87	.94	1.63	.10	1.09	.30	.06	1.09	4.76	.63	.25
Lysine	.27	.00	.53			.27	.13					.23	.50	.98	.13	.79	.34	.11	.22	.40	4.25	.16
Histidine	.21	.24	.26			.49	.11					.30	1.60	1.53	.11	1.33	.41	.53	.78	1.32	.30	5.41

Table 10. Levels of N^{15} in liver protein amino acids, calculated on the basis of the excess in glutamic acid as 1.00.

Administered compound	Insoluble humin	Soluble humin	Leuc. + isoleuc.	Leucine	Isoleucine	Phenylalanine	Val., meth., tyr.	Val. + meth.	Valine	Methionine	Tyrosine	Proline	Alanine	Glutamic acid	Threonine	Aspartic acid	Serine	Glycine	Ammonia	Arginine	Lysine	Histidine
Glut. a.	.14	.13	.40	.31		.14		.24	.20	.48	.20	.12	.74	1.00	.03	.50	.46	.19	.23	.34	.04	.02
Ammonia	.11	.13	.14		.13	.08		.14			.24	.05	.85	1.00	.04	.65	.12	.16	1.49	.66	.01	.02
Asp. a.	.19		.33	.26	.59	.14		.20		.07	.20	.15	.67	1.00	.01	.53	.22	.20	.31	.32	.08	.13
Alanine	.15	.25		.50	.52	.11	.42	.45	.38		.13	.20	1.30	1.00	.01	.57	.30	.27	.35	.43	.06	.04
Proline	.14	.16		.19	.21	.06	.21	.24	.20		.10	4.27	.78	1.00	.01	.61	.19	.13	.24	.50	.07	.03
Threonine	.76	.85		.11	.33	.25	.49	.54	.66		.11	.24	.89	1.00	16.85	.82	3.36	2.34	1.09	.77	.22	.05
Serine	.45	.27	.25	.23	.18	.17		.24	.16		.44	.15	.88	1.00	1.05	.67	7.41	3.72	.99	.63	.06	.04
Glycine	.54	.12	.14			.15	.11					.07	.86	1.00	.03	.65	4.69	5.34	.83	.62	.02	.08
Leucine	.45	.48	2.56	3.36	.83	.10			.40	.21	.23		.83	1.00	.02	.52	.24	.25	.32	.36	.00	.05
Isoleuc.	.61	.95	2.05	1.19	3.52	.27	.94	1.09	.55	1.38	.43	.39	.80	1.00	.02	.51	.30	.29	.29	.35	.15	.11
Valine	.46			1.34	1.18	.14	2.47		2.92	.42	.18	.20	.84	1.00		.54	.35	.28	.49	.39	.04	.07
Phenylal.		.15		.14	.23	4.14	.82		.12	.15	3.06	.08	.76	1.00	.02	.50	.12	.10	.17	.40	.03	.09
Tyrosine	.31	.35		.19	.21	1.89	1.02	.22	.17		4.26	.14	.71	1.00	.02	.54	.16	.19	.23	.38	.07	.08
Arginine	.17	.14	.21			.32	.28	.24			.29	.53	.57	1.00	.06	.67	.19	.04	.67	2.91	.39	.15
Lysine	.28		.54			.27	.13					.23	.51	1.00	.14	.80	.34	.11	.22	.40	4.32	.16
Histidine	.14	.16	.17			.32	.01					.20	1.04	1.00	.07	.87	.27	.35	.51	.86	.19	3.53

the experiment with injected lysine, the lysine compound found in the threonine fraction was separated from threonine (as in the experiment with administered threonine), so that the figure for threonine does not include the highly labeled lysine compound.

Tables 9 and 10 show approximately the same pattern for N^{15} distribution from the basic amino acids as from the other acids. Some differences can be seen, but the fact that the N^{15} is distributed throughout the molecule of the injected compound could be expected to produce some variations. Also it must be remembered that there was a low excess of N^{15} in the injected compound, so that the direct figures were quite low and were multiplied by a factor of about 22 to obtain the figures based on 100 % N^{15}. In view of this, many of the differences, such as can be seen in figures for alanine, aspartic acid and ammonia are probably not significant. Of course, significantly high excesses are found in the injected amino acid in each experiment. It is possible that the figure for proline from injected arginine may be high due to a conversion of ornithine to proline, as reported by Stetten and Schoenheimer [6]. The actual difference observed, however, is small.

From the present data it is impossible to tell whether the N^{15} found in the isolated compounds is derived from the α-amino groups of the injected compounds or from labeled nitrogen from other parts of the molecules. In the work of Weissman and Schoenheimer [19] with lysine marked with N^{15} in the α-position, the N^{15} was found to be distributed to the other amino acids tested, and when γ-labeled histidine was used as a tracer by Tesar and Rittenberg [20], this nitrogen was likewise distributed among the various amino acids investigated. On the basis of these studies it might be assumed that nitrogen from any part of the basic amino acids could take part in transfer reactions. There is no way of knowing which nitrogen atoms account for the transfer of N^{15} observed in our experiments with these amino acids, but all three have distributed their nitrogen to the same extent as have any of the other amino acids. However, as pointed out earlier, neither lysine nor histidine ever received any significant amount of N^{15} from any of the amino acids injected.

GENERAL DISCUSSION

Previous workers [5, 6, 10, 13–15, 19–24] have found N^{15} to be incorporated into almost all amino acids from labeled ammonia and from every N^{15}-marked amino acid thus far tested, indicating extensive exchange of the amino group in metabolism. This is confirmed by the present investigation, in which fifteen different amino acids and ammonia, labeled with N^{15}, have been administered separately to rats, and the content of isotope determined in all amino acids isolated from total liver protein. As can be seen in the tables already presented, each of the administered compounds has distributed its nitrogen to all the amino acids isolated except threonine, lysine and histidine.

When the tables are compared with each other, similarities can be observed in the figures. The highest excess of N^{15} is always found in the injected compound (except in the case of aspartic acid), but in the figures for N^{15} transferred to the other amino acids a high value is always observed for glutamic acid. Moreover, the excesses of N^{15} in the other amino acids show a fairly constant relationship to each other. This can be seen more clearly in Table 10, in which the figures for N^{15} excess have been recalculated for each experiment on the basis of the N^{15} excess in glutamic acid as 1.00. This recalculation eliminates certain variations between experiments and makes the figures more comparable. Since it has been necessary to use so few animals for each experiment, differences in the transfer of N^{15} from the injected amino acids could be due partly to biological variation between animals in the rate of amino acid metabolism. Such variation is eliminated by setting the figures for all experi-

ments equivalent to a common level of nitrogen transfer. Variation due to differences in the amount of amino acid injected is also rendered negligible.

When the figures for different experiments are recalculated on a common basis in this fashion, and compared as in Table 10, the similarities in ratios are quite apparent, and a picture can be constructed of a general pattern for nitrogen distribution in amino acids. This indicates that amino acid metabolism, as judged by nitrogen exchange, may follow a common pathway. The simplest explanation for this is that, from each injected compound, the path of nitrogen transfer proceeds over one compound in central position, which then distributes the nitrogen to other amino acids. Such a compound must, of course, show a higher incorporation of isotope than the compounds to which it subsequently donates nitrogen. Glutamic acid seems a likely possibility for this central compound because of its high incorporation of N^{15} in all experiments. It might thus be implied that amino acid nitrogen transfer proceeds in general by some common metabolic pathway over glutamic acid. The figures in several experiments which are significantly higher than the levels found in the general pattern have then been assumed to indicate specific direct pathways between the injected compound and these particular amino acids. Some of these direct conversions have been observed previously.

The choice of glutamic acid as the compound in central position may seem arbitrary. The constancy of ratios among N^{15} levels in the different amino acids would naturally be apparent no matter what amino acid was taken as a basis of calculation. Even if glutamic acid does show a very high level of isotope, we can not say whether this is a result of a rapid, primary uptake of N^{15} by this amino acid, or whether it merely signifies that labeled glutamic acid is incorporated into protein more rapidly than are the other amino acids. Another explanation could be that total liver protein is made up of various individual proteins, in which the summary of amino acid compositions and rates of turnover favors the importance of glutamic acid (*i.e.*, the protein containing most glutamic acid may also have the highest turnover rate). These factors could affect not only glutamic acid but all the isolated labeled compounds. Their contribution could be clarified by carrying out this sort of study on each of several individual proteins. If the same pattern was found in all cases, one might conclude that it represented a general pattern of intermediary metabolism and not merely reflected protein compositions and turnovers.

However, the present experiments do offer some direct evidence for the correctness of the theory which places glutamic acid in a central metabolic position. This exists in the results of the experiment with administered proline. As already pointed out, the pattern of ratios in this experiment does not differ

significantly from that in any other, in spite of the existence of a known direct conversion of this amino acid to glutamic acid [6]. This supports the hypothesis that the metabolic transfer of nitrogen from each amino acid proceeds to glutamic acid, and that the distribution of isotope to the other amino acids depends on the level in glutamic acid.

In such theories about intermediary metabolism of amino acids nothing has been stated about the mechanisms involved. Glutamic acid has generally been recognized to maintain an important position in metabolism, especially with respect to transamination reactions. The extensive literature on this subject has been ably reviewed by Braunstein [25]. In connection with this, it is of interest to observe that alanine and aspartic acid also show high figures for incorporation of N^{15} in all of these experiments and close correlation with the glutamic acid value. It is probable that the similarity in the levels of N^{15} in these three amino acids is a reflection of their close equilibrium with respect to nitrogen exchange, due to the known transamination reactions between them. However, very little is definitely known about similar reactions between glutamic acid and the other amino acids which could explain the amounts of N^{15} found in them. Perhaps the most logical mechanism to be expected would be a transfer of nitrogen from the injected amino acid to α-keto-glutaric acid and from the resulting glutamic acid to the respective keto acids of the others. However, from the present data it can not be decided whether or not the reactions proceed in this way, nor, if they do, whether the mechanism involved is as direct as that implied in the usual conception of transamination.

It is equally unjustified to postulate mechanisms for the specific conversions believed to be indicated by variations from the general pattern. Here again it must be remembered that all the figures for levels of N^{15} are subject to other variables than uptake from the injected compound, and may therefore be misleading, but it is highly probable that these large differences do really indicate direct pathways. These conversions have already been pointed out; some have been observed earlier but others have not been reported previously. It must be kept in mind that, even for these direct pathways, the figures do not indicate whether or not the carbon skeletons of the amino acids take part, but merely the extent of nitrogen transfer in each case.

The observation in these experiments that no significant amount of N^{15} is incorporated into lysine confirms previous work [21–24] with several administered labeled amino acids. Similarly, no significant uptake of N^{15} into histidine has been found here from any of the administered compounds, although it has been reported earlier that histidine incorporated a small amount of N^{15} in the α-position after the feeding of labeled ammonia [26]. Other workers

have found no transfer of isotope to threonine from N^{15}-marked glycine[13] or leucine[15]. The present results show this to be true for threonine no matter what amino acid is administered. It would then appear that none of these three amino acids can receive nitrogen from the common metabolic transfers, and that they may therefore be essential even with respect to the amino group of the molecule. However, all three of these acids have distributed nitrogen in line with the general pattern, so that processes of transfer apparently do occur. We can not say which nitrogen atoms of lysine or histidine have participated in these reactions. The transfer from threonine is less extensive than from any other amino acid.

It would be valuable if one could use the figures from these experiments as a quantitative index of the extent of metabolic activity of these amino acids. Unfortunately, however, one can not do this with certainty, because various factors influence the figures and it is almost impossible to distinguish between the effects of different ones. The levels of N^{15} found in the amino acids isolated are affected by the rate of incorporation of the different amino acids into protein and by the protein compositions and turnovers, as has already been pointed out. In addition, the isotope levels must be influenced by the amounts of amino acid present in free state in the animal at the time of isotope administration, which would dilute the injected labeled compounds. However, it might be assumed that this amount of free amino acid would be negligible in each case, in comparison with the large amount of amino acid injected, and variations between amino acids as a result of this may be insignificant. The most important variable which we can not know and control is the fact that all figures for isotope level reflect the extent of both the production of the amino acid, as indicated by its incorporation of nitrogen, and the breakdown or utilization, as evidenced by its transfer of nitrogen. Within the limits of variations due to incorporation rates, the level of N^{15} in the amino acids in the general pattern are an index of the extent to which the amino acids receive nitrogen. The best indication of the extent to which the amino acids transfer nitrogen is a comparison of the direct figures for the amount of N^{15} contributed by each of them to one particular amino acid, for example glutamic acid, which shows an appreciable incorporation of isotope in all experiments. The validity of this comparison is somewhat questionable due to the fact that the amounts of amino acids injected have been different. In both these comparisons small differences can not be considered decisive, and one can, at best, draw conclusions only from the extremes, since both transfer and incorporation of nitrogen proceed simultaneously and one can not determine to what extent each has been operative. The amino acids which transfer most nitrogen to glutamic acid are aspartic acid and alanine, indi-

cating a high extent of deamination of these amino acids in metabolism. The comparatively low figures for N^{15} remaining in the injected compound in the experiments with the administration of these amino acids may also reflect this, as well as an extensive production of amino acid. The production of new molecules dilutes the isotope level and extensive breakdown of the amino acid, as in these cases, makes this dilution effect more evident. This dilution by newly formed molecules is absent for the amino acids threonine, lysine and histidine, which have been shown not to incorporate nitrogen; the metabolism of these three amino acids seems therefore to be limited to transfer of nitrogen. A high figure for N^{15} remaining in the administered compound could therefore be expected in the experiments with these amino acids. Injected threonine can be seen to maintain a very high excess of N^{15}, in agreement with this expectation. Since the incorporation of isotope into glutamic acid from injected threonine is extremely low, it would appear that threonine is also poorly deaminated; both transfer and incorporation of nitrogen are low for this amino acid. Unfortunately, the figures for lysine and histidine are complicated by the nitrogen in the molecule other than the α-amino group, and by the fact that the molecule is labeled with N^{15} throughout. It is therefore impossible to draw such definite conclusions from them. However, we can perhaps cite aspartic acid and alanine, on the one hand, and threonine, on the other, as the extremes in extent of metabolic activity (both formation and breakdown) among the amino acids studied here.

SUMMARY

Figures are presented for the N^{15} uptake into eighteen different compounds (fifteen amino acids, ammonia, and soluble and insoluble humin) isolated from normal total liver protein after the administration of each of fifteen different amino acids and ammonia, labeled with N^{15}. In each case the isotope has been found to be distributed to all of the compounds isolated except threonine, lysine and histidine. Similarities in all the experiments in the ratios of N^{15} contents of the different compounds are discussed as being indicative of a common pathway of amino acid metabolism which always results in approximately the same pattern of nitrogen distribution. Evidence is presented and discussed for a central position of glutamic acid in this common metabolic transfer of nitrogen. Figures which are significantly higher than the usual levels in the general pattern are considered as pointing to specific conversions between the administered tracer and these particular compounds. On the basis of this evidence the direct pathways believed to be indicated by these figures are a reversible interconversion of glycine and serine, a non-

reversible transformation of threonine to glycine, a reversible interconversion of phenylalanine and tyrosine, a reversible interconversion between leucine and isoleucine and a probably reversible pathway between valine and either or both of the leucines. The figures in these experiments are also considered to be in agreement with a direct transformation of proline to glutamic acid, although the evidence is of a somewhat different nature.

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Amino Acid Interrelationships during Growth, Studied with N^{15}-Labeled Glycine in Regenerating Rat Liver

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Previous studies have been made in this laboratory on various metabolic interrelationships of amino acids in normal rat liver, as indicated by experiments with N^{15}-labeled amino acids [1]. It was therefore of interest to investigate certain of these relationships in regenerating rat liver, in order to compare this with the normal tissue and perhaps gain some insight into the changes in amino acid metabolism occurring during accelerated growth and protein synthesis. Such a study has been carried out in conjunction with an investigation of the relationship between formation of protein and of nucleic acid bases in regenerating liver. The experiments were made with N^{15}-labeled glycine as tracer. The results of the comparisons of nucleic acid and protein syntheses have already been published [2]; the present paper deals with the relationships indicated by the isotope distribution in the amino acids isolated from the liver protein. The proteins from cell nuclei and cytoplasm have been studied separately.

EXPERIMENTAL

The experimental procedures were those described in the previous publication [2]. Partial hepatectomy was performed on albino rats weighing 180–200 g. The technique used for hepatectomy has been reported earlier [3]. Throughout the experiments the animals were fed on a diet of milk, bread and oats, and were allowed to eat *ad libitum*. The animals were allowed to live for different periods of time after hepatectomy and the total time from operation to death was termed the regeneration stage; the stages chosen were 11, 26, 32, 56 and 170 hours. The labeled glycine (31 % excess N^{15}) was injected subcutaneously at a level of 100 mg per 100 g body weight (weight at time of operation). The tracer was divided into four doses which were given at two-hour intervals over the last eight hours of the regeneration time. The animals were killed two hours after the last injection of isotope.

Table 1. Incorporation of N^{15} from injected glycine into amino acids of liver cytoplasm protein during regeneration.

Regeneration stage *	Insoluble humin	Soluble humin	Leuc. + isoleuc.	Phenylalanine	Val., meth., tyr.	Val. + meth.	Valine	Methionine	Tyrosine	Proline	Alanine	Glutamic acid	Threonine	Aspartic acid	Serine	Glycine	Ammonia	Arginine	Lysine	Histidine	Cystine	Total hydrolys.
11 hours	**	.13	.15	.07	.20	.12			.44	.09	.39	.46	.04	.28	5.20	5.93	1.09	.29	.13	.06	1.24	.73
26 »	.38	.10	.18	.08	.22		.17	.24	.47	.10	.66	.74	.03	.49	7.92	8.14	1.41	.63	.10	.07		1.19
32 »	.61	.21	.15	.10	.23	.14			.53	.12	.73	.92	.02	.68	6.38	7.68	1.12	.74	.14			1.17
56 »	.57	.13	.22	.11	.22	.16	.24	.55	.55	.13	1.23	1.00	.03	.87	8.56	9.66	1.55	.90	.17	.06	1.73	1.38
170 »	.40	.12	.16	.09	.17	.15	.18	.51	.30	.13	.68	.82		.65	4.71	5.15	1.07	.69	.05	.03	1.24	.71
Normal	.46	.10	.12	.13	.10					.06	.73	.85	.00	.55	3.97	4.53	.71	.52	.01	.07		.75

* Regeneration stage is total hours from partial hepatectomy to death.

** Figures are expressed as % excess N^{15}, calculated on the basis of 100 % N^{15} in the administered glycine.

The livers from all rats in each regeneration stage were pooled, and the cell nuclei were separated from cytoplasm in citric acid according to the technique described earlier [3]. The tissue from both sources was treated as in previous experiments [2] to remove lipid material and to extract polynucleotides (method of Hammarsten [4], followed by extraction with hot trichloroacetic acid after Schneider [5]). The protein was taken as the final dry, TCA-insoluble residue. This was hydrolyzed in acid and the amino acids separated by starch chromatography and analyzed for N^{15}. Details of these procedures have already been reported by Åqvist [6].

RESULTS

Table 1 shows the figures for excesses of N^{15} found in the compounds isolated from the cytoplasm protein. Results are given for normal, non-regenerating liver and for liver at the different stages of regeneration. Values are included for fifteen different amino acids, ammonia and soluble and insoluble humin. The figures for the N^{15} excesses in the total hydrolysates of the proteins are also given. The amino acids are listed in the order in which they emerge from the starch column. All figures are expressed on the basis of 100 % N^{15} in the injected glycine.

In the case of the protein from cell nuclei only glycine and serine were analyzed for N^{15}. The figures are presented in Table 2 for the same regeneration stages. The excesses in the total protein hydrolysates are also given. The figures are expressed in the same way as for Table 1.

The figures in Table 1 show that the highest level of N^{15} is found in the isolated glycine in both normal and regenerating liver, as would be expected

Table 2. Incorporation of N^{15} from injected glycine into amino acids of liver nuclei protein during regeneration.

Regeneration stage *	Serine	Glycine	Total hydrolysate
11 hours	**	5.62	.76
26 »	9.29	10.18	1.40
32 »	8.17	8.21	1.19
56 »	7.91	9.01	1.30
170 »	4.30	4.86	.71
Normal			.74

* Regeneration stage is total hours from partial hepatectomy to death.

** Figures are expressed as % excess N^{15}, calculated on the basis of 100 % N^{15} in the administered glycine.

from glycine as precursor. A very high value is also found in serine in each case. High values can also be seen in ammonia, especially in the regenerating liver. The cystine values which are included are also high. Unfortunately, the amounts of cystine isolated by these procedures were always too small to allow any really exact determination of N^{15} content, and the values can not be considered quantitatively accurate. However, a significant content of N^{15} in cystine does seem to be indicated by these consistently high figures.

From Table 1 it can be seen that except for glycine, serine and ammonia the amino acids of the cytoplasm protein show levels of N^{15} which are in general lower for 11-hours regeneration than for non-regenerating liver, although a few are not significantly different. Most of the figures increase from 11 to 26 hours of regeneration, but some are still below the normal levels at 26 hours. The figures increase further with longer regeneration time and seem to approach a common maximum at 56 hours. They decrease again at the last regeneration stage, in which they approach the levels in non-regenerating liver. The figures for glycine, serine and ammonia are somewhat different, because in addition to the 56-hour maximum they show another lower peak at 26 hours and a drop at the intermediate point of 32 hours. This pattern can also be seen in the excesses in the total protein hydrolysates, indicating a predominant influence by these compounds on the N^{15} level in the total protein.

For the cell nuclei protein, only values for glycine, serine and total protein hydrolysate are given. They are very much the same as those from the cytoplasm protein, except that the maximum at 26 hours is higher than that at

56 hours. The same intervening drop at 32 hours is observed, and the figures for both 11 hours and 170 hours are low. The same pattern is seen in both amino acids and in the total hydrolysate. There are no values for glycine and serine from nuclear protein of non-regenerating liver but the figure for the total hydrolysate i s the same as that of cytoplasm and very close to the 11-hour and 170-hour figures in both cases.

In the previous investigation on metabolic interrelationships among amino acids in non-regenerating liver [2], there were indications of a common pattern of nitrogen distribution no matter what labeled amino acid was administered. A high incorporation of isotope into glutamic acid was always observed, and the ratios of levels of N^{15} in the different amino acids were very much the same in each case. A central position of glutamic acid in this common metabolism was postulated, and certain specific conversions were indicated by large increases in the levels of N^{15} above the usual ones shown in the general pattern. The general similarities of ratios and the large differences indicating direct metabolic pathways were made clearer in that study by recalculating the figures found in each experiment on the basis of the excess in glutamic acid as 1.00. The same thing is done for the figures in these experiments; the results are shown in Table 3.

The figures for non-regenerating liver in Table 3 have been presented in earlier work [1], along with other amino acid experiments, and are typical of the general pattern of nitrogen distribution found from all administered amino acids, except for the high values in glycine, serine and ammonia. These values would seem to indicate a direct conversion of glycine to serine, and a direct

Table 3. Levels of N^{15} in amino acids of liver cytoplasm protein, calculated on the basis of the excess in glutamic acid as 1.00.

Regeneration stage *	Insoluble humin	Soluble humin	Leuc. + isoleuc.	Phenylalanine	Val., meth., tyr.	Val. + meth.	Valine	Methionine	Tyrosine	Proline	Alanine	Glutamic acid	Threonine	Aspartic acid	Serine	Glycine	Ammonia	Arginine	Lysine	Histidine	Cystine
11 hours		.28	.32	.16	.42	.25			.95	.20	.84	1.00	.08	.61	11.22	12.80	2.35	.63	.29	.13	2.68
26 »	.52	.14	.24	.11	.30		.23	.33	.64	.14	.89	1.00	.04	.66	10.68	10.97	1.89	.85	.13	.09	
32 »	.66	.23	.17	.11	.25	.16			.57	.13	.79	1.00	.02	.73	6.93	8.34	1.21	.80	.15		
56 »	.57	.13	.22	.11	.22	.16	.24	.55	.55	.13	1.23	1.00	.03	.87	8.56	9.66	1.55	.90	.17	.06	1.73
170 »	.50	.15	.20	.10	.21	.18	.22	.62	.37	.15	.83	1.00		.80	5.76	6.29	1.31	.84	.06	.04	1.51
Normal	.54	.12	.14	.15	.11					.07	.86	1.00	.03	.65	4.69	5.34	.83	.62	.02	.08	

* Regeneration stage is total hours from partial hepatectomy to death.

contribution of either or both of them to ammonia. In non-regenerating liver the conversion of glycine to serine results in an N^{15} level in isolated serine which is about 88 % of that in the isolated glycine. This ratio of serine to glycine varies somewhat throughout the regeneration stages. The ratio is very nearly the same as this at the first and at the last two regeneration stages for both cytoplasm and nuclei protein, but there are variations in the ratio around 26 and 32 hours of regeneration. (Cytoplasm protein — — 11 hours—0.88, 26—0.97, 32—0.83, 56—0.88, 170—0.92; nuclei protein — — 26—0.91, 32—0.995, 56—0.88, 170—0.88.)

In Table 3 one can compare the relative distribution of N^{15} at each regeneration point with that for non-regenerating liver. It can be seen that the pattern of distribution is much the same in all cases and is very close to the general pattern observed earlier [1]. Of the compounds other than glycine, serine, ammonia and cystine, the highest levels of N^{15} are found in glutamic acid, alanine, aspartic acid, arginine and tyrosine, and insignificant amounts of N^{15} are seen in threonine and histidine. All of these levels are in line with the general pattern except possibly those in tyrosine. The figures for lysine in regenerating liver also seem a bit high, for lysine, like threonine and histidine, has not been found to incorporate any significant amount of isotope from any administered labeled amino acid in non-regenerating liver. It is hard to say whether or not this increase in lysine is significant and is a result of a difference due to regeneration.

However, in all the figures larger variations can be seen than have been observed in the previous experiments. These seem especially large in the ratios for glycine, serine and ammonia, but can also be seen even in such cases as aspartic acid and alanine which have earlier been found to have the most constant ratios. The figure for alanine at 56 hours regeneration is particularly high, but the ratios are in general somewhat high in this group and may be partly a result of too low a figure for glutamic acid.

DISCUSSION

The figures presented here for cytoplasm protein from non-regenerating liver have also been included and discussed in the previous study on N^{15} distribution in the same compounds isolated from normal liver after the administration of various amino acids [1]. The very high value for serine in all these experiments with injected glycine confirms previous reports of a direct conversion of glycine to serine [7–10], as has also been discussed in the previous paper. The high figures for ammonia are probably due to a rather extensive decomposition of highly-labeled serine during acid hydrolysis [1, 11]. The values

are much higher in the regenerating liver than in normal liver, but they parallel the levels of N^{15} in serine. It is probable, therefore, that these figures represent an artifact more than a metabolic transfer of nitrogen. The highest cystine value also corresponds to the glycine-serine maximum. It has already been stated that these values are somewhat doubtful, quantitatively, but the high figures may well be explained by a conversion of serine to cystine, as discussed by Stetten [12].

A variable but high incorporation of N^{15} into glutamic acid is seen for all the stages of regenerating liver as well as for normal liver, and, except for the cases of special contributions already discussed, the ratios of the other amino acids to glutamic acid for the most part follow the general pattern previously observed [1]. This can be seen more clearly from Table 3, where variations in the extent to which glycine transfers nitrogen to glutamic acid are eliminated. Since the conditions in regenerating liver are those of extensive protein synthesis, variations in this metabolic distribution of nitrogen from glutamic acid to the other compounds might be expected, but there do not seem to be appreciable changes.

From the figures in Table 3 for level of N^{15} in glycine, it can be seen that the amount of nitrogen retained by glycine, with respect to transfer to glutamic acid, is greatest at the first regeneration stage, appreciably above the value for non-regenerating liver, and that there is a continuous decrease in this level with increasing hours of regeneration, approaching the normal figure at 170 hours. The direct figures in Table 1 show that the incorporation of nitrogen into glutamic acid falls below normal at 11 hours and then rises to reach a maximum above the normal level at 56 hours. The isolated glycine, meanwhile, shows immediately a sharp increase in N^{15} level, above that of non-regenerating liver, which reaches a peak at 26 hours, dips and then rises to a maximum, also at 56 hours. The utilization of the injected compound, *i.e.*, its transfer of nitrogen to glutamic acid, is apparently lower during early stages of regeneration. The nitrogen transfer in liver seems to be somewhat inhibited just after hepatectomy, although the incorporation of glycine into protein proceeds at once, as the formation of new protein begins.

The various factors in non-regenerating liver affecting the N^{15} levels in the isolated amino acids, other than uptake from the administered compound, have been quite extensively discussed in the previous study [1]. Among these are the differences in rate of incorporation of the respective amino acids into protein, and the summary of amino acid compositions and turnover rates of the different proteins which make up total liver protein (*i.e.*, glutamic acid may have a high level of N^{15} because it is incorporated into protein faster, or because some individual proteins in the total liver protein which have a high

turnover rate also have a high content of glutamic acid). Such factors may become even more important under the conditions of rapid protein formation in regenerating liver. Another important variable can be found in the differences in extent both of utilization of the amino acid (nitrogen transfer) and of its formation (incorporation of nitrogen). An extensive production of new molecules of the amino acid serves to dilute the isotope content and a rapid breakdown or utilization of the amino acid makes this dilution effect more evident. Thus the very high levels in glycine during early stages of regeneration could be due partly to the decreased utilization to form glutamic acid. However, the N^{15} level in the glycine in free state has been shown not to be appreciably affected by such variations. A separate experiment, in which free glycine existing in the liver tissue has been isolated and analyzed for N^{15}, has shown very small variations in the isotope level at different regeneration stages. It is likely, therefore, that this rise in level of N^{15} in the glycine in protein reflects mostly an increased incorporation of glycine during the formation of new protein. Some increase of this sort can be seen in the figures for all the amino acids, which reach a common maximum at 56 hours. The effect on the other amino acids is less than on glycine (and on the compounds formed directly from glycine) because the others depend on the N^{15} level in glutamic acid, which shows a later and much smaller increase. The figures for serine and ammonia parallel those for glycine, and in Table 3 the ratios for these materials can be seen to drop with increasing regeneration time, just as do those for glycine. A similar tendency can be seen in tyrosine, but it is hard to say whether this indicates a closer relation of tyrosine to glycine or serine than heretofore suspected, or whether the high value at 11-hour regeneration is merely in error.

The factors of protein compositions and turnovers may help to explain the double peak observed in the figures for glycine, serine and ammonia. These factors may be accentuated, not only by the rate of formation of protein, but by the possibility that the different individual proteins may start to regenerate at different times and/or show maxima of synthesis or turnover at different periods of time after hepatectomy. The peak at 26 hours may be the maximum for a different protein (or proteins) than the one represented by the 56-hour peak. The total cell nuclei protein may contain a larger proportion of the first protein(s) and so show a higher maximum at 26 hours, in contrast to the total cytoplasm protein, which may be richer in the second protein(s), which has its peak at 56 hours. The figures for glutamic acid and for the other amino acids may not show this double peak because the intervening dip is obscured by the increase in the extent of transfer of nitrogen from glycine to glutamic acid (and thence to the other amino acids). This

contribution of different proteins could affect any of the amino acid figures and may account for some of the variations observed at these 26- and 32-hour regeneration stages. It may help to explain the altered serine to glycine ratios at 26 and 32 hours. In general it is interesting that the cell nuclei protein resembles the cytoplasm protein as closely as is indicated by the figures for glycine, serine and total hydrolysate. From these figures the total protein in both parts of the liver cell seem to have much the same turnover.

SUMMARY

Figures are reported for the uptake of N^{15} from injected glycine into various amino acids isolated from the protein of both cytoplasm and nuclei in normal and regenerating liver. The N^{15} has been found to be distributed to all of the compounds isolated from these proteins, the extent of transfer being for the most part in line with a general pattern of nitrogen distribution observed earlier for protein of normal rat liver after administration of various labeled amino acids. Contents of N^{15} higher than those in the general pattern support a direct conversion of glycine to serine and serine to cystine. These high levels are found in all these experiments with administered glycine. A high incorporation of isotope into glutamic acid can be seen in all cases, and the ratios of the levels in other amino acids to that in glutamic acid are about the same at all the different regeneration stages as in normal liver. However, the ratio of the level in glycine to that in glutamic acid is very high at early stages of regeneration and decreases steadily with increasing hours of regeneration to approach the ratio found in normal, non-regenerating liver. It is concluded that the utilization of the administered tracer is poorer at early stages of regeneration, with respect to N^{15} transfer to glutamic acid, but that the nitrogen distribution from glutamic acid to the other amino acids proceeds to about the same extent in all cases.

The actual levels of N^{15} in glycine are considered to be indicative of the extent of synthesis of new protein. For cytoplasm protein these show a maximum at 56 hours of regeneration and a lower peak at 26 hours. For cell nuclei protein the maximum is found at 26 hours and the second peak at 56 hours is slightly lower. These differences may well be due to contributions of different individual proteins which have maxima of synthesis or turnover at different periods of regeneration.

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The Effect of Heat and Urea Treatment on Crystalline Horse Erythrocyte Catalase

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The variability in the activity of various crystalline erythrocyte catalases preparations [1–5] has recently led to the suggestion that this enzyme as usually employed should not be considered a native protein [5]. The preliminary results of denaturation studies also suggested this and it was hoped that further work along these lines might disclose essential differences in various catalase preparations that would be of future value in preparing more uniform material. Such studies as applied to catalase are particularly attractive for one can use a fairly sensitive and rapid activity test for denaturation without having to resort to conditions leading to marked solubility changes as must be so often done with many proteins.

In this investigation the denaturation of several horse erythrocyte catalase preparations by heat and urea treatment showed no evidence of this enzyme being a single chemical entity. Loss of activity as effected by dilution, heating and treatment with urea all suggest the presence of molecules of various labilities.

EXPERIMENTAL

Crystalline horse erythrocyte catalases were prepared by modifications of the method of Bonnichsen [3] as previously described [5]. Enzyme assays were carried out by the rapid titration method of Bonnichsen, Chance and Theorell [6] at 18–20° C except in one experiment in which the method of von Euler and Josephson [7] was employed. Various experimental conditions were applied to catalase solutions of such concentration that 0.1 ml of a 1–10 dilution would give a suitable assay sample. This 1–10 dilution prior to assay was always carried out with pH 6.8, *M*/15 sodium phosphate buffer and served to dilute out the urea, adjust the pH to the desired assay level and the experimental solution to room temperature, etc. The actual protein concentration of the catalase solutions subjected to various conditions ranged from 0.5 to 1.0 mg per ml depending on the particular enzyme preparations used. These were three in number and showed Kat.F. values of

61 000, 117 000 and 76 000. They will be referred to as catalase no. 1, 2, and 3 respectively. Catalase no. 2 was the material described previously [5]. The amount of enzyme in any given experiment will always be noted in terms of the first order reaction constant given by the amount of catalase used in the assay.

Absorption spectra were determined by means of a Beckman spectrophotometer. A Zeiss pocket spectroscope was employed for locating the absorption maxima of the CO hemochromogens which were formed in tests for the presence of free heme resulting from treatment of catalase with urea.

EXPERIMENTAL RESULTS

Effect of temperature

In previous work it was found that catalase showed an initial rapid loss of activity upon dilution [5]. Consequently all studies on the effect of temperature were controlled by running a reaction at either 0° or 20° C employing as nearly as possible the identical conditions of assay as were used with the experimental sample. As will be noted later various catalase preparations showed variable effects as regards the amount of activity lost on dilution.

Little or no denaturation was evidenced at temperatures below 60° C. It was actually found that the loss of activity resulting from dilution was retarded at temperatures between 20° and 60° C. It often appeared that slight decreases in activity in the initial five minutes after dilution tended to be reversed with further incubation and in some cases at 50° C the initial activity was found after 30 minutes. The marked loss of activity on dilution was as a rule associated with relatively fresh material. On aging for several weeks in the cold this dilution effect was usually diminished and often small amounts of precipitate had developed in the solutions. The dilution effect was not abolished by use of glass distilled water or of serum albumin solutions as the diluent. If the loss of activity after dilution is due to absorption as has been suggested [8] the effect of temperature above 20° must be to diminish such absorption. It is also possible that there is an equilibrium between native and denatured catalase which is markedly influenzed by temperature. The effect of temperature on the loss of activity of catalase no. 1 is shown in Fig. 1. The same type of result was observed for preparation no. 2. It is apparent from Fig. 1 that the rate of the denaturation process falls off rapidly with time and that the greater portion of the catalase is stabile at 60° C over a 60 minute interval. There appear to be a series of catalase molecules of graded lability as regards their resistance to temperature denaturation. Such an effect will also be noted in the experiments involving urea. The reaction rate of the denaturation process in the initial stage was approximated by extrapolating the denaturation rate (calculated as a first order reaction) for the 5 and 10

minute period to zero time. No correction was applied for losses in activity as experienced at 0° and 20° C since the results at 50° C indicated that for these catalase preparations they were largely abolished at the higher temperatures. It would appear likely that the main destructive process at 60° and 65° C is due primarily to temperature as opposed to the activity loss at 20° C. Various thermodynamic data were calculated from the extrapolated denaturation reaction rates and are shown in Table 1.

Table 1. The reaction rates, heat, free energy and entropy change of activation in the heat denaturation of two catalase preparations as studied at 60° and 65° C in pH 6.8, M/150 phosphate buffer.

	Catalase no. 1	Catalase no. 2
k_{60}	4.9×10^{-4}	6.2×10^{-4}
k_{65}	13.5×10^{-4}	17.7×10^{-4}
$\Delta H^{\ddagger}$	44 540	46 840
$\Delta F^{\ddagger}$	24 800	24 200
$\Delta S^{\ddagger}$	+ 60	+ 68

It is to be emphasized that these data are somewhat qualitative in nature and moreover in view of the results shown in Fig. 1 are data for the more labile portions of the catalase preparations. Catalases no. 1 and 2 while showing some differences in the initial denaturation rate gave quite similar values for the heat of reaction, free energy change and the entropy change of the activation process. It is interesting that these values are of similar magnitude to those reported for the heat denaturation of some other enzymes [9].

Effect of pH

The effect of hydrogen ion concentration at various salt concentrations at 60° C was also investigated. Since the loss in activity in the first five minutes at this temperature appeared to be most representative of the denaturation process measurements were restricted to this interval. A control sample at 0° C was also run. The results are shown in Table 2 and indicate that catalase is most stable at 60° C near its isoelectric point (pH 5.5) when the salt concentration is low. It can be seen that there is a considerable loss of activity even at 0° C. It must be emphasized again that the destruction at 0° C may be adsorptive in nature and may bear no counterpart to the activity loss occurring at 60° C. Results for the control sample were almost identical when carried out at 20° instead of 0° C.

Table 2. Effect of pH on the denaturation of catalase no. 2 at 60° C.

Molarity of buffer		pH	Temperature	Percent activity loss in 5 minutes
0.067	A	4.2	0°	30
0.067	A	4.2	60°	50
0.05	A	5.6	0°	28
0.05	A	5.6	60°	31
0.5	A	5.6	0°	37
0.5	A	5.6	60°	86
0.067	P	6.8	0°	29
0.067	P	6.8	60°	40
0.067	P	7.8	0°	33
0.067	P	7.8	60°	70

A = Sodium acetate
P = Sodium phosphate

There is usually little difference in the amount of destruction of catalase on dilution in the temperature range from 0° to 30° C.

Effect of urea

Preliminary experiments indicated that at room temperature 5 *M* urea would effect approximately 50 % destruction of the catalase activity in 60 minutes at neutral pH. An initial experiment utilizing this concentration of urea in the presence of pH 6.8, *M*/150 sodium phosphate buffer showed a rapid initial decrease in activity followed by a much decreased rate of destruction. The results of this experiment corrected for the loss of a control sample are shown in Fig. 2. It is apparent that a relatively rapid destruction is effected within the first hour with this catalase preparation (no. 1). The destruction showed no evidence of being a first order reaction over the prolonged period. Further experiments with urea utilized an incubation period of an hour or less.

Data for the reaction of catalase no. 1 at various urea concentrations in pH 6.8, *M*/150 phosphate buffer at 20° C were plotted to give Fig. 3. The loss of activity is very rapid initially and then shows only relatively slight decreases. This is reminiscent of the effect of temperature as shown in Fig. 1. The influence of increased concentrations of urea is readily seen from Fig. 3. In another experiment at 18° C and in the same buffer the denaturation reaction rate was determined for various urea concentrations. As in the case of the

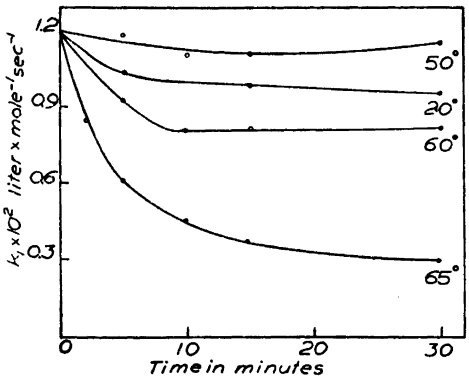

Fig. 1. Effect of temperature on loss of catalase activity in pH 6.8, M/150 phosphate buffer.

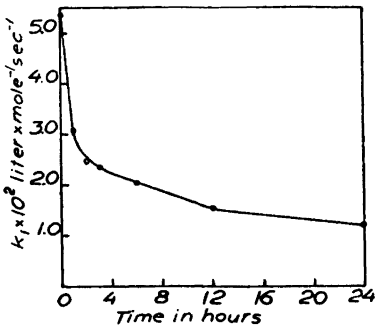

Fig. 2. Loss in catalase activity in 5 M urea, pH 6.8 M/150 phosphate buffer at 20°C. The catalase assay in this experiment was carried out by the method of von Euler and Josephson (7).

experiments involving temperature destruction only the results for the 5 and 10 minute incubation periods were utilized. The denaturation rate, calculated as a first order reaction, was extrapolated to zero time and data for various urea concentrations are given in Table 3. These data as well as those to be given shortly for the effect of temperature variation on the urea denaturation of catalase are subjected to the same limitations as pointed out earlier.

Table 3. The reaction rate for the denaturation of catalase no. 1 at various urea concentrations and in pH 6.8, M/150 phosphate buffer at 20° C.

Urea molarity	k_1 denaturation
3	1.0×10^{-4}
5	3.2×10^{-4}
6	11.0×10^{-4}
7	17.5×10^{-4}
7.5	25×10^{-4}

Lauffer [10] has calculated that the velocity constant for urea denaturation at constant temperature is proportional to a power of the urea concentration. The slope of a plot of the above type of data is indicative of the number of urea molecules bound. The data of Table 3 was plotted in such a manner to give Fig. 4 which, while not showing a completely satisfactory straight line

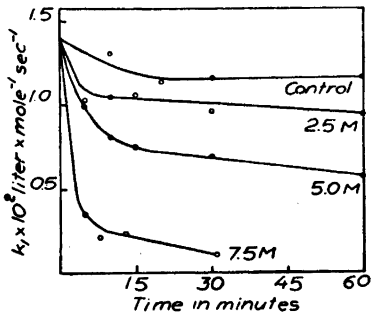

Fig. 3. Effect of various concentrations of urea on catalase in pH 6.8 M/150 phosphate buffer at 20° C.

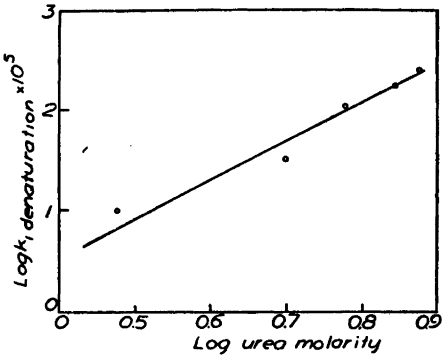

Fig. 4. The logarithm of the extrapolated denaturation velocity constant for catalase plotted against the logarithm of the urea concentration.

relationship, indicates that nearly four urea molecules are bound per molecule of protein at 20° C. This is less than that found by Lauffer [10] for tobacco mosaic virus but somewhat higher than that found by Mihályi [11] for bovine fibrinogen.

Effect of salt concentration

The denaturation of catalase in 5 *M* urea at 20° C was also studied in the presence of pH 6.8 phosphate from 0.05 to 1.0 *M*. A very slight increase in the denaturation rate was seen at the higher phosphate concentrations. This is in keeping with the effect of acetate on the heat denaturation at pH 5.6.

Reversal of urea denaturation

Attempts were made to determine whether losses in catalase activity were reversible upon dilution of the urea. Catalase preparations that had undergone from 50—75 % decrease in activity as a result of treatment with 5 and 7.5 *M* urea were diluted 1—10 with *M*/150, pH 6.8 phosphate buffer. After periods up to 18 hours after dilution there sometimes appeared to be a slight recovery of activity as compared with the appropriate controls. This recovery never exceeded 10 % of the initial activity and was usually of such a low magnitude as to fall within the realm of experimental error. The results, however, were always in the direction of increased activity.

Catalase samples treated with 9 *M* urea at 20° show precipitation upon dilution with the phosphate buffer. Catalase solutions more concentrated than

1 mg protein per ml when treated with 5 *M* urea at 30—40° C often also show precipitation upon dilution.

Effect of temperature

The denaturation of catalase no. 1 in 5 *M* urea and in pH 6.8, *M*/150 phosphate buffer was also studied over a temperature range of 0—50° C. The denaturation reaction rate was determined by extrapolation of the data obtained in the initial stages of the reaction to zero time as previously described. The various thermodynamic data calculated for catalase under the above conditions are given in Table 4.

Table 4. The reaction rates, heat, free energy and entropy change of activation in the denaturation of catalase no. 1 in 5 M urea in pH 6.8, M/150 phosphate buffer at various temperatures.

Temperature	$k_1$denaturation	$\Delta H^{\ddagger}$	$\Delta F^{\ddagger}$	$\Delta S^{\ddagger}$
0	2.1×10^{-4}			
10	2.94×10^{-4}	3 300	21 200	− 63
20	3.5×10^{-4}	3 900	21 000	− 58
30	5.3×10^{-4}	6 800	22 200	− 51
40	16.7×10^{-4}	20 400	22 300	− 6
50	120×10^{-4}	91 400	21 900	+ 215

The heat of activation of the denaturation process is lowered considerably by the presence of urea between 0° and 40° C. A marked rise, however, is noted at 40° C and this becomes quite large at 50° C. The urea-protein complex formation is exothermic and the number of molecules of urea molecules bound should decrease with a rise in temperature. Correspondingly the entropy and heat of activation will rise with increasing temperature and the latter value should approach that found in the absence of urea at the higher temperatures. Actually the heat of activation at 50° C in 5 *M* urea is much higher than that found at 60—65° C in the absence of urea. The reason for this, while not readily apparent, may be the result of the destruction of molecules of quite different chemical reactivities in the two cases. This is quite possible for in the case of 5 *M* urea approximately 96 % of the activity is destroyed within 5 minutes as 50° C whereas the data of Fig. 1 show that at 60° and 65° C only 20—50 % of the catalase activity is lost in the absence of urea in the same time interval. The data from 0° through 30° C gives a fairly good Arrhenius plot but deviates markedly above the latter temperature. At these higher temperature

conditions the more stable catalase molecules are undergoing destruction and it would appear that the heat of activation for the denaturation of these molecules is relatively high. In these studies it must be remembered that we are dealing with data representing an average for molecules of various labilities. This is especially true for the higher temperature, where an increasingly greater percentage of the more stable catalase molecules are undergoing destruction. The free energy change of the activation remains relatively constant since the heat and the entropy of activation show a compensating rise.

Effect of pH

These studies were carried out on catalase no. 3. This material showed a very high activity loss on dilution but the use of adequate controls allows for the demonstration of the effect of pH on the denaturation process by urea. The experiments utilized 5 *M* urea at a temperature of 20° C. Acetate and phosphate buffers of low concentration (0.035—0.05 *M*) were employed to regulate the pH. The results are shown in Table 5 and indicate that as in the case of the temperature denaturation (Table 2) there is a greater stability of catalase at pH 5.6. At this hydrogen ion concentration the loss of activity on dilution of the control is also less and the presence of the 5 *M* urea actually appears to retard this loss in the initial stages. This may suggest that the dilution loss and the urea denaturation do not concern identical molecules. It is difficult to accept the very large dilution loss of catalase no. 3 as one due to absorption on the walls of the container. No change in extinction coefficient at 405 mμ is seen on dilution under the same conditions that give huge activity decreases. Apparently a variable portion of the catalase preparations contain molecules of extreme lability. After standing for two weeks at 2—5° C catalase no. 3 showed a marked decrease in the dilution effect and a decrease in activity. This was synchronous with the appearance of a small amount of precipitate in the sample.

Spectrophotometric studies

Since the denaturation kinetics suggested that the catalase preparations employed were made up of a series of molecules of varying reactivities it appeared desirable to attempt to determine whether the enzyme showed any changes in spectral properties after treatment with urea. It was found that urea caused a decrease in the absorption at the 405 and 620 mμ maxima but had little or no effect on the protein absorption (280 mμ). Catalase solutions of the same concentration or lower than those used in the previously discussed

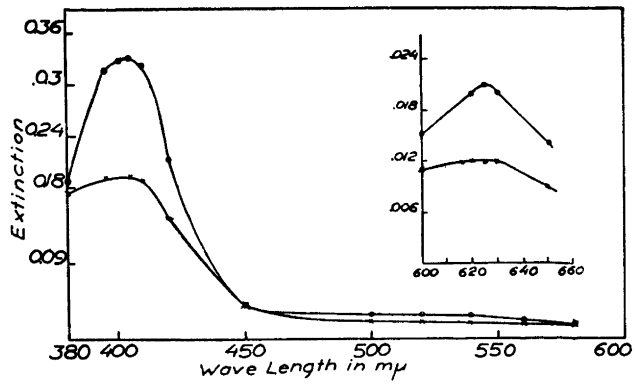

Fig. 5. Absorption spectrum of catalase in 5 M urea in pH 6.8, M/150 phosphate buffer at 20° C.

—O—O—O—O = *at zero time*
—×—×—×—× = *after 60 minutes incubation.*

denaturation studies were employed because definite turbidities developed in 5 *M* urea with a consequent marked increase in light absorption in the lower spectral region (below 350 mμ) at higher protein concentrations. The absorption spectrum of catalase no. 1 after 60 minutes in 5 *M* urea is shown in Fig. 5. It is to be noted that the relative decreases in extinctions at 405 and 625 mμ are identical. The rate of the extinction change with time was also recorded and is plotted to give Fig. 6. No change in absorption at 280 mμ was apparent. The rate of decrease in the extinction at 405 mμ is qualitatively similar to the activity decrease for this preparation under the same conditions as shown by inspection of Fig. 3. After incubation for 60 minutes with 5 *M* urea the decrease in extinction coefficient was 43 %. This is to be compared to the activity decrease of 60 %. Usually the decrease in activity was somewhat greater than the change in extinction coefficient. Different catalase preparations, however, show varying reactivities as regards the rate of spectral change in 5 *M* urea. Whereas catalase no. 1 showed a 43 % decrease in the absorption at 405 mu after 60 minutes, catalase no. 2 showed only a 25 % decrease. Preparation no. 3 was similar to no. 2 in this respect.

A single experiment on the effect of 5 *M* urea on the absorption spectrum of human hemoglobin and of horse myoglobin was also carried out. Both of these proteins showed a decrease (approximately 15—20 %) in the Soret absorption maxima. Contrary to the results for horse erythrocyte catalase they showed an increase in the absorption from 600—650 mμ on treatment with urea. The decrease in the absorption maxima in the Soret region for the above

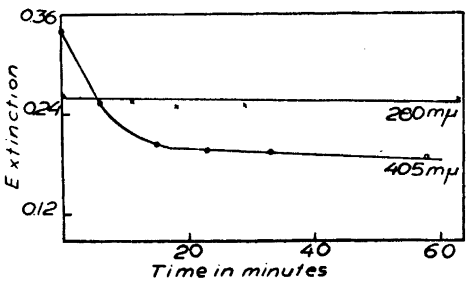

Fig. 6. Rate of change of absorption at 280 and 405 mμ by catalase in 5 M urea in pH 6.8, M/150 phosphate buffer at 20° C.

two hemoproteins appeared to be almost instantaneous in contrast to the catalase reaction. No further studies of the effect of urea on hemoglobin or myoglobin were carried out.

The decrease in the absorption maxima of the catalase at 405 and 625 mμ suggested that heme was being liberated as a result of the urea treatment. Proof of this was obtained in the following way. A catalase solution was treated with 5 *M* urea at 30° C for approximately one hour. The sample was then adjusted to pH 9 and dithionite added. The latter will reduce ferri heme to the ferro compound but is unable to effect this reduction of the iron in catalase. Following this CO was passed into the solution to form the CO ferroheme complex which at pH 9 should be rapidly converted into the hemochromogen. The controls for this experiment were a sample of pure heme and a solution of catalase to which urea was added immediately before treatment with CO. Inspection of these solutions with a hand spectroscope showed the presence of free heme in the catalase sample treated with urea. Attempts to put this test on a quantitative basis so that the decrease in absorption coefficient at 405 and 625 mμ could be compared directly with the rate of appearance of free heme in the solution were unsuccessful.

DISCUSSION

With few exceptions protein denaturation and biological inactivation have been reported to be first-order reaction rate processes. In our experience catalase does not obey such a reaction although it is possible that various species of catalase molecules may show this property individually. The nature of the heat and urea denaturation processes for horse erythrocyte catalase indicate the presence of molecules of widely varying reactivities in crystalline and supposedly pure enzyme preparations.

Catalase shows a strong positive temperature coefficient for the urea denaturation process as has been reported for such proteins as fibrinogen [11,12],

a carboxyhemoglobin [13] and chorionic gonadetrophin [14]. Although $\alpha\beta$-lactoglobulin [15] and egg albumin [16,17] show a negative temperature coefficient in this respect at high urea concentrations, Clark [18] has found a positive coefficient at lower urea concentrations for the latter protein. He has suggested that apparent negative temperature coefficients may be the result of secondary solubilizing reactions at the higher urea concentrations [19]. In this respect the use of an enzyme such as catalase avoids such considerations of solubility for one can utilize activity as a reaction guide. It is quite likely in the case of catalase that activity losses indicate earlier protein modifications than those represented by loss of solubility for when the latter occurred in this study almost all enzyme activity had disappeared. Furthermore, marked loss of activity in most cases was not synonomous with loss of solubility under the conditions utilized in this work.

Catalase was found to be most stable to both heat and urea denaturation near its isoelectric point. Apparently low concentrations of salt are to be preferred under any conditions which tend to give denaturation. The application of these two conditions to the more or less rigorous fractionation steps used in the preparation of catalase may possibly result in recovery of more uniform material.

A point particularly of future interest in the urea denaturation of catalase should be to determine whether this reagent causes a molecular dissociation. Horse hemoglobin dissociates to half molecules in the presence of 6.6 *M* urea [20–22]. Various other proteins [23–27] also show this type of reaction but substances such as egg albumin [20], serum albumin [28], gliadin [29], zein [24], pepsin [22] and several hemoglobins other than the equine form show no such change.

If horse erythrocyte catalase like its hemoglobin counterpart and the latter proteins undergoes a molecular dissociation in strong urea solutions it will be interesting to determine the effect of this on enzyme activity. Horse hemoglobin which has been split in this manner shows differences in oxygen equilibrium as compared to the intact molecule [30]. It was noted that catalase activity losses in urea were higher than the decreases in absorption at 405 and 625 mμ. It is possible that along with the removal of heme from the protein by the action of urea there is likewise a dissociation into two or more molecules and that in this form the catalase shows a diminished or loss of activity.

It would appear that such investigations would best await the development of fractionation methods giving more uniform catalase preparations of Kat.F. at least 120 000. Consistent evidence exists for the presence of material of this activity in erythrocytes. However, it is possible (but not likely) that erythrocyte catalases exist in nature as a series of molecules of varying activity and that such high activity material represents the more active portions of the enzyme.

Table 5. The effect of pH on the loss of activity of catalase no. 3 in 5 M urea at 20° C.

pH	Minutes incubation	$k_1 \times 10^2$		$\frac{k_{1\text{urea}} \times 10^2}{k_{1\text{control}}}$
		Control	Urea	
	0	2.93	2.93	
	5	1.12	0.98	88
4.0	10	1.01	0.79	78
	15	1.02	0.68	67
	0	2.93	2.93	
	5	1.41	1.46	104
5.6	10	1.31	1.02	78
	15	1.31	0.96	73
	0	2.93	2.93	
	5	1.13	1.01	89
6.8	10	1.10	0.91	83
	15	1.12	0.85	76
	0	2.93	2.93	
	5	1.46	1.53	105
7.8	10	1.46	0.65	45
	15	1.42	0.55	39

SUMMARY

Various crystalline horse erythrocyte catalase preparations appear to consist of a series of molecules of various reactivities. The enzymatic inactivation of this enzyme by heat and urea does not follow a first order reaction. The enzyme is most stable near its isoelectric point and in the presence of urea is more readily inactivated by the presence of high salt concentrations. Urea causes a decrease in the absorption maxima at 405 and 625 mμ that appears to be synonomous with the release of heme from the molecule.

The author wishes to acknowledge his indebtedness to Professor Hugo Theorell for his interest and helpful suggestions during the course of this investigation.

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Microbiological Determination of Free Amino Acids in Human Plasma

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Microbiological determinations of free amino acids have been carried out in deproteinized plasma of a number of species[1–5]. The assay procedure is particulary well suited for routine analyses of material containing small amounts of amino acid. However, such materials may contain minimal amount of peptides[6] and the lactic acid bacteria may also utilize an essential amino acid in combined form[7, 8]. The use of these methods without preliminary hydrolysis may accordingly be criticized. Nevertheless, such methods are useful when comparative results are desired and they might conceivably become a useful tool for investigations of pathological conditions.

Hier and Bergeim[4] used 16 ml of human plasma from 40—50 ml of blood for the analysis of the amino acids in tungstic acid filtrates. The volume of the final medium was 2 ml per tube. By diminishing the final volume of the medium to a tenth of this amount, it seemed probable that a determination of the 18 amino acids, (which are analyzed) by microbiological methods could be carried out on 4 ml of plasma from about 10 ml of blood. However, the repeated withdrawal of 10 ml of blood from the organism may be a serious draw-back, especially when working with small animals. A micro adaptation of the microbiological procedure was accordingly[9] found where the final developed volume of basal medium corresponded to a drop, 45 ml. Assays of 16 amino acids in a casein hydrolysate indicated that values could be obtained which closely agreed with those obtained in macroprocedures. In the studies reported here this method has been employed for the determination of free * amino acids in human plasma deproteinized with tungstic acid, trichloracetic acid, ferric sulfate, or ethanol.

* Free as used throughout implies microbiologically available amino acids, and may include combined forms as well as free amino acids.

MATERIAL AND METHODS

Preparation of plasma filtrates

The human subjects were volunteer medical students who had not fasted previous to removal of the 10 ml sample used for test. Clotting was prevented by the use of heparin. The blood from 20 students was pooled and centrifuged for 20 minutes. The plasma was divided in four parts. The first was precipitated with sulfuric acid and sodium tungstate as described by Hier and Bergeim [3]. The precipitate was removed by centrifugation. The pH was adjusted with 2 *N* NaOH to pH 6.8. A few drops of sodium hydroxide were usually adequate, thus having a negligible effect on the volume. Each ml filtrate represented 0.33 ml of original plasma. The filtrate was concentrated *in vacuo* to a third of the original volume. Each ml thus represented 1 ml of original plasma. The manometric α-amino nitrogen value was 0.061 mg per ml.

A second part of the pooled plasma was diluted with an equal volume of destilled water and a half volume of a fresh 50 per cent solution of trichloracetic acid was slowly added with mixing. The final concentration of trichloracetic acid was 10 per cent. The reagent was mainly removed by four extractions with ethyl ether in a separating funnel as previously described [10]. There was no trace of amino acids in the ether washings. The pH of the water phase was pH 5 and was brought to pH 7 with a few drops of 0.1 N NaOH. The water solution was concentrated *in vacuo*. 1 ml of the concentrated filtrate corresponded to 1 ml of plasma. The manometric α-amino nitrogen value was 0.069 mg per ml.

A third part of plasma, 20 ml, was deproteinized with ferric sulfate and sodium hydroxide according to Somogyi [11]. By this procedure the plasma filtrate contains a comparatively large amount of sodium sulfate which was practically removed as previously described [10] by concentrating the filtrate to 10 ml, followed by addition of 10 ml of absolute ethanol. The precipitate of sodium sulfate was redissolved in a minimal volume of water and precipitated twice at an ethanol concentration of 50 per cent. The combined ethanol centrifugates were concentrated to dryness and dissolved in destilled water. Each ml of solution corresponded to 1 ml of plasma. The manometric α-amino nitrogen value was 0.061 mg per ml.

A fourth part of the plasma was precipitated with four volumes of absolute ethanol. The precipitate was centrifuged and washed with 80 per cent ethanol. The combined centrifugate were concentrated *in vacuo* to a small volume and finally to dryness in a desiccator over P_2O_5. The dried material was extracted twice with dry ethyl ether and dissolved in destilled water. Each ml of solution represented 1 ml of original plasma. The manometric α-amino nitrogen value was 0.034 mg per ml. The four concentrated filtrates were preserved in a frozen state under toluene until analyzed.

Assay procedure

The general procedure has previously been described [9]. The sterilized concentrated filtrates were aseptically measured into the micro tubes either directly or in dilutions where 1 ml represented 0.66 or 0.33 ml of original plasma. The measured volume ranged from 25 to 3 μl and water was added to bring the volume to 25 μl in all tubes. 25 μl of the double strength, sterilized and inoculated basal medium were aseptically added to each tube. Microorganisms, basal medium, ranges of standard curves, and incubation

Table 1. Experimental conditions for the microbiological analyses.

Amino acid	Medium	Microorganism	Standard curve mμg per 50 μl	Incubation time, hours
Alanine	Dunn *et al.*[12]	*L. citrovorum* (8081)	125–1000	48
Aspartic acid	Henderson and Snell [13]	*L. mesenteroides* P–60	62–500	48
Cystine	» » »	*L. arabinosus* 17–5	15–125	24
Glutamic acid	» » » *	» » »	125–1000	»
Glycine	Dunn *et al.* [12]	*L. citrovorum* (8081)	62–500	48
Leucine	Henderson and Snell	*L. arabinosus* 17–5	31–250	48
Proline	» » » *	*L. mesenteroides* P–60	62–250	48
Serine	» » » **	*L. casei* (7469)	125–1000	48
Threonine	» » » ***	*L. fermenti* 36	125–750	48
Valine	» » »	*L. arabinosus* 17–5	62–500	48

* pH of basal medium 6.0.

** Single-strength medium diluted with an equal volume of water [9,14].

*** Citrate exchanged for acetate [21].

times are given in Table 1. The procedures followed for the culture and inoculum have been described in previous papers [9, 14, 15]. A casein hydrolysate was always included as an extra control in the determinations of the amino acids. For each amino acid several separate assays were carried out with an average deviation of approximately ± 10 per cent for all amino acids. In each series, five assay levels were used. The amino acids used as standards were dried *in vacuo*, at room temperature, and kept *in vacuo* in a desiccator containing silica gel. DL-forms of threonine and valine were employed. The natural isomers of the others were used.

RESULTS AND DISCUSSION

Three of the investigated amino acids, leucine, threonine, and valine were analyzed to facilitate a comparison with data obtained by Hier and Bergeim [4] on free amino acids in human plasma. The remaining seven compounds have not been determined in human plasma with microbiological methods, but alanine and glycine have been analyzed by Gutman and Alexander [16] by means of chemical methods. It was accordingly possible to control the reliability of several of the results obtained with the micro method used in the present investigation.

It was also possible to determine whether the use of tungstic acid filtrates, three times as concentrated as those used by Hier and Bergeim [3], had any effect on the microbiological data relative to that obtained with plasma

filtrates prepared by thrichloracetic acid, ferric, sulfate, and alcohol precipitation, where the precipitating agents had been removed from the filtrate. Such a comparison would be of interest since it could be supposed that some of the amino acids to be analyzed, aspartic acid, cystine and serine were present in such small amounts that it was necessary to use concentrated tungstic acid filtrates. The results obtained are summerized in Table 2.

Table 2. Amino acid content of normal human plasma precipitated in four different ways.

Reported as microgram of amino acid per ml of plasma.

Amino acid	Tungstic acid filtrate (I)	Trichloracetic acid filtrate (II)	Ferric hydroxide filtrate (III)	Filtrate from ethanol precipitation (IV)	Filtrate * dilution
Alanine	36.0	42.0	44.0	16.2	1.0
Aspartic acid	6.2	4.0	7.2	2.4	1.0
Cystine	7.0	6.6	0.8	4.2	0.33
Glutamic acid	36.4	35.4	40.0	7.5	0.33
Glycine	22.0	18.0	18.0	6.2	0.66
Leucine	17.2	11.8	19.5	6.5	0.33
Proline	41.0	38.2	36.5	13.5	0.66
Serine	5.6	9.8	8.2	2.8	1.0
Threonine	14.0	—	13.5	5.4	1.0
Valine	29.4	28.0	26.8	7.5	0.33

* The figures 0.33, 0.66 and 1.0 means that each ml of filtrate represents the corresponding volumes of human plasma.

The data indicate several interesting points. With tungstic acid precipitation, and in the filtrate dilution, 0.33, used by Hier and Bergeim [4] the remaining part of this acid in the filtrate does not inhibit the growth of the tested microorganism. The leucine and valine values obtained with filtrates I—III agree fairly well and also with those obtained by Hier and Bergeim. Cystine has not previously been determined in human plasma filtrate. Hier [18] using a microbiological method reports values ranging from 5 to 15 μg per ml plasma in dogs. The low value obtained in the ferric hydroxide filtrate may depend upon an oxidation of cystine to a microbiologically inactive form. The glutamic acid values may include a part of the glutamine fraction present in our filtrates. The conversion of biologically active glutamine to inactive pyrrolidonecarboxylic acid during the sterilization of the neutralized filtrates may only be partial [17].

Two amino acids have been determined in a filtrate dilution of 0.66, glycine and proline. The glycine values are in good agreement with those obtained by Gutman and Alexander with a chemical method [16]. Proline has not previously been determined in human plasma filtrate *, Henderson *et al.*[5] using a microbiological method obtained a value of 43 μg per ml of plasma in rats, more in accordance with our values.

Finally, four amino acids have been determined directly in the concentrated filtrates. Agreeing values were obtained practically throughout with filtrates I—III. The mean value for threonine given by Hier and Bergeim [4] is 20.8 μg $\pm$ 4.9 with several values around 12 μg in the series in good agreement with the values reported here. With a chemical method Gutman and Alexander obtained 37 μg of alanine per ml human plasma from non-fasting subjects. This value has been confirmed in these studies. According to the previous investigators [5, 19], the amounts of aspartic acid in human and dog plasma should be negligible. Our experience is that a small aspartic acid spot is a rather constant phenomenon on two-dimensional chromatograms of human plasma [20]. As shown in that paper human plasma also semms to contain a small amount of asparagine. The microbiological aspartic acid values reported in this paper may accordingly include some asparagine. Serine has previously not been determined in human plasma filtrate.

In accordance with the low α-amino-nitrogen value in the ethanol filtrate the microbiological determinations of the different amino acids carried out with this filtrate gave low results. Generally, the yield was about a third of that obtained with filtrates I—III. This result may be stressed with regard to the numerous attempts made to quantitatively evaluate the ninhydrin spots on two-dimensional chromatograms of ethanol filtrates from plasma and extracts of different organs.

SUMMARY

1. The micro adaptation of the usual microbiological procedure for the determination of amino acids previously reported has been used for analysis of amino acids in human plasma filtrate.

2. A comparison has been made of the amino acid content in filtrates from precipitation with tungstic acid, trichloracetic acid, ferric hydroxide, and ethanol. Agreeing result were obtained with the three first mentioned filtrates.

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* Alexander [19] quotes from a personal communication by S. W. Hier that the proline concentration in plasma should be negligible.

3. The presence of tungstic acid in the filtrates did not markedly influence the growth of the microorganism even when concentrated filtrates were analyzed.

4. Values are reported for cystine, proline and serine in human plasma filtrates. These amino acids have not previously been determined.

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The Reaction between Diphenylchloroacetylmethylurea and Alcohols in the Presence of Alkali

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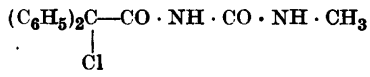
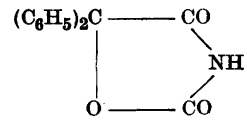
In 1943 Aspelund and Holmberg [1] published results of work concerning the reactions between diphenylchloroacetyl chloride and substituted ureas. They found that when N-diphenylchloroacetyl-N′-methylurea (I) reacted with alkali in approximately 22 per cent aqueous ethyl alcohol, there were formed a small amount of 5,5-diphenyloxazolidinedione-2,4 (II) and a relatively large amount of a chlorine-free substance melting at 106° after purification. This substance was not then examined, but when the experiment was repeated this year it was established that the chlorine-free substance was N-diphenylethoxyacetyl-N′-methylurea (III). This was done by allowing N-diphenylchloroacetyl-N′-methyl-



I



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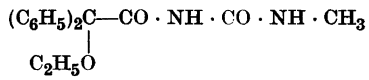
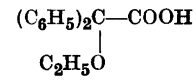


III



IV

urea and ethyl alcohol to react in a solution containing alkali and only a small amount of water and by treating the product with aqueous alkali. The first operation gave the chlorine-free substance and no 5,5-diphenyloxazolidinedione-2,4 and from the alkaline solution diphenylethoxyacetic acid (IV) was isolated.

In order to prove clearly that the chloro substituted urea had reacted with the solvent, the reaction was performed in n-propyl and isopropyl alcohol and the corresponding alkoxy derivatives were isolated. Alkaline degradation of these substances gave diphenyl-*n*-propoxyacetic acid and diphenylisopropoxyacetic acid, respectively.

One of the unusual features of this etherification reaction is that it occurs in 22 per cent ethyl alcohol, and comes to the end within five minutes. Diphenylchloroacetylmethylurea accordingly reacts much faster with alcohol than with water.

EXPERIMENTAL

Action of alkali on N-diphenylchloroacetyl-N′-methylurea in 22 per cent ethanol

The experiment of Aspelund and Holmberg was repeated and could be verified in detail. The chlorine-free neutral substance (m. p. 106°) proved to be N-difenylethoxyacetyl-N′-methylurea.

$C_{18}H_{20}O_3N_2$ Calc. N 8.97 Found N 9.10

One fourth of a gram of this substance was mixed with 20 ml of 1 N sodium hydroxide solution, and the mixture was first heated on the water bath for 23 hours and then boiled for four hours. When the clear solution had cooled a slight turbidity appeared, but it was removed by ether extraction. Hydrochloric acid was added to the aqueous solution whereupon 0.15 g of diphenylethoxyacetic acid precipitated. Its identity was established by a combustion analysis and from a mixed melting point determination with a sample of pure diphenylethoxyacetic acid.

$C_{16}H_{16}O_3$ Calc. C 74.98 H 6.29
» 75.03 » 6.28

Synthesis of N-diphenylethoxyacetyl-N′-methylurea in 94.5 per cent ethyl alcohol

Five grams of diphenylchloroacetylmethylurea were dissolved in 50 ml of 96 per cent ethyl alcohol and to this solution 1.85 ml of 50 per cent potassium hydroxide solution were added. Potassium chloride separated almost at once. The mixture was boiled for five minutes and the solvent was then evaporated under reduced pressure. Water, ether, and 0.6 ml of 50 per cent potassium hydroxide solution were added and the mixture was thoroughly shaken. After separation the ether layer was dried with sodium sulfate. When the ether had been evaporated, there remained 4.99 g of an oil which crystallized after some time. The solid substance was treated with water and filtered. After recrystal-

lization it melted at 105—106°. The yield was 3.22 g. The identity of this substance with the chlorine-free substance obtained in the experiment of Aspelund and Holmberg was established by the mixed melting point method.

When the alkaline aqueous layer was acidified with hydrochloric acid no substance separated.

Synthesis of N-diphenyl-n-propoxyacetyl-N′-methylurea

The synthesis was performed in the way described above from 2 g of diphenylchloroacetylmethylurea, 25 ml of n-propyl alcohol, and 0.74 ml of 50 per cent potassium hydroxide solution. Even in this case nothing could be isolated from the alkaline aqueous solution. The ether solution contained 2.13 g of an oil, which was treated with water after it had crystallized. The substance obtained was dissolved in 4 ml of ethyl alcohol and precipitated by adding 3 ml of water. The yield was 1.60 g and the substance, diphenyl-n-propoxymethylurea, melted at 109—110°.

$C_{19}H_{22}O_3N_2$ Calc. N 8.58 Found N 8.46

Eight-tenths of a gram of this substance was mixed with 5 g of potassium hydroxide dissolved in 100 ml of water. After the mixture had been heated for 23 hours on the water bath it was boiled for 6 hours. After cooling a slight precipitate was filtered and hydrochloric acid was added to the filtrate. Diphenyl-n-propoxyacetic acid separated and was filtered when the crystallization was complete. It melted at 114—115° and the yield was 0.45 g.

$C_{17}H_{18}O_3$	Calc.	C	75.53	H	6.71
		»	75.64	»	6.97

Synthesis of N-diphenylisopropoxyacetyl-N′-methylurea

This synthesis was performed as described in the preceding synthesis, isopropyl alcohol being used instead of n-propyl alcohol. The crude product did not crystallize when it was treated with water. The oil was therefore dissolved in a small amount of ethyl alcohol. When water was added 1.00 g of diphenylisopropoxyacetylmethylurea, m. p. 100—101°, precipitated.

$C_{19}H_{22}O_3N_2$ Calc. N 8.58 Found N 8.66

Half a gram of this substance was degraded with a solution of 4 g of potassium hydroxide in 80 ml of water for 23 hours. To complete the reaction the mixture was boiled for 6 hours. After acidifying, 0.30 g of diphenylisopropoxyacetic acid, m. p. 143—144°, could be isolated.

$C_{17}H_{18}O_3$	Calc.	C	75.53	H	6.71
	Found	»	75.30	»	6.85

SUMMARY

The neutral chlorine-free substance that Aspelund and Holmberg obtained when they treated N-diphenylchloroacetyl-N′-methylurea with alkali in 22 per cent aqueous ethanol is N-diphenylethoxyacetyl-N′-methylurea. The corresponding n-propoxy and isopropoxy derivatives have been prepared.

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On the Alkaline Alcoholysis of Benzenesulphonyl Chloride

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In a previous paper [1], in connection with the analytical procedure, attention was paid to the fact that the hydroxyl ion considerably accelerates the solvolysis of sulphonyl chlorides. This observation is not a new one. In the preparation of sulphonic esters, bases have generally been used to make the alcoholysis of sulphonyl chlorides more rapid. In general, the process has been carried out according to the Schotten-Baumann reaction by using alcohol and sulphonyl chloride in equimolar proportions in the presence of sodium methoxide, trimethyl amine, pyridine, or a concentrated aqueous solution of sodium hydroxide [2]. After a gradual mixing of the reagents, often in the presence of an indifferent liquid, the reaction mixture has been left to stay, during which period, if needed, it has been shaken. The reaction time has varied from ten minutes to several months. High yields of esters have thus been obtained at temperatures in the 0—15° range. Menalda [3] has investigated the influence of temperature and of the water content of the alcohol upon the yield of ester and the corresponding acid in Schotten-Baumann reaction. In this connection also the reaction with benzenesulphonyl chloride has been studied.

Since the alkaline alcoholysis of sulphonyl chlorides is of great importance as a method of preparation of sulphonic esters, and as on the other hand it influences in a special way the following of the course of the alcoholysis by acid-base titration, we have carried out some experiments on the velocity of the alcoholysis of benzenesulphonyl chloride in the presence of bases. The preparation and purification of the materials and the experimental method are, in their main features, described in the previous paper. Reactions conducted in the presence of sodium hydroxide, sodium ethoxide, or sodium acetate were stopped by a to 0° C precooled mixture of 70 ml of acetone and of a slight excess of 0.02 *N* aqueous hydrochloric acid. As an indicator in the titration of solutions containing acetate bromothymol blue was used, and

cresol red in all the other cases. The degree of advancement of reaction was calculated to give the percentage of the number of moles acid formed in the reaction compared with the number of moles sulphonyl chloride in the beginning of the reaction.

EXPERIMENTS

Experiment 1. Benzenesulphonyl chloride was weighed in the side tube of the reaction vessel [4], and sodium hydroxide solution in absolute methanol was placed in the bulb, and thermostated. After mixing the concentrations of both reactants were 0.05 moles/liter. Temperature 25.00° C.

	Time	Advancement of reaction
a.	960 sec.	98.9 per cent
b.	60	97.2
c.	17	98.1

Experiment 2. Solvent absolute methanol. Concentrations after mixing: benzenesulphonyl chloride 0.025, NaOH 0.05 moles/liter. Temperature 25.00° C

Time	Advancement of reaction
30 sec.	105.2 per cent

Experiment 3. Solvent absolute ethanol. Concentrations after mixing: benzenesulphonyl chloride 0.0394, sodium ethoxide 0.0394 moles/liter. Temperature 25.00° C

Time	Advancement of reaction
21 sec.	99.0 per cent

Experiment 4. Abs. ethanol. Concentrations: benzenesulphonyl chloride 0.0166, sodium ethoxide 0.0394 moles/liter. Temperature 25.00° C

Time	Advancement of reaction
25 sec.	105.0 per cent

Experiment 5. Benzenesulphonyl chloride 0.05 moles/liter, pure ethanol containing water 0.05 moles/liter. Temperature 25.00° C.

Time	Advancement of reaction
300 sec.	0.67 per cent

Table 1. Benzenesulphonyl chloride 0.05 moles/liter, Na-acetate 0.05 moles/liter in absolute methanol, t = time in minutes, p = percentage change, k_1 in sec.$^{-1}$ calculated from the first order and k_2 in $l \cdot mole^{-1} \cdot sec.^{-1}$ calculated from the second order formula.

t	25.00° C			17.00° C		0.00° C	
	p	$k_1 \cdot 10^4$	$k_2 \cdot 10^3$	t	p	t	p
5	15.5	5.60	12.2	8	12.5	60	14.7
10	21.2	3.97	8.99	30	25.5	168	25.8
21	33.8	3.28	8.12	32	28.4	260	33.1
30	40.4	2.88	7.54	61	37.9	391	39.3
51	51.5	2.37	6.95	91	46.9	985	61.7
60	56.1	2.29	7.09	120	52.9	1374	70.7
90	66.1	2.00	7.21	406	72.6		
122	72.7	1.77	7.27				

Table 2. Benzenesulphonyl chloride 0.05 moles/liter, Na-acetate 0.05 moles/liter in absolute ethanol, t = time in minutes, p = percentage change, k_1 in sec.$^{-1}$ calculated from the first order formula and k_2 in $l \cdot mole^{-1} \cdot sec.^{-1}$ calculated from the second order formula.

t	25.00° C			17.00° C		0.00° C	
	p	$k_1 \cdot 10^4$	$k_2 \cdot 10^3$	t	p	t	p
8	14.6	2.89	7.10	10	9.8	143	16.8
15	20.8	2.59	5.82	16	12.1	173	18.6
20	24.0	2.28	5.26	22	15.0	282	25.5
40	35.6	1.83	4.60	36	21.6	472	31.9
59	41.6	1.52	4.02	62	34.0	1129	47.2
81	47.2	1.32	3.68	120	40.1	2496	58.4
93	50.2	1.25	3.61	174	46.0	3952	58.7
121	53.6	1.06	3.18	312	54.9		
165	58.7	0.89	2.87	354	53.7		
245	62.5	0.67	2.27				

Experiments 6 and 7. Solvent acetone-water containing 50 volume per cent acetone. Concentration of benzenesulphonyl chloride 0.05 moles/liter. Temperature 0.00° C. 6. Pure solvent. 7. Sodium hydroxide, 0.05 moles/liter.

	Time	Advancement of reaction
6.	180 sec.	0.19 per cent
7.	180 »	50.12 »

Table 3. Benzenesulphonyl chloride 0.05 moles/liter, Na-acetate 0.032 moles/liter in absolute n-*propanol. t = time in minutes, p = percentage change.*

25.00 °C		17.00° C		0.00 °C	
t	*p*	*t*	*p*	*t*	*p*
10	13.2	31	15.3	156	14.8
23	20.2	78	28.0	314	21.4
40	28.2	119	34.1	513	28.0
79	41.6	217	45.2	1395	48.2
133	50.8	363	54.4	1411	48.6
315	66.1	472	58.8		
		590	63.2		

DISCUSSION

The acidity of the reaction mixture, except in cases where acetate was used, may be changed by the following over-all reactions:

(*a*) $RSO_2Cl + R'OH \rightarrow RSO_2OR' + HCl$
(*b*) $RSO_2Cl + R'OH \rightarrow RSO_2OH + R'Cl$
(*c*) $RSO_2OR' + R'OH \rightarrow RSO_2OH + R'OR'$
(*d*) $RSO_2OR' + HOH \rightarrow RSO_2OH + R'OH$
(*e*) $RSO_2Cl + 2NaOH \rightarrow RSO_2ONa + NaCl + H_2O$
(*f*) $RSO_2Cl + R'ONa \rightarrow RSO_2OR' + NaCl$
(*g*) $RSO_2OR' + NaOH \rightarrow RSO_2ONa + R'OH$
(*h*) $RSO_2OR' + R'ONa \rightarrow RSO_2ONa + R'OR'$
(*i*) $R'Cl + NaOH \rightarrow R'OH + NaCl$

In the absence of alkali the solvolysis (reactions (*a*) and (*b*)) at the temperatures used is slow [1]. Preparative experiments show that in the absence [5] as well as in the presence * of a base the reaction (*b*) has hardly any significance. Hence, the reactions of the alkyl chloride may be neglected. Thus, in p u r e a l c o h o l practically only the reaction (*a*) occurs, and the velocity experimentally measured is that of this reaction. In the presence of sodium hydroxide or sodium ethoxide the solvolysis of the sulphonyl chloride is so fast that the reactions (*b*), (*c*), (*d*), (*g*), (*h*), and (*i*) may be neglected [4].

* *E. g.*, we dropped rapidly a cooled 5 per cent solution of sodium methoxide in abs. methanol in an equivalent amount of benzenesulphonyl chloride which was cooled by keeping the vessel in ice-water mixture. The temperature was not allowed to rise above 20° C. Methanol was immediately evaporated in a vacuum, water added to the mixture, and the ester taken in ether. After drying with anhydrous sodium sulphate the ester was distilled at reduced pressure. The yield was about 95 per cent of theoretical (*Cf.*[11]).

The experiments 1 and 3 show that even within the shortest times possible in the measurements sodium hydroxide or sodium ethoxide has been entirely used. In the presence of an excess of either of these reagents (experiments 2 and 4) it may be said that processes (*a*) and (*f*) go in a few moments into completion. That the degree of advancement of the over-all reaction, as defined above, in both cases is a little over 100 per cent, is due to reactions [4] (*g*) and (*h*). If in experiments 1 and 2 as a fast step were the reaction (*e*), the degree of advancement of the over-all reaction must have been about 200 per cent instead of about 100 per cent. Thus the fastest step, even in the presence of sodium hydroxide, is the formation of ester. From experiment 5 we see that the great increase in reaction velocity is not due to water formed in the reaction $R'OH + NaOH \rightleftharpoons R'ONa + H_2O$. Experiment 6 shows that in acetone-water the reaction between sulphonyl chloride and water is so slow at 0° C that it does not appreciably occur during the titration of reaction mixtures in which the reaction is stopped by diluting with acetone precooled to 0° C [1]. Experiment 7 shows that sodium hydroxide accelerates greatly also the hydrolysis of sulphonyl chloride.

The experiments referred to above showed that in the presence of sodium hydroxide, or sodium alkoxide the alcoholysis of benzenesulphonyl chloride is so fast that it was not possible to follow the reaction by the method used. Therefore, the influence of sodium acetate was investigated, the reaction now proceeding with a measurable velocity. The results are given in Tables 1—3. The first order or the second order equation does not give a constancy for k (Tables 1 and 2, columns 3 and 4). Assuming that besides the pure alcoholysis, which is of the first order [1], a bimolecular reaction takes place between sulphonyl chloride and acetate ion (*j*), we should obtain:

$$\frac{dx}{dt} = k_1 (a—x) + k_2 (a—x) (b—x) \qquad (1)$$

Table 4. Benzenesulphonyl chloride 0.05 moles/liter, Na-acetate 0.05 moles/liter in absolute methanol, or in absolute ethanol, p = percentage change from curves, dx/dt = the slope of the tangent in mole · l^{-1} · sec.$^{-1}$ k_2 = second order rate constant in equation (1) in l · mole^{-1} sec.$^{-1}$, 25.00° C.

p	Methanol		Ethanol	
	$dx/dt \cdot 10^5$	$k_2 \cdot 10^3$	$dx/dt \cdot 10^5$	$k_2 \cdot 10^3$
10	2.03	8.48	1.02	5.49
20	1.21	4.49	0.702	3.88
30	0.828	3.53	0.438	2.99
40	0.548	3.05	0.268	2.29
50	0.387	2.65	0.149	1.40

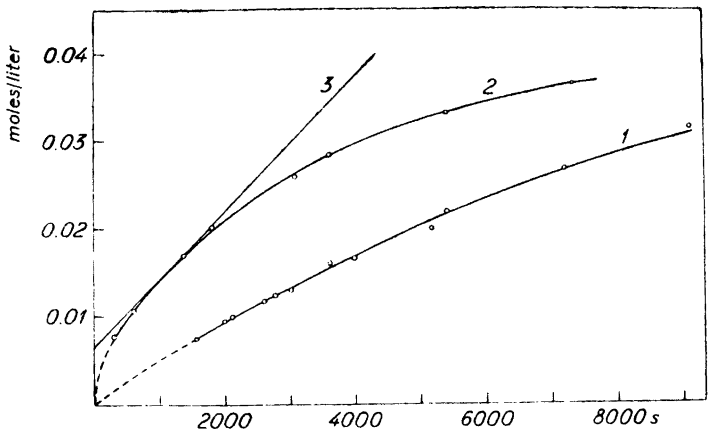

Fig. 1. *1. Benzenesulphonyl chloride (0.05 moles/liter) in pure methanol, 25° C. Data taken from the previous work (Ref. 1).*
2. Benzenesulphonyl chloride (0.05 moles/liter) + sodium acetate (0.05 moles/liter) in methanol, 25° C.
3. Tangent drawn to curve 2. Its slope represents dx/dt *at 30 per cent change.*

The value of k_1 is known from the previous work [1]. For the calculation of k_2, the integral of this equation is very complicated. By the graphical method, from the slope of the tangents drawn to the curve which represents x as a function of time (Fig. 1), however, dx/dt may be roughly evaluated at different times and k_2 so obtained as a function of time. Table 4 shows that no constancy for k_2 is thus obtained. This shows that the change of the composition of the reaction mixture is not governed only by these two reactions, or by one of them. The esterification of acetic acid formed in the reaction influences the acidity of the mixture, too, but this reaction is very slow [6], especially in the beginning, when the concentration of the acetic acid is small, and is, therefore, in the above consideration and in all following considerations neglected.

That the acetate ion reacts with the sulphonyl chloride has previously been shown [7] by heating solid sodium acetate with benzenesulphonyl chloride.

As a further test we carried out the following experiment. 10 g of benzenesulphonyl chloride were dissolved in 50 g of acetic acid made free from water and acetic anhydride according to Kilpi [8], and 10 g of dry sodium acetate (an excess) were mixed to this solution, a part of the salt remaining undissolved. After five days' staying at room temperature, a considerable amount of solid sodium chloride was formed. A quantitative determination of the chloride ion showed that about 70 per cent of the sulphonyl chloride had reacted. The filtered solution was distilled at reduced pressure. The distillate contained, besides acetic acid, acetic anhydride, and the solid residue (10 g) was composed of sodium acetate and a large amount of sodium benzenesulphonate.

According to the general scheme [1] the mechanism of the reaction may be supposed to be:

$$(j)' \qquad R \cdot SO_2Cl + CH_3COO^- \rightarrow Cl^- + RSO_2 \cdot O{-}CO \cdot CH_3 \xrightarrow[\text{or } CH_3COO^-]{CH_3COOH}$$

$$RSO_3^- + CH_3CO \cdot O \cdot COCH_3 + Cl^- \; (+ H^+).$$

In an alcohol the second step (fast) involves alcohol molecule instead of acetic acid or acetate ion:

$$(j)'' \qquad RSO_2 \cdot O - CO \cdot CH_3 + R'OH \longrightarrow RSO_3^- + CH_3COOR' + H^+.$$

In the alcohol solution, the equilibrium

$$R'OH + CH_3COO^- \rightleftharpoons R'O^- + CH_3COOH \qquad (2)$$

produces a small concentration of alkoxide ions the concentration of which in course of the reaction is given by the (approximate) formula

$$[R'O^-] = \frac{K_{alc.}}{K_{HAc}} \cdot \frac{b - x}{x} \qquad (3)$$

where b is the initial concentration of the added acetate and x the concentration of the acid liberated. $K_{alc.}$ is the ionic product of the alcohol, and K_{HAc} the acid constant of acetic acid in the alcohol used. Since the reaction (f) is very fast, as stated above, even the small concentration of $R'O^-$ produced by (2) must influence the measured over-all velocity.

If all the three reactions (a), (f), and (j) are taken into the consideration the rate equation will be

$$\frac{dx}{dt} = k_1 (a - x) + k_2 (a - x)(b - x) + k' \frac{(b - x)}{x} (a - x). \qquad (4)$$

As given above, k_1 is known from the previous work. The values of k_2 and k' could be obtained by evaluating dx/dt graphically at two points corresponding to different change percentages p_1 and p_2, and by resolving the system of the two equations. However, the possible errors in the drawing of the curves and their tangents are so large that the dispersion of the values of k' is too great to allow any accurate results of their constancy. Thus only very rough values may be obtained. In methanol solution, *e.g.*, at 25° C $k_2 \approx 1 \cdot 10^{-3}$, $k' \approx 3 \cdot 10^{-5}$. By using the values of the ionic product of methanol [9], $K_{MeOH} =$

2.2×10^{-17}, and the acid constant of acetic acid in methanol[10], $K_{HAc} = 2.24 \cdot 10^{-10}$, at 25° C, we obtain by means of the equation (3) for the bimolecular rate constant of the reaction (*f*) $k_f = \frac{K_{HAc}}{K_{alc.}} \cdot k' \approx 300$. For the reaction with ethoxide ion, the rate constant is roughly of the same order.

From preparative and analytical viewpoints, on the ground of the above results the following general conclusions can be made:

1. The formation of ester in systems formed by mixing benzenesulphonyl chloride, an aliphatic alcohol, and sodium hydroxide, or sodium alkoxide, judged on the ground of the formation of hydrochloric acid, is too rapid to be measured by means of the method used here. In the presence of sodium acetate the speed of the reaction is measurable, but the total reaction is complicated, and thus only rough evaluations of the rate of the reaction between benzenesulphonyl chloride and alkoxide ion are possible.

2. In preparations of sulphonic esters from benzenesulphonyl chloride and alcohols in presence of bases much shorter reaction times could be used than has been customary until now.

3. The proceeding of the reaction between sulphonyl chlorides and alcohols can be followed by the method used in the previous work[1].

SUMMARY

The alkaline alcoholysis of benzenesulphonyl chloride has been investigated and reactions possible in the system discussed.

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Studies on Liver Alcohol Dehydrogenase

I. Equilibria and Initial Reaction Velocities

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When reduced diphosphopyridine nucleotide (DPNH) forms a compound with horse liver alcohol dehydrogenase (ADH) the absorption maximum of DPNH shifts from 340 mμ to 325 mμ [1]. This observation opened up a possibility to carry out a more complete study on the stoichiometry, equilibria and kinetics in an enzyme-DPN system than had before been feasible. The reversible reaction studied in these two papers is:

$$\text{Alcohol} + \text{DPN} \underset{\text{ADH}}{\rightleftharpoons} \text{Acetaldehyde} + \text{DPNH} + \text{H}^+$$

The equilibrium constant,

$$K = \frac{[\text{DPNH}]\,[\text{Acetaldehyde}]\,[\text{H}^+]}{[\text{DPN}]\,[\text{Ethanol}]}$$

earlier determined by Negelein and Wulff [2] at pH 7.9, was recently redetermined at different pH values by Racker [3], using small concentrations of yeast alcohol dehydrogenase. He found about the same value as Negelein and Wulff at pH 7.9, $K = 1.15 \times 10^{-11}$, and furthermore found K to be independent of pH within the investigated range, in agreement with the above formula.

The kinetic properties of yeast alcohol dehydrogenase were studied to some extent by Negelein and Wulff [2]. They assumed that alcohol and acetaldehyde, as well as DPN and DPNH, combined reversibly with the protein, and found the "dissociation" constant to be 2.4×10^{-2} for ethanol-enzyme and 1.1×10^{-4} for acetaldehyde-enzyme; 9×10^{-5} for DPN-enzyme and 3×10^{-5} for DPNH-enzyme. However, in the theory developed by Negelein and Wulff no distinction was made between Michaelis constants and true dissociation constants.

As all the experiments of Negelein and Wulff were carried out at pH 7.9 the effect of variation in pH upon the kinetics was not investigated. Since hydrogen ions take part in the reaction it seemed to us to be of great interest to investigate the equilibria and reaction velocities at varied pH values.

There was another problem in this connection that attracted our attention. Practically all kinetic work on pyridine nucleotide enzyme systems has hitherto been carried out with very small concentrations of the enzymes, compared with the DPN or TPN concentrations. A rough calculation, however, shows that this ratio may be entirely unphysiological. Since the liver contains about 1 g of enzyme (ADH) per kg wet weight [4] and the molecular weight (see below) is 73 000 the liver alcohol dehydrogenase concentration is of the order of 10^{-5} M. If the ADH is located to parts of the liver cells, its concentration must be still higher there. The total concentration of DPN is higher [5] but we have to bear in mind that the DPN has to serve as prosthetic group for many other enzymes, so that the concentration of free DPN and DPNH is probably very low in the living cells. A difference between the dissociation constants of DPN-ADH and DPNH-ADH will cause the equilibrium constant K to be dependent on the ADH concentration. It might thus turn out to be a question of enzyme concentration whether the reduction of acetaldehyde or the oxidation of alcohol is preferred.

The substrates react only with the undissociated coenzyme-enzyme compounds. The redox potentials of these are a function of the ratio between the dissociation constant for the DPN-ADH complex (D_{ox}) and the DPNH-ADH complex (D_{red}). This follows from the simple fact that the enzyme-bound part of the coenzymes must be in potential equilibrium with the free coenzymes. The ratios between the concentrations of oxidized and reduced coenzyme in free and bound form, however, will evidently be a function of the dissociation constants of the complexes. Clark and his associates [6] have studied this effect in metalloporphyrin complexes with bases and derived the equations for different cases, but insufficient attention has still been paid to its implications in the pyridine- or flavine nucleotide enzyme systems. In the DPN-DPNH systems the following formula is found to obtain (at 30°):

$$E'_0 \text{ (complex)} = E''_0 \text{ (free DPN-DPNH)} + 0.030 \log \frac{D_{ox}}{D_{red}}$$

The assumption is made that the radical formation constant is low. If the ratio $\frac{D_{ox}}{D_{red}}$ is > 1 the redox potential in the case of ADH will be raised from the low level for the free DPN, DPNH equilibrium (— 0.282 V at pH 7 and 30° /Borsook [7]/) to a level nearer the normal potential of alcohol, acetaldehyde

(— 0.163 V at pH 7, 25° /Kalckar [8]/). The reaction velocity in the system would probably be favoured by the diminished potential difference, and the equilibrium shifted so that the oxidation of ethanol would be favoured.

We therefore undertook to study the equilibria between the reaction partners at pH 7, 8, 9 and 10 by the aid of spectrophotometry in the region 310—350 mμ. When the ADH concentration was increased to the "physiological" level around 1 mg per ml very marked effects on the equilibrium constant appeared.

Calculating the stoichiometry of the reactions required the molecular weight of the pure ADH to be known. Dr. K. O. Pedersen, Institute of Physical Chemistry, Upsala, kindly made this determination by ultracentrifugation and diffusion.

Further it was essential to determine the number of coenzyme molecules bound to one molecule of ADH. This was accomplished by titrating a certain amount of ADH with increasing amounts of DPNH, spectrophotometric readings being made at 350 and 310 mμ. Such experiments were carried out at pH 7.0 and 10.0. Further data were obtained from the equilibrium experiments at pH 7, 8, 9 and 10. It was found that one molecule of ADH (M = 73 000) combined with 2 molecules of DPNH at pH 7—9; at pH 10 the value was nearer to 1 mol DPNH/1 mol ADH.

The kinetics of the ADH-reactions were studied in different ways. The Michaelis constants for DPN, ethanol, DPNH and acetaldehyde, were determined spectrophotometrically in Beckman cuvettes from the initial reaction velocities of the appearance respectively disappearance of the 340 mμ band.

Most of the experiments on reaction velocities reported in this paper were carried out in Stockholm, but were completed with another set of determinations in Philadelphia. The results of the experiments with rapid spectrophotometry and the theory of reaction velocities in the ADH-system will be given in part II by H. T. and Britton Chance.

EXPERIMENTAL

Molecular weight. The sedimentation constant was found in two runs to be $s_{20} = 4.86$ and 4.90 Svedberg units, the diffusion constant, D_{20}, was found = 6.5×10^{-7} cm²/sec by the "normal" method, 7.3×10^{-7} by the "moment" method. We determined the partial specific volume to 0.751. From these values $M = 73\,000$.

It may be mentioned that a sample of yeast alcohol dehydrogenase, crystallized according to Negelein and Wulff [2] gave $s_{20} = 7.61$ which indicates its molecular weight to be about twice that of liver ADH.

The enzyme used in these experiments was prepared according to an earlier description [4,9]. The concentration of the protein was measured from its light absorption at 280 mμ, 1 mg of protein per ml in a 1 cm cell giving an extinction of 0.455 [4].

The activity of the enzyme was followed with a Beckman spectrophotometer by measuring the density of the band of DPNH at 340 mμ. The extinction value $\log I_0/I$, 340 mμ, d = 1 cm = 0.938 for a solution containing 100 μg per ml of DPNH was used [10].

This value can be recalculated to $\varepsilon = 6.25\ \text{cm}^{-1} \times \text{mM}^{-1}$. The activity determination has not before been given in detail. 3 ml solution in a Beckman cuvette is made up to contain: glycine-NaOH-buffer m/10, pH 9.6; 0.75 mg DPN; semicarbazide m/100; 0.05 ml ethanol. The extinction at 340 mμ is determined. A suitable quantity of enzyme, containing 15—20 μg of pure ADH in a small volume, is placed on a stirring rod and mixed into the cuvette at $t = 0$. The extinction at 340 mμ is read after 3 minutes. Our best preparations have given a density increase of 0.036 in 3 minutes per μg ADH at room temperature ($\sim$ 20°). This gives the maximal turnover number = 140 mol DPN/mol ADH $\times$ min.

This value is considerably lower than the value 220 given by Bonnichsen and Wassén [9]. Their value, however, was calculated on the basis of a too high extinction coefficient for ADH at 280 (1.0 instead of 0.455), and the assumption of M = 70 000. The recalculated value, on the basis of M = 73 000 and ε_{280} = 0.455, for their turnover number would be = 110. The difference from our somewhat higher value, 140, is due to the fact that ADH gives beautiful crystals together with some inactive protein that can be removed by repeated recrystallization.

Fig. 1 shows that the reaction velocity with DPNH and acetaldehyde as substrate is strictly proportional to the enzyme concentration. The same applies to the system DPN + ADH + ethanol at higher pH.

The actual concentration of ADH is computed according to the activity test to be

$$[\text{ADH}] = \frac{\mu\text{M DPNH/sec } \mu\text{M}}{2.34}$$

where 2.34 is the turnover number per sec.

The purity of the enzyme can be determined by dividing this value with the concentration of ADH determined from the extinction at 280 mμ:

$$[\text{ADH}] = \frac{\varepsilon_{280} \times 10^3}{33.3}\ \mu\text{M}$$

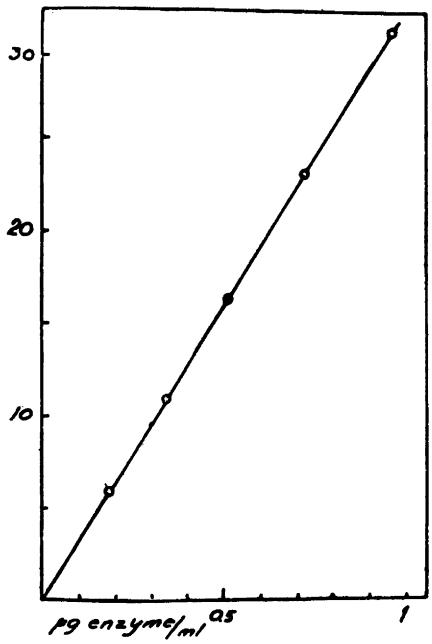


Fig. 1. Initial reaction velocity in μg DPNH per ml per minute with different quantities of ADH of 0.7 degree of purity. pH = 6.9, 0.05 M phosphate buffer. DPNH: 30 μg/ml. Acetaldehyde 3 × 10^{-3} M.

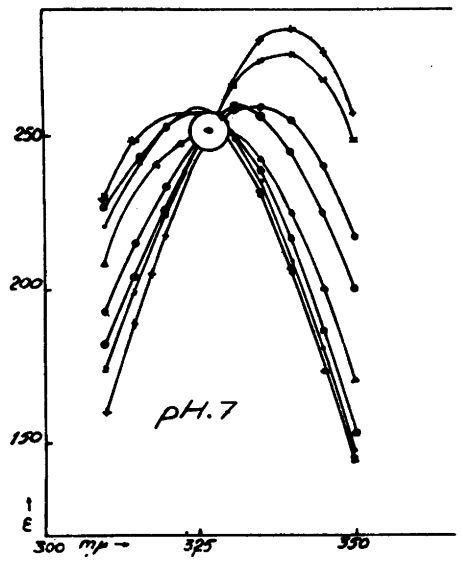


Fig. 2. Light absorption of DPNH with varied concentrations of ADH.
[DPNH] = 0.449 × 10^{-4} M
× [ADH] = 0.395 »
✳ » = 0.295 »
● » = 0.197 »
○ » = 0.148 »
△ » = 0.099 »
◇ » = 0.049 »
· » = 0.025 »
+ [ADH] extrapol. to 0

where ε_{280} is the optical density (cm^{-1}) at 280 mμ and 33.3 is the millimolar extinction coefficient of pure ADH. The optical density at 280 mμ would include protein impurities.

The DPN was prepared according to LePage [11]; the purity has varied from 20 to 65 %. DPN prepared by the method of Neilands [12], 60 to 85 % pure, has also been used; we found no difference in the results with 20 or 80 % pure DPN. The measurements of the DPN concentration and the preparation of DPNH were both made with the enzymatic method [13].

A number of experiments on the *equilibria at varied pH* and ADH concentration were carried out in 1 cm Beckman cuvettes in a thermostat at 20°. 0.05 m phosphate, or pyrophosphate, or glycine buffers of pH 7, 8, 9 or 10 were mixed with ADH that had been dialyzed against the same buffer, and suitable amounts of dilute ethanol added to make the volume = 3 ml. The

light absorption was measured at every 5 mμ in the region 310—350 mμ. At $t = 0$, 0.3 ml of DPN solution was added and readings taken at the shortest possible intervals.

The equilibrium was established instantaneously with high concentrations of ADH and then a further slow reduction of DPN followed. In such cases we extrapolated our reading to $t = 0$. When the ADH concentration was low we had to wait until the equilibrium was established. In these cases no further reduction occurred; it was thus very probably caused by some small amount of reducing material in the ADH.

After having made enough equilibrium readings we added more ethanol (0.1 ml, diluted 1 : 10) and semicarbazide to a final concentration of 0.01 *M*. Full reduction of the remaining DPN was obtained in a few seconds with the high, in 30—60 minutes with the lowest ADH concentrations. The light absorption in the 310—350 mμ region was read and corrected for the absorption determined before adding DPN. The dilution factors were taken into account.

Fig. 2 shows the extinction values ($\times 10^3$) obtained for the DPNH with increasing amounts of ADH at pH 7. It is seen how the absorption band 340 mμ of the free DPNH gradually moves towards 325 mμ, and decreases a little. All the curves intersect at 328 mμ; this is thus an isosbestic point for free and ADH-bound DPNH. The millimolar extinction coefficients for bound and free DPNH from $\lambda = 310$ to $\lambda = 350$ mμ are given in Table 1. The greatest difference in extinction occurs at 350 mμ (2.4 $cm^{-1} \times mM^{-1}$), whereas the difference at 310 mμ is somewhat smaller (1.5 $cm^{-1} \times mM^{-1}$). The values in Table I are plotted in Fig. 3. (corr. for the light absorption of ADH!).

The results of the equilibrium experiments at pH 7, 8, 9 and 10 are given in the Tables 2-5. The extinction coefficients are not included in the tables; instead the DPNH extinction coefficients at 310 and 350 mμ after full reduction are plotted against the molarity of the ADH in Fig. 4.

Table 1. Millimolar extinction coefficients for free, respectively ADH-bound DPNH from 310 to 350 mμ.

λ, mμ	310	315	320	325	328	330	335	340	345	350
Free DPNH	3.6	4.1	4.8	5.3	5.65	5.8	6.1	6.25	6.1	5.7
Bound DPNH	5.1	5.5	5.7	5.8	5.65	5.6	5.3	4.7	4.1	3.3

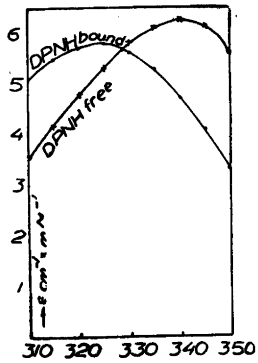


Fig. 3. Millimolar extinction of free and ADH-bound DPNH.

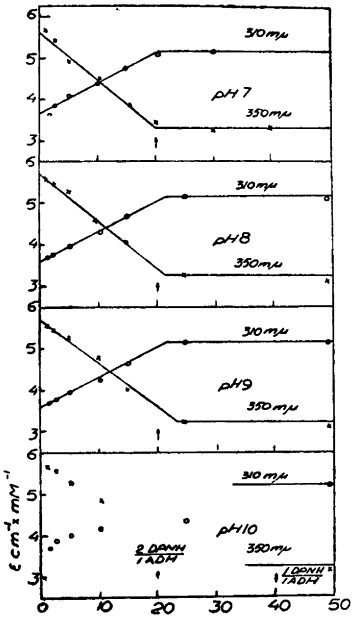


Fig. 4. The extinction coefficients of DPNH calculated from the equilibrium experiments in Tables 2—4. The abscissa gives the micromolarity of the added ADH (M = 73000) per 40 μM DPNH.

The Fig. 4 clearly shows that two molecules of DPNH are bound to each molecule of ADH at pH 7 and 8 and possibly somewhat less at pH 9. The values at pH 10 were more uncertain, but not far from 1 mol DPNH per mol ADH. It is further evident that both molecules of DPNH are so tightly bound to the ADH at pH 7—9 that no dissociation constants can be calculated at concentrations of the components. These determinations therefore had to be carried out at much higher dilutions with a more sensitive instrumental arrangement (See part II).

Some direct spectrophotometric titrations of a certain amount of ADH with increasing amounts of DPNH were carried out at 310 and 350 mμ as well in Stockholm as in Philadelphia. These experiments confirmed that 2 DPNH were bound per 1 ADH at pH 7. The values obtained at pH 10 seem to indicate that only 1 DPNH is bound per 1 ADH at this pH, and that the complex is more dissociated than at pH 7—9. A couple of these experiments are plotted in the Figures 5 and 6.

It is seen in the Tables 2—5 that the *equilibrium constant K* is increased with increasing ADH concentration to a new level in the presence of ADH in excess, around 200 times its initial value at pH 7. With increasing pH this effect is diminished, so that at pH 10 very little, if any, is left, see Fig. 7. The slight increase in K with the highest ADH amounts at pH 10 may as well

Table 2. Equilibria at pH = 7.0, 20°.

[ADH] × 10^4	[C_2H_5OH] × 10^4	[DPN]$_{Tot}$ × 10^4	% Red	$K \times 10^{11}$	log K+11	log [ADH] + 7
0.003	10 400	0.40	79.6	1.20	0.08	0.48
0.003	1 040	0.40	40.0	1.02	0.01	0.48
0.012	1 560	0.45	48.2	1.29	0.11	1.08
0.025	1 560	0.45	50.4	1.47	0.17	1.39
0.050	780	0.45	46.8	2.2	0.35	1.70
0.099	260	0.45	46.4	6.7	0.83	2.00
0.099	26.8	0.45	20.4	13	1.11	2.00
0.148	104	0.45	51.2	23	1.36	2.17
0.197	31.2	0.45	40.0	39	1.59	2.30
0.197	5.4	0.34	26.5	80	1.90	2.30
0.296	20.8	0.45	48.2	97	1.99	2.47
0.395	10.4	0.45	46.8	181	2.26	2.60
0.395	5.4	0.40	32.5	146	2.16	2.60
0.790	5.4	0.35	35.5	192	0.28	2.90

Table 3. Equilibria at pH 8.0, 20°.

[ADH] × 10^4	[C_2H_5OH] × 10^4	[DPN]$_{Tot}$ × 10^4	% Red	$K \times 10^{11}$	log K + 11	log [ADH] + 7
0.003	1040	0.40	71.4	0.69	− 0.16	0.49
0.003	104	»	35.1	0.73	− 0.14	0.49
0.012	520	»	65.5	0.96	− 0.02	1.09
0.025	520	»	66.3	1.00	± 0	1.39
0.050	156	»	53.5	1.60	0.20	1.70
0.099	26	»	33.0	2.50	0.40	2.00
0.148	10.4	»	40.4	11	1.04	2.17
0.247	5.2	»	50.4	41	1.61	2.40
0.493	5.2	»	64.9	96	1.98	2.70

Table 4. Equilibria at pH 9.0, 20°.

[ADH] × 10^4	[C_2H_5OH] × 10^4	[DPN]$_{Tot}$ × 10^4	% Red	$K \times 10^{11}$	log K + 11	log [ADH] + 7
0.003	104	0.40	78.0	1.08	+ 0.03	0.49
0.003	10.4	»	38.8	1.03	+ 0.01	0.49
0.012	31.2	»	58.4	1.06	+ 0.03	1.09
0.050	31.2	»	56.9	0.98	− 0.01	1.70
0.099	10.4	»	40.0	1.05	+ 0.02	2.00
0.148	5.2	»	45.8	3.1	0.49	2.17
0.247	3.6	»	49.5	5.0	0.70	2.40
0.493	2.6	»	54.9	10.0	1.00	2.70

Table 5. Equilibria at pH 10.0, 20°.

[ADH] × 10^4	[C_2H_5OH] × 10^4	[DPN Tot] × 10^4	% Red	$K \times 10^{11}$	log K + 11	log [ADH] + 7
0.003	10.4	0.4	63	0.45	− 0.35	0.49
0.003	1.04	»	41	1.35	+ 0.13	0.49
0.012	3.12	»	50.4	0.70	− 0.15	1.09
0.025	3.12	»	48.5	0.63	− 0.20	1.39
0.050	2.08	»	46.0	0.81	− 0.09	1.70
0.099	2.08	»	47.6	0.92	− 0.04	2.00
0.247	2.08	»	54.5	1.4	0.15	2.40
0.493	2.08	»	62.2	2.3	0.36	2.70

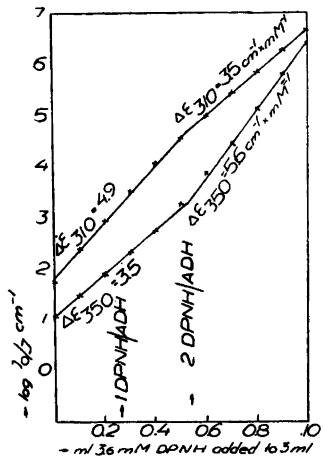


Fig. 5. Spectrophotometric titration of ADH with increasing amounts of DPNH, pH = 7.

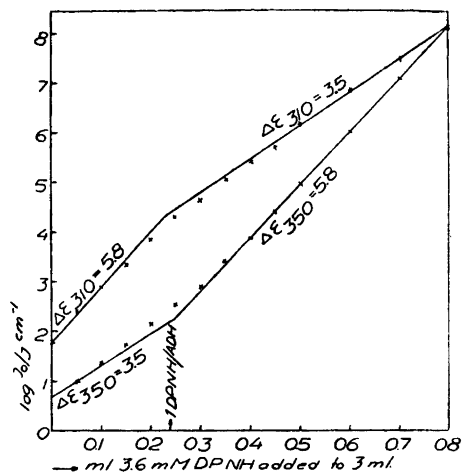


Fig. 6. Spectrophotometric titration of ADH with increasing amounts of DPNH, pH = 10.

be caused by traces of ethanol in the ADH-solution; a few micrograms would be enough to cause this effect at pH 10, but would have no significance at the lower pH-values.

The equilibrium constant, K, at low ADH-concentrations (0.004×10^{-4} M) was found to be independent of pH and $= 0.86 \times 10^{-11}$ on an average. This value agrees reasonably well with that found by Racker [3], 1.15×10^{-11}. He used yeast ADH; the difference between the two values may be a temperature effect, since Racker does not give any temperature data. It should be pointed out that K is independent of the ratio [DPNH] : [DPN] at low and high [ADH].

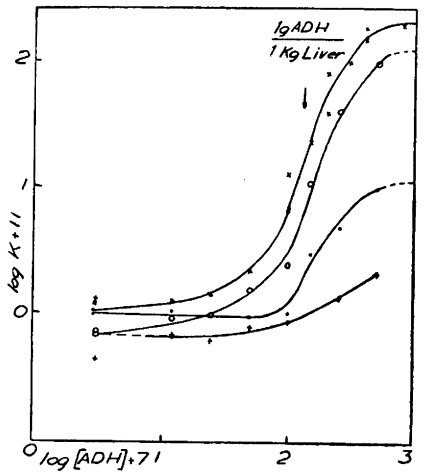


Fig. 7. Equilibrium constant, K, at varied [ADH] (M = 73000) and pH. Log K + 11 plotted against log [ADH] + 7.
× pH = 7
○ pH = 8
· pH = 9
+ pH = 10

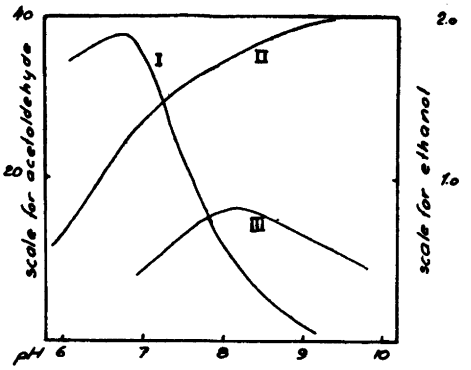


Fig. 8. Initial reaction velocity at different pH values. Buffers = 0.05 M phosphate and pyrophosphate. 0.5—1 µg/ml of ADH and 50 µg/ml of DPNH were used in the experiments with acetaldehyde; 4—8 µg/ml of ADH and 180 µg/ml of DPN with ethanol.

$v = \frac{c_0 - c_t}{t \times e} \cdot min^{-1}$

I = Acetaldehyde 10^{-3} M
II = Ethanol 2×10^{-2} M
III = » 5×10^{-4} M

but not at intermediate [ADH]. This is the reason why a considerable dispersion of the K values occurs in this region. Two determinations of K at pH 6.4, with excess of ADH, gave the values 128 and 134 $\times$ 10^{-11}.

If Borsook's (7) value for the redox potential of the DPN-DPNH system, — 0.282 V. at 30°, pH 7, is recalculated to 20°, we obtain — 0.275 V. Kalckar [8] calculated the potential of acetaldehyde-ethanol from Negeleins and Wulffs data; he obtained the value $E'_0 = -0.163$ V. at 25°. This corresponds to — 0.156 V at 20°. Our value of $K = 0.86 \times 10^{-11}$ at pH 7, and 20° would give the following value for the redox potential of acetaldehyde-ethanol:

E'_0 (20° C, pH 7) $= -0.275 + 0.0291 \log \frac{1}{0.86 \times 10^{-4}} = -0.156$ V. This is in exact agreement with Negeleins and Wulffs experimental data and Kalckars calculation.

The redox potentials for the ADH-bound coenzymes at pH 6.4—10 were calculated from the maximum values of the equilibrium constants in Tables 2—5, obtained with excess of ADH. The reasonable assumption was made that the slope 0.029 V/pH for the free DPN and DPNH, and the slope 0.058 V/pH for acetaldehyde and ethanol obtains in this pH range. The results are given in Table 6.

Table 6. Values calculated from the equilibrium constants for the redox potentials of the ADH-bound DPN—DPNH system.

pH	$E'_{0,\ \text{DPN, DPNH, free}}$	$E'_{0,\ \text{DPN, DPNH bound}}$	$E'_{0,\ \text{ethanol, acetaldehyde}}$
6.4	— 0.258	— 0.196	— 0.121
7.0	— 0.275	— 0.208	— 0.156
8.0	— 0.304	— 0.244	— 0.214
9.0	— 0.333	— 0.302	— 0.272
10.0	— 0.361	— 0.351	— 0.330

It is interesting to notice that the E'_0 for the enzyme complex approaches the level of the aldehyde-ethanol potential very considerably so that at pH 8 the difference is only 30 mV. The slope of the calculated E'_0 curve for the complex is partly steeper than for the free coenzymes, 0.058 V/pH from pH 8 to 9.5. The significance of this observation will be discussed later (p. 1121).

The K-values indicate that the equilibrium states for [DPN · ADH] = [DPNH-ADH] will be ethanol: acetaldehyde = 60 at pH 7, and = 10 at pH 8, instead of respectively $\sim$ 10 000 and 1 000 for the equilibria with small amounts of ADH. The coupling of the coenzymes to the enzyme thus in this case greatly favours the oxidation of ethanol to acetaldehyde — a reaction that would seem rather improbable without this effect. It should be noticed that acetaldehyde, when formed in the liver, is rapidly oxidized by aldehyde oxidase and thus removed from the equilibrium.

The *experiments on initial reaction velocities* were carried out as follows: The Beckman cuvettes were first charged with buffer, coenzyme and substrate. The enzyme was pipetted into a small excavation on the end of a small glass rod and stirred into the solution at $t = 0$. Readings were taken at 15 second intervals. Since the reaction velocity rapidly decreases with time the initial velocity had to be determined by extrapolating to zero time, and all activities of the enzyme referred to initial velocity. As an expression for initial velocity we use $\frac{c_0 - c_t}{t \times e}$ min.$^{-1}$, $c_0 = \gamma$ DPN/ml at zero time, $c_t = \gamma$ DPN/ml at time t, $e = \gamma$ enzyme/ml.

All the buffers used were made 0.1 % with respect to glycine in order to stabilize the enzyme; our stock solutions of enzyme contained 5—6 mg of

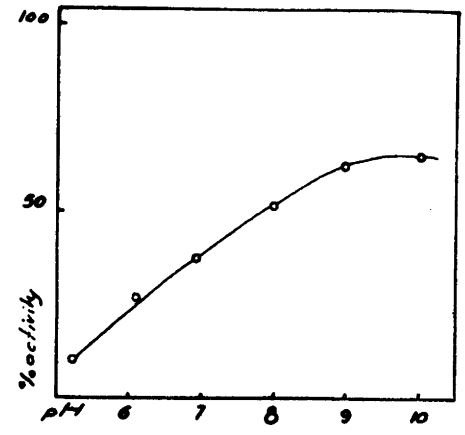


Fig. 9. Inhibition of initial velocity by DPNH. "100 % activity" is = initial velocity with 130 μg DPN. The inhibition experiments were carried out by mixing 25 μg of DPNH and 130 μg of DPN and measuring the initial velocity in phosphate and pyrophosphate buffers.

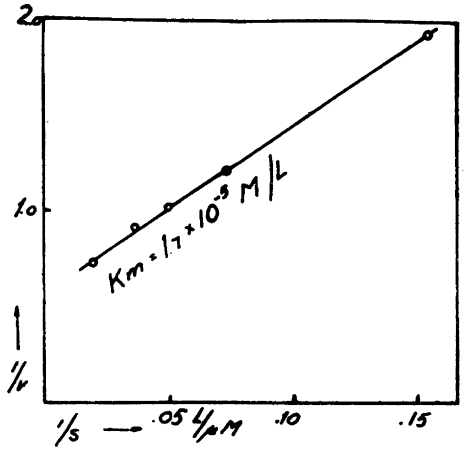


Fig. 11. Michaelis constant K_m for DPN. Alcohol 5.10^{-3} M, phosphate buffer pH 10.

$$V = \frac{C_0 - C_t}{t \times e} \; min^{-1}, \; S = [DPN]$$

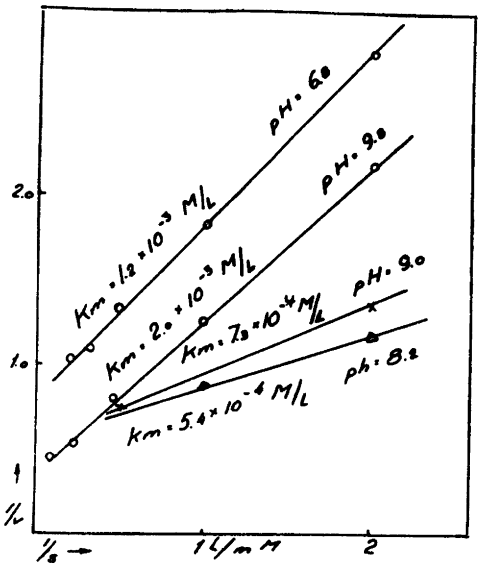


Fig. 10. Michaelis constant, K_m, for alcohol at different pH levels. DPN 180 μg of enzyme per ml.

$$V = \frac{C_0 - C_t}{t \times e} \; min^{-1}, \; S = [C_2H_5OH]$$

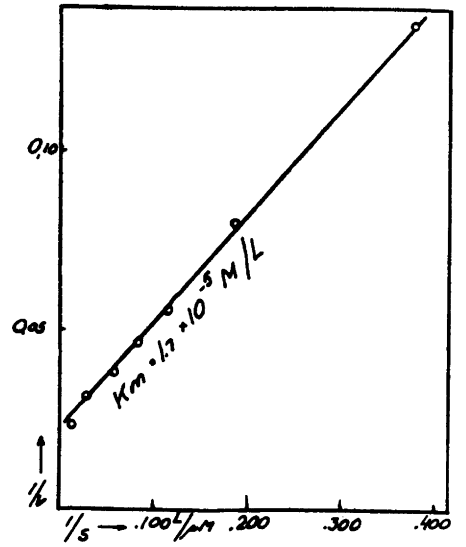


Fig. 12. Michaelis constant K_m for DPNH.
Phosphate buffer pH 10.
Acetaldehyde 5.10^{-3} M.

$$V = \frac{C_0 - C}{t \times e} \; min^{-1}, \; S = [DPNH]$$

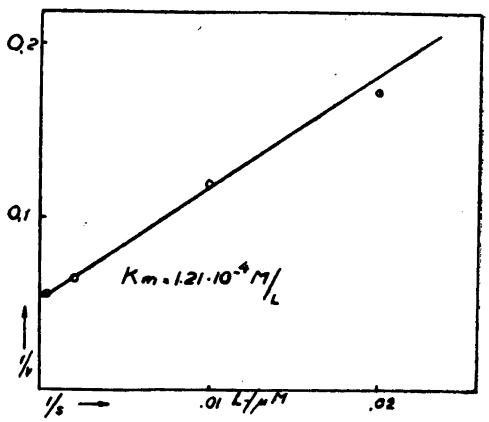


Fig. 13. Michaelis constant for acetaldehyde. Phosphate buffer pH 10; DPNH 6.12 μg per ml. 5 cm cuvettes were used.

$$V = \frac{C_0 - C_t}{t \times e} \; min^{-1}, \; S = [CH_3CHO]$$

enzyme per ml. Dilutions from this stock solution were made for each set of experiments, since solutions containing less than 50 μg per ml retained their full activity for only a few hours. When alcohol was used as substrate the buffers were made 0.01 M with respect to semicarbazide in order to bind the acetaldehyde.

The Fig. 8 shows the results of some experiments on the initial reaction velocities under varied conditions described in the figure legend. It is seen that DPNH and acetaldehyde are brought to rapid reaction by the ADH at pH 6 and 7. At higher pH-values the velocity drops sharply (curve I).

The curves II and III show that the reaction velocity in the system DPN + ethanol + ADH depends upon the pH in a somewhat complicated way. High concentrations of ethanol, far exceeding the Michaelis constant (see below) cause the reaction velocity to increase continuously from pH 6 to between 9 and 10 (curve II), whereas small concentrations (500 μM, curve III) lower than K_m give a velocity maximum around pH 8. The explanation is the difference in limiting factors in the two cases, as will be shown below and in part II.

The initial reaction velocity in the system DPN + ethanol + ADH is decreased in a pH-dependent way by the addition of DPNH (see Fig. 9). The effect is most pronounced at lower pH-values. This experiment shows that DPN and DPNH compete about the same position at the enzyme. It further illustrates the higher affinity to ADH of DPNH compared with DPN.

The Figs. 10—13 illustrate the results of some measurements on the initial reaction velocities under conditions given in the figure legends and plotted in the way suggested by Lineweaver and Burke [14]. These experiments were made in Stockholm in the way described above. In the figures, c_o, c_t and e are given

Table 7.

I	II	III	IV	V	VI
Compound varied in concentration	pH	K_m μM		V_{max} sec^{-1} corr. ($= k_3$)	Max. turnover number Mol. substrate / Mol. ADH min^{-1}
		Theorell and Bonnichsen	Theorell and Chance		
Ethanol	6.8	1.200		1.1 *	132
	8.2	540		1.5	180
	9.0	730		1.5	180
	9.8	2.000		5.5 *	330
Other alcohols	10	220—410		6.6	400
DPN	7		12		
	8		7		
	9		15		
	10	17	30	4.6	280
Acetaldehyde	7	120		45 [1]	5400
	8		52	17	2040
	9		2500	8	960
	10		1000	4.5	270
DPNH	7	17		39	4700
	7		13		
	8		4		
	9		—		
	10		6		

[1] K_m for DPNH taken as 15 μM.

in μg (γ) per ml. They were completed by some determinations in Philadelphia in the cases where low concentrations and high sensitivity were required (*cf.* part II). The results are summarized in the Table 7.

The values of K_m in column III are those given in the Figs. 10—13. The maximal reaction velocities, V_{max}, found in these experiments were converted to k_3 (for definition see part II) by using the known molecular weights of DPNH and ADH, and by converting minutes to seconds:

$$k_3 = \frac{1}{663} \times 73\,000 \times \frac{1}{60} V_{max} = 1.8\ V_{max}\ sec^{-1}$$

This value was used at pH 9.8—10, where ADH binds 1 DPNH; but where ADH binds 2 DPNH, thus at pH 6.8—9, the effective molecular weight of ADH is $\frac{73000}{2}$, and

$$k_3 = 0.9\ V_{max}\ sec^{-1}$$

In some cases the concentration of the reaction partner that was kept constant was found to have been insufficient to give maximal reaction velocity at high concentration of the variable partner. The values marked * in column V were then corrected by the aid of the known K_m for the constant partner.

The col. VI gives the maximal turnover numbers of substrate per mol. ADH (M = 73 000) and minute.

The K_m-values in col. IV are to be regarded as preliminary, especially in the case of acetaldehyde and DPNH, because these may to some extent react directly with one another even without ADH.

It is seen from the Table 7 that the K_m for ethanol passes a minimum around pH 8, and the same is true for DPN and acetaldehyde, whereas the K_m for DPNH shows a minimum at pH 9, too low to be determined with the available technique.

The Michaelis constants, compared with the values for the maximal velocities, readily explain the experimental results shown in Fig. 8. The reaction velocity with acetaldehyde and DPNH (curve I) decreases very rapidly when the pH increases from pH 7 to 10, because the K_m increases at the same time as V_{max} decreases. The reaction velocity in the DPN + ethanol system increases continuously with increasing pH in the presence of high concentrations of ethanol (II) because V_{max} increases, and because even the comparatively high K_m at pH 10 does not play any role. At low ethanol concentrations (III) the influence of the minimum in K_m at pH 8 predominates so that a velocity maximum at this pH will result. A pH-value of 9.2—10 is, however, to be preferred for determining ethanol quantitatively [15].

SPECIFICITY

The pure ADH does not react with methanol + DPN to any measurable extent. This is surprising since Røe [16] in clinical cases found ethanol to act as an antidote in methanol poisoning, supposedly by displacing "methyl alcohol from the inner surface of the cells". Zatman [17] in experiments with impure ADH, prepared according to Lutwak-Mann [18], found methanol to be oxidized at one ninth of the rate for ethanol. A competitive inhibition of methanol oxidation by ethanol was observed. The only possible explanation

seems to be that methanol oxidation *in vivo* requires some catalyst besides ADH + DPN.

The higher homologues of ethanol react if they contain the group $-\overset{|}{\underset{|}{C}}-CH_2-OH$. Thus secondary butanol reacts, but not tertiary butanol or isopropanol. Ethylene, glycol, and β-hydroxybutyric acid do not react, neither does acetone (with DPNH + ADH). It is obviously important for the practial use of the ADH-method [15] for determining ethanol in blood samples or organs that methanol, acetone, acetoacetic acid and β-hydroxybutyric acid do not react.

Some experiments on the initial reaction velocity on higher aliphatic alcohols are shown in Fig. 14. The Michaelis constants are lower for these alcohols than for ethanol, but the maximal reaction velocity is practically the same, as required by the theory (*cf.* part II). Lutwak-Mann [18] in experiments with impure horse liver ADH used several alcohols as substrate, and found the highest turnover with ethanol and slightly lower values for propanol. The reaction with amyl alcohol was slow and with methanol very slow.

In 1949, professor R. A. Morton, Liverpool, sent us a sample of crystalline retinene with the suggestion to try whether it could react with DPNH + ADH to give vitamin A. Before our experiments on this problem were carried out, Bliss [19] showed that liver extracts could convert the alcohol vitamin A to the corresponding aldehyde, retinene, and discussed the possibility that the ADH present in his extracts might be responsible for the reaction. This was confirmed in experiments on the reverse reaction with pure ADH + DPNH + retinene, carried out by one of us (R. B.) and R. Hubbard at the Carlsberg Laboratory in Copenhagen. It seems therefore possible that ADH may be present in the retina exerting another important physiological function in the formation mechanism of rhodopsin.

DISCUSSION

It is interesting to compare the normal potential values calculated here with some other oxidoreduction enzymes:

Cytochrome b:	+ 0.04 V
"Old" yellow ferment:	— 0.06 »
Free riboflavin:	— 0.185 »
ADH—DPN(H)	— 0.21 »
DPN(H)	— 0.28 »

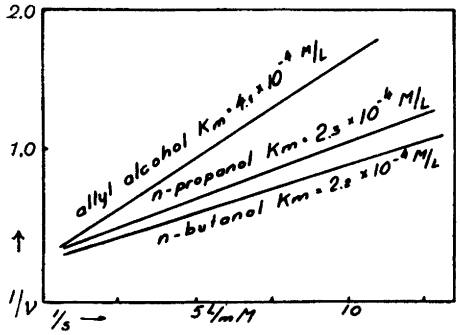


Fig. 14. Michaelis constants for various alcohols. Glycine-NaOH buffer pH 10. DPN 180 μg per ml. Enzyme 8 μg per ml.

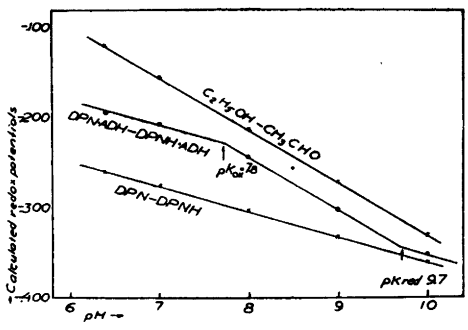


Fig. 15. Redox potentials in the ADH-system. The straight lines for $C_2H_5OHCH_3$-CHO and DPN-DPNH are calculated according to Borsook (7) and Kalckar (8). The points (o) on the DPN · ADH—DPNH · ADH line are calculated from the equilibrium data at high [ADH].

Unsuitably large potential differences seem thus to be overbridged by nature by the different dissociation constants in the coenzyme-enzyme systems.

In the flavin enzymes it is a well-known fact that absorption maximum at 445—450 mμ in the free riboflavinmono-or dinucleotides are displaced somewhat towards longer wavelengths when they are coupled to different enzyme proteins, for instance to 465 mμ in the "old" yellow ferment [20]. Kuhn and Boulanger [21] assumed this shift to be due to a linkage between the protein component and the NH (3) group in the riboflavin. In fact we were lead by analogy to look for an analogous shift in the 340 mμ band in DPNH-ADH.

Since it is a definitely established fact that the 340 mμ band is caused by the reduced pyridine ring in DPNH, the shift in absorption maximum on coupling with ADH must be caused by some group in the protein being linked in one way or the other to the pyridine. This would give an explanation to the differences in the dissociation constants of the DPN-ADH and the DPNH-ADH compounds.

The calculated redox potentials of the enzyme-coenzyme compound at different pH give additional evidence of the existence of an acid group in the ADH that is linked to the pyridine. As will be seen on Fig. 15 the slope $\frac{d E_0'}{d\,pH}$ is = 0.029 from pH 6.38 to pH 7.8, 0.058 up to pH about 9.7, and

probably 0.029 above this value, though further experimental data will be needed to verify this. The observed slopes indicate that an acid group in the DPNH · ADH with pK' 10 is influenced by the oxidation of DPNH · ADH, so that its pK' is changed to 7.8. This is readily understandable since the tertiary, uncharged pyridine-N in DPNH becomes a quaternary $\backslash\overset{+}{\underset{|}{N}}\diagup$ on oxidation. The difference in pK', two pH-units, is of the expected order of magnitude in such a case. If the DPNH-linked group in the protein is titrated, with p$K' = 10$, so that the bond to the DPNH is broken, one would expect ADH to bind half as much DPNH at pH 10 as at lower pH values, as in fact was found experimentally.

Proteins may contain three kinds of groups with pK-values around 10, amino groups, tyrosine hydroxyls and sulfhydryl groups. G. Wald and R. Hubbard kindly informed us very recently that the ADH-activity was strongly inhibited by *p*-chloromercuribenzoic acid, and that this inhibition was reversed by glutathione. We therefore measured the extinction coefficient of the DPNH—ADH complex at pH 7 before and after the addition of *p*-chloromercuribenzoic acid (0.0003 M). *The maximum at 325 mμ was immediately shifted to 340 mμ upon the addition of the inhibitor.* Monoiodoacetic acid, that according to Lutwak-Mann [18] does not inhibit the ADH activity, had no influence on the position of the band, nor had chloroacetophenone.

These observations seem to demonstrate that 1) *a sulfhydryl group in the ADH is linked to the dihydropyridine in DPNH*, 2) that this linkage causes the band shift from 340 to 325 mμ, and 3) that this linkage is essential for the enzymatic activity of the DPNH—ADH complex. ADH gives no color in the nitroprusside test. The sulfhydryl groups if present are thus masked in some way ("b-groups", Hellerman *et al.*[22]) so that they do not react with this reagent nor with monoiodoacetic acid or chloroacetophenone.

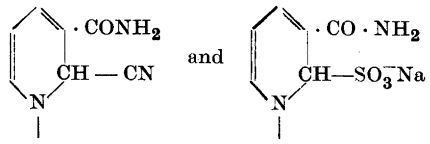
The activity of the alcohol dehydrogenase from yeast is according to Lutwak-Mann [18] inhibited by monoiodoacetic acid. It is therefore possible that this ADH contains unmasked sulfhydryl groups coupling to DPNH in an analogous way as in liver-ADH. We hope to be able to report shortly if the 340 mμ band of DPNH is displaced by the complex formation with yeast ADH.

As far as we know the possibility of a direct interaction of enzyme SH-groups with coferments like DPN and TPN has never before been considered. Work on other DPN-linked enzyme systems than ADH is in progress. It is interesting to recall that Hellerman, Lindsay and Bovarnich [23] found *d*-aminoacid oxidase to be inhibited by p-chloromercuribenzoic acid in competition with the prosthetic group, flavineadenine-dinucleotide. A

similar mechanism thus seems to operate in this flavine enzyme as in the ADH-DPN.

Evidently the linkage HS- to pyridine can stabilize the bond between DPNH and ADH, so that the dissociation constant of the DPNH · ADH becomes 200 times lower than for DPN · ADH at pH 7. It is further to be expected that this difference would be diminished at higher pH for the following reasons. As will be shown experimentally in part II, the dissociation constant, D_{red}, of the ADH · DPNH is 10^{-7} at pH 7 and 3×10^{-6} at pH 10. The D_{ox} moves the opposite way with pH, from 2×10^{-5} at pH 7 to a lower value near 3×10^{-6} at pH 10.

Meyerhof, Ohlmeyer and Möhle [24] found that DPN forms addition compounds with cyanide and bisulfite, presumably of the formulas



The reactions were reversible and, in the case of bisulfite, rapid. "Bisulfit reagiert so rasch dass in seiner Gegenwart die enzymatische Reoxydation von CzH_2 spektroskopisch nicht gemessen werden kann." The absorption band in the near ultraviolet *had its maximum at 320 mμ* instead of 340 mμ. Our DPNH-ADH complex may thus be analogous with the bisulfite-DPN addition product.

It would be tempting to speculate upon the different possibilities implied in these observations. However, we still lack information on the amino acid composition and the nature of the sulfur compounds in ADH. Some preliminary experiments showed that simple sulfhydryl compounds like thioglycolic acid, cysteine, and glutathione do not give any spectroscopically observable addition compounds with DPN at pH 7—10. The nitroprussid test remained negative after the addition of an equivalent amount of DPNH to ADH at pH 9.5.

DPN and DPNH may further be assumed to be linked by way of their phosphoric acid components to some unknown group or groups in the ADH. This linkage, however, would probably not be influenced to any great extent by the oxidation state of the pyridine component.

Much more work has to be done in order to elucidate the chemical mechanism of the interaction between ADH, DPN and alcohol. Such work on the basis of the observations presented above is in progress.

THE PHYSIOLOGICAL OXIDATION OF ALCOHOL

Ethanol is combusted in the human body, mainly in the liver, at a rate of 7 g per hour, at 70 kg body weight. The rate is independent of the ethanol concentration down to the limit of the Widmark test, around 0.1 $^0/_{00}$ in the blood[26]. In terms of micromolarity this figure corresponds to 2 000 μM, around three times the K_m at physiological pH. It is thus obvious that a rectilinear course of the physiological ethanol combustion is to be expected from the kinetic data down to values too low to be determined by the Widmark method, under the assumption that the k_3 of human DPNH-ADH is not largely different from horse DPNH-ADH. The content of ADH in the horse liver can now be calculated under the assumption that enough of free DPN is available to give maximal turnover of ethanol. If 1 molecule of ADH, M = 73 000, turns over 140 mols of ethanol per minute, and the observed rate is 7 g ethanol per hour, 1.32 g ADH would be required. This figure agrees very well with the value estimated from the preparative yields, about 1 g per kg liver, since a human liver weighs about 1.5 kg. It thus seems reasonable to assume that the ADH content will be about the same in horse and human liver. The turnover number is very probably higher at 37° than at 20°, so that less ADH would be required, but on the other hand it is not probable that the ADH works quite at maximal velocity, with excess of DPN, under physiological conditions. These effects would more or less compensate one another.

The results discussed above seem to give convincing evidence to prove that ADH is the enzyme essentially responsible for the physiological combustion of ethanol. The possibility of an oxidation of alcohol by catalase and hydrogen peroxide has been considered by Chance[27]. In the case of ethanol, such a mechanism may not play any great role because the rectilinear course of ethanol oxidation is scarcely compatible with the catalase kinetics. On the other hand the physiological oxidation mechanism of methanol actually proposed by Chance[27], is rendered more probable by these data which show no methanol activity for liver ADH.

SUMMARY

1. The molecular weight of horse liver alcohol dehydrogenase (ADH) is = 73 000, that of yeast alcohol dehydrogenase is around twice as high.

2. When increasing quantities of ADH are added to DPNH, the absorption maximum shifts from 340 to 325 mμ and becomes somewhat lower. There is an isosbestic point for free and ADH-bound DPNH at 328 mμ. The millimolar extinction coefficients for both forms in the region 310 to 350 mμ are given.

3. By the aid of these extinction differences it was possible to establish that two molecules of DPNH are bound to one molecule of ADH from pH 7—9, at pH 10 around 1 DPNH/1 ADH.

4. The equilibrium constant

$$K = \frac{[\text{DPNH}]\,[\text{CH}_3\text{CHO}]\,[\text{H}^+]}{[\text{DPN}]\,[\text{C}_2\text{H}_5\text{OH}]}$$

was found to be raised on the addition of increasing amounts of ADH to new levels when ADH was in excess over DPNH. K became around 200 times higher at pH 6.4—7.8, around 20 times higher at pH 9, and only slightly higher at pH 10.

5. This increase in K is due to the DPNH being more firmly bound to ADH than DPN. The redox potentials of the coenzyme-enzyme complex were calculated from the equilibrium data and were found to approach the level of the potentials for the ethanol-acetaldehyde. The conclusion is drawn that the coupling of DPN and DPNH to ADH greatly facilitates the oxidation of ethanol to acetaldehyde.

6. Data for the Michaelis constants and the maximal reaction velocities, and their variations with pH are given.

7. The substrate specificity of the ADH was studied. It is especially interesting that the ADH-system does not react with methanol, but with many higher primary alcohols, including vitamin A.

8. *p*-Chloromercuribenzoic acid inhibits the enzyme activity (G. Wald and R. Hubbard). We found the same reagent to cause an immediate shift of the 325 mμ band of the DPNH-ADH complex to 340 mμ. It is concluded that sulfhydryl groups of the ADH are linked to DPNH in the active complex, most probably to the pyridine ring, since the whole absorption band derives from this part of the molecule. This seems to be the first observation of pyridine nucleotides being activated by enzyme SH-groups.

9. The concentration of ADH in the liver, around 1 g per kg wet weight, fits very well to the known rate of the physiological ethanol oxidation. The Michaelis constant gives an explanation of its rectilinear course with time. It is concluded that the ADH-system, and not catalase + H_2O_2, is the physiological catalyst for ethanol oxidation.

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Studies on Liver Alcohol Dehydrogenase

II. The Kinetics of the Compound of Horse Liver Alcohol Dehydrogenase and Reduced Diphosphopyridine Nucleotide

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The elucidation of the mechanism of enzyme action by means of direct measurements of enzyme-substrate compounds has previously been limited to the peroxide compounds of the hemoproteins [1]. In catalases and peroxidases, the addition of substrate causes changes of absorption spectra that are readily measurable with rapid and sensitive spectrophotometric methods [2]. Theorell and Bonnichsen [3] have recently found a small shift in the spectrum of reduced diphosphopyridine nucleotide (DPNH) upon the addition of liver alcohol dehydrogenase (ADH) **. This paper describes kinetics and equilibrium studies of the enzyme-substrate compound, ADH-DPNH, in the reduction of aldehyde (Ald) to alcohol (Alc). The reactions of this enzyme-substrate complex are outlined by the following equations,

$$\text{ADH} + \text{DPNH} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{ADH-DPNH} \qquad (1)$$

$$\text{H}^+ + \text{ADH-DPNH} + \text{Ald} \underset{}{\overset{k_4}{\rightleftharpoons}} \text{ADH-DPN}^+ + \text{Alc} \qquad (2)$$

$$\text{ADH-DPN}^+ \underset{k_5}{\overset{k_3}{\rightleftharpoons}} \text{ADH} + \text{DPN}^+ \qquad (3)$$

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** The symbol ADH is here taken to represent the portion of the alcohol dehydrogenase molecule that binds one molecule of DPNH.

and the assumptions and simplifications represented by these equations are discussed below. The values of the equilibrium constant for Eqn. 1, and for the values of the reaction velocity constants, k_1 and k_4, are determined experimentally and are compared with values computed from studies of the overall activity of this enzyme.

PREPARATIONS

ADH and DPNH were prepared as described by Bonnichsen[4, 5] and had purities of 70—100 % and 69 % respectively. The actual concentration of ADH was computed according to part I.

The concentration of acetaldehyde was tested enzymatically with ADH and excess DPNH at pH 7.0. The concentration of formaldehyde was tested by the method of MacFadyen[6].

METHODS

The spectroscopic data of Theorell and Bonnichsen show that the largest changes of molecular extinction coefficient ($\Delta\varepsilon$) of the DPNH spectrum caused by the addition of ADH occur at 310 and 350 mμ. The actual values of $\Delta\varepsilon$ are small, 1.5 and 2.4 $cm^{-1} \times mM^{-1}$ at 310 and 350 mμ respectively. There is an isosbestic point between the DPNH and ADH-DPNH spectra (after subtraction of the ADH absorption) at 328 mμ. But there is no single wavelength where the formation of ADH-DPNH may be recorded without recording changes in [DPNH] at the same time.

In equilibrium studies of the ADH-DPNH complex (part I) it is possible to correct for the DPNH or ADH absorption, but it is neither practical nor accurate to do this in kinetic studies.

Thus the problem of measuring the reaction kinetics of the ADH-DPNH complex is a much more formidable one that that of measuring the hydrogen peroxide compounds of catalases and peroxidases. In the latter case the substrate has negligible absorption in the region of the enzyme and thus the measurements of the enzyme kinetics are not interfered with. In addition the molecular extinction coefficients of catalases and peroxidases change by about 50 $cm^{-1} \times mM^{-1}$ at 405 mμ on combination with peroxide. In the case of the ADH · DPNH complex, the change of extinction coefficient is much less than that of the hemoproteins, namely 2.4 $cm^{-1} \times mM^{-1}$ at 350 mμ and the DPNH absorption is relatively large at this wavelength ($\varepsilon_{350} = 5.7$ $cm^{-1} \times mM^{-1}$). It has been necessary to develop a spectrophotometric method that will respond only to the formation and disappearance of the ADH-DPNH complex and reject the changes of light absorption caused by DPNH alone.

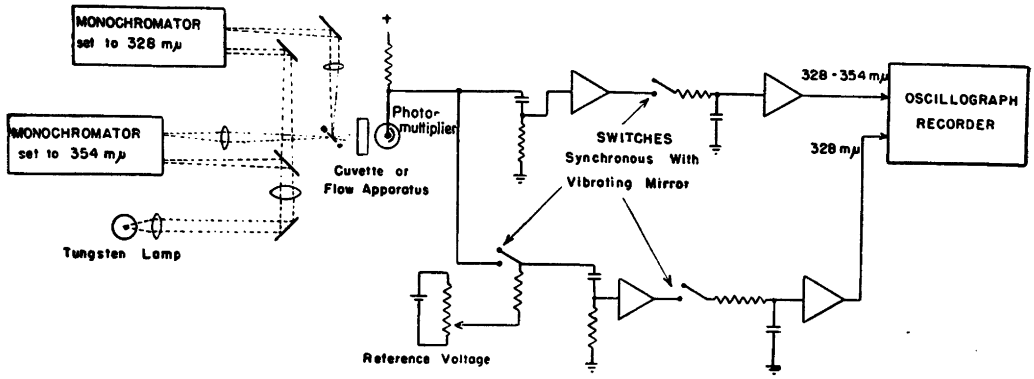

Fig. 1. A block diagram of the double beam system for measuring the difference of light absorption at 328 and 354 mμ as well as the light absorption at 328 mμ only. All parts of the optical system, except the lamp bulb, are quartz. The mirror and the switches vibrate at 60 cps. The symbol —▷— represents an amplifier. (MD-13.)

A consideration of the spectrum of DPNH shows that the extinction coefficients of DPNH are very nearly equal at the wavelengths 328 and 354 mμ. Thus a spectrophotometric method that measures only the difference of light absorption at these two wavelengths effectively rejects changes in concentration of DPNH. On the other hand the difference of extinction coefficients of the ADH-DPNH complex at these two wavelengths is measurable (about 1.4 $cm^{-1} \times mM^{-1}$ — see Table 1 of part I).

By alternately flickering light of a wavelength of 328 and 354 mμ through the ADH + DPNH solution and thence to a photocell, an alternating current wave is obtained whose amplitude represents only the difference of light absorption caused by the ADH-DPNH complex. And by more complex electronic circuits [2], it is possible to record simultaneously the change of light absorption at 328 mμ which is a measure of the [DPNH] since the ADH-bound and the free DPNH spectra have an isosbestic point at this wavelength. Fig. 2 illustrates the performance of this circuit. The lower trace indicates the deflection recorded by the circuit operating at 328 mμ when buffer alone and buffer plus 8.4 μM DPNH are alternately placed in the light path. The upper trace shows that the change of [DPNH] causes a change of only 1 % in the circuit measuring the difference of light absorption at 328 and 354 mμ. The wavelength readings for optimum rejection vary about ± 0.5 mμ. Since the wavelengths must be set accurately in order to achieve good rejection of DPNH, an experimental test similar to that of Fig. 2 is made before each experiment and the longer wavelength is adjusted to give optimum rejection (see Fig. 3). Thereby thermal drift of the monochromators is eliminated.

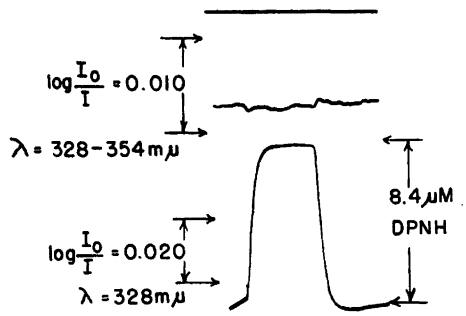

Fig. 2. The measurement of 8.4 μM DPNH by the optical density change at 328 mμ (lower trace) and the nearly complete rejection of this optical density change by a differential measurement between 328 and 354 mμ (upper trace) (Expt. 7b).

Under actual experimental conditions the ratio [DPNH] : [ADH] rarely exceeds 2 and the error in the measurement of [ADH · DPNH] caused by [DPNH] is about 2 %.

The sensitivities used in Fig. 2 are representative of those necessary for the measurement of the equilibrium and kinetics of the reactions of ADH and DPNH. The random fluctuations of the upper trace correspond to an optical density increment (log I_0/I) of 5×10^{-4}. In view of the small errors caused by random fluctuations and by the change of [DPNH], it is possible to use concentrations of ADH and DPNH as low as a few micromolar.

A typical record of the titration of dilute ADH with DPNH is given in Fig. 3. In this case an open 3 ml cuvette is used and 0.01 ml additions of 0.63 *mM* DPNH are made with a stirring rod. The first deflection of the traces only represents the stirring of the solution and it is seen that no net change of optical density results. Each of the successive deflections (except the last

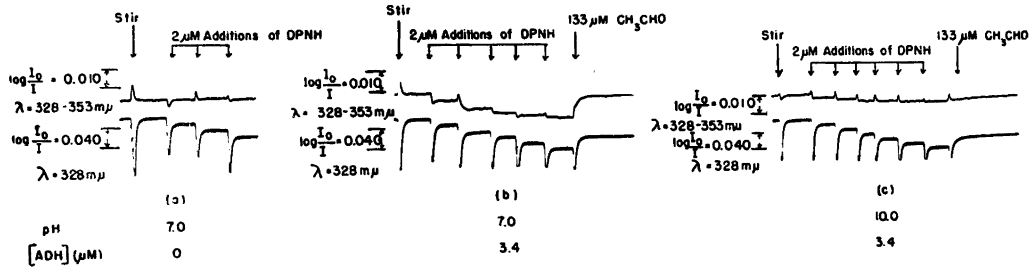

Fig. 3. Illustrating the titration of ADH with DPNH. The difference between the absorption at 328 and 354 mμ is recorded on the upper trace and the absorption at 328 mμ is recorded on the lower trace. Record a is a control experiment by which the rejection of DPNH by the circuit operating at 328—354 mμ is tested with [ADH] = 0. A downward deflection represents an increase in light absorption at 328 mμ and a decrease of absorption at 328—354 mμ. 3.4 μM ADH, pH = 7.0, 0.01 M PO_4''', pH = 10, 0.01 M glycine-NaOH buffers. (Expt. 5c, 33c—35.)

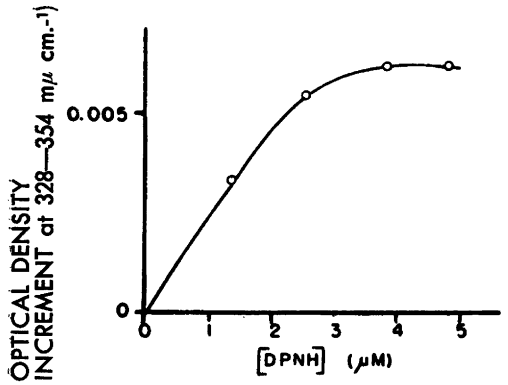

Fig. 4. The results of the titration of very dilute ADH with DPNH at pH = 7.0. The performance of the apparatus used in this experiment is given by Fig. 2. 1.17 μM ADH, pH = 7.0, 0.01 M PO_4''' buffer. (Expt. 6f.)

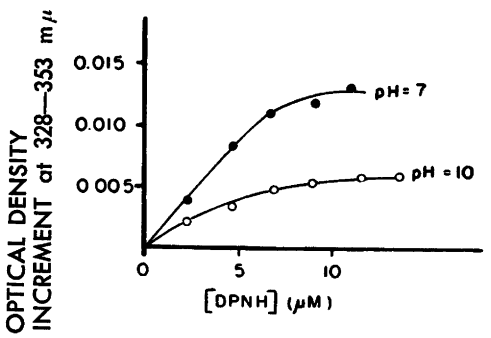

Fig. 5. The results of a titration of ADH with DPNH at pH = 7 and 10 plotted from the experimental data of Fig. 3. (Expt. 5c, 33c—35.)

one) correspond to the addition of 2 μM DPNH (0.01 ml of 0.6 mM) and it is seen that the deflections increase regularly at 328 mμ. In fact the exact amount of DPNH added is determined from this trace. In record (a) no ADH is present and the addition of DPNH causes no effect upon the circuit recording at 328—353 mμ. In record (b) 3.4 μM is present and differences between the absorption at 328 and 353 mμ are now recorded. On the first few additions of DPNH, a relatively large decrease of optical density is measured, but further additions cause relatively smaller increases. On the last addition, acetaldehyde (133 μM) is added which reacts with the ADH-DPNH complex and liberates ADH, causing the trace at 328—353 mμ to return to the original base-line. These records are discussed in detail below.

It was found that the combination of ADH with DPNH is too fast to be measured by ordinary mixing methods and a special flow apparatus having a rectangular observation tube with an optical path of 1 cm was used. Control experiments on the rate of formation of the peroxidase hydrogen peroxide complex show that the mixing is adequate for the measurement of reactions whose half-times are greater than 0.01 sec [2]. By using very dilute ADH and DPNH the half-times of the reactions actually measured were of the order of 0.1—0.05 sec for which this type of apparatus performs satisfactorily.

In summary, it may be said that the measurement of the kinetics and equilibrium of ADH and DPNH has been made possible by new spectrophotometric and rapid reaction techniques that have been used up to the limit of

their sensitivity and time resolution. The accuracy of the results is therefore not as great as might be desired but is surely adequate for a preliminary exploration of the mechanism of the reaction.

THE EQUILIBRIUM OF DPNH AND ADH

The titrations reported earlier in part I were necessarily carried out at such high concentrations of ADH and DPNH that no measurable dissociation of DPNH occurred. Those experiments were, however, excellently suited to a determination of the stoichiometry of the ADH-DPNH reaction. With the more sensitive techniques described above it is possible to reduce the [ADH] by a factor of 10 to 100 and Fig. 4 shows that a measurable dissociation constant can be obtained when 1.17 μM ADH (see footnote p. 1127) is titrated with DPNH. The average of the two values of dissociation constant computed from Fig. 4 is about 10^{-7} M (based on the fact that ADH binds 2 DPNH at pH = 7.0 (part I). The affinity of ADH for DPNH is indeed very high.

The effect of pH upon the optical density change caused by the formation of the ADH-DPNH complex is shown by the original data of Fig. 3 which are plotted in Fig. 5. Since somewhat larger [ADH] must be used to give adequte deflections at pH = 10, the titration at pH = 7.0 gives a scarcely measurable dissociation constant. But the value of the dissociation constant at pH = 10 can be calculated to be roughly 3×10^{-6} M (based on the fact that ADH binds 1 DPNH at pH = 10 (part I). Thus there is an increased dissociation of DPNH from ADH in alkaline solutions.

THE RELATIONSHIP BETWEEN THE ADH-DPNH COMPLEX AND THE REDUCTION OF ALDEHYDE

The experiment in Fig. 3 at pH = 7 shows that the ADH-DPNH complex rapidly disappears upon the addition of acetaldehyde. In fact, the reaction with acetaldehyde is so rapid that it has been difficult to increase the acetaldehyde concentration much above a stoichiometric equivalent of DPNH without causing the life-time of the ADH-DPNH complex to be too short for accurate measurement. Fortunately, it has beens found that formaldehyde reacts with the ADH-DPNH complex much more slowly than acetaldehyde and therefore the most satisfactory kinetic experiments have been carried out on the reduction of formaldehyde by the system ADH + DPNH.

A typical example of the formation and disappearance of the ADH-DPNH complex in the presence of excess formaldehyde is given in Fig. 6. As usual, the disappearance of DPNH is measured simultaneously at 328 mμ. These

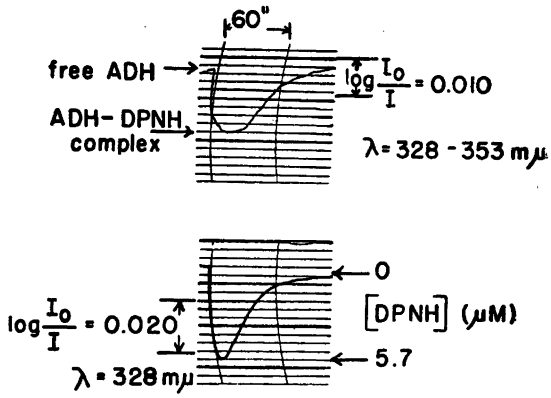

Fig. 6. The kinetics of formation and disappearance of the ADH · DPNH complex measured at 328—354 mμ (upper trace) and the simultaneous disappearance of DPNH measured at 328 mμ (lower trace). 1 cm optical path flow apparatus. 1.17 μM ADH, 8.8 μM DPNH (initial), 66 μM HCHO pH = 7.0, 0.01 M PO_4'''. (Expt. 7c—5.)

experiments were carried out in the flow apparatus by mixing ADH with DPNH plus HCHO. Since the experiment shown in Fig. 6 is the second of a duplicate set, the record begins with "free ADH" (+DPN) remaining from the previous test. On initiating the flow of reactants, the fairly rapid formation of the ADH-DPNH complex starts immediately as is indicated by the fall of the 328—354 mμ trace. The complex exists in a steady state for some seconds, and then decomposes into "free ADH". At 328 mμ, the initiation of the flow replaces the spent DPNH solution and causes the abrupt downward deflection (increase in optical density). The chemical reaction causing the disappearance of DPNH then proceeds and the linear upward sweep of the trace is sustained until the [DPNH] falls to a very low value.

One of the obvious criteria of an enzyme-substrate complex that follows the theory of Michaelis and Menten is that the half-time for the cycle of the enzyme-substrate complex should be twice the half-time for the overall reaction[7]. The values of the half times are 51 and 25 sec. for the cycle and the overall reaction respectively and, according to this criterion, verify the role of the ADH-DPNH complex to be that indicated by Eqns. 1 and 2.

The velocity constant for the reaction of ADH-DPNH with formaldehyde (k_4) is computed directly from experiments similar to those of Fig. 6 by measurement of the kinetics of the ADH-DPNH complex. The value of k_4 is computed according to the following equation (4)

$$k_4 = \frac{[\text{DPNH}]_0}{[\text{ADH} \cdot \text{DPNH}]_m \; t_{\frac{1}{2}\ \text{off}} \; [\text{HCHO}]_0} \tag{4}$$

$[\text{DPNH}]_0$ is the initial [DPNH] (M)
$[\text{HCHO}]_0$ is the initial [HCHO] (M)

Table 1. A summary of values for the velocity of the reaction of formaldehyde and acetaldehyde with the ADH-DPNH complex. 1.17 μM ADH, pH = 7.0, 0.01 M PO_4'' 27° C, (Expt. 7c)
(note: 1.17 μM ADH gives 2.34 μM complex.)

$[DPNH]_0$ μM	8.8	3.7	7.4	7.4	$[DPNH]_0$ μM	7.5	15
$[HCHO]_0$ μM	66	66	132	330	$[CH_3CHO]_0$ μM	33	66
$[ADH\text{-}DPNH]_m$ μM	2.3	2.0	2.3	2.0	$[ADH\text{-}DPNH]_m$ μM	0.56	0.89
$t\frac{1}{2}$ off (sec.)	52	22	19	8.5	$t\frac{1}{2}$ off (sec.)	2	3
$k_4 \times 10^{-3}$ ($M^{-1} \times sec.^{-1}$)	1.1	1.3	1.3	1.3	$k_4 \times 10^{-5}$ ($M^{-1} \times sec.^{-1}$)	2	0.9

$[ADH\text{-}DPNH]_m$ is the maximum concentration of the complex formed in the particular experiment (M).* $t\frac{1}{2}$ off is the time interval between formation and half-disappearance of the ADH-DPNH complex (sec).

The value of k_4 for several experimental conditions is given in Table 1. The average value for formaldehyde is 1.3×10^3 $M^{-1} \times sec^{-1}$ at pH = 7.0.

Considerable difficulty was experienced in obtaining satisfactory "cycles" of the ADH-DPNH complex in the presence of a reasonable excess of acetaldehyde over DPNH for under those conditions the complex concentration ($[ADH\text{-}DPNH]_m$) is too small for accurate measurement. The preliminary value of k_4 for acetaldehyde based on the data of Table 1 is 10^5 $M^{-1} \times sec^{-1}$.

THE VELOCITY CONSTANT FOR THE FORMATION OF THE ADH-DPNH COMPLEX

The rapid formation of the ADH-DPNH complex is verified by records such as Fig. 6 which show that ADH and DPNH even in extremely dilute solution combine in much less than 1 sec. The flow method has therefore been used and a typical record is shown in Fig. 7. Formaldehyde is present so that the baseline before starting the flow corresponds to the optical density of free ADH. The flow is then started momentarily and the formation of the complex is observed. The flow is then restarted and is held near its maximum velocity for over one second in order to allow the photoelectric circuits to respond

* The concentration of ADH used in this computation is twice the actual molarity of ADH because two molecules of DPNH are bound to one molecule of ADH at pH = 7.0[3].

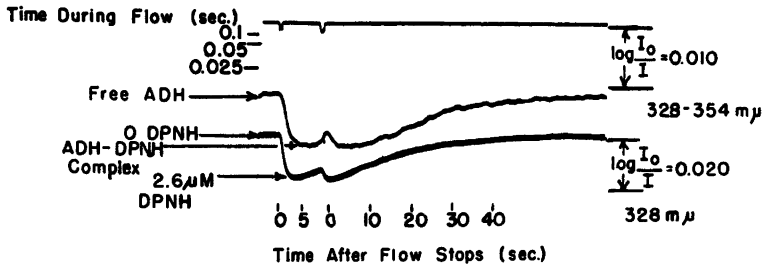

Fig. 7. A measurement of the speed of combination of ADH and DPNH. 1 cm path flow apparatus. 1.17 μM ADH, 3.7 μM DPNH, 66 μM HCHO, pH = 7.0, 0.01 M PO_4'''. (Expt. 7c—10.)

fully to the change of optical density. The trace clearly indicates that the maximum amount of complex is not reached during the flow. As soon as the flow stops, the maximum amount of complex is formed.

The trace at 328 mμ serves as a good control, in this case the second initiation of the flow should only restore the [DPNH] to its full value — and so it does.

The velocity constant for the formation of the ADH-DPNH complex (k_1) may be computed according to the second order equation on the assumption that the two DPNH molecules are bound independently and that the effective molarity of ADH is twice its actual concentration

$$k_1 = \frac{2.3}{t([\text{DPNH}]_0 - [\text{ADH}]_0)} \log \frac{[\text{ADH}]_0([\text{DPNH}]_0 - [\text{ADH-DPNH}]_t)}{[\text{DPNH}]_0([\text{ADH}]_0 - [\text{ADH-DPNH}]_t)} \quad (5)$$

The subscript 0 denotes initial concentrations and t denotes the concentration at time t.

A summary of the values of k_1 obtained with several values of $[\text{DPNH}]_0$ is given in Table 2. The average of all values is $k_1 = 4 \times 10^6$ M^{-1}, $\times$ sec^{-1}, although the values obtained at higher [DPNH] are considered less accurate.

DISCUSSION

These experiments show that the combination of DPNH with liver alcohol dehydrogenase is a rapid reaction and that the DPNH is tightly bound to the protein. An estimate of the value of the velocity constant for the dissociation of DPNH molecules from the protein is given by the product of the dissociation constant and the velocity constant for the combination of DPNH and ADH

Table 2. A summary of data on the velocity constant for the combination of ADH and DPNH. 1.17 μM ADH, pH = 7.0, 0.01 M PO_4'', 27° C, (Expt. 7c) (note: 1.17 μM ADH gives 2.34 μM complex).

$[DPNH]_0$ (μM)	4.3	3.7	7.4	7.4
$[ADH\text{-}DPNH]_m$ (μM)	1.6	1.9	1.7	2.0
t (sec.)	0.067	0.12	0.12	0.095
$k_1 \times 10^{-6}$ ($M^{-1} \times sec.^{-1}$)	5.2	5.4	1.6	3.2

($4 \times 10^6 \times 10^{-7} = 0.4$ sec.$^{-1}$). Thus the half-time for the dissociation reaction is about 1.7 sec. $(\frac{0.693}{0.4})$.

The complex of ADH and DPNH also reacts fairly rapidly with aldehydes, the velocity constants are 1.3×10^3 and 10^5 $M^{-1} \times sec^{-1}$ for formaldehyde and acetaldehyde respectively.

The results of these kinetic tests may be summarized according to the following equations for the case of acetaldehyde:

$$\text{ADH} + \text{DPNH} \underset{k_2 = 0.4 \text{ sec}^{-1}}{\overset{k_1 = 4 \times 10^6 M^{-1} \times sec^{-1}}{\rightleftharpoons}} \text{ADH-DPNH} \qquad (6)$$

$$\text{ADH} \cdot \text{DPNH} + \text{Ald} + H^+ \overset{k_4 \approx 10^5 \ M^{-1} \times sec^{-1}}{\rightleftharpoons} \text{ADH-DPN}^+ + \text{Alc} \qquad (7)$$

The velocity constant k_4 represents the value for the reaction of ADH-DPNH and aldehyde at pH = 7.0.

At pH = 7, the reaction of alcohol and ADH-DPN is very slow and is neglected.

In the presence of various small formaldehyde concentrations, the values of k_4 are reasonably constant and it is probable that the dissociation of DPN from the complex is not a rate limiting step with dilute formaldehyde. In this case the reaction mechanism is identical to that previously studied for catalases and peroxidases:

$$E + S \rightleftharpoons ES \qquad (8)$$

$$ES + A \rightleftharpoons E + P \qquad (9)$$

and the methods of computing k_4 derived previously are valid [8].

When more concentrated aldehyde solutions are used an accumulation of the ADH-DPN complex is to be expected. Studies of the overall reaction are in accord with this explanation because a definite maximum in the aldehyde-activity relationship is obtained [3]. Although such a maximum might be attributed to an ADH-aldehyde complex, the accumulation of the ADH-DPN complex would provide an equally satisfactory explanation. The next step in the reaction is then assumed to be

$$\text{ADH-DPN}^+ \underset{k_5}{\overset{k_3}{\rightleftharpoons}} \text{ADH} + \text{DPN}^+ \tag{10}$$

In order to determine whether the mechanism outlined by the Eqns. 6, 7 and 10 is compatible with the observed data on the overall activity of ADH, it is necessary to determine the relationships between the reaction velocity constants as defined by these equations and the values of activity and the "Michaelis constants" for DPNH and acetaldehyde. Eqns. 6, 7, and 10 are comverted into the usual form for computations of enzyme-substrate kinetics:

$$\begin{array}{ccccc} \text{E} & + \text{S}_\text{I} & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & \text{ES}_\text{I} & \\ e - p_1 - p_2 & x_1 & & p_1 & \end{array} \tag{11}$$

$$\begin{array}{ccccccc} \text{ES}_\text{I} & + & \text{A} & \overset{k_4}{\rightleftharpoons} & \text{ES}_\text{II} & + & \text{AH} \\ p_1 & & a_1 & & p_2 & & a_2 \end{array} \tag{12}$$

$$\begin{array}{ccccc} \text{ES}_\text{II} & \overset{k_3}{\rightleftharpoons} & \text{E} & + & \text{S}_\text{II} \\ p_2 & k_5 & e - p_1 - p_2 & & x_2 \end{array} \tag{13}$$

In this form the first two steps of the reaction mechanism are seen to be a form of the simple mechanism shown to apply, for example, to catalase, alkyl-hydrogen peroxides, and alcohol [9]. But in this case the enzyme is not released until ES_II is dissociated. As usual, the concentrations of the various reactants at any time are written underneath the appropriate symbols. Since only a steady state analysis is required, only the differential equations for p_1, p_2, and a are required.

$$\frac{dp_1}{dt} = k_1\ x_1\ (e - p_1 - p_2) - (k_2 + k_4a_1)\ p_1 \tag{14}$$

$$\frac{dp_2}{dt} = k_4a_1p_1 - k_3p_2 + k_5x_2\ (e - p_1 - p_2) \tag{15}$$

$$\frac{da_1}{dt} = k_4a_1p_1 \tag{16}$$

In the steady-state both $\frac{dp_1}{dt}$ and $\frac{dp_2}{dt}$ are negligible and p_1 and p_2 can be computed.

$$p_1 = \frac{k_1\ x_1\ (e - p_2)}{k_1x_1 + k_2 + k_4a_1} = \frac{e - p_2}{1 + \frac{K_{m_1}}{x_1}} \tag{17}$$

$$K_{m1} = \frac{k_2 + k_4a_1}{k_1} \tag{18}*$$

$$p_2 = \frac{k_4\ a_1p_1 + k_5\ x_2\ (e - p_1)}{k_3 + k_5x_2} = \frac{e - p_1\,(1 - \frac{k_4a_1}{k_5x_2})}{1 + \frac{K_{m_2}}{x_2}} \tag{19}$$

$$K_{m_2} = \frac{k_3}{k_5} \tag{20}$$

K_{m_2} is actually the dissociation constant of the ADH-DPN complex and is about 2×10^{-5} M. See below p. 1141.

$$\frac{p_1}{e} = \frac{1}{(1 + \frac{x_2}{K_{m_2}})\ (1 + \frac{K_{m_1}}{x_1}) - \frac{x_2}{K_{m_2}} + \frac{k_4a_1}{k_3}} \tag{21}$$

The overall activity is usually measured by the rate of disappearance of DPNH, $-\frac{da_1}{dt}$, and the turnover number is $\frac{1}{e} \times \frac{da_1}{dt}$
From Eqn. 16,

* K_{m_1} is usually regarded as a Michaelis constant.

$$\frac{1}{e} \times \frac{da_1}{dt} = k_4 a_1 \times \frac{p_1}{e} \tag{22}$$

and on substituting for $\frac{p_1}{e}$ its value given in Eqn. 21,

$$\frac{1}{e}\frac{da_1}{dt} = \frac{1}{k_4 a_1 [(1 + \frac{x_2}{K_{m_2}})\ (1 + \frac{K_{m_1}}{x_1})] - \frac{x_2}{K_{m_2}} + \frac{1}{k_3}} \tag{23}$$

Under the conditions of these experiments a very small [DPN] is formed since the initial [DPNH] is very low. And in studies of the overall activity, the initial rate is measured (part I) and again very little [DPN] forms. Thus the amount of DPN under both these conditions is probably negligible compared to K_{m_2} ($\sim 1 \times 10^{-5}$ M). Eqn. 23 is therefore simplified as follows

$$\frac{1}{e}\frac{da_1}{dt} = \frac{1}{\frac{1}{k_1 x_1} + \frac{1}{k_4 a_1}(1 + \frac{k_2}{k_1 x_1}) + \frac{1}{k_3}} \tag{24}$$

The values of [DPNH] giving maximal turnover number then depends upon the [aldehyde]. For large [aldehyde],

$$\frac{1}{e}\frac{da_1}{dt} = \frac{1}{\frac{1}{k_1 x_1} + \frac{1}{k_3}} \tag{25}$$

and the maximum turnover number for large [DPNH] is

$$\frac{1}{e}\frac{da_1}{dt} = k_3 \tag{26}$$

The [DPNH] giving half maximal activity in the presence of excess aldehyde is

$$(x_1)_{\frac{1}{2}} = \frac{k_3}{k_1} \tag{27}$$

Thus at *high* [aldehyde], the "Michaelis constant"for DPNH depends upon the rate of dissociation of DPN from ADH, and not at all upon the rate of dissociation of DPNH from ADH.

For *low* [aldehyde] ($k_3 \gg k_4 a$)

$$\frac{1}{e}\frac{da_1}{dt} = \frac{1}{\frac{1}{k_1 x_1} + \frac{1}{k_4 a_1}\left(1 + \frac{k_2}{k_1 x_1}\right)} \tag{28}$$

and the maximum turnover number is

$$\frac{1}{e}\frac{da_1}{dt} = k_4 a_1 \tag{29}$$

The [DPNH] giving half maximal activity in the presence of low [aldehyde] is

$$(x_1)_{\frac{1}{2}} = \frac{k_2 + k_4 a_1}{k_1} = K_{m_1} \tag{30}$$

Thus only at very low [aldehyde] will the true dissociation constant for the ADH-DPNH complex be measured (when $k_2 \gg k_4 a_1$).

At large [DPNH], the effect of aldehyde upon the activity will be as follows:

$$\frac{1}{e}\frac{da_1}{dt} = \frac{1}{\frac{1}{k_4 a_1} + \frac{1}{k_3}} \tag{31}$$

For large [aldehyde] and [DPNH], the maximum turnover number is k_3 as already given by Eqn. 26. The [aldehyde] giving half maximal activity is

$$(a_1)_{\frac{1}{2}} = \frac{k_3}{k_4} \tag{32}$$

These formulas and the relevant assumptions are summarized in Table 3.

The values $(a_1)_{\frac{1}{2}}$ and $(x_1)_{\frac{1}{2}}$ corresponding to the conditions of Eqns. 27 and 32 as well as the value of k_3, have already been published by Theorell and Bonnichsen, see Table 7 part I.

Since no overall data are available on the reactions of ADH, DPNH, and formaldehyde, some new data are plotted in Fig. 8 and are summarized

Table 3. Summary of equations for calculation of reaction velocity constants (x_2, $a_2 \approx 0$).

Equation number	26	27	29	30	31	32
x_1	∞	$\frac{k_3}{k_1}$	∞	$\frac{k_2 + k_4 a_1}{k_1}$	∞	∞
a_1	∞	∞	small	small	variable	$\frac{k_3}{k_4}$
$\frac{1}{e}\frac{da_1}{dt}$	k_3	$\frac{k_3}{2}$	$k_4 a_1$	$\frac{k_4 a_1}{2}$	$\frac{1}{\frac{1}{k_4 a_1} + \frac{1}{k_3}}$	$\frac{k_3}{2}$

in Table 4. The values of the reaction velocity constants k_1 and k_4 are computed from the overall data on acetaldehyde and formaldehyde and are compared with the values obtained by direct measurements of the reaction kinetics of ADH-DPNH in Table 5.

The agreement of the overall and direct data is especially close in the tests in which dilute formaldehyde was used. And this corresponds most closely to the conditions used in the direct studies of the ADH-DPNH complex. In view of the fact that there is over 100 fold difference in the [ADH] for the two studies, the agreement is considered to be satisfactory.

If the mechanism described here applies to the oxidation of alcohol by ADH and DPN, the formulae derived for the computation of the reaction velocity constants can be used. Although no direct kinetic measurements have been made on the ADH-DPN complex, the value of k'_3 for the alcohol-DPN system (the velocity constants for this system are designated by primes) should be the same as the value of k_2 for the ADH-DPNH. The value of k'_3 computed from the data of Theorell and Bonnichsen at pH = 6.8 is 1.1 sec^{-1} and is in fair agreement with our calculated value of k_2 (0.4 sec^{-1}).

The value of K_m of Eqn. 20 ($K_m = \frac{k'_3}{k'_5}$) has already been determined by Theorell and Bonnichsen (part I) to be about 200 times greater than the dissociation constant of the ADH-DPNH complex at pH = 7.0, thus $\sim 2 \times 10^{-5}$. Since k_3 is already known (see Table 7, part I, $k'_3 = 39$—45 sec^{-1} at pH = 7.0), k'_5, the velocity constant for the combination of ADH and DPN, is computed to be 2×10^6 $M^{-1} \times sec^{-1}$. This value is in remarkably good agreement with the value of k_1, the velocity constant for the combination of ADH and

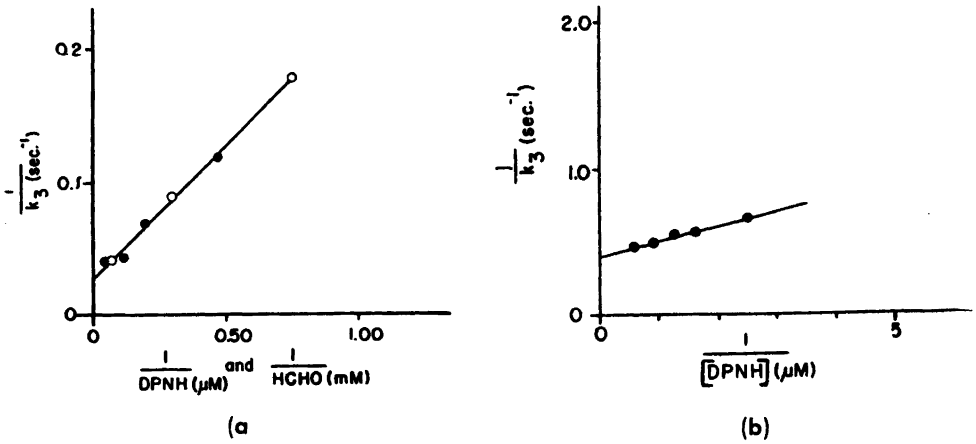

Fig. 8. The effect of [DPNH] and [HCHO] upon the activity of ADH. In Fig. 8 a the solid circles represent the values obtained with varying [DPNH] for [HCHO] = 10 mM. The open circles represent the values obtained with varying [HCHO] and [DPNH] = 8.6 μM. [ADH] = 3 × 10⁻⁹ M. In Fig. 8b, the effect of varying [DPNH] for 0.67 mM HCHO is shown. [ADH] = 15 × 10⁻⁹ M. pH = 7.22, 0.01 M PO_4''' for all experiments. (Expts. 9e, 9g.)

Table 4. Summary of overall data on the reaction of ADH, DPNH, and HCHO (pH = 7.22, 26°).

Substance	K_m (M)	k_3 (sec.$^{-1}$)	k_4a_1 (sec.$^{-1}$)	[HCHO] (mM)	[DPNH] (μM)
Formaldehyde	8×10^{-3}	19	—	—	8.6
DPNH	8×10^{-6}	19	—	10	—
DPNH	2.6×10^{-7}	—	1.25	0.67	—

DPNH, in view of the uncertainties of the various quantitites. The combination of ADH with DPN or DPNH apparently occurs at about the same speed, but their dissociation velocities differ considerably, DPNH being bound much more tightly. And on this basis the different activities of ADH towards alcohol and aldehyde are readily explained.

The close agreement of the reaction velocity constants for the combination of ADH with DPN and DPNH is reasonable in view of the similarity of the latter two molecules and in view of the probability that they combine at the

Table 5. Velocity constants for the reactions of ADH computed on the basis of the mechanism of equations 6, 7, and 10.

	Substrate used	k_1 ($M^{-1} \times sec.^{-1}$)	k_4 ($M^{-1} \times sec.^{-1}$)	k_2 ($sec.^{-1}$)
Computed from overall data	Acetaldehyde	1.6×10^6	2.2×10^5	—
	Formaldehyde (10 μM)	2.4×10^6	2.4×10^3	—
	Formaldehyde (0.67 μM)	4.8×10^6	1.9×10^3	—
	Alcohol	—	—	1.1
Measured directly from the kinetics of ADH-DPNH	Formaldehyde	4×10^6	—	0.4
	Formaldehyde	—	1.3×10^3	—
	Acetaldehyde	—	10^5	—

same position on the protein. The latter supposition is supported by preliminary experiments on the competition between DPN and DPNH for ADH.

There are many aspects of these reactions that require further study but these preliminary results encourage us to believe that the detailed analysis of the mechanism of action of catalases and peroxidase based on direct studies of enzyme-substrate compounds may be applied to the reactions of many enzyme systems.

SUMMARY *

1. A rapid spectrophotometric method for measuring the formation and disappearance of the compound of ADH and DPNH without appreciable interference from the absorption of DPNH has been developed.

2. A titration of very dilute ADH (1.17 μM) with DPNH gives a dissociation constant for the ADH-DPNH complex of 10^{-7} M at pH = 7.0.

3. The velocity constant for the formation of the ADH-DPNH complex is 4×10^6 $M^{-1} \times sec^{-1}$ at pH = 7.0.

4. The velocity constant for the dissociation of DPNH from ADH is computed to be 0.4 sec^{-1}.

5. The velocity constant for the reaction of the ADH-DPNH complex with formaldehyde is 1.3×10^3 $M^{-1} \times sec^{-1}$ at pH = 7.0. The values of k_4

* The experiments were carried out at 26° C.

are constant over a reasonable range of experimental conditions. A preliminary value for the velocity constant for acetaldehyde is 10^5 $M^{-1} \times sec^{-1}$ at pH = 7.0.

6. The ADH-DPNH complex in the presence of dilute formaldehyde fulfils the requirements for a Michaelis intermediate.

7. On the basis of the following mechanism for the action of ADH,

$$\text{ADH} + \text{DPNH} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{ADH} \cdot \text{DPNH}$$

$$\text{H}^+ + \text{ADH} \cdot \text{DPNH} + \text{Aldehyde} \underset{k_3}{\overset{k_4}{\rightleftharpoons}} \text{ADH-DPN}^+ + \text{alcohol}$$

$$\text{ADH-DPN}^+ \underset{k_5}{\overset{k_3}{\rightleftharpoons}} \text{ADH} + \text{DPN}^+,$$

the velocity constants k_1, k_4, and k_2 have been computed from data on the overall activity of the enzyme in very dilute solutions and values of 3×10^6 $M^{-1} \times sec^{-1}$, 2.2×10^5 $M^{-1} \times sec^{-1}$ (for acetaldehyde), 1.3×10^3 $M^{-1} \times sec^{-1}$ (for formaldehyde) and 1.1 sec^{-1} respectively are obtained and agree reasonably well with the values obtained by direct measurements of the kinetics of the ADH · DPNH complex. This agreement has been obtained without assuming the formation of compounds of ADH with aldehyde or alcohol.

8. The velocity constant for the combination of ADH and DPN is calculated to be about 2×10^{-6} M^{-1} sec^{-1} at pH = 7.0 and it is concluded that DPN and DPNH are bound by ADH at about the same speed and on the same place.

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Some Derivatives of Certain Basically Substituted Phenyl-alkyl-carbinols

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During a search for new compounds with analgesic and spasmolytic properties we have prepared a number of derivatives of the following carbinols:

(See Table 1.)

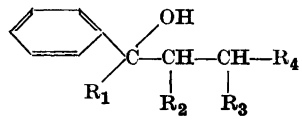


Table 1.

Comp.	R_1	R_2	R_3	R_4	M.p base	°C HCl-salt	Empirical formula of HCl-salt	% Cl in HCl-salt calc.	found
1.	C_6H_5	H	H	$-N(CH_3)_2$	161	204	$C_{17}H_{22}ClNO$	12.30	12.15
2.	C_6H_5	CH_3	H	$-N(CH_3)_2$	92	238	$C_{18}H_{24}ClNO$	11.60	11.50
3.	C_6H_5	H	CH_3	$-N(CH_3)_2$	123	202	$C_{18}H_{24}ClNO$	11.60	11.65
4.	C_6H_5	CH_3	H	$-N(CH_2)_5$	115	266	$C_{21}H_{28}ClNO$	10.29	10.21
5.	C_6H_5	H	CH_3	$-N(CH_2)_5$	76	213	$C_{21}H_{28}ClNO$	10.29	10.19
6.	C_6H_{11}	CH_3	H	$-N(CH_3)_2$	94	244	$C_{18}H_{30}ClNO$	11.42	11.45
7.	C_6H_{11}	CH_3	H	$-N(CH_2)_5$	112	267	$C_{21}H_{34}ClNO$	10.09	10.00

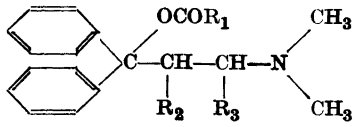
The carbinols were synthesized by the Grignard-Reaction, using phenyl magnesium bromide or cyclohexyl magnesium bromide and, in cases where R_3 was hydrogen, the corresponding phenylketones. These were obtained from acetophenone or propiophenone using the Mannich reaction with dimethylamine hydrochloride or piperidine hydrochloride. In cases where R_3 was a methyl group the carbinols were prepared by the Grignard-reaction from the corresponding aminoacid-ethylesters. These esters were obtained from allyl-

cyanide, which was condensed with the amine to the aminoacid nitrile which in turn was converted directly to the ester by hydrolysis in abs. ethanol saturated with hydrogen chloride.

None of the carbinols prepared, were found to exhibit any significant spasmolytic or analgesic effect in animal experiments.

Hydrochlorides of some esters of a number of these carbinols with acetic, propionic, butyric and benzoic acid, were prepared by treating an etheral solution of the free carbinols with the acid chloride. The following esters were prepared:

(See Table 2.)



Tabel 2.

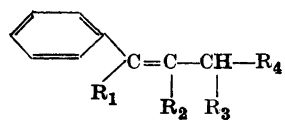
Comp	R_1	R_2	R_3	Mp. of HCl-salt	Empirical formula of HCl-salt	% Cl in HCl salt calc.	found.
8.	C_2H_5	H	H	183	$C_{20}H_{26}ClNO_2$	10.20	10.12
9.	C_2H_5	CH_3	H	187	$C_{21}H_{28}ClNO_2$	9.84	9.90
10.	C_2H_5	H	CH_3	158	$C_{21}H_{28}ClNO_2$	9.84	9.79
11.	CH_3	CH_3	H	102	$C_{20}H_{26}ClNO_2$	10.20	10.16
12.	C_3H_7	CH_3	H	80	$C_{22}H_{30}ClNO_2$	9.47	9.41
13.	C_6H_5	H	CH_3	176	$C_{25}H_{28}ClNO_2$	8.65	8.69

The free bases of any of these esters were immediately hydrolyzed in aqueous solution, and the hydrochlorides were, in aqueous solution, hydrolyzed to the carbinol and the acid in a few hours even at room temperature.

These new compounds, therefore, are of no interest from a practical point of view, although some of them, especially 9 and 10, show spasmolytic and analgetic properties (of very short duration).

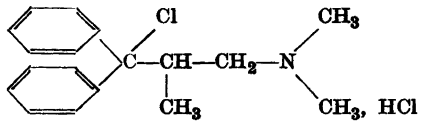
In order to obtain more stable derivatives, attempts were made to prepare alkylethers of some of the carbinols, but without success. In sodium-alcoholate alcohol the free carbinols failed to react with alkylhalides.

By treating the carbinols with thionyl chloride in order to substitute the OH group with chlorine, water was in most cases removed and an unsaturated compound of the following formula was formed:



In a single case the hydrochloride of a chlorine-compound could be isolated: 1,1-Diphenyl-1-chloro-2-methyl-3-dimethyl-amino-propane.

Comp. 14.



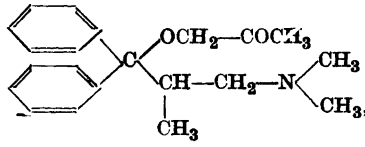
M.p. 131° C.

This compound was extremely readily hydrolyzed to the carbinol, and like the esters the free base could not be isolated. By dissolving the hydrochloride in cold water both chlorine atoms could be estimated as ionised chlorine.

Treating the above hydrochloride with sodium propylate in abs. propanol gave no ether but liberation of formaldehyde and formation of 1,1-diphenyl propene-1 indicating destruction of the molecule.

By treating an etheral solution of the carbinol 1,1-diphenyl-2-methyl-3-dimethylamino-propanol with bromoacetone, the hydrobromide of the following ether was formed:

Comp. 15.



M.p. 107—110° C.

As this compound was almost as unstable in aqueous solution as the esters, no other compounds of this type were prepared. The compound showed spasmolytic properties of a high degree.

The olefins prepared by dehydration of the carbinols or by removing hydrogen chloride from the intermediately formed chlorine compounds were, as expected, quite stable in an aqueous solution of the hydrochloride salts. The following were prepared:

(See Table 3.)

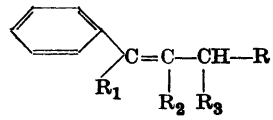


Table 3.

Comp.	R_1	R_2	R_3	R_4	M.p. base	°C HCl-salt	Empirical formula of HCl-salt	% Cl in HCl-salt calc.	found
16.	C_6H_5	CH_3	H	$-N(CH_3)_2$	56	184	$C_{18}H_{22}ClN$	12.33	12.21
17.	C_6H_5	H	CH_3	$-N(CH_3)_2$	oil	161	$C_{18}H_{22}ClN$	12.33	12.30
18.	C_6H_5	CH_3	H	$-N(CH_2)_5$		222	$C_{21}H_{26}ClN$	10.88	10.73
19.	C_6H_5	H	CH_3	$-N(CH_2)_5$		88	$C_{21}H_{26}ClN$	10.88	10.68
20.	C_6H_{11}	CH_3	H	$-N(CH_3)_2$	oil	158	$C_{18}H_{28}ClN$	12.07	12.01
21.	C_6H_{11}	CH_3	H	$-N(CH_2)_5$		183	$C_{21}H_{32}ClN$	10.62	10.56

All the compounds showed spasmolytic properties and several of them of high degree.

EXPERIMENTAL

Preparation of the carbinols 1, 2, 4, 6 and 7

The general procedure employed here is suitably illustrated by the preparation of 1-cyclohexyl-1-phenyl-2- methyl-3-piperidino-propanol- 1. (compound 7).

α-methyl-*β*-piperidino-propiophenone was prepared as described for *β*-dimethylamino-propiophenone [1]: 80 g propiophenone, 100 g piperidine hydrochloride and 20 g para-formaldehyde in 100 ml of 96 % ethanol containing 1 ml conc. hydrochloric acid gave 67 g *α*-methyl-*β*-piperidino-propiophenone bp. 30 mm: 174—180° C.

$C_{15}H_{21}NO$ Calc. 6.06 % N Found 6.10 % N

In the same way [2] the intermediate *α*-methyl-*β*-dimethylamino-propiophenone used for the carbinols 4 and 7 was obtained in 75 per cent yield. bp_{10} mm 160°—164° C. HCl-salt Mp. 147—149° C.

$C_{12}H_{17}NO$ Calc. 7.33 % N Found 7.36 % N

1-cyclohexyl-1-phenyl-2-methyl-3-piperidino-propanol-1. *α*-Methyl-*β*-piperidino-propiophenone (50 g) in 100 ml dry ether was added drop by drop to a stirred etheral solution of cyclohexyl magnesium bromide prepared from 7 g magnesium and 48 g cyclohexyl bromide in 150 cc dry ether. When the addition was completed, the mixture was stirred and heated under reflux for two hours. The mixture was then allowed to stand for two hours.

The mixture was poured onto cracked ice and acidified with concentrated hydrochloric acid. The hydrobromide of 1-cyclohexyl-1-phenyl-2-methyl-3-piperidino-propanol-1 separated and was isolated and washed with ether. White to slight yellow crystals Mp 264—268°. The free base was obtained as a white cryst. powder, Mp 110—112°, by treating the hydrobromide with 25 per cent ammonia. The hydrochloride was obtained by adding dry hydrogen chloride to a solution of the base in acetone-ether. White crystals Mp. 265—267° C.

Preparation of the carbinols 3 and 5

The procedure is illustrated by the preparation of 1,1-diphenyl-3-dimethylamino-butanol-1 (Comp. 3).

Ethyl 3-dimethylamino-butyrate was prepared according to Breckpot[3] from 3-dimethylamino-butyronitrile, which was obtained from allylcyanide and dimethylamine. The corresponding 3-piperidino ester used in the synthesis of Comp. 5 was prepared in the same way[4].

1,1-Diphenyl-3-dimethylamino-butanol-1. Ethyl 3-dimethylamino-butyrate (44.5 g) in 100 ml dry ether was added slowly under cooling and vigorous stirring to an etheral solution of phenylmagnesium bromide prepared from 130 g bromobenzene in 300 ml dry ether and 20 g magnesium. After the addition was completed the mixture was allowed to stand for 12 hours. The mixture was then poured onto crushed ice and acidified with concentrated hydrochloric acid. The hydrobromide of the 1,1-diphenyl-3-dimethylamino-butanol separated as a white crystalline substance. It was isolated and washed with ether. M.p. 208° C. The free base was obtained as a slight yellow cryst. substance. M.p. 122—123° C, by treating the salt with aqueous ammonia, yield 45 g. By dissolving the base in ether-acetone and adding dry hydrogen chloride, the hydrochloride was obtained as white crystals M.p. 202°.

Preparation of the esters 8—13

The procedure is illustrated by the preparation of compound 9.

1,1-Diphenyl-2-methyl-3-dimethylamino-propanol-1 (10 g) was dissolved in 75 ml dry ether and freshly distilled propionyl chloride (5 g) was added. The mixture was allowed to stand for 24 hours. Carbinol hydrochloride (6.5 g) separated and was removed by filtering. To the filtrate was added dry hydrogen chloride in ether. The ester (4,2 g) was separated as a white crystalline substance. Mp. 170—175°. Recrystallisation from acetone-ether raised the Mp. to 187° C. In contrast to the hydrochloride of the carbinol, the hydrochloride of the ester is very soluble in acetone.

Preparation of compound 14

1,1-Diphenyl-1-chloro-2-methyl-3-dimethylamino-propane. 1,1-Diphenyl-2-methyl-3-dimethylamino-propanol-1, HCl (10 g) was suspended in 50 ml chloroform and 10 ml thionyl-chloride added. The mixture was heated under reflux for 1 hour when the excess of thionyl chloride and the chloroform were removed by distillation. Traces of thionyl chloride were removed by adding more chloroform and distilling in vacuum. The residue was dissolved in acetone and by subsequent addition of ether the hydrochloride of the chlorocompound was separated as a white crystalline substance. Mp. 125—131°. Recrystallisation from acetone-ether raised the Mp. to 131°. Yield 10,5 g.

$C_{18}H_{23}NCl_2$ Calc. 21.90 % Cl Found 21.76 % Cl.

Preparation of compound 15

Hydrobromide of acetonyl-(1,1-diphenyl-2-methyl-3-dimethylamino-) propyl-ether.

1,1-Diphenyl-2-methyl-3-dimethylaminopropanol-1 (10 g) was dissolved in 75 ml dry ether and fresh distilled bromoacetone (8 g), was added. The mixture was allowed

to stand for 72 hours. The crystals which separated were recrystallised from acetone-ether. Yield 8 g. Mp. 107 – 110°.

$C_{21}H_{28}O_2NBr$ Br calc. 19.71 % Br found 19.65 %

Preparation of the olefines 16 – 21.

The procedure is illustrated by the preparation of compound 20. *1-Phenyl-1-cyclohexyl-2-methyl-3-dimethylaminopropene-1.*

The carbinol 1-phenyl-1-cyclohexyl-2-methyl-3-dimethylaminopropanol-1 (30 g) dissolved in 100 ml chloroform, was converted to the hydrochloride by adding dry hydrogen chloride. Thionyl chloride (30 g) was then added and the mixture heated under reflux for 3 hours. Thionyl chloride and chloroform were removed by distillation in vacuum, and the residue heated with 25 ml pyridine for 1 hour. After removing the pyridine by distillation in vacuum, the residue was dissolved in water and extracted with ether. The 1-phenyl-1-cyclohexyl-2-methyl-3-dimethylaminopropene-1 formed was separated from the aqueous solution as an oil by adding sodium hydroxide. The base was extracted with ether and traces of pyridine removed from the etheral solution by shaking with water. The etheral solution was then treated with activated carbon and dried with potassium carbonate. After filtering the hydrochloride of the compound was separated from the ether by adding dry hydrogen chloride. The hydrochloride was recrystallized from acetone ether. White needles M.p. 158° C. Yield 19 g.

SUMMARY

The preparation of seven α,α-diphenyl- or α-phenyl-α-cyclohexyl-γ-dimethylamino- or γ-piperidino propyl or butyl alcohols is described.

Six carboxylic esters of some of these carbinols and a number of the corresponding olefins are described.

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2. Knott, E. B., *J. Chem. Soc.* (1947) 1190.
3. Breckpot, *Bull. soc. chim. Belges* **32** (1923) 414.
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On the Complex Chemistry of the Uranyl Ion

V.* The Complexity of Uranyl Sulfate

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From his early cryoscopic and conductometric investigations, Dittrich [5] concluded that uranyl sulfate was incompletely dissociated. Since then however only one attempt has been made to carry out a quantitative investigation of this complex system, *viz.* by Betts and Michels [6]. Unfortunately serious objections must be raised to their investigation.

Betts and Michels use the method of Job [7] for an extinctiometric determination of the composition of the complex solution. This method can however not be applied if more than one complex is formed. As it is very difficult to decide with certainty, if this is the case or not, the method has thus a very limited applicability. Moreover those complex systems which have been thoroughly investigated have, as a rule, been shown to involve more than one complex (see *e.g.* Bjerrum [8], Leden [9], Fronaeus [10,11] Sillén [12]). The method of Job may therefore be used only in rare cases, which are, moreover, not easy to indicate. The attempt of Vosburgh and Cooper [13] to extend this method to systems involving more than one complex is not useful, as their extension is accompanied by a number of limiting conditions which cannot possibly be fulfilled in practice.

The measurements of Betts and Michels are in addition performed in solutions of a $[H^+] = 2\,C$. These solutions contain the sulfate chiefly as hydrosulfate ions, which are considered not to form any uranyl complexes. The reason given for this opinion [6 p.S289] is however not conclusive as the same behaviour would be observed if both sulfate and hydrosulfate ions form complexes.

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* The preceding papers of this series (Ahrland [1–4]) are in the following referred to as I–IV. Unless otherwise stated, the symbols in the present paper refer to the same quantities as those mentioned in the previous papers.

In the present investigation the complexity of uranyl sulfate is determined potentiometrically as well as extinctiometrically.

The potential measurements are performed according to the method of ligand displacement, developed by Fronaeus [11]. This is the only known potentiometric method which is applicable here. A suitable displacing ligand has been found in the acetate ion. As shown in IV, this ion forms strong complexes with the uranyl ion, and the experimental conditions can be chosen so that the hydrolysis of the uranyl ion and its complexes does not interfere with the acetate complex formation.

The extinction measurements are carried out according to the method described in II and IV.

In all the measurements, the ionic medium is built up in the same manner as in I—IV, having a ionic strength = 1 C with $NaClO_4$ as the supplementary neutral salt. The temperature is 20° C.

Chemicals used: Baker's analyzed *sodium sulfate* is used without further purification. A stock solution with $C_A \approx {}^1/_3$ C is prepared. It is analyzed by a) drying a sample and weighing the residue and b) passing a sample through an ion exchange column saturated with hydrogen ions and then titrating alkalimetrically. Both the methods give C_A = 334 mC.

The *other chemicals* are of the same preparations as in I and IV.

THE POTENTIAL MEASUREMENTS

a. Equations for the calculation of the complexity constants

At measurements made by the method of ligand displacement the solutions contain two competing ligands A and B. Thus the central group M may form complexes with A or B as well as mixed complexes with both A and B. Provided that the complexity of the B-system is stronger than that of the A-system it is then possible to calculate not only the constants of the complexes MA_j (j = 1, 2,.., N) but also those of the mixed complexes of the type MA_jB (j = 1, 2 ... N—1), by measuring merely the free ligand concentration [B].

From [B], the ligand number with respect to B, $\bar{n}_B$, is calculated. At a given [B], this quantity is smaller in solutions containing A than in solutions free from A, owing to the competing complex formation between M and A. The difference, $\Delta\bar{n}_B$, will, however, grow smaller and smaller, as [B] is increased at a constant value of [A] = $[A]_m$, and it will cease completely within the random errors at a finite value of [B], b, if the complexity of the B-system is sufficiently strong. Practically all A-ligands of the complexes are then replaced

by B-ligands. The value of b will depend on the mutual strength of the complex systems, and, of course, on the value of $[A]_n$ chosen.

In order to get $\Delta\bar{n}_B$ for solutions of a constant and known $[A]_n$ it is necessary to make measurements at different C_M and then extrapolate to $C_M = 0$, where $C_A = [A]_n$. By this, the influence of polynuclear complexes is also removed, and the method therefore permits a correct determination of all mononuclear constants even if polynuclear complexes exist.

From the data thus measured the function $X([A]_n)$ is obtained by the equation (Fronaeus [11 p.75])

$$\ln X([A]_n) = \int_0^b \left(\frac{\Delta\bar{n}_B}{[B]}\right)_{C_M = 0} \cdot d[B] \tag{1}$$

The integration is carried out graphically and from $X([A_n])$ thus found the functions $X_1([A]_n)$, $X_2([A]_n)$ and $X_3([A]_n)$ are formed and the constants β_j of the complexes MA_j calculated in the usual way (II p. 787).

The mixed constants $\beta_{j,1}$ of the complexes MA_jB are then calculated according to (Fronaeus [11 p.76]):

$$\lim_{[B]\to 0}\left(\frac{\bar{n}_B}{[B]}\right)_{C_M = 0} = \frac{\sum_{j=0}^{N-1} \beta_{j,1}\,[A]^j}{X([A])} \tag{2}$$

Here $X([A])$ is known from (1), and $\beta_{0,1}$ is the first constant of the B-system, which is known in advance. The other constants $\beta_{j,1}$ are then calculated according to an extrapolation method analogous to the one employed when the constants β_j are calculated from the X-functions. Thus the function $U_1([A])$ is formed according to

$$U_1([A]) = \frac{X([A]) \cdot \lim_{[B]\to 0}\left(\frac{\bar{n}_B}{[A]}\right)_{C_M = 0} - \beta_{0,1}}{[A]} = \sum_{j=1}^{N-1} \beta_{j,1}\,[A]^{j-1} \tag{3}$$

The extrapolation of $U_1([A])$ to $[A] = 0$ gives $\beta_{1,1}$. We are then able to form a new function

$$U_2([A]) = \frac{U_1([A]) - \beta_{1,1}}{[A]} = \sum_{j=2}^{N-1} \beta_{j,1}\,[A]^{j-2} \tag{4}$$

the extrapolation of which to $[A] = 0$ gives $\beta_{2,1}$, etc.

b. The calculation of [B] and n_B

The experiments are quite analogous to those of IV. In both cases, the quantities immediately searched for are the free acetate ion concentration (here = [B]) and the ligand number with respect to acetate (here = $\bar{n}_B$). They are calculated from measurements of [H$^+$] of an acetate buffer. These measurements are very accurately and conveniently performed by means of the quinhydrone elctrode. As stated in IV, the influence of the hydrolysis of the uranyl species can be neglected in a buffer with $C'_{HB}/C'_B = 5.0$. Therefore this buffer is used here. The [H$^+$] of the solutions then becomes so high however, that the formation of hydrosulfate ions must be taken into consideration, and so the equations of IV have to be accordingly modified when used here.

As in IV, two solutions are compared, the first one (denoted ′) containing no uranyl, the second one with the uranyl concentration C_M. The law of mass action then gives the following equation for the dissociation of the acetic acid:

$$\frac{[H^+]' \ (C'_B + [H^+]' + [HSO_4^-]')}{C'_{HB} - [H^+]' - [HSO_4^-]'} = \frac{[H^+][B]}{C'_{HB} - [H^+] + C_H^o - C_s - [HSO_4^-]} \quad (5)$$

We introduce $C'_{HB} = \delta \cdot C'_B$ and

$$[HSO_4^-] = [H^+][SO_4^{2-}]/K_2 \approx [H^+] \cdot C_A/K_2 \quad (6)$$

As $[HSO_4^-]$ is a correction term, the approximation $[SO_4^{2-}] \approx C_A$ may be permitted. Further, the term C_s is put = 0, according to IV, pp. 203, 205. So we obtain for [B]:

$$[B] = \frac{[H^+]'}{[H^+]} \cdot \frac{(C'_B + [H^+]'(1 + C_A/K_2))(\delta \cdot C'_B - [H^+](1 + C_A/K_2) + C_H^o)}{\delta \cdot C'_B - [H^+](1 + C_A/K_2)} \quad (7)$$

or, approximately, with the value $\delta = 5.0$ inserted:

$$[B] = \frac{[H^+]'}{[H^+]} (C'_B + 1.2 \ \vartheta' - 0.2 \ \vartheta + 0.2 \ C_H^o) \quad (8)$$

if the expressions [H$^+$] $(1 + C_A/K_2)$ and [H$^+$]$'(1 + C_A/K_2)$ are denoted by ϑ and ϑ' respectively.

For $\bar{n}_B$ we obtain the expression (*cf.* IV p. 203):

$$\bar{n}_B = \frac{C'_B + [H^+] - C_H^o + C_s + [HSO_4^-] - [B]}{C_M} \quad (9)$$

Table 1. Determination of E' as a function of C'_B at different C'_A. The calculated values of ϑ' ($[H^+]_0 = 10.19$ mC).

$C'_A \rightarrow$ mC	25		50		100		150		200	
C'_B mC	E' mV	ϑ' mC	E' mV	ϑ' mC	E' mV	ϑ' mC	E' mV	ϑ' mC	E' mV	ϑ' mC
10.37	110.4	0.17	110.1	0.21	109.4	0.29	109.1	0.38	108.8	0.47
20.20	110.0	0.17	109.6	0.21	108.8	0.30	108.5	0.39	107.9	0.48
29.55	109.8	0.17	109.4	0.21	108.6	0.30	108.2	0.40	107.5	0.49
38.4	109.7	0.2	109.3	0.2	108.6	0.3	108.0	0.4	107.3	0.5
46.9	109.7	»	109.3	»	108.5	»	107.9	»	107.2	»
66.5	109.6	»	109.2	»	108.4	»	107.7	»	107.0	»
99.7	109.6	»	109.2	»	108.3	»	107.6	»	106.8	»
138.9	109.6	»	109.2	»	108.3	»	107.6	»	106.8	»
177.3	109.6	»	109.2	»	108.3	»	107.6	»	106.7	»
206.0	109.7	»	109.3	»	108.4	»	107.6	»	106.7	»
228.0	109.7	»	109.3	»	108.4	»	107.7	»	106.7	»
260.0			109.5	»	108.6	»	107.8	»	106.8	»

which gives, when simplified according to the above:

$$\bar{n}_B = \frac{C'_B - (C^o_H - \vartheta) - [B]}{C_M} \tag{10}$$

In these equations of [B] and $\bar{n}_B$, the quantities $[H^+]'$, $[H^+]$, C^o_H and $[H^+]' / [H^+]$ are calculated exactly as in IV, *i.e.* from the equations (16), (17) and (18) of II, respectively. It should be noted, that the quantity $E' - E$ ($= E_A$ of II) is called E_B here, as it is the emf caused by the complex formation of the acetate ions (here = B). C'_B, δ and C_A are stoichiometrically known. K_2 has been determined by Fronaeus [11 p.80] to $8.4 \cdot 10^{-2}$ C for the medium and temperature used.

c. Experimental data

The complex solutions are prepared by mixing the solutions (*cf.* I p. 383):

$$S = \begin{cases} C'_M \text{ mC } UO_2(ClO_4)_2 \\ C'_A \text{ mC } Na_2SO_4 \\ (1\,000 - 3C'_M - 3C'_A) \text{ mC } NaClO_4 \end{cases} \qquad T = \begin{cases} C'_A \text{ mC } Na_2SO_4 \\ 400 \text{ mC NaAc*} \\ 2\,000 \text{ mC HAc} \\ (400 - 3C'_A) \text{ mC } NaClO_4 \end{cases}$$

* Ac = acetate.

Table 2. Determination of corresponding values of $\bar{n}_B/[B]$ and $[B]$ at different C'_A and C'_M.

			C'_A = 25 mC				C'_A = 50 mC			
No	C_M mC	C'_B mC	E_B mV	$C^0_H - \vartheta$ mC	[B] mC	$\bar{n}_B/[B]$ C^{-1}	E_B mC	$C^0_H - \vartheta$ mC	[B] mC	$\bar{n}_B/[B]$ C^{-1}
					C'_M = 25 mC					
1	24.35	10.37	42.8	0.22	1.93	175	38.3	0.03	2.33	141.5
2	23.75	20.20	41.0	0.24	4.03	166.5	36.6	0.06	4.81	134.5
3	23.15	29.55	39.6	0.25	6.19	161	35.3	0.07	7.36	129.5
4	22.6	38.4	38.1	0.3	8.59	150.5	33.9	0.1	10.12	123.5
5	22.05	46.9	36.0	»	11.35	140.5	32.3	»	13.1	116.5
6	21.55	55.1	33.5	0.4	14.69	127	30.0	0.2	16.85	105
7	20.85	66.5	29.5	»	20.75	105	26.4	»	23.5	87.5
8	19.75	84.0	22.8	0.5	34.2	73	20.9	0.3	36.9	64.5
9	18.75	99.7	17.8	»	49.4	54	16.5	0.4	52.0	48.5
10	17.85	114.0	14.4	»	64.6	42.5	13.8	»	66.2	40
11	17.05	127.0	12.0	»	79.2	35	11.6	»	80.5	33.5
12	16.3	138.9	10.4	»	92.3	30.5	10.0	0.3	93.8	29.5
13	15.0	159.6	8.1	»	116.1	24.5	7.8	»	117.5	24
14	13.9	177.3	6.5	0.4	137.4	20.5	6.3	»	138.3	20
15	12.1	206.0	4.8	»	170.6	17	4.7	0.2	171.4	16.5
16	10.7	228.0	3.9	0.3	195.9	15	3.6	»	198.1	14
17	8.72	260.0								
					C'_M = 50 mC					
18	48.7	10.37	61.6	0.29	0.927	203	57.7	0.04	1.084	175
19	47.5	20.20	58.7	0.42	2.005	186.5	54.6	0.18	2.36	157.5
20	46.3	29.55	57.1	0.46	3.11	180.5	53.1	0.23	3.66	151.5
21	45.2	38.4	55.9	0.5	4.24	176	51.9	0.3	4.96	148
22	44.1	46.9	54.9	»	5.37	173	50.9	»	6.28	145.5
23	43.1	55.1	53.6	»	6.65	167.5	49.5	»	7.80	140
24	41.7	66.5	51.4	0.6	8.73	157	47.4	0.4	10.21	131.5
25	39.5	84.0	47.1	0.7	13.06	136.5	43.2	0.5	15.25	113.5
26	37.5	99.7	41.6	0.8	19.23	110	38.1	0.6	22.3	92
27	35.7	114.0	35.7	0.9	27.85	86	33.0	0.8	31.0	74.5
28	34.1	127.0	30.2	1.0	38.5	67	28.1	»	41.9	59
29	32.6	138.9	25.2	»	51.4	51.5	24.1	»	55.8	45.5
30	30.0	159.6	18.8	»	76.0	36.5	18.3	»	77.3	35
31	27.8	177.3	14.9	»	98.6	28.5	14.6	»	99.8	27.5
32	24.2	206.0	10.6	0.8	135.5	21	10.2	0.7	137.7	20.5
33	21.4	228.0	8.0	0.7	166.3	17	7.9	0.6	167.1	17
34	17.45	260.0								

Table 2 (continued).

No	C'_A = 100 mC				C'_A = 150 mC				C'_A = 200 mC			
	E_B mV	$C^0_H-\vartheta$ mC	[B] mC	$\bar{n}_B/[B]$ C^{-1}	E_B mV	$C^0_H-\vartheta$ mC	[B] mC	$\bar{n}_B/[B]$ C^{-1}	E_B mV	$C^0_H-\vartheta$ mC	[B] mC	$\bar{n}_B/[B]$ C^{-1}
					C_M = 25 mC							
1	32.5	−0.34	2.94	108.5	27.8	−0.61	3.56	85.5	24.5	−0.83	4.07	72
2	31.3	−0.34	5.94	103.5	26.9	−0.62	7.10	81.5	23.5	−0.84	8.13	67
3	30.3	−0.32	9.00	100	26.0	−0.60	10.69	78.5	22.8	−0.83	12.17	65
4	29.3	−0.3	12.14	97	24.9	−0.6	14.49	75	21.9	−0.8	16.29	62.5
5	27.6	»	15.81	90	23.8	−0.5	18.45	71.5	20.8	»	20.75	59
6	25.8	−0.2	20.0	82	22.3	»	22.9	66.5	19.6	−0.7	25.6	55
7	23.2	»	26.7	72	20.0	−0.4	30.3	58	17.8	»	33.1	49.5
8	18.9	−0.1	39.9	56	16.6	»	43.7	47	15.0	−0.6	46.7	41
9	15.6	0.0	54.0	45	13.9	−0.3	57.7	39	12.6	−0.5	60.8	34.5
10	13.1	»	68.1	38	11.9	»	71.5	32	10.9	»	74.3	30.5
11	11.2	»	81.8	32.5	10.3	−0.2	84.7	29.5	9.5	»	87.7	26.5
12	9.9	»	94.2	29	8.8	»	98.4	25.5	8.2	−0.4	100.7	23.5
13	8.0	»	116.4	25	7.4	»	119.4	22.5	6.6	»	123.0	20
14	6.7	»	136.5	21.5	6.0	»	140.3	19	5.6	»	142.6	18
15	5.1	»	168.7	18.5	4.3	»	174.2	15	4.0	»	176.2	14
16	4.0	»	195.0	16	3.5	»	198.6	14	3.1	»	202.3	12
17					2.5	»	236.0	12	2.2	»	238.7	10.5
					C'_M = 50 mC							
18	49.7	−0.51	1.49	130	43.9	−0.96	1.87	104	39.3	−1.30	2.25	86
19	46.8	−0.37	3.21	114	41.5	−0.85	3.96	91	37.2	−1.22	4.71	75
20	45.5	−0.32	4.93	109.5	40.3	−0.81	6.05	87	36.2	−1.20	7.13	71.5
21	44.6	−0.3	6.62	107	39.2	−0.8	8.20	84	35.3	−1.2	9.59	69
22	43.7	»	8.36	105	38.2	−0.7	10.40	81	34.5	−1.1	12.08	67.5
23	42.5	−0.2	10.28	101.5	37.3	»	12.65	79	33.5	»	14.72	65.5
24	40.6	»	13.40	95.5	35.5	−0.6	16.37	74	31.9	−1.0	18.88	62
25	36.7	0.0	19.7	83	32.2	−0.5	23.55	65.5	29.2	−0.9	26.5	56
26	32.5	0.1	27.6	69.5	28.8	−0.3	32.0	57	26.0	−0.7	35.8	48
27	28.7	0.2	36.7	59.5	25.4	−0.2	41.8	48.5	23.2	−0.6	45.7	42
28	24.8	0.3	47.6	49	22.3	−0.1	52.7	41.5	20.6	−0.5	56.5	37
29	21.7	»	59.0	41.5	19.7	»	63.8	36	18.4	−0.4	67.3	33
30	16.8	0.4	82.2	31	15.6	0.0	86.3	28.5	14.8	»	89.1	26.5
31	13.7	»	103.3	25.5	12.8	»	107.2	23.5	12.3	−0.3	109.1	22.5
32	9.6	0.3	141.3	19	9.1	»	144.2	18	9.0	»	144.5	17.5
33	7.6	»	169.0	16.5	7.4	»	170.7	15.5	7.2	»	171.8	15.5
34	5.1	0.2	212.8	12.5	5.0	−0.1	213.8	12.5	5.0	»	213.8	12.5

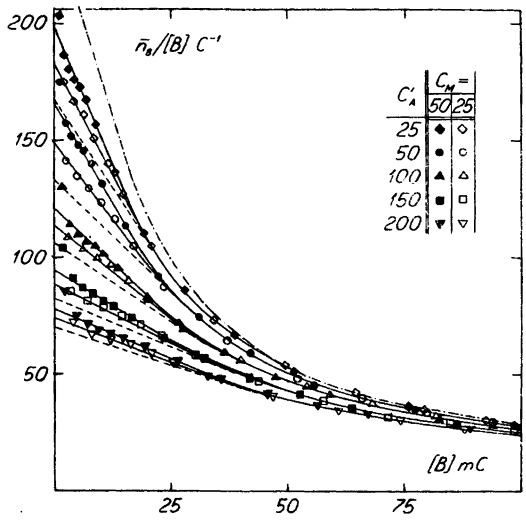

Fig. 1. $\bar{n}_B[B]$ *as a function of* [B]. *The curve of dots and dashes refers to pure acetate complex formation (IV Fig. 2). The fulldrawn curves are drawn according to the experimental points (scheme on the fig.) The dashed curves give the result of the extrapolations to* $C_M = 0$.

Thus, when T is added to S, the resulting solution has always a constant C_A', while C_B' increases and C_M decreases. The ionic strength is constant apart from changes owing to the complex formation.

Five different values of C_A' are used *viz.* $C_A' = 25$, 50, 100, 150 and 200 mC. For every C_A'-series, titrations are performed with $C_M' = 50$ and 25 mC, besides the titration with $C_M = 0$. The reference electrode, RE, has in the present case $[H^+]_0 = 10.19$ mC.

The titrations of different C_A' with $C_M = 0$ are collected in Table 1. From the course of E' found, it is plain that an exchange of perchlorate for sulfate implies a greater change of the ionic medium than the corresponding exchange for acetate (*cf.* IV p. 204).

The main titrations are collected in Table 2. In Fig. 1, $\bar{n}_B/[B]$ is drawn as a function of [B] for the different C_A' and C_M' used. The value of the function for $C_A = 0$ (as determined in IV) is also introduced. The $\bar{n}_B$ / [B]-functions are extrapolated to [B] = 0.

At high values of [B], the curves of different C_M' coincide for a given C_A' (Fig. 1). They thus represent the function $(\bar{n}_B / [B])_{C_M = 0}$ ultimately searched for (p. 1153). Only at low [B] is it necessary to make an extrapolation. As the difference between the C_M'-curves is relatively slight even there, it is certainly allowed to perform this extrapolation linearly (Table 3). The

Table 3. Determination of $(\bar{n}_B/[B])_{C_M=0}$ *as a function of* [B] *at the different values of* $C'_A = C_A = [A]_n$.

[B] mC	C_M mC	$\bar{n}_B/[B]$ C^{-1}	C_M mC	$\bar{n}_B/[B]$ C^{-1}	$\left(\frac{\bar{n}_B}{[B]}\right)_{C_M=0}$ C^{-1}	C_M mC	$\bar{n}_B/[B]$ C^{-1}	C_M mC	$\bar{n}_B/[B]$ C^{-1}	$\left(\frac{\bar{n}_B}{[B]}\right)_{C_M=0}$ C^{-1}
	C'_M = 50 mC		C'_M = 25 mC			C'_M = 50 mC		C'_M = 25 mC		
	C'_A = 25 mC					C'_A = 50 mC				
0	50.0	198	25.0	182.5	167	50.0	165	25.0	149	133
5	44.5	174	23.5	163.5	152	45.2	148	23.7	136	122.5
10	41.0	151	22.3	144.5	137	41.8	131.5	22.6	123	113
15	38.9	128	21.5	125.5	122.5	39.6	114	21.8	109.5	104
	C'_A = 100 mC									
0	50.0	121	25.0	113.5	601					
5	46.3	111.5	23.95	5.501	99					
10	43.2	102	23.0	98	5.39					
15	41.1	92.5	22.2	89.5	86					
20	39.5	83.5	21.55	81.5	79					
	C'_A = 150 mC					C'_A = 200 mC				
0	50.0	94.5	25.0	88.5	82.5	50.0	78	25.0	74	70
5	46.9	88.5	24.1	83.5	78	47.35	74	24.25	70.5	66
10	44.3	82.5	23.25	78.5	73.5	45.0	70	23.45	66.5	63
15	42.2	76	22.55	73.5	70.5	42.9	65.5	22.8	63	60.5
20	41.1	70	21.9	68 5	67	41.4	61	22.15	59	57
30	38.0	59.5	20.9	58.5	57.5	38.7	52.5	21.0	51.5	50.5

values of the limes functions $(\bar{n}_B / [B])_{C_M=0}$ thus found are introduced in Fig. 1 as dashed curves.

For these limes functions, we have $[A]_n = C_A$ (*cf.* above, p. 1153). C_A is connected with C'_A according to $C_A = C'_A - [HSO_4^-]'$. From Table 1, we are able to calculate the ratio $[HSO_4^-]'/C'_A \approx [HSO_4^-]'/[SO_4^{2-}]' = [H^+]'/K_2 \approx 1.5\,\%$. Thus we may very well neglect $[HSO_4^-]'$ in comparison with C'_A in the pure buffer, and so put $[A]_n = C_A = C'_A$ for the limes functions.

From Fig. 1, it is now possible to determine the upper limits b of the integration and they are tabulated in Table 4. Unfortunately, these values

Table 4. The X-functions, as obtained by graphical integration according to equ. (1). The ligand number and the composition of the system as calculated from the complexity constants found.

$\beta_1 = 50 \pm 10\ C^{-1}$ $\beta_2 = 350 \pm 150\ C^{-2}$ $\beta_3 = 2\,500 \pm 1000\ C^{-3}$

$[A]_n$ mC	b mC	$\ln X([A])$	$X([A])$	$X_1([A])$ C^{-1}	$X_2([A])$ C^{-2}	$X_3([A])$ C^{-3}	$\bar{n}_A$	α_0 %	α_1 %	α_2 %	α_3 %
0				*50*	*330*	*2 500*					
10							0.38	65	32.5	2.5	0
25	60	0.902	2.465	58.6	340		0.72	40	50	8.5	1.5
50	120	1.575	4.83	76.6	530		1.11	21.5	53.5	18.5	6.5
100	180	2.445	11.53	105.3	550	2 200	1.62	8.5	42	28.5	21
150	240	3.185	24.15	154.3	700	2 500	1.96	4	30	31.5	34.5
200	300	3.815	45.4	222.0	860	2 600	2.18	2	22.5	31	44.5

Table 5. Corresponding values of $[A]_n$, $X([A]_n)$ *and* $\lim_{[B]\to 0}\left(\frac{\bar{n}_B}{[B]}\right)_{C_M = 0}$ *The calculated U-functions and mixed complexity constants.*

$\beta_{1,1} = 6\,000 \pm 1\,500\ C^{-2}$ $\beta_{2,1} = 40\,000 \pm 15\,000\ C^{-3}$

$[A]_n$ mC	$X([A]_n)$	$\lim_{[B]\to 0}\left(\frac{\bar{n}_B}{[B]}\right)_{C_M=0}$ C^{-1}	$U_1([A])$ C^{-2}	$U_2([A])$ C^{-3}
0		241	*6 000*	*40 000*
25	2.465	167	6 800	
50	4.83	133	8 000	40 000
100	11.53	106	9 800	38 000
150	24.15	82.5	11 700	38 000
200	45.4	70	14 700	43 500

cannot be determined with the amount of accuracy which is desired, as the $(\bar{n}_B / [B])_{C_M = 0}$ — curves of different $[A]_n$ converge rather slowly. Evidently the complexity of uranyl sulfate is rather strong, and therefore the acetate ions force the sulfate ions out of their complexes only with difficulty. It would have been more advantageous to use a stronger displacing ligand, but, unfortunately, no such stronger ligand is known.

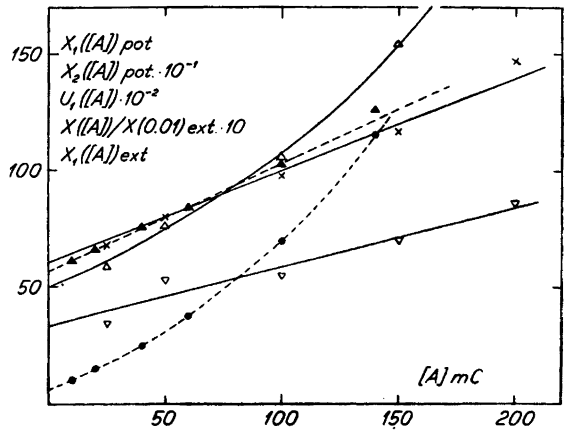

Fig. 2. The functions 1. $X_1([A])$ *pot.* (△), *2.* $X_2([A])$ *pot.* (▽), *3.* $U_1([A])$ (×), *4.* $X([A])/X(0.01)$ *ext.* (●) *and 5.* $X_1([A])$ *ext.* (▲). *The functions found potentiometrically are fulldrawn, those found extinctiometrically dashed.*

With the courses of the functions $(\bar{n}_B / [B])_{C_M = 0}$ known, it is possible to perform the graphical integration of (1) using the identity:

$$\int_0^b \left(\frac{\Delta\bar{n}_B}{[B]}\right)_{\substack{C_M = 0 \\ [A] = [A]_n}} \cdot d[B] = \int_0^b \left(\frac{\bar{n}_B}{[B]}\right)_{\substack{C_M = 0 \\ [A] = 0}} \cdot d[B] - \int_0^b \left(\frac{\bar{n}_B}{[B]}\right)_{\substack{C_M = 0 \\ [A] = [A]_n}} \cdot d[B] \qquad (11)$$

The X-functions hence obtained are found in Table 4 and $X_1([A])$ and $X_2([A])$ are also found in Fig. 2. Their extrapolation to [A] = 0 gives β_1 and β_2. $X_3([A])$ has an almost constant value ($= \beta_3$), thus complexes with more than three ligands cannot be proved within the range of [A] used.

With the constants obtained, $\bar{n}_A$ is calculated ((2) of II) for the values of $[A]_n$ used before, and in addition for [A] = 10 mC (Table 4 and fulldrawn

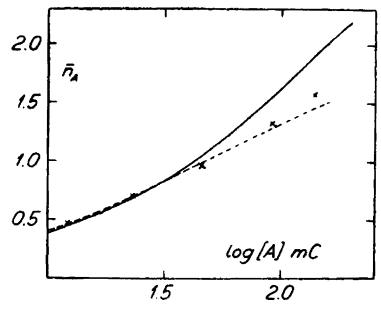

Fig. 3. The complex formation curves. The fulldrawn curve is calculated from the complexity constants found potentiometrically and the dashed one from those found extinctiometrically. × = *the experimental values of the extinctiometric investigation (Table 7).*

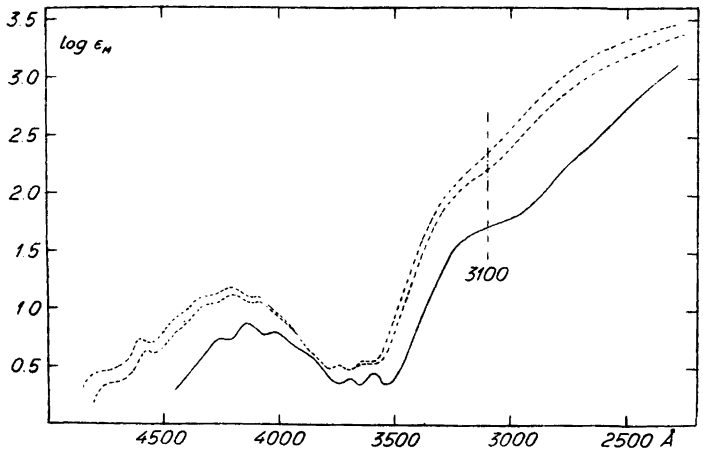

Fig. 4. Extinction curves of a. uranyl ion (fulldrawn) and b. complex solutions with C_M = 10 mC, C'_A = 50 mc (lower dashed) and C_M = 10 mC, C'_A = 200 mC (upper dashed).

curve of Fig. 3). For the same [A] the composition of the system is also calculated ((8) of II) and given in Table 4.

Then the U-functions are formed according to (3) and (4) and the constants of the mixed complexes MA_jB hence calculated. (Table 5). $U_1([A])$ is also drawn in Fig. 2. Its extrapolation to [A] = 0 gives $\beta_{1,1}$. Within the random errors, $U_2([A])$ is a constant ($= \beta_{2,1}$), only the first two complexes MA_jB can thus be proved.

THE EXTINCTION MEASUREMENTS

a. Selection of a wave-length suitable for measurement

To select a suitable wave-length λ, the extinction curves of the following solutions have been measured *:

1. C_M = 10 mC, C'_A = 50 mC
2. C_M = 10 mC, C'_A = 200 mC

I = 1 by $NaClO_4$

The curves are found in Fig. 4, together with that of UO_2^{2+} (I p. 377). The extinction of the sulfate and hydrosulfate ions can be neglected in the λ-range used. [15]

* As in IV, all extinction measurements are performed with a Beckman Quartz Spectrophotometer DU, improved as described by Adell [14] p. 3.

As seen from the curves, the addition of sulfate causes a considerable change of ε_M in different parts of the λ-range. In the visible and near UV regions, however, ε_M is so small that very high C_M have to be used to the d:s available. This would imply a not allowed change of the ionic medium. On the other hand, ε_M is so great at very short λ that only very low C_M can be used there. But as the present complex system is of medium strength only no marked differences of [A] would then arise between the series of different C_M. Thus no differences would be measured between their ε_M either, and the calculation of $\bar{n}_A$ would then be impossible. Thus the middle UV-region must be used, and 3 100 Å is selected as a suitable value of λ.

The solutions are not influenced if kept some days in the dark or in the diffuse day-light (*cf.* II p. 784, 804). The same ε_M:s are always found, within the limits of error of the Spectrophotometer.

b. The measurements at $\lambda = 3\,100$ Å

At $\lambda = 3\,100$ Å, the hydrolysis of the uranyl ion has a great influence on ε_M (*cf.* I 377). Therefore, so much perchloric acid is added to the complex solutions that those of the lowest C'_A acquire a $[H^+] \approx 3$ mC, the experimental determination of which is performed by quinhydrone electrode using the same RE as above. At this $[H^+]$, the hydrolysis can be neglected even in not complex solutions (I p. 377), while the formation of hydrosulfate is not yet very large. If it is assumed that the slight $[HSO_4^-]$ does not give any appreciable complex formation, (21) of II is valid and may be applied to the determination of [A] and $\bar{n}_A$ in the present system.

Absorption cells of $d = 0.1$, 0.3 and 1 cm are available. As in IV (p. 212), every cell is used in connection with a constant C_M which is chosen so that $C_M \cdot d$ is always a constant, too. At the ε_M of the present λ, $C_M = 30$, 10 and 3 mC give suitable values of E. As before, (*cf.* IV p. 212), the differences of transparancy between the cells as well as the ratios between their thicknesses are determined by separate measurements.

For every C_M, C'_A has been varied between 10 (or 25) and 300 mC. A further increase of C'_A causes an increase of ε_M which is too small to be useful (*cf.* IV p. 216). C'_A lower than those used here would give a very small E, and, moreover, there would be an increase of the influence of that hydrolysis which possibly remains.

The function searched for is $\varepsilon_M = f(C_A)$. To find C_A, (14) of II is applied with the simplification $C_s = 0$, *i.e.* the hydrolysis is neglected (*cf.* p. 1154). We thus put $[HSO_4^-] = C'_A - C_A = C^o_H - [H^+]$. This manner of estimating $[HSO_4^-]$ can be applied here, but not in the potential measurements above

Table 6. E_M as a function of C_A at the different values of C_M.

$C_M \rightarrow$ mC	30		10		3	
$d \rightarrow$ cm	0.1		0.3		1	
C'_A mC	ε_M C^{-1}. cm^{-1}	C_A mC	ε_M C^{-1}. cm^{-1}	C_A mC	ε_M C^{-1}. cm^{-1}	C_A mC
10			87.1	9.8	93.7	9.8
25	102.5	24.7	120.8	24.6	129.0	24.6
50	136.5	49.4	154.8	49.3	161.0	49.3
100	175.1	98.7	187.5	98.8	191.3	98.9
150	195.8	148.2	203.3	148.5	206.0	148.7
200	207.4	197.9	214.2	198.4	216.0	198.5
300	219.9	297.6	224.4	298.1	226.3	298.3

where acetate buffer is present. It gives lower values of $[HSO_4^-]$ than (6) does, on account of the approximation $[SO_4^{2-}] \approx C_A$ introduced there. At high C_A, however, where this approximation is a good one, the two methods give much the same result.

The functions $\varepsilon_M = f(C_A)$ thus found are tabulated in Table 6, and graphically given in Fig. 5. They are now cut at five constant ε_M, the highest value of ε_M chosen being 205 $cm^{-1} \cdot C^{-1}$. Cuts performed at still higher ε_M would give too uncertain values of C_A, on account of the slower and slower rise of the ε_M-curves. For every ε_M chosen, C_A is found to be a linear function of C_M, which indicates a mononuclear complex formation (*cf.* II p. 803). These linear

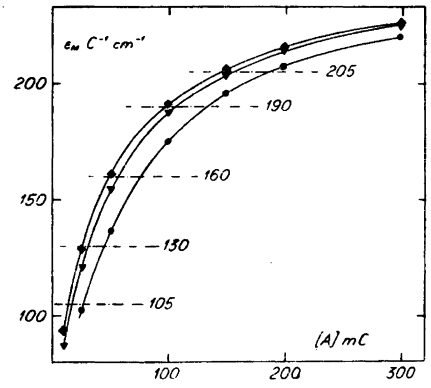

Fig. 5. ε_M as a function of C_A at C_M = 30 (●), 10 (▼) and 3 mC (•). The curves are cut at five constant ε_M, each representing a certain constant value of $[A]$ and $\bar{n}_A$.

Table 7. C_A as a function of C_M at the selected values of ε_M and the obtained values of $[A]$ and $\bar{n}_A/[A]$.

$C_M \rightarrow$ mC	30	10	3	0		
ε_M $C^{-1}.cm^{-1}$	C_A mC			$C_A = [A]$ mC	$\bar{n}_A$	$\bar{n}_A/[A]$ C^{-1}
105	26.3	16.6	13.6	12.1	0.47	39
130	44.2	30.0	25.1	22.9	0.71	31.0
160	74.2	55.1	48.0	45.2	0.97	21.5
190	131.6	105.1	95.6	91.6	1.33	14.5
205	186.0	155.5	144.2	139.6	1.57	11.3

functions have the intercept on the C_A-axis = [A], while their slope gives the corresponding $\bar{n}_A$. The results are found in Table 7 and Fig. 3.

The X-function are then computed by graphical integration of the $\bar{n}_A/[A]$-function (Table 8). As no values of [A] are determined in the immediate neighbourhood of the $\bar{n}_A/[A]$-axis, the integration is performed with $[A]_0 =$ 10 mC as the lower limit (*cf.* II p. 786). The integration then gives $X([A])/X(0.01)$. This function, Fig. 2, is extrapolated to $[A] = 0$ and gives $1/X(0.01) = 0.62 \pm 0.03$. Thus $X([A])$ is known, and hence $X_1([A])$ and $X_2([A])$ are formed. The extrapolation of $X_1([A])$ to $[A] = 0$ gives β_1. Indeed, this function is almost linear within the limited range of [A] available. Thus

Table 8. The X-functions, as obtained by graphical integration of the extinctiometrically determined $\bar{n}_A/[A]$-function. The complexity constants and the ligand number obtained from these constants.

	$\beta_1 = 56 \pm 6$ C^{-1}			$\beta_2 = 450 \pm 50$ C^{-2}		
[A] mC	$\ln \frac{X([A])}{X(0.01)}$	$\frac{X([A])}{X(0.01)}$	$X([A])$	$X_1([A])$ C^{-1}	$X_2([A])$ C^{-2}	$\bar{n}_A$
0		*0.62*		*56.5*	*465*	
10		1.000	1.61	61	450	0.40
20	0.3657	1.441	2.32	66	475	0.64
40	0.9122	2.490	4.01	75.5	475	0.93
60	1.3199	3.743	6.03	84	460	1.10
100	1.9455	7.00	11.3	103	465	1.32
140	2.4481	11.57	18.7	126	495	1.45

$X_2([A])$ is almost a constant $= \beta_2$. Possibly a slight deviation at the highest [A] may be regarded as an indication of the existence of a third complex, but no information can be gained about β_3.

Ultimately, the difficulty of proving the third complex extinctiometrically is certainly due to the circumstance that ε_3 has much the same value as ε_2, as can be understood by the course of the ε_M-curves at high C_A.

With the constants obtained, $\bar{n}_A$ is calculated according to (2) of II, and given in Fig. 3 as a dashed curve. This curve is seen to fit the experimental points of Table 7 very well, except at the highest [A], where, as said above, the third complex may have some influence.

COMPARISON BETWEEN THE RESULTS OF THE TWO METHODS USED

On comparison between the results gained in potentiometric and extinctiometric way, it is seen that the two complex formation curves found coincide at low [A]. In harmony with this, consistent values of β_1 and β_2 are obtained according to both methods. At high [A], on the other hand, the curves run apart, the potentiometric curve then being the steepest one. In connection with this, a high value of β_3 is found potentiometrically, whereas it is not possible to determine any value of β_3 extinctiometrically.

The discrepancy between the curves is no doubt partly due to the difficulty of determining high [A] extinctiometrically, but it is also certain that activity changes are of great importance. From Table 1 it is seen that the exchange of perchlorate for sulfate causes a perceptible change of the medium. It is obvious that this change may affect the two methods in different measure. Moreover, the perchlorate is partly exchanged for acetate buffer in the potentiometric investigation but not in the extinctiometric one, and this may also have some influence even if it is not very large to judge from the results of IV p. 204.

SUMMARY

The complexity of uranyl sulfate is investigated potentiometrically as well as extinctiometrically. The measurements are performed at 20° C, and the ionic strength is kept = 1 C by addition of sodium perchlorate.

The potentiometric investigation is carried out according to the method of ligand displacement [11], with acetate as the displacing ligand B. As a buffer of acetate is used, [B] can be determined by measurements of $[H^+]$, carried out by quinhydrone electrode. The acidity of the buffer is chosen so that the hydrolysis of the uranyl species may be neglected. The constants β of the

sulfate compexes MA_j as well as the constants $\beta_{j,1}$ of the mixed complexes MA_jB are computed:

$$\beta_1 = 50 \pm 10\ C^{-1} \qquad \beta_2 = 350 \pm 150\ C^{-2} \qquad \beta_3 = 2\,500 \pm 1\,000\ C^{-3}$$

$$\beta_{1,1} = 6\,000 \pm 1\,500\ C^{-2} \qquad \beta_{2,1} = 40\,000 \pm 15\,000\ C^{-3}$$

The extinctiometric investigation is performed according to the method previously used by the author [2,4]. From a study of the extinction curves of uranyl sulfate solutions, 3 100 Å is found to be a convenient wave-length for the main measurements. Perchloric acid is added in sufficient large amounts to suppress the hydrolysis while $[HSO_4^-]$ is still low. The first two constants β_j can be computed:

$$\beta_1 = 56 \pm 6\ C^{-1} \qquad \beta_2 = 450 \pm 50\ C^{-2}$$

A value of β_3 cannot be calculated, but the measurements indicate a value much lower than that of the potentiometric investigation. This may be due partly to the experimental difficulties, partly to activity effects.

On the whole, however, the agreement between the methods employed is good. This proves, firstly that the potentiometric method works well and, secondly that the extinctiometric method gives the right result, which in turn, implies that the complex formation really is mononuclear.

My thanks are due to Försvarets Forskningsanstalt (FOA), Stockholm, which has given this work a liberal financial support.

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Über die Umsetzung von Formaldehyd mit Allylcarbinol

II.* Zur Kenntnis des 4-Oxytetrahydropyrans und des Δ^3-Dihydropyrans

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Im Zusammenhang mit Versuchen zur synthetischen Darstellung von 2-Desoxypentosen und verschiedener Vertreter der Patulinreihe wurde das aus Allylcarbinacetat und Formaldehyd[1] leicht zugängliche 4-Oxytetrahydropyran näher untersucht. Besonders sollte festgestellt werden, ob das bei der Reaktion unmittelbar entstehende 4-Acetoxytetrahydropyran nach dem früher für andere Verbindungen beschriebenen Verfahren durch Essigsäureabspaltung in das Δ^3-Dihydropyran überführbar ist. Dieses nimmt nämlich für den vorliegenden Zweck insofern eine Schlüsselstellung ein, als sich bei dessen Hydroxylierung die Bildung des 3,4-Dioxytetrahydropyrans, die reduktive Vorstufe einer 2-Desoxypentose, voraussehen liess. Andererseits vermuteten wir im Δ^3-Dihydropyran eine geeignete Ausgangssubstanz für den Aufbau reduktiver Vorstufen des Patulins bzw. patulinähnlicher Stoffe.

Für die Bereitung grösserer Mengen 4-Acetoxytetrahydropyran haben wir die etwas abgewandelte Vorschrift Baker's[2] mit unserer eigenen (*l.c.*) in der Weise kombiniert, dass wir das aus Propylen erhaltene Gemisch aus 4-Acetoxytetrahydropyran und Butandiol-(1,3)-diacetat trennten, letzteres in Allylcarbinacetat überführten und dieses erneut mit Formaldehyd zu 4-Acetoxytetrahydropyran umsetzten. Das 4-Acetoxytetrahydropyran lieferte bei der Umsetzung mit Acetylbromid in Gegenwart von Zinkchlorid 1-Brom-pentandiol-(3,5)-diacetat. Wie wir fanden, geht das 4-Acetoxytetrahydropyran bei der Destillation mit p-Toluolsulfonsäure glatt und in guter Ausbeute in das Δ^3-Dihydropyran über.

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* 9. Mitt. über Formaldehyd-Olefin-Reaktionen.

Das Δ^3-Dihydropyran wurde erstmalig 1947 von Paul und Tchelitcheff[3] bei der Destillation des 3-Bromtetrahydropyrans mit überschüssigem Kaliumhydroxyd erhalten. Durch Bromaddition gewannen sie daraus das 3,4-Dibromtetrahydropyran. Die von Paul und Tchelitcheff für das Δ^3-Dihydropyran und dessen Dibromid angegebenen Konstanten stimmen mit den von uns ermittelten befriedigend überein. Wir haben das Δ^3-Dihydropyran in 90 %-iger Ameisensäure mit Wasserstoffperoxyd hydroxyliert und gelangten dabei zum 3,4-Dioxytetrahydropyran, das als Bisphenylurethan identifiziert wurde. Bei der Umsetzung des Glykols mit Benzoylchlorid in Pyridin entstand das 3,4-Dibenzoxytetrahydropyran (Schmp. 87—90°). Bei der Darstellung des Chlor-tetrahydropyranols haben wir uns eines für die Bereitung von Chlorhydrinen u.W. bisher nicht beschriebenen Weges bedient. Wie gefunden wurde, reagiert eine Mischung aus Wasserstoffperoxyd und Salzsäure mit Olefinen und Olefinderivaten unter Anlagerung der Elemente der unterchlorigen Säure an die olefinische Doppelbindung und Entstehung des entsprechenden Chlorhydrins*. Bei der Anwendung dieser Reaktion auf Δ^3-Dihydropyran entstand eine fast farblose Flüssigkeit vom $Sdp._9$ 89.5°, die sich durch ihre Molrefraktion, ihren Chlorgehalt und die Bildung eines Phenylurethans als 3-Chlor-tetrahydropyranol-(4) bzw. 4-Chlor-tetrahydropyranol-(3) zu erkennen gab.

EXPERIMENTELLER TEIL

Darstellung von 4-Acetoxytetrahydropyran und Butandiol-(1,3)-diacetat

In eine auf 90° erhitzte Lösung von 200 g Paraformaldehyd und 30 ml konz. Schwefelsäure in 1000 ml Eisessig leitete man Propylengas bis zu einer Gewichtszunahme von 185 g ein. Das braune Reaktionsgemisch versetzte man mit 59 g wasserfreier Soda und destillierte an der Brücke unter vermindertem Druck zunächst die überschüssige Essigsäure, dann die entstandenen Reaktionsprodukte möglichst vollständig vom Salz ab. Durch zweimalige Destillation an der Raschigkolonne erhielt man 109 g 4-Acetoxytetrahydropyran, $Sdp._{10}$ 71—71,5°, V. Z. 361,9 (ber. 389), 17 g Zwischenlauf, $Sdp._{10}$ 71,5—89°, 290 g Butandiol-(1.3)-diacetat, $Sdp._{10}$ 89°, V. Z. 620,4 (ber. 645) und 30 g Rückstand.

1-Brom-pentandiol-(3,5)-diacetat**

100 g 4-Acetoxytetrahydropyran wurden mit 65 ml Acetylbromid und 0.5 g Zinkchlorid unter Rückflusskühlung allmählich auf 150° erwärmt. Das braunschwarze Reaktionsgemisch gab nach dreimaliger Destillation 150 g 1-Brom-pentandiol-(3,5)-diacetat vom $Sdp._{10}$ 151—153° V. Z. 617,3 (ber. 630,1).

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* Über verschiedene Anwendungen der Reaktion wird in Kürze berichtet.

** Experimentell bearbeitet von Frl. Gyda Svenneby.

Δ^3-Dihydropyran

163 g 4-Acetoxytetrahydropyran wurden mit 10 g *p*-Toluolsulfonsäuremonohydrat an der Widmerspirale bei gewöhnlichem Druck destilliert. Sobald die Destillationsgeschwindigkeit nachliess, fügte man 10 g *p*-Toluolsulfonsäure hinzu. Bei einer Badtemperatur von ca. 280—310° erhielt man 142,2 g Destillat vom Sdp. 92—119°. Das Destillat wurde mit Äther versetzt und die Ätherlösung zur Entfernung der Essigsäure mit Sodalösung gewaschen. Die Ätherlösung destillierte man bei gewöhnlichem Druck über gebranntem Kalk und erhielt 55,5 g (= 60 % der Theorie) Δ^3-Dihydropyran vom Sdp. 92—93°, $n_D^{24,5} = 1{,}4428$

C_5H_8O (84.1)	Ber.	C	71,39	H	9,59
	Gef.	»	70,72	»	9,46

Zur Darstellung des Dihydropyrans ist es nicht erforderlich, das aus Propylen erhaltene Gemisch aus 3-Acetoxytetrahydropyran und Butandiol-(1,3)-diacetat zu trennen. Man kann dieses Gemisch auch direkt mit *p*-Toluolsulfonsäure destillieren und das Reaktionsgemisch, nach dem Auswaschen der Essigsäure, an einer wirksamen Kolonne auf Dihydropyran und Allylcarbinacetat fraktionieren.

3,4-Dibrom-tetrahydropyran

Eine Lösung von 50 g Δ^3-Dihydropyran in 75 ml Chloroform wurde unter Kühlung vorsichtig mit einer Lösung von 31 ml Brom in 100 ml Chloroform versetzt. Die Bromlösung wurde fast völlig verbraucht. Nach Abdestillieren des Lösungsmittels erhielt man 145 g einer farblosen Flüssigkeit vom $Sdp._{13}$ 106.5°.

$D_4^{22,5} = 1.9354$ $n_D^{22,8} = 1.550$ (Paul und Tchelitcheff geben an: $Sdp._9$ 102—103°, $D_4^{19,5} = 1{,}932$ $n_D^{19.5} = 1{,}5513$).

$C_5H_8OBr_2$ (243,9)	Ber.	C	24,62	H	3,31	Br 65,52
	Gef.	»	24,80	»	3,33	» 65,19

3,4-Dioxy-tetrahydropyran

60 g Δ^3-Dihydropyran wurden mit 194 ml 90 %iger Ameisensäure und 78 ml Perhydrol unter Rückflusskühlung vorsichtig bis zur eben einsetzenden Selbstreaktion erwärmt. Durch Kühlen wurde dafür gesorgt, dass die Temperatur nicht über 70° stieg. Nach vollständigem Abklingen der Reaktion destillierte man nach Zusatz von 50 ml Wasser überschüssiges Lösungsmittel im Vakuum ab.* Den farblosen Rückstand kochte man 1 Stde mit 2 %iger methylalkoholischer Salzsäure. Nach Abdestillieren des Lösungsmittels erhielt man nach zweimaliger Destillation 60 ml eines dickflüssigen gelben Öles vom $Sdp._{11}$ 148°, $D^{19,5} = 1{,}232$, $n_D^{19,5} = 1{,}4820$, Molrefr. = 27,30 (ber. 27,60). (Paul und Tchelitcheff geben an: $Sdp._9$ 158—159°, $D_4^{18} = 1{,}225$, $n_D^{18} = 1{,}4873$.

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* Wegen der Möglichkeit zur Bildung von Peroxyden ist es aus Sicherheitsgründen zweckmässig, vor der Destillation diese und einen eventuellen Überschuss von Hydrogenperoxyd zu beseitigen.

Bisphenylurethan feine Nadeln aus Aceton-Benzol Schmp. 206—208° (Paul und Tchelitcheff geben an: Schmp. 212°):

$C_{19}H_{20}O_5N_2$ (356,4)	Ber.	C 64,03	H 5,66	N 7,86
	Gef.	» 64,27	» 5,53	» 7,54

Dibenzoat. Farblose Krystalle vom Schmp. 87—90°

$C_{19}H_{18}O_5$ (326,3)	Ber.	C 69,93	H 5,56
	Gef.	» 69,63	» 5,39

Addition von unterchloriger Säure an Δ^3-Dihydropyran mittels Wasserstoffperoxyd-Salzsäure

In eine Mischung von 64,5 g Δ^3-Dihydropyran und 90 ml Perhydrol (Dihydropyran bildet die obere Schicht!) wurden unter Rückflusskühlung und lebhaftem mechanischem Rühren ca. 75 ml konzentrierte Salzsäure allmählich zugetropft, wobei man durch gelegentliches Kühlen dafür sorgte, dass die in der Flüssigkeit gemessene Temperatur nicht über 40° stieg. Nach ca. 8 Stden wurde die untere (!) organische Schicht (A) abgetrennt und die wässrige Schicht nach Zusatz überschüssigen Natriumbisulfites (zur Beseitigung von möglicherweise gebildeten Peroxyden und nicht umgesetztem Wasserstoffperoxyd) wiederholt mit Chloroform ausgeschüttelt. Die Chloroformauszüge vereinigte man mit der Flüssigkeit A, wusch erneut mit Bisulfitlösung und trocknete über wasserfreiem Natriumsulfat. Nach dem Abdestillieren des Chloroforms erhielt man durch zweimalige Destillation an der Widmerspirale ca. 30 g *Chlortetrahydropyranol* als eine farblose Flüssigkeit vom $Sdp._9 89{,}5°$, $D_4^{21} = 1{,}2719$, $n_D^{21} = 1{,}4896$,

$M_R = 31{,}03$ (ber. 31,13)

$C_5H_9O_2Cl$ (136.6)	Ber.	Cl 25,96	Gef. Cl 25,29

Phenylurethan aus Benzol Schmp. 152 — 153°.

$C_{12}H_{14}O_3NCl$ (255,7)	Ber.	Cl 13,88	N 5,48
	Gef.	» 13,10	» 5,50

ZUSAMMENFASSUNG

Das 4-Acetoxytetrahydropyran gibt bei der Destillation mit *p*-Toluolsulfonsäure Δ^3-Dihydropyran. Der im 4-Acetoxytetrahydropyran vorliegende Ring lässt sich durch Acetylbromid unter Bildung von 1-Brom-pentandiol-(3,5)-diacetat öffnen. Das Δ^3-Dihydropyran liefert bei der Hydroxylierung 3,4-Dioxytetrahydropyran, bei der Behandlung mit einem Gemisch aus

Wasserstoffperoxyd und Salzsäure 3-Chlor-tetrahydropyranol-(4) bzw. 4-Chlor-tetrahydropyranol-(3).

Dem Fridtjof Nansen-Fond danke ich für finanzielle Unterstützung. Frau Birgit Haugen bin ich für gelegentliche geschickte Hilfe verbunden.

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Über Tri-*n*-propyl-amino-silan und einige verwandte Verbindungen

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In einigen früheren Untersuchungen *, die in dem organisch-chemischen Institut der Chalmers Technischen Hochschule ausgeführt worden sind, wurden u.a. die folgenden Si-N-Verbindungen dargestellt:

$(CH_3)_3Si \cdot NH \cdot Si(CH_3)_3$	$(CH_3)_3Si \cdot NH \cdot R'$
$(C_2H_5)_3Si \cdot NH_2$	$(C_2H_5)_2Si \cdot NH \cdot R'$
$(CH_3)Si_2(NH_2)_2$	$(CH_3)_2Si(NH \cdot R')_2$
$(t\text{–}C_4H_9O)_2Si(NH_2)_2$	$(t\text{–}C_4H_9O)_2Si(NH \cdot R')_2$

Diese Untersuchungen hatten hauptsächlich den Zweck, die Anwendbarkeit der doppelten Umsetzungen

$$R_3Si \cdot NH \cdot R' + R'' \cdot NH_2 \longrightarrow R_3Si \cdot NH \cdot R'' + R' \cdot NH_2$$
$$R_2Si(NH \cdot R')_2 + 2\ R'' \cdot NH_2 \longrightarrow R_2Si(NH \cdot R'')_2 + 2\ R' \cdot NH_2$$

zur Darstellung von N-substituierten Amino-silanen zu zeigen und wurden mit R = CH_3, C_2H_5 und t-C_4H_9O durchgeführt. Die Verbindungen $(CH_3)_3Si \cdot NH \cdot Si(CH_3)_3$, $R_3Si \cdot NH \cdot R'$ und $R_2Si(NH \cdot R')_2$ können in wässriger Alkohollösung oder Acetonlösung mit Salzsäure (Methylrot) titriert werden, wenn das Amin $R' \cdot NH_2$ titrierbar ist. Ob diese Titrierbarkeit von einer sehr raschen Hydrolyse des Amino-silans in Silanol und Amin oder von der Basenstärke des Amino-silans abhängig ist, ist noch nicht näher untersucht. Es scheint jedoch, als ob die Bindung zwischen den Silizium- und Stickstoffatomen so leicht hydrolysiert wird, dass der Stickstoff bei dem Endpunkt der Titration als $R' NH_3^+$ vorliegt.

* R, R′ und R″ sind Alkyl-, Aryl- oder Aralkyl-gruppen. R kann auch eine t-Butoxygruppe sein.

Es ist zu vermuten, dass die Festigkeit der Bindung zwischen den Silizium- und Stickstoffatomen in einer Verbunding $R_3Si \cdot NH \cdot R'$ sowohl durch R als auch R' beeinflusst wird. Es wäre daher von Interesse, die erwähnte doppelte Umsetzung und Titrierbarkeit der Silizium-Stickstoff-Verbindungen näher zu untersuchen, wenn R eine Aryl-, Aralkyl- oder höhere Alkyl-gruppe ist. Von denselben Gesichtspunkten aus würde es vom Interesse sein, einige Verbindungen $R_3Si \cdot N\begin{smallmatrix}R'\\R''\end{smallmatrix}$ darzustellen und zu untersuchen.

Als Teil eines nach diesen Gesichtspunkten geplanten Arbeitsprogrammes wurde die folgende Untersuchung über Tri-*n*-propyl-amino-silan und einige seiner N-substitutionsderivate vorgenommen.

Die folgenden Verbindungen mit Silizium-Stickstoff-Bindungen wurden hergestellt:

I $(n\text{-}C_3H_7)_3Si \cdot NH_2$
II $(n\text{-}C_3H_7)_3Si \cdot NH \cdot CH_3$
III $(n\text{-}C_3H_7)_3Si \cdot NH \cdot CH_2 \cdot CH_3$
IV $(n\text{-}C_3H_7)_3Si \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_3$
V $(n\text{-}C_3H_7)_3Si \cdot NH \cdot CH(CH_3)_2$
VI $(n\text{-}C_3H_7)_3Si \cdot NH \cdot C_6H_5$
VII $(n\text{-}C_3H_7)_3Si \cdot NH \cdot CH_2 \cdot C_6H_5$
VIII $(n\text{-}C_3H_7)_3Si \cdot NH \cdot CH_2 \cdot CH_2 \cdot C_6H_5$
IX $(n\text{-}C_3H_7)_3Si \cdot N(CH_3)_2$
X $(n\text{-}C_3H_7)_3Si \cdot N(CH_2 \cdot CH_3)_2$.

Die Verbindungen I—V, IX und X wurden aus Tri-*n*-propyl-chlor-silan und Ammoniak oder dem betreffenden Amin dargestellt. Die doppelte Umsetzung zwischen I und Anilin, Benzylamin bzw. β-Phenäthylamin ergab VI, VII bzw. VIII. VII wurde ausserdem aus IV und Benzylamin dargestellt. Die Verbindungen I—V und VII—X können mit Salzsäure (Methylrot) in wässriger Alkohollösung titriert werden.

Die Verbindung IV ergibt mit Methanol Tri-*n*-propyl-methoxy-silan. Überhaupt dürften die Verbindungen I—X mit Alkoholen unter Bildung von Tri-*n*-propyl-alkoxy-silan reagieren können.

Tri-*n*-propyl-chlor-silan, das als Ausgangsmaterial bei einigen von den Synthesen verwendet wurde, ist nicht früher in der Literatur beschrieben. Wir haben es etwa in derselben Weise dargestellt, wie DiGiorgio, Strong, Sommer und Whitmore[2]. Triäthyl-chlor-silan darstellten. Tetraäthoxy-silan ergab mit *n*-Propylmagnesiumchlorid im Überschuss und nach Hydrolyse des Reaktionsproduktes ein Gemisch von Tri-*n*-propyl-silanol, Tri-*n*-propyl-äthoxysilan und Hexa-*n*-propyl-disiloxan, aus dem in konzentrierter Schwefelsäure mit Ammoniumchlorid Tri-*n*-propyl-chlor-silan erhalten wurde.

BESCHREIBUNG DER VERSUCHE

Tri-*n*-propyl-chlor-silan

175,1 g (7,2 G.-Atome) Magnesiumspäne in 400 ml abs. Äther wurden unter Rühren bei höchstens 70° mit 565 g (7,2 Mol) *n*-Propylchlorid grignardiert. Das Reaktionsgemisch wurde im Laufe von 4 Stunden unter Rühren mit 172,8 g (0,85 Mol) Tetraäthoxy-silan versetzt. Dann wurde es während 2 Stunden siedend gehalten, wonach 200 ml Toluol zugesetzt und der grösste Teil des Äthers im Laufe von etwa 3,5 Stunden abdestilliert wurde. Das Reaktionsgemisch wurde dann während 11 Stunden unter Rühren (Temperatur 100—110°) erwärmt und nach dem Erkalten allmählich mit 5 *N* Salzsäure im Überschuss zugesetzt. Die Toluollösung wurde abgetrennt und die Wasserlösung mit Äther extrahiert. Aus der Ätherlösung wurde der Äther abdestilliert und der Rückstand mit der Toluollösung vereinigt. Die Toluollösung wurde mit Sodalösung und Wasser gewaschen und dann mit wasserfreiem Natriumsulfat getrocknet. Das Toluol wurde bei Athmosphärendruck abgetrieben und der Rückstand unter vermindertem Druck destilliert. Es wurden 125 g eines Gemisches von Tri-*n*-propyl-silanol, Tri-*n*-propyl-äthoxy-silan und Hexa-*n*-propyl-disiloxan vom Sdp. 65—155° (16 mm) erhalten.

125 g dieses Gemisches wurden unter Rühren und Kühlen mit Eis in 250 ml konz. Schwefelsäure gelöst. Die Lösung wurde unter Rühren mit 95 g Ammoniumchlorid in kleinen Portionen versetzt. Nachdem alles Ammoniumchlorid zugesetzt worden war, bildete das Tri-*n*-propyl-chlor-silan eine obere leichte Schicht. Diese wurde abgetrennt und destilliert. Es wurden 115 g Tri-*n*-propyl-chlor-silan vom Sdp. 67—71° (8 mm) erhalten. Auf Tetraäthoxy-silan berechnet betrug die Ausbeute 70 %.

$(C_3H_7)_3SiCl = C_9H_{21}ClSi$ (192,8)	Ber.	Cl 18,4		Si 14,6	
	Gef.	» 18,4		» 14,9	

Tri-*n*-propyl-amino-silan (I)

Zu einem mit Kohlensäureschnee und Alkohol gekühlten Gemisch von 200 g flüssigem Ammoniak und 200 ml abs. Äther wurde eine Lösung von 77,1 g (0,4 Mol) Tri-*n*-propyl-chlor-silan in 200 ml abs. Äther zugetropft. Nach fünfstündigem Stehenlassen in dem Kältegemisch liess man das Ammoniak langsam abdunsten, wonach das Ammoniumchlorid unter Feuchtigkeitsabschluss abfiltriert und mit Äther sorgfältig gewaschen wurde. Bei der Destillation der Ätherlösung wurden 61,6 (ber. 69,3) g Tri-*n*-propyl-amino-silan vom Sdp. 70—72° (9 mm) erhalten.

$(C_3H_7)_3Si \cdot NH_2 = C_9H_{23}NSi$ (173,3)	Ber.	Si 16,2		Äquiv.-Gew.	173,3
	Gef.	» 15,8		» »	174,0

Tri-*n*-propyl-N-methylamino-silan (II)

Zu einer Lösung von 19,2 g (0,1 Mol) Tri-*n*-propyl-chlor-silan in 50 ml abs. Äther wurden unter Rühren und Kühlen auf — 20° 6,2 g (0,2 Mol) Methylamin in 50 ml abs. Äther zugetropft. Die Hydrochloridfällung wurde unter Feuchtigkeitsabschluss abfil-

triert und mit Äther gewaschen. Aus den Ätherlösungen wurden bei der Destillation 13,8 (ber. 18,7) g Tri-*n*-propyl-N-methylamino-silan vom Sdp. 195—196° erhalten.

$(C_3H_7)_3Si \cdot NH \cdot CH_3 = C_{10}H_{25}NSi$ (187,4) Ber. Si 15,0 Äquiv.-Gew. 187,4
Gef. » 14,9 » » 188,8

Tri-*n*-propyl-N-äthylamino-silan (III)

Zu einer Lösung von 19,2 g (0,1 Mol) Tri-*n*-propyl-chlor-silan in 50 ml abs. Äther wurden 9,0 g (0,2 Mol) Äthylamin in 50 ml abs. Äther unter Kühlen zugetropft. Danach wurde das Reaktionsgemisch während 3 Stunden bei Zimmertemperatur gerührt. Die Hydrochloridfällung wurde unter Feuchtigkeitsabschluss abfiltriert und mit Äther gewaschen. Die Ätherlösung ergab bei der Destillation 13,0 (ber. 20,1) g Tri-*n*-propyl-N-äthylamino-silan vom Sdp. 75—76° (3 mm).

$(C_3H_7)_3Si \cdot NH \cdot CH_2 \cdot CH_3 = C_{11}H_{27}NSi$ (201,4) ber. Si 13,9 Äquiv.-Gew. 201,4
Gef. » 13,4 » » 204,6

Tri-*n*-propyl-N-*n*-propylamino-silan (IV)

Zu einer Lösung von 38,5 g (0,2 Mol) Tri-*n*-propyl-chlor-silan in 50 ml abs. Äther wurden 23,6 g (0,4 Mol) *n*-Propylamin in 50 ml abs. Äther zugesetzt, wonach das Reaktionsgemisch während 2 Stunden siedend gehalten wurde. Die Hydrochloridfällung wurde in üblicher Weise abfiltriert und gewaschen. Die Ätherlösung ergab bei der Destillation 27,2 (ber. 43,1) g Tri-*n*-propyl-N-*n*-propylamino-silan vom Sdp. 95° (6 mm).

$(C_3H_7)_3Si \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_3 = C_{12}H_{29}NSi$ (215,4) Ber. N 6,6 Si 13,0 Äquiv.-Gew. 215,4
Gef. » 6,7 » 12,8 » » 216,2

Tri-*n*-propyl-N-*i*-propylamino-silan (V)

19,2 g (0,1 Mol) Tri-*n*-propyl-chlor-silan in 50 ml abs. Äther und 11,8 g (0,2 Mol) *i*-Propylamin in 50 ml Äther ergaben wie in der vorangehenden Synthese 16,0 (ber. 21,5) g Tri-*n*-propyl-N-*i*-propylamino-silan vom Sdp. 79—80° (9 mm).

$(C_3H_7)_3Si \cdot NH\ CH(CH_3)_2 = C_{12}H_{29}NSi$ (215,4) Ber. Si 13,0 Äquiv.-Gew. 215,4
Gef. » 12,7 » » 217,3

Tri-*n*-propyl-N-phenylamino-silan (VI)

15,6 g (0,09 Mol) Tri-*n*-propylamino-silan und 16,8 g (0,18 Mol) Anilin wurden gemischt. Das Reaktionsgemisch wurde während 1,5 Stunden unter Rückfluss zum Sieden erwärmt. Die darauf folgende Fraktionierung ergab 19,5 (ber. 22,4) g Tri-*n*-propyl-N-phenylamino-silan vom Sdp. 142—143° (5 mm).

$(C_3H_7)_3Si \cdot NH \cdot C_6H_5 = C_{15}H_{27}NSi$ (249,4) Ber. H 10,9 Si 11,25
Gef. » 10,9 » 11,2

Tri-*n*-propyl-N-benzylamino-silan (VII)

a) Ein Gemisch von 10,4 g (0,06 Mol) Tri-*n*-propylamino-silan und 12,9 g (0,12 Mol) Benzylamin ergab nach dem Sieden während 2,5 Stunden bei der Destillation 10,1 (ber. 15,8) g Tri-*n*-propyl-N-benzylamino-silan vom Sdp. 147—148° (6 mm).

$(C_3H_7)_3Si \cdot NH \cdot CH_2 \cdot C_6H_5 = C_{16}H_{29}NSi$ (263,5)	Ber.	N 5,3	Si 10,7
	Gef.	» 5,1	» 10,7

b) Aus einem Gemisch von 10,7 g (0,05 Mol) Tri-*n*-propyl-N-*n*-propylamino-silan und 10,7 g (0,1 Mol) Benzylamin wurde während 2,5 Stunden *n*-Propylamin abdestilliert, wonach der Rückstand fraktioniert wurde. Es wurden dabei 10,6 (ber. 13,2) g Tri-*n*-propyl-N-benzylamino-silan vom Sdp. 145—146° (5 mm) erhalten.

Tri-*n*-propyl-N-β-phenäthylamino-silan (VIII)

Ein Gemisch von 10,4 g (0,06 Mol) Tri-*n*-propylamino-silan und 14,5 g (0,12 Mol) β-Phenäthylamin ergab wie in dem vorangehenden Versuch 10,0 (ber. 16,7) g Tri-*n*-propyl-N-β-phenäthylamino-silan vom Sdp. 154—155° (3 mm).

$(C_3H_7)_3Si \cdot NH \cdot CH_2 \cdot CH_2 \cdot C_6H_5 = C_{17}H_{31}NSi$ (277,5)	Ber. Si 10,1	Äquiv.-Gew. 277,5
	Gef. » 9,8	» » 276,0

Tri-*n*-propyl-N-dimethylamino-silan (IX)

Zu einer auf etwa — 20° gekühlten Lösung von 9,0 g (0,2 Mol) Dimethylamin in 50 ml abs. Äther wurden unter Rühren 19,2 g (0,1 Mol) Tri-*n*-propyl-chlor-silan in 50 ml abs. Äther zugetropft. Das Reaktionsgemisch wurde dann während 4 Stunden unter Kühlen gerührt, wonach die Hydrochloridfällung in üblicher Weise abfiltriert wurde. Die Ätherlösung ergab bei der Destillation 14,2 (ber. 20,1) g Tri-*n*-propyl-N-dimethylamino-silan vom Sdp. 58—60° (3 mm).

$(C_3H_7)_3Si \cdot N(CH_3)_2 = C_{11}H_{27}NSi$ (201,4)	Ber.	Si 13,9	Äquiv.Gew. 201,4
	Gef.	» 13,9	» » 201,0

Tri-*n*-propyl-N-diäthylamino-silan (X)

27,7 g (0,5 Mol) Diäthylamin in 50 ml abs. Äther und 19,2 g (0,1 Mol) Tri-*n*-propylamino-silan in 50 ml abs. Äther ergaben wie in dem vorangehenden Versuch 18,0 (ber. 22,9) g Tri-*n*-propyl-N-diäthylamino-silan vom Sdp. 95—96° (7 mm).

$(C_3H_7)_3Si \cdot N(C_2H_5)_2 = C_{13}H_{31}NSi$ (229,4)	Ber.	Si 12,2	Äquiv.-Gew. 229,4
	Gef.	» 12,0	» » 229,5

Tri-*n*-propyl-methoxy-silan

Ein Gemisch von 5,0 g (0,15 Mol) Methanol und 10,7 g (0,05 Mol) Tri-*n*-propyl-N-*n*-propylamino-silan wurde während 2 Stunden zum Sieden erwärmt und dann destilliert. Es wurden 6,5 (ber. 9,4) g Tri-*n*-propylmethoxy-silan vom Sdp. 63° (4 mm) erhalten.

$(C_3H_7)_3Si \cdot OCH_3 = C_{10}H_{24}OSi$ (188,4)	Ber. Si	14,9	OCH_3	16,5
	Gef. »	14,5	»	16,7

ZUSAMMENFASSUNG

Es wurde eine Reihe Amino-silane von der allgemeinen Formel $(n\text{-}C_3H_7)_3 Si \cdot NH \cdot R$ mit R = H, C_2H_5, $n\text{-}C_3H_7$, $i\text{-}C_3H_7$, C_6H_5, $C_6H_5 \cdot CH_2$, $C_6H_5 \cdot CH_2 \cdot CH_2$ dargestellt. Weiter wurden $(n\text{-}C_3H_7)_3 Si \cdot N(CH_3)_2$ und $(n\text{-}C_3H_7)_3 Si \cdot N(C_2H_5)_2$ hergestellt. Durch Methanolyse von $(n\text{-}C_3H_7)_3 Si \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_3$ wurde $(n\text{-}C_3H_7)_3 Si \cdot OCH_3$ erhalten.

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Infra-Red Absorption Spectra of Some Organic Phosphoryl Compounds

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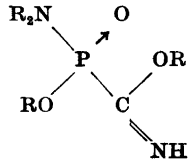
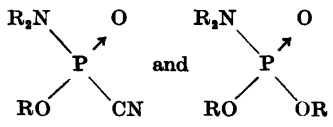
During the last fifteen years organo-phosphorus compounds have more and more attracted the interest of chemists. From 1934 and onwards G. Schrader [1] is said to have synthetized over 2000 new compounds submitted to bioassay. Some of these were so toxic that they were intended to be used as war-gases during World War II. Many of them have superseded the valuable nicotine as insecticides. Lately they have been used therapeutically in some diseases *e.g.* myasthenia gravis and glaucoma.

The phosphorus compounds of this type are potent enzyme inhibitors. The enzyme cholinesterase, vital to the transmission of nerve impulses in the body, is completely inhibited even by very minute amounts [2].

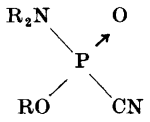
One of the most active substances is dimethylamido-ethoxy-phosphoryl cyanide (TABUN), the method of synthesis of which can be traced back to Schall [3]. However, he never seems to have recognized the true, chemical constitution of his compound. According to him the compound was an imino-ether with the following structure:



Michaelis [4] in 1903 suggested that Schall had obtained a mixture of



Further work on the synthesis of this compound has been done by Schrader who adopted the formula



published in Protar (1948)[5]. Because the method of synthesis of dimethylamido-ethoxy-phosphoryl cyanide still leaves some doubt to its chemical structure, we have made an infra-red spectroscopical examination of this compound and some related derivatives. This method is a very suitable way to elucidate problems of this kind. In addition the purpose of the present study has been to determine the characteristic frequencies of the P-N linkage. Similar measurements of alkyl esters of phosphorus oxy-acids have recently been made for the P→O and P-O linkages by Meyrick and Thompson[6].

EXPERIMENTAL

The absorption spectra of the phosphoryl compounds between 2.5 and 15.0 μ were recorded on a Beckman Infrared Spectrophotometer. It was equipped with rock salt optics and rock salt prism. The absorption cell sealed with an amalgam gasket had a thickness of the liquid sample of 0.10 mm. The compounds were dissolved in carbon tetrachloride which is a very good solvent owing to its weak absorption in this spectral region except between 12 and 14 μ where instead benzene solutions were used. The concentration of the solutions was 10 % except in the range 9 — 11 μ where 4 % solutions were used to get a better resolution of the very strong absorption bands in this part of the spectrum.

The compounds studied were synthetized at the Medical Section of the Research Institute of National Defence[2]:

	n_D^{20}	B. p.
Diethoxy-phosphoryl chloride	1.4177	68—70°/3 mm
Diethoxy-phosphoryl cyanide	1.4015	91.5—94°/11 mm
Diethoxy-phosphoryl amide	—	140°/3 mm
Dimethylamido-phosphoryl dichloride	1.4080	58—60°/4—5 mm
Dimethylamido-ethoxy-phosphoryl chloride	1.4380	70—74°/4—6 mm
Dimethylamido-ethoxy-phosphoryl cyanide (TABUN)	1.4250	100—108°/9 mm
Tetraethyl pyrophosphate (TEPP)	1.4184	132°/1.3
sym-Bis-(dimethylamido)-diethylpyrophosphate	1.4392	130°/1.5 mm

RESULTS AND DISCUSSION

Figs. 1 and 2 show the infra-red absorption spectra of the compounds examined. From these spectra one can observe some absorption frequencies which can be correlated with specific atomic configurations.

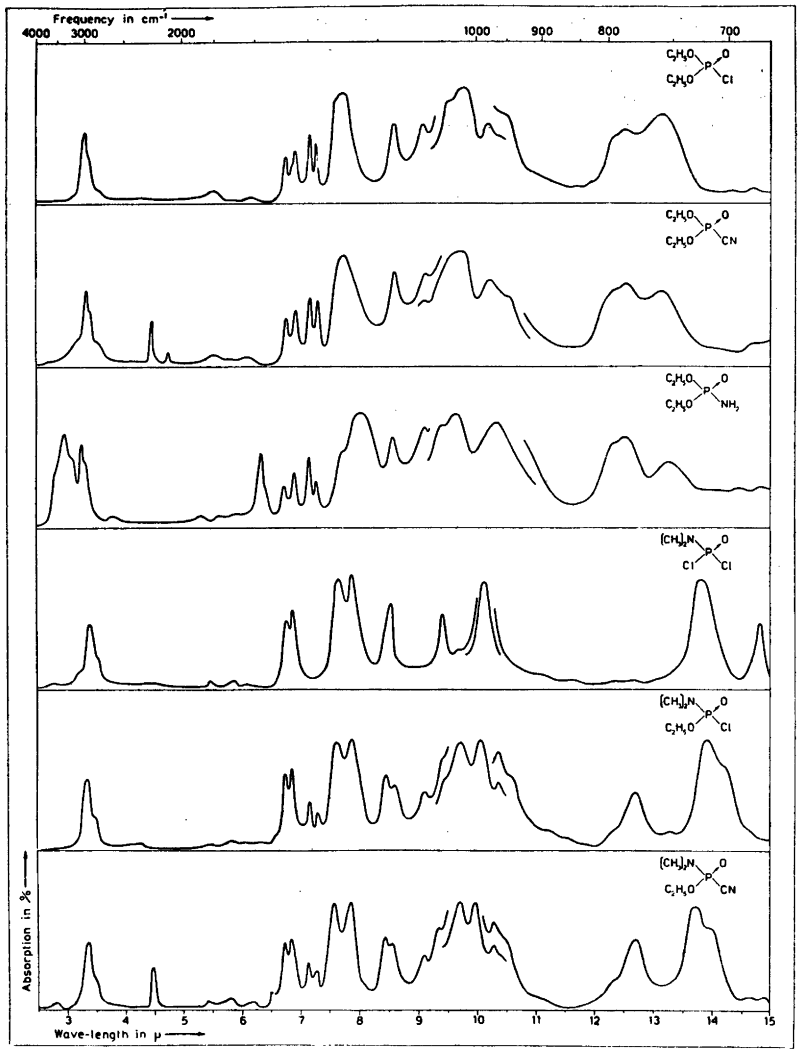


Fig. 1. Absorption curves of the phosphoryl compounds.

The absorption bands caused by the vibrations in the $>CH_2$ and $-CH_3$ groups occur in the normal positions: bondstretching vibrations in $>CH_2$ and $-CH_3$ at 3.27—3.55 μ (3058—2817 cm^{-1}), bondbending vibrations in $-CH_3$ at 6.72—6.94 μ (1488—1440 cm^{-1}) and bondbending vibrations in $>CH_2$ at 7.14—7.31 μ (1400—1368 cm^{-1}).

The absorption band at 4.48 μ (2232 cm^{-1}), occurring only in the spectrum of the two cyanogen compounds, can be assigned to the bondstretching vib-

Table 1. Absorption bands of the phosphoryl compounds.

λ = Wavelength in μ
ν = Frequency in cm^{-1}
s = strong, m = medium, w = weak

Diethoxy-phosphoryl chloride		Diethoxy-phosphoryl cyanide		Diethoxy-phosphoryl amide	
λ	ν	λ	ν	λ	ν
3.33	3 003 m	3.20	3 125 w	2.98	3 356 s
3.40	2 941 m	3.35	2 985 m	3.10	3 226 m
5.54	1 805 w	3.42	2 924 m	3.27	3 058 s
6.77	1 477 m	3.55	2 817 w	3.35	2 985 m
6.87	1 456 m	4.48	2 232 m	3.80	2 632 w
6.93	1 443 m	4.76	2 101 w	5.32	1 880 w
7.18	1 393 m	5.55	1 802 w	5.65	1 770 w
7.29	1 372 m	6.10	1 639 w	6.34	1 577 m
7.62	1 312 s	6.77	1 477 m	6.73	1 486 m
7.74	1 292 s	6.94	1 441 m	6.91	1 447 m
8.60	1 163 m	7.19	1 391 m	7.16	1 397 m
9.09	1 100 m	7.31	1 368 m	7.28	1 374 m
9.54	1 048 s	7.75	1 290 s	7.73	1 294 m
9.78	1 022 s	8.61	1 161 s	8.04	1 244 s
10.20	980 s	9.11	1 098 s	8.57	1 167 s
10.45	957 s	9.70	1 031 s	9.10	1 099 s
12.35	810 m	10.22	978 s	9.40	1 064 s
12.53	798 m	10.55	947 s	9.64	1 037 s
13.15	761 s	12.35	810 m	10.34	967 s
		12.55	797 s	12.35	810 s
		13.14	761 m	12.52	799 s
				13.26	754 m

rations in the —C≡N linkage. The diethoxy-phosphoryl cyanide shows a band at 4.76 μ (2101 cm^{-1}) also, which may be interpreted either as vibrations in the isocyanide group —N = C obtained as a by-product in the synthesis or as vibrations in HCN formed by a partial hydrolysis of the compound. Both these groups may be surmised to cause absorption in this region.

All the phosphoryl derivatives show an absorption band in the region 7.57—8.04 μ (1321—1244 cm^{-1}). Most of them cause a doubling of the band. This absorption can be correlated with the stretching of the P→O bond, which has previously been established by Meyrick and Thompson. They also have attributed the double band at 12.20—12.70 μ (820-788 cm^{-1}) to the stretching vibrations of the P-O linkage in the skeleton P-O-C.

Dimethylamido-phosphoryl dichloride		Dimethylamido-ethoxy-phosphoryl chloride		Dimethylamido-ethoxy-phosphoryl cyanide	
λ	ν	λ	ν	λ	ν
3.23	3 096 w	3.35	2 985 m	2.83	3 534 w
3.40	2 941 m	3.49	2 865 w	3.37	2 967 m
3.55	2 817 w	5.48	1 825 w	3.50	2 857 w
5.48	1 825 w	5.86	1 706 w	4.48	2 232 m
5.88	1 701 w	6.56	1 524 w	5.44	1 838 w
6.77	1 477 m	6.75	1 481 m	5.82	1 718 w
6.88	1 453 m	6.86	1 458 m	6.18	1 618 w
7.65	1 307 s	7.17	1 395 m	6.52	1 534 w
7.88	1 269 s	7.30	1 370 m	6.74	1 484 m
8.54	1 170 s	7.61	1 314 s	6.85	1 460 m
9.41	1 062 m	7.88	1 269 s	7.15	1 399 m
9.68	1 033 m	8.46	1 182 m	7.29	1 372 m
10.11	989 s	8.60	1 163 m	7.57	1 321 s
13.82	723 s	9.11	1 098 m	7.86	1 272 s
14.81	675 m	9.40	1 064 s	8.44	1 185 m
		9.70	1 030 s	8.57	1 167 m
		10.05	994 s	9.10	1 099 m
		10.36	966 s	9.34	1 071 s
		10.60	944 m	9.70	1 031 s
		12.35	810 w	9.96	1 004 s
		12.70	788 m	10.28	973 s
		13.30	752 w	10.45	956 m
		13.92	718 s	12.32	811 w
		14.25	702 s	12.70	788 m
				13.70	730 s
				13.95	716 s

In all the spectra of the dimethylamido compounds two absorption bands appear, which are absent from the spectra of all other derivatives. One occurs at 9.94—10.11 μ (1006—989 cm^{-1}) and the other, which in more complex compounds is split, at 13.70—14.25 μ (730—702 cm^{-1}). The latter absorption may be a result of the stretching vibrations of the P-N bond in the combination P-N$\begin{smallmatrix}\diagup\mathrm{C}\\ \diagdown\mathrm{C}\end{smallmatrix}$ analogously to the absorption of the P-O linkage mentioned above. The origin of the former absorption is still more difficult to derive, because the spectrum in this region is so complex. This absorption may possibly depend on the vibrations in the C-N bond. If the two methyl radicals in the amido group are exchanged for hydrogen, both the absorption bands disappear,

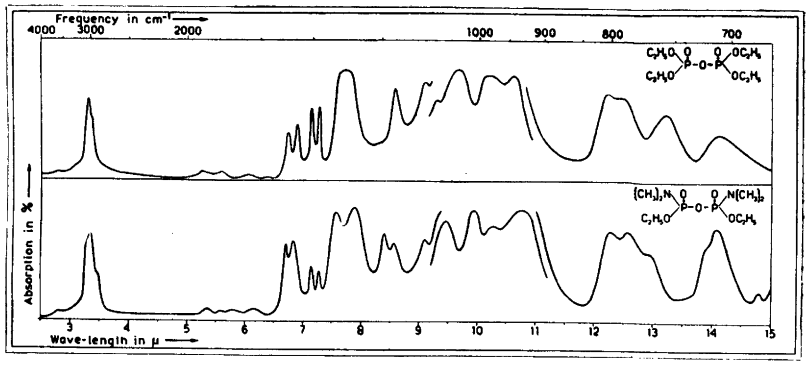


Fig. 2. Absorption curves of the derivatives of pyrophosphoric acid.

and instead the normal absorption bands of the $—NH_2$ bond are obtained at 2.98 μ (3356 cm $^{-1}$) and 3.10 μ (3226 cm $^{-1}$) caused by bondstretching vibrations and a band at 6.34 μ (1577 cm $^{-1}$) attributed to the bondbending vibrations. To some extent this argument supports the hypothesis of the correlation between the P-N resp. C-N linkages and the stated absorption bands.

The absorption spectra of the two derivatives of pyrophosphoric acid show a close resemblance to the other spectra. However, an obvious difference is present at about 14.10 μ (710 cm $^{-1}$). In order to verify whether this absorption may be associated with the stretching in the P-O linkage in the skeleton P-O-P, too few spectra have been recorded.

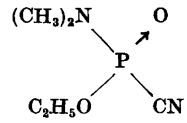
A rough computation by the aid of Hooke's law indicates that the absorption band arising from the stretching in the P-Cl bond can be expected to appear at about 14 μ . An absorption referring to this vibration seems to be found only in the spectrum of dimethylamido-phosphoryl dichloride at 14.81 μ (675 cm $^{-1}$). Analogously to the absorption of the C-Cl bond a displacement of the absorption of the P-Cl linkage to longer wavelengths is expected, and for that reason this absorption of the other chlorine compounds probably lies above 15 μ.

From these results one can conclude, that the formula given by Schall is incorrect, because no absorption band assigning the imino group has been detected, while on the other hand the existence of the $—C \equiv N$ bond is evident. Neither can it be a question of a mixture, which was proposed by Michaelis. This is obvious on comparison between the spectra of dimethylamido-ethoxy-phosphoryl cyanide and of dimethylamido-ethoxy-phosphoryl chloride. These compounds are synthetized by two quite different methods, but their absorption spectra coincide perfectly with the exception of the absorption of the $—C \equiv N$ linkage. If the first mentioned compound should

Table 2. Absorption bands of the derivatives of pyrophosphoric acid.

Tetraethyl pyrophosphate				*sym*-Bis-(dimethylamido)-diethyl pyrophosphate			
λ	ν	λ	ν	λ	ν	λ	ν
3.10	3 226 w	8.56	1 168 s	2.80	3 571 w	8.57	1 167 m
3.30	3 030 s	9.07	1 103 s	3.28	3 049 m	9.10	1 096 m
3.37	2 967 m	9.29	1 076 s	3.35	2 985 s	9.46	1 057 s
5.25	1 905 w	9.65	1 036 s	3.46	2 890 m	9.94	1 006 s
5.60	1 786 w	10.18	982 s	5.36	1 866 w	10.26	975 s
6.74	1 484 m	10.60	943 s	6.16	1 623 w	10.74	931 s
6.90	1 449 m	12.20	820 s	6.72	1 488 m	12.27	815 s
7.14	1 401 m	12.40	806 s	6.85	1 459 m	12.56	796 s
7.27	1 376 m	13.18	759 m	7.15	1 399 m	12.92	774 m
7.70	1 299 s	14.10	709 m	7.28	1 373 m	13.90	719 m
				7.57	1 321 s	14.08	710 s
				7.90	1 266 s	14.80	675 w
				8.40	1 190 m		

be composed of a mixture of dimethylamido-ethoxy-phosphoryl cyanide and diethoxy-phosphoryl dimethylamide, this would be apparent by a stronger absorption of the $>CH_2$ group at 7.14—7.31 μ. However, this is not the case and the structure of dimethylamido-ethoxy-phosphoryl cyanide can be written:



The results obtained indicate the possibility of following the complete course of the hydrolysis, the knowledge of which is of great toxicological interest. By means of determination of liberated cyanide and simultaneous toxicity tests it has been possible to get a rough view of the inactivation of the described compounds by hydrolysis. Recently performed investigations in infrared made by us have confirmed that besides the splitting off of the cyanide group the P-N bond is split. More detailed examinations in this field are now in progress.

SUMMARY

The infra-red absorption spectra of six phosphoryl compounds and two derivatives of pyrophosphoric acid have been recorded and correlated with the molecular structure. Further the characteristic bands for the C-N and P-N linkages in the skeleton P-N$\begin{smallmatrix}\diagup\mathrm{C}\\ \diagdown\mathrm{C}\end{smallmatrix}$ at about 10.0 μ resp. 14.0 μ have been discussed and an absorption at about 14.1 μ has been surmised to be characteristic for the P-O bond in the combination P-O-P.

The authors are indepted to the Chief of the Research Institute of National Defence, Dept. 1, Professor Gustav Ljunggren, for permission to publish the results.

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An Apparatus for the Wet Combustion of Organic Compounds for C 14 Assay

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In the last two years direct mounting of organic compounds for the assay of C 14 has been used in several instances (Entenman, Lerner, Chaikoff and Dauben [1], Wilk, Barnet and Ackerman [2], Popjak [3]). The direct assay, however, requires relatively large amounts of material, *i.e.* about 25 mg/sq. cm. area, if it is to be assayed in infinite thickness. When only small amounts of material are available, the conversion to $BaCO_3$ is the method of choice, unless the direct assay of CO_2 in windowless counter is used. Besides the direct mounting is troublesome when working with oily substances.

In connection with work on the metabolism of C 14 labelled fatty acids we needed a method enabling us to run many combustions simultaneously. Many different methods have been described but a search through the literature revealed the need of a more easily handled apparatus satisfying the above demands.

A modification of the combustion tubes described by Baxter (Calvin, Heidelberger, Reid, Tolbert and Yankwich [4]) has been developed. The samples are combusted in van Slyke-Folch fluid in vacuum at 230° in an oilbath for 1 ½ hour and the CO_2 formed is collected in barium hydroxide. The amount of bariumcarbonate formed is estimated by titrating the excess of barium-hydroxide. A simple shaking device is described that enables us to run a number of combustions simultaneously. A device similar to that described here has recently been published by Claycomb, Hutchans and van Bruggen [5].

METHOD OF OPERATION

The sample to be combusted, containing 5—20 mg of carbon, is weighed in a glass cup 12 × 12 mm, which is then placed in the combustion flask A (see Fig. 1). 10 ml of van Slyke-Folch combustion fluid (without potassium

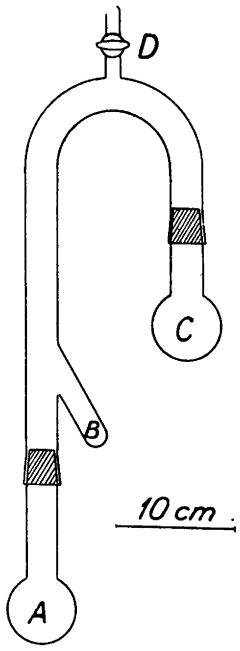

Fig. 1. Wet combustion apparatus (for description see text).

iodate) is pipetted into side tube B, while the apparatus is held in an upright position. C is filled with 12 ml of 0.25 *N* barium hydroxide. The apparatus is assembled and evacuated through D with an oil pump while carefully shaking to avoid splashing of the barium hydroxide solution in C. The combustion fluid is used as a joint lubricant at the lower joint and high-vacuum stopcock grease at the upper one. After the evacuation the combustion fluid in B is transferred to A by gently tipping the tube, and the tube is placed in an oil-bath at 230° for one hour and a half. The oil-bath here described (see Fig. 2) can hold six combustion tubes and is provided with a thermoregulator and an arrangement for automatic shaking of flasks C, in order to facilitate rapid adsorption of the CO_2. A fan is used to cool the tubes over A, thus preventing the SO_3 of the combustion fluid from reaching C.

For the samples containing no nitrogen or halogen the amount of carbon dioxide formed is determined by titrating the excess of barium hydroxide with 0.25 *N* HCl, using phenolphtalein as indicator. The $BaCO_3$ is then collected and washed by centrifugation.

ACCURACY OF THE METHOD

The method has hitherto been used for combustion of fatty acids and cholesterol.

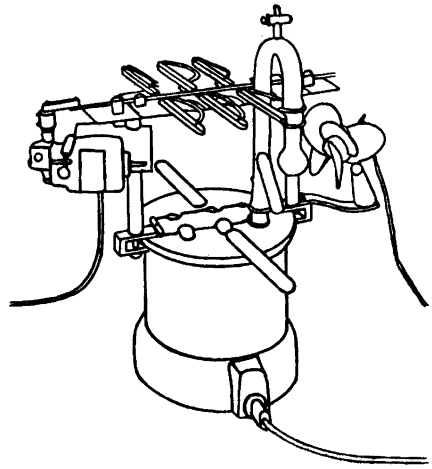

Fig. 2. Oil bath with shaking device and fan for six combustion tubes.

Ten determinations on 14.0 mg of stearic acid gave a mean value of 13.4 (95.6 per cent) S.D. $\pm$ 0.2.

SUMMARY

An apparatus is described for the wet combustion of organic compounds to be used in C-14 assay, convenient for serial analyses.

I wish to express my indeptedness to professor E. Hammarsten, Stockholm, for the time spent in his laboratory learning the isotope technic used there.

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A Note on the Synthesis of Cholesterol in the Animal Organism

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Recent work has shown that cholesterol is synthesized not only in the liver but also in the extra-hepatic tissues. Srere, Chaikoff, Treitman and Burstein[1] found cholesterol synthesis from C—14 labeled acetate in the eviscerated rat and in *in vitro* experiments they demonstrated conversion of C—14 acetate to cholesterol by the following isolated tissues of adult rat: liver, kidney, testis, small intestine and skin. The most rapid synthesis occurred in skin and liver. Srere, Chaikoff and Dauben[2] had earlier shown the adrenal cortex to be a site for cholesterol synthesis.

The synthesis of cholesterol in the rabbit has been studied by Popjak and Beeckmans[3] with the aid of deuterium oxide and C—14 acetate. They found the D and C—14 content of the cholesterol of the small intestine and ovaries higher than that of the liver indicating a synthesis of cholesterol in the former organs. They also found the interesting fact that the specific activity of the free cholesterol was higher than that of esterified cholesterol, being about 1.5 times higher in liver and about 3 times higher in small intestine.

In connection with a study of the metabolism of C-14-1-stearic acid in the rat[4] we have made similar observations. We determined the specific activities of the free cholesterol of liver, small intestine and blood, and of total cholesterol of heart, kidney, lung, brain and carcass at different intervals after peroral administration of C-14-1-stearic acid to adult rats (Table 1). The highest specific activities were found in the free cholesterol of the small intestine, indicating that part of the absorbed stearic acid is degraded in the intestinal mucosa presumable to acetate that is utilized for cholesterol synthesis. The specific activities of the cholesterol of all other organs tested were lower than that of the intestine, the liver having the next highest activity.

Table 1. Specific activity of free cholesterol in counts per min. after a single peroral administration of l—C 14-stearic acid to rats: 1/20 mmole/sq dm² body surface. Specific activity 90000 c/min./mg as $BaCO_3$.

Standard deviation in counting < ± 7 %. Background 20 c/min.

	Hours after administration				
	2	6	12	24	48
Liver	8	13	25	39	25
Small intestine	11	17	34	65	24
Blood	(2)	9	23	38	21

In another type of experiment we found that the intestinal wall contributed a quantitatively important part of the blood cholesterol via the lymphatic system at least during fat absorption and that part of this cholesterol is synthesized in the intestinal wall from ingested fatty acids.

Two adult rats with the intestinal lymphatics cannulated according to Bollman *et. al.* [5] were given C-14 labeled fat and the intestinal lymph was collected for 24 hours. The free and esterified cholesterol were separated on columns of aluminum oxide [6]. After saponification of the esterified cholesterol, the two cholesterol fractions were precipitated as digitonides according to Srere *et al.* [2]. and the C-14 acitivities assayed after wet combustion. The results are summarized in Table 2.

Table 2. C^{14} content of esterified and free cholesterol and neutral fat fatty acids of intestinal lymph in rat after peroral administration of 0.5 ml corn oil + 18.0 mg C^{14}-l-stearic acid (specific activity 150000 c/min./mg as $BaCO_3$). Total administrated activity about 2.7 · 10^6 c/min. Specific activity of the fatty acid mixture after hydrolysis about 7000 c/min./mg as $BaCO_3$.

	Weight mg	Specific activity c/m	Per cent of administered activity
Esterified cholesterol	6.8	109	0.027
Free cholesterol	9.4	112	0.039
Neutral fat fatty acids	367	5360	73.8
Esterified cholesterol	3.6	58.5	0.0078
Free cholesterol	5.8	107	0.016
Neutral fat fatty acids	117	4050	17.5

The findings here reported are in accord with the results of Popjak and Beeckmans[3] on rabbit and show the active part played by the small intestine in the metabolism of fatty acids and cholesterol. However, Popjak and Beeckmans found higher activities of esterified than of free cholesterol of small intestine. Our figures for intestinal lymph cholesterol show about the same or lower specific activities for esterified cholesterol compared with those for free cholesterol.

This work is part of an investigation supported by a grant from the Medical Faculty of the University of Lund.

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Cryoscopic Measurements in Fused Salts at Elevated Temperatures

Preliminary Report

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The calculation of chemical equilibria involving fused salt mixtures at present involves many uncertain assumptions, mainly due to the fact that our knowledge of the thermodynamic properties of fused salts is still very limited. Reliable data on heats of fusion and specific heats in the fused state is scarce, and very little is known about the deviations from ideal solutions of fused salt mixtures.

Kelley and collaborators [1] have carried out very extensive calculations of the heat of fusion of inorganic salts from the phase diagrams. The phase diagrams, however, are for many purposes not sufficiently accurately determined, as they are normally given with rather wide limits of error (more than 2° C). This deficiency becomes particularly clear if one attempts to estimate activity coefficients from the diagrams. Also, there remain unsolved some questions concerning the ionic species present in the salt mixture questions which are of fundamental importance in calculations of chemical equilibria involving fused salts. In the following a method will be described which allows a more precise determination of the liquidus curve of the phase diagram than may be obtained by the usual thermal analysis, thus leading to more accurate thermodynamic data for the fused salts.

The principle of the method is to separate the solid and liquid phases by filtration at the equilibrium temperature and then to determine the composition of the liquid phase after cooling, by chemical analysis. The original idea was taken from a paper by Mason, Hiskey and Ward [2]. They used the principle in their methods of studying the ion distribution equilibria related

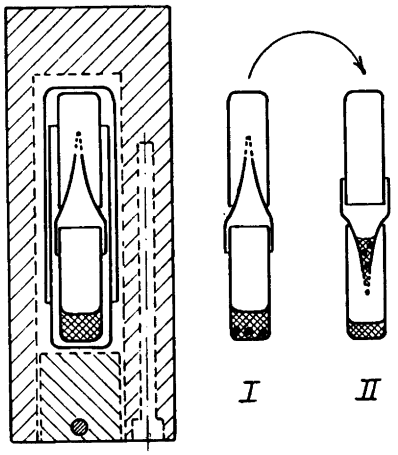

Fig. 1. Apparatus for high temperature filtration.

to infra red sensitive phosphors. Our apparatus has a different form as it serves a different purpose (see Fig. 1).

A platinum crucible contains the salt mixture. On top of this crucible is placed an inverted platinum filter, and a second inverted platinum crucible, is placed above the filter.

The salt mixture is kept for a long time at a temperature where solid is in equilibrium with liquid, then the whole arrangement of crucibles and filter is turned upside down by inverting the furnace in which it is placed. Most of the liquid phase then flows through the filter into the second crucible.

The crucibles and filters are placed in a quartz capsule which fits into a solid copper cylinder whose function is to prevent the occurrance of temperature gradients between the thermo-couple, the crucible and the filter. The whole apparatus is placed in an electric furnace, which is swept out with oxygen-free gas to prevent oxidation of the copper cylinder. In order to maintain a constant temperature in the furnace, the electric current is taken from a constant voltage supply. The temperature was measured by means of a Pt-Pt 10 % Rh thermocouple and a compensator from Otto Wolf. The melting point depressions could be measured with an accuracy of 0.1—0.2 ° C and the accuracy of the absolute value of the temperature is estimated to be $\pm$ 0.5°C.

Two systems were investigated —Na_2SO_4—NaCl and Na_2SO_4-NaBr. Only the sulphate side of the phase diagram was determined. The system Na_2SO_4-NaCl has been investigated by Jänecke, Wolters [4], Koltchko [5], and the system Na_2SO_4-NaBr by Rea [6]. Both are simple eutectics and no solid solution has been found.

The calculation from the phase diagram of the heat of fusion and the deviation from ideal solution do not offer any theoretical problems which have not already been worked out in classical physical chemistry. The formulas used will be briefly reviewed.

The liquidus curve on the sulphate side of the phase diagram gives the composition of the liquid phase which is in equilibrium with solid Na_2SO_4. If f_s is the fugacity of solid Na_2SO_4, and f_m is the fugacity of Na_2SO_4 in the fused mixture, then

$$f_s = f_m$$

Using pure liquid Na_2SO_4 as the standard state, the activity of Na_2SO_4 in the fused mixture will be

$$a_m = \frac{f_m}{f^0} = \frac{f_s}{f^0}$$

where f^0 is the fugacity of pure liquid Na_2SO_4.

Further

$$RT \ln a_m = RT \ln \frac{f_s}{f^0} = -\Delta F$$

where ΔF is the change in free energy on fusing the pure sulphate at a temperature T. ΔF may be expressed in terms of the heat of fusion ΔH and the temperature. If we neglect the variation with temperature of the heat of fusion, then

$$\Delta F = -RT \ln a_m = \Delta H \left(1 - \frac{T}{T_0}\right)$$

where T_0 is the melting point of sodium sulphate.

Introducing $a_m = N_1\gamma_1$ where N_1 and γ_1 are the mole fraction and activity coefficient of Na_2SO_4 we obtain

$$\ln N_1 + \ln \gamma_1 = -\frac{\Delta H}{R}\left(\frac{1}{T} - \frac{1}{T_0}\right)$$

Plotting $\ln N_1$ as a function of $\frac{1}{T}$ should give a straight line in the region where deviations from an ideal solution are small, the heat of fusion being determined by the slope of the line. Deviations from the straight line should give information about the activity coefficients in the mixture.

Table 1. Corresponding values of the liquidus phase composition and temperature for the systems Na_2SO_4-NaCl and Na_2SO_4-NaBr.

	Temperature °C	$\frac{1}{T}\,10^3$	Molefraction of Na_2SO_4 (N_1) in liquid phase	$-\log_{10} N_1$
System Na_2SO_4-NaCl	857.5	0.8844	0.9391	0.0273
	835.3	0.9021	0.8870	0.0521
	802.7	0.9294	0.8185	0.0869
	683.2	1.0455	0.5801	0.2365
Melting point (extrapolation)	885.0		1.0000	
System Na_2SO_4-NaBr	843.1	0.8958	0.9052	0.0433
	804.9	0.9275	0.8231	0.0845

The experimental data are given in Table 1, and Fig. 2 shows $\log_{10}N_1$ as a function of $\frac{1}{T}$ for the systems Na_2SO_4-NaCl and Na_2SO_4-NaBr. The two systems give practically the same value for the heat of fusion of sodium sulphate. The three points of the system Na_2SO_4-NaCl, in which the NaCl content is less than 20 mol%, give 6 040$\pm$70 cal/mol for the heat of fusion. The melting point is found by extrapolation to be 885 ° C. Using this melting point, and the two measurements from the system Na_2SO_4-NaBr, the heat of fusion is found to be 6 070 cal, thus verifying the value calculated from the system Na_2SO_4-NaCl.

The value of the heat of fusion above may be compared with other values found from earlier phase diagram measurements[1, p. 115].

System	Investigator	
Na_2SO_4-NaCl	Jänecke	5 870
»	Klotchko	5 970
»	Wolters	5 790
»	Present measurements	6 040 ± 70
Na_2SO_4-NaBr	» »	6 070

In these calculations the difference in the specific heats of solid and fused Na_2SO_4 is not taken into account as the specific heat of the salt in the liquid state is not known. The difference in the specific heats of the solid and liquid states is of the order of 1 cal/° C for most inorganic salts[1, p. 4]. This will increase the above values of the heat of fusion by about 60 cal, giving *6 100 cal* as the

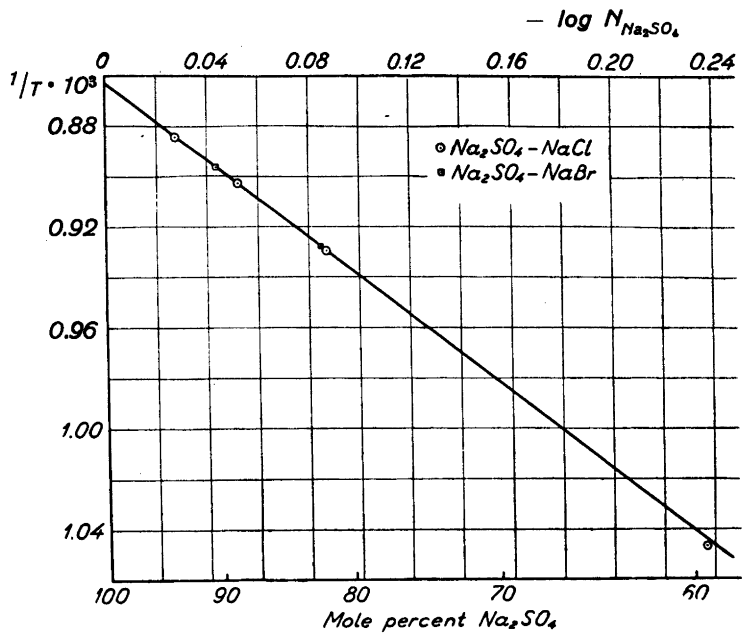

Fig. 2. The relationship between molefraction of sodium sulphate (— $\log_{10} N_{Na_2SO_4}$) and inverse temperature ($^1/_T$) for the systems Na_2SO_4-NaCl and Na_2SO_4-NaBr.

corrected figure. It will also bring the point at about 40 mol% NaCl in Fig. 2 close to the straight line.

Furthermore, measurements in Fig. 2 show that the linear relationship between $\log N_1$ and $\frac{1}{T}$ is valid over a very wide interval, indicating that the systems Na_2SO_4-NaCl and Na_2SO_4-NaBr form ideal mixtures.

SUMMARY

A method has been worked out which permits an accurate determination of the liquidus curve of phase diagrams at high temperatures. The methode is based on a separation of a fraction of liquid from solid + liquid by filtration at the equilibrium temperature.

The sulphate sides of the binary systems Na_2SO_4-NaCl and Na_2SO_4-NaBr have been investigated by this method.

From these measurements, the heat of fusion of Na_2SO_4 is estimated to be 6 100 cal, and the two systems are found to form ideal mixtures in the liquid state.

Our investigations have been supported by grants from *Statsminister Gunnar Knudsen og hustru Sophie f. Cappelens Familielegat* (*Borgestads Legat IV*) for which we wish to express our sincere gratitude.

The experiments were carried out in the laboratories of Institutt for uorganisk kjemi, Norges tekniske høgskole and we express our gratitude to the director, Professor M. Ræder.

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Potentiometric and Spectrophotometric Studies on 8-Quinolinol and Its Derivatives. I. Ionization of 8-Quinolinol in Aqueous Solutions of Potassium Chloride

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For an investigation of 8-quinolinol chelates of metals in aqueous salt solutions reliable values for the ionization constants of 8-quinolinol were required. The earlier determinations, however, have been carried out at one ionic strength only. Moreover, the values obtained by different authors are not in good agreement [1]. Therefore the determination of these constants was necessary.

In aqueous solutions 8-quinolinol HOx (8-hydroxyquinoline or oxine) reacts as follows:

$$HOx + H_3O^+ = H_2Ox^+ + H_2O \quad (1)$$

and

$$HOx + H_2O = H_3O^+ + Ox^- \quad (2)$$

The law of mass action gives

$$[H^+]\ [HOx] / [H_2Ox^+] = K_1 \quad (3)$$

and

$$[H^+]\ [Ox^-] / [HOx] = K_2 \quad (4)$$

where the symbols in brackets denote concentrations (in this paper moles per liter solution). K_1 and K_2 are concentration dissociation constants of the acids H_2Ox^+ and HOx. In the present paper also the "mixed" constants:

$$(H^+)\ [HOx] / [H_2Ox^+] = K_1' \quad (5)$$

and

$$(H^+)\ [Ox^-] / [HOx] = K' \quad (6)$$

are needed. Here (H^+) denotes the activity of hydronium ion. The thermodynamic constants are defined as follows:

$$K_{1,0} = (f_{H^+} f_{HOx} / f_{H_2Ox^+}) K_1 = (f_{HOx} / f_{H_2Ox^+}) K_1' \quad (7)$$

and

$$K_{2,0} = (f_{H^+} f_{Ox^-} / f_{HOx}) K_2 = (f_{Ox^-} / f_{HOx}) K_2' \quad (8)$$

where f's are the activity coefficients. In addition the constant

$$[OH^-]\,[HOx] / [Ox^-] = K_w/K_2 = K_{2b} \quad (9)$$

was used. Here K_w is the ionic product of water.

Two potentiometric and one spectrophotometric methods were used:

The first potentiometric method was the familiar one, in which equations (5) and (6) are used. These equations give

$$pK_1' = pH - \log ([HOx] / [H_2Ox^+]) \quad (10)$$

and

$$pK_2' = pH - \log ([Ox^-] / [HOx]) \quad (11)$$

When the quotients $[HOx] / [H_2Ox^+]$ and $[Ox^-] / H[Ox^-]$ do not differ too much from unity we can write with sufficient accuracy

$$[HOx] / [H_2Ox^+] = (c_B + c_{Ox} - c_A + [H^+]) / (c_A - c_B - [H^+]) \quad (12)$$

and

$$[Ox^-] / [HOx] = (c_B - c_A - [OH^-]) / (c_A + c_{Ox} - c_B + [OH^-]) \quad (13)$$

where c_B is the stoichiometric concentration of added sodium hydroxide, c_A that of a strong acid (hydrochloric acid in this study), and c_{Ox} that of 8-quinolinol. The experimental procedure comprises the preparation of a solution in which the quotient (12) or (13) does not differ too much from unity and the measurement of pH of this solution.

The second potentiometric method is based on the determination of the buffer capacity at the inflection points which occur about where $c_A - c_B = c_{Ox}$ and $c_B - c_A = c_{Ox}$. The determination of the buffer capacity was performed according to Näsänen's [2] method, using the equations derived by him:

$$\frac{dE}{dm} = \Delta E_{max} + \frac{1}{6}\left|(\Delta E_{max} - \Delta E_1) + (\Delta E_{max} - \Delta E_2)\right| - \frac{(\Delta E_{max} - \Delta E_1)\,(\Delta E_{max} - \Delta E_2)}{2\,(\Delta E_{max} - \Delta E_1) + (\Delta E_{max} - \Delta E_2)} \quad (14)$$

and

$$P = \frac{\Delta V C k}{V dE/dm} \quad (15)$$

where ΔE_1, ΔE_{max} and ΔE_2 are the three successive potential jumps of which ΔE_{max} is the greatest, m is the number of equal increments of the titrating reagents (in this case a solution of hydrochloric acid), $k = 2.303\ RT/nF$, ΔV is the volume of an incre-

ment, V is the volume of the titrated solution, C the concentration of the titrating reagent and P the buffer capacity. The dissociation constant can be calculated by means of the equation [2, 3]

$$x = \sqrt{Kc\ (1-K/x)\ /\ (1 + K/x)^3} \tag{16}$$

$$K = (0.434\ P-x)\ (x + K)^2\ /\ xc \tag{17}$$

by the iterative method. In these equations c denotes the total concentration of the weak acid or base. By means of this method the constants K_1 and K_{2b} can be determined. In the first case $x = [H^+]$ and in the second case $x = [OH^-]$.

The spectrophotometric method is based on the general equation

$$\varepsilon - \varepsilon_{HOx} + (\varepsilon - \varepsilon_{H_2Ox^+})\ (H^+)\ /\ K_1' + (\varepsilon - \varepsilon_{Ox^-})\ K_2'\ /\ (H^+) = 0 \tag{18}$$

where $\varepsilon = e/c$. Here e is the extinction of a c molar 8-quinolinol solution as a 1 cm layer (in the reference cell the same solution without 8-quinolinol), and ε's are molar extinction coefficients. In a sufficiently acidic medium $\varepsilon_{H_2Ox^+} = \varepsilon$, in a sufficiently alkaline medium $\varepsilon_{Ox^-} = \varepsilon$ and near the isoelectric point approximately $\varepsilon_{HOx} \sim \varepsilon$, as is seen from equation (18). By the measurement of ε in sufficiently acidic and alkaline solution of 8-quinolinol it is thus possible to obtain the values of $\varepsilon_{H_2Ox^+}$ and ε_{Ox^-}. By measurement of ε near the isoelectric point the approximate value of ε_{HOx} is obtained. The measurement of ε at pH $\sim$ 5 gives K_1' and the measurement at pH $\sim$ 10 gives K_2'. In the first case the term containing K_2' and in the second case the term containing K_1' can be neglected. Now it is possible to calculate a more accurate value for ε_{HOx} as well as for $\varepsilon_{H_2Ox^+}$ and ε_{Ox^-} if necessary. Thereafter the calculation of K_1' and K_2' is repeated *etc.*

EXPERIMENTAL

The potentiometric measurements were performed in a water thermostat controlled with an accuracy of $\pm$ 0.01° C. A glass electrode (Beckman types 4990, 42 and E) and a saturated calomel electrode were used. A Radiometer PHM3 potentiometer was used. The glass electrode was standardized against 0.05 molar solutions of potassium acid phthalate or sodium tetraborate. The titration vessel was carefully sealed and provided with a cover, which has holes for the electrodes, the stirrer, the inlet and outlet of nitrogen as well as the addition of reagents.

The chemicals used were the best obtainable. Since 8-quinolinol dissolves slowly and relatively poorly in water, it was generally dissolved in an equivalent amount of a dilute solution of hydrochloric acid. In the spectrophotometric investigation hydrochloric acid, acetate, phosphate and borate buffers were used. The pH of the solutions were calculated and in addition the values obtained were checked by measurement with a glass electrode.

The measurement of the light absorption was carried out with a Beckman Model DU spectrophotometer with 1 cm silica cells. The measurements were performed in a constant temperature room. Temperature of the mounting block of the spectrophotometer was also regulated with a termostat.

RESULTS

The results by the first potentiometric method are summarized in Table 1. Each value for the dissociation constant is a mean value of four measurements. The concentration of 8-quinolinol c_{Ox} was never higher than about

Table 1. Determination of the dissociation constant K_1' at 20° C and K_2' at 25° C in the aqueous solutions of potassium chloride.

$\sqrt{I}$	pK_1'	$\sqrt{I}$	pK_2'
0.095	5.056	0.0984	9.765
0.168	5.084	0.168	9.726
0.402	5.164	0.398	9.663
0.696	5.221	0.685	9.610
0.971	5.268	0.985	9.579
1.251	5.309	1.226	9.558
1.524	5.340	1.503	9.548
1.772	5.351	1.746	9.553

0.005 because it was observed that the pH was anomalous too near the saturation point. In most experiments the quotients [HOx] / [H_2Ox^+] and [Ox^-] / [HOx] were unity but some other values were also used. The results can be presented by Debye-Hückel equation. The calculation by means of the method of least squares gave

$$p(f_{H_2Ox^+} / f_{HOx}) = \frac{0.505\sqrt{I}}{1 + 0.907\sqrt{I}} \tag{19}$$

$$p(f_{Ox^-} / f_{HOx}) = \frac{0.509\sqrt{I}}{1 + 1.018\sqrt{I}} - 0.022\ I \tag{20}$$

and

$$pK_{1,0} = 5.011\ (20°),\ pK_{2,0} = 9.805\ (25°).$$

The results for the constant K_1 obtained by the second potentiometric method are recorded in Table 2. Equation (7) can be written in the form

$$pK_1 = pK_{1,0} + p(f_{H_2Ox^+} / f_{HOx}) - pf_{H^+} \tag{21}$$

Table 2. Potentiometric determination of dissociation constant K_1 in potassium chloride solutions at 20° C.

$\sqrt{I}$	$c \cdot 10^3$	$P \cdot 10^3$	$[H^+] \cdot 10^4$	pK_1
0.0670	3.25	0.768	1.57	5.019
0.0947	6.51	1.087	2.26	5.035
0.169	6.51	1.073	2.23	5.047
0.412	6.48	1.025	2.14	5.086
0.700	6.43	0.933	1.95	5.165
0.985	6.30	0.837	1.77	5.261
1.275	6.21	0.739	1.56	5.360
1.553	6.06	0.640	1.36	5.488
1.818	5.98	0.533	1.16	5.615

When equation (19) was taken into consideration the data of Table 2 gave

$$pf_{H^+} = \frac{0.505\sqrt{I}}{1 + 2.201\sqrt{I}} - 0.129\,I \qquad (22)$$

and

$$pK_{1,0} = 5.025 \ (20° \text{ C})$$

The data in Table 2 are mean values obtained from four identical experiments.

In table 3 the results for the constant K_{2b} are summarized. In this case also each P value is a mean value obtained from four identical experiments. The method of least squares gave

$$pK_{2b} = 4.177 + 0.0614\,I \qquad (23)$$

The thermodynamic constant is thus

$$pK_{2b,O} = 4.177 \ (25° \text{ C})$$

Adopting $pK_{W,O} = 13.996$ in accordance with Harned and coworkers [4] we obtain

$$pK_{2,O} = pK_{W,O} - pK_{2b,O} = 9.819 \ (25° \text{ C})$$

The spectrophotometric measurements were made at the wavelengths 350—400 mμ, since at this range the light absorption of HOx is slight but that of the other constituents much greater. This is seen from Fig. 1, in which the extinction of a 0.0003 molar solution of 8-quinolinol is represented as a func-

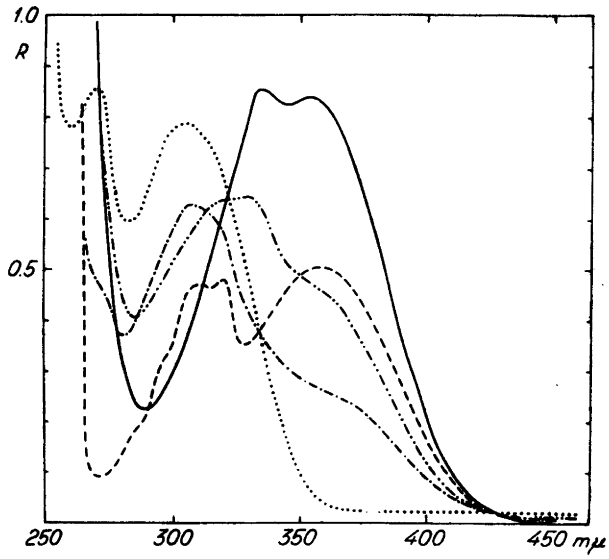

Fig. 1. Ultra-violet absorption spectra of 8-quinolinol in aqueous buffer solutions.
pH = 12.40 ——; pH = 9.80 · · — · ·; pH = 6.96 · · · · ·; pH = 5.02 · — · — ;
pH = 1.44 — — — ; I = 0.04.

Table 3. Potentiometric determination of dissociation constant K_{2b} in potassium chloride solutions at 25° C.

$\sqrt{I}$	$c \cdot 10^3$	$P \cdot 10^3$	$[OH] \cdot 10^4$	pK_{2b}
0.113	5.03	2.323	4.26	4.182
0.180	5.03	2.330	4.26	4.180
0.409	5.01	2.323	4.26	4.180
0.701	4.96	2.250	4.15	4.207
0.976	4.92	2.169	4.02	4.241
1.259	4.80	2.068	3.87	4.272
1.533	4.68	1.959	3.70	4.314
1.779	4.55	1.812	3.45	4.376

tion of wavelength at various pH values. The molar extinction coefficients of the three constituents were obtained by light absorption measurement of the following solutions:

	I	c_{KCl}	pH
$\varepsilon_{H_2Ox^+}$	0.0025—4.00	0.139—3.89	0.71— 2.67
ε_{HOx}	0.0025—4.00	0.139—3.89	6.85— 7.13
ε_{Ox^-}	0.04—1.96	0.034—1.90	12.42—12.50

Hydrochloric acid, phosphate and sodium tetraborate-sodium hydroxide buffers were used. In Tables 4 and 5 the results concerning K_1' and K_2' are recorded. In these cases acetate and sodium tetraborate-sodium hydroxide buffers were used. The ions of the buffers are seen to represent a high proportion of the ionic strength at low ionic strengths. All light absorption measurements were performed the range 350—400 mμ at intervals of 5 mμ. The values of the quotients [HOx] / [H_2Ox^+] and [Ox^-] / [HOx] obtained from the spectrophotometric measurements are therefore mean values from eleven experiments. The calculation by means of the method of least

Table 4. Spectrophotometric determination of the constant K_1' in potassium chloride solutions at 25° C. $c_{Ox} = 3.00 \cdot 10^{-4}$.

I	c_{KCl}	pH	pK_1'
0.0024	0.00029	4.862	4.966
0.0099	0.00549	4.947	4.966
0.0244	0.0109	4.996	4.976
0.0400	0.0156	5.018	5.004
0.0900	0.0371	5.031	5.018
0.250	0.193	5.035	5.060
0.490	0.432	5.014	5.091
1.00	0.948	4.960	5.165
1.96	1.92	4.849	5.203
2.89	2.86	4.747	5.236
4.00	3.98	4.625	5.282

squares gave

$$\mathrm{p}(f_{H_2Ox^+} / f_{HOx}) = \frac{0.509\sqrt{I}}{1 + 0.972\sqrt{I}} \tag{24}$$

Table 5. Spectrophotometric determination of the constant K_2' in potassium chloride solutions at 25° C. $c_{Ox} = 3.00 \cdot 10^{-4}$.

I	c_{KCl}	pH	pK_2'
0.040	0.0061	9.800	9.736
0.090	0.0226	9.822	9.698
0.250	0.132	9.882	9.664
0.490	0.372	9.843	9.621
1.00	0.882	9.797	9.581
1.96	1.84	9.772	9.561

and

$$\mathrm{p}(f_{Ox^-} / f_{HOx}) = \frac{0.509\sqrt{I}}{1 + 1.256\sqrt{I}} \tag{25}$$

For thermodynamic constants the values

$$pK_{1,0} = 4.910 \ (25° \text{ C}) \text{ and } pK_{2,0} = 9.814 \ (25° \text{ C})$$

were obtained with this method.

The effect of temperature on the constant pK_2' was preliminarily investigated over the range 15°—40° C. The decrease of pK_2' was on average 0.0179 when temperature was increased one degree.

DISCUSSION

For the thermodynamic constants the following values were thus obtained:

Method	$pK_{1,0}$	(20° C)	$pK_{2,0}$	(25° C)
Potentiometric I	5.011		9.805	
Potentiometric II	5.025		9.819	
Spectrophotometric	(4.910	(25° C)	9.814	
Mean value	5.017	(20° C)	9.813	(25° C)

The agreement of the values obtained by means of the different methods is very satisfactory. The first potentiometric method is based on the pH value of the 0.05 molar acid potassium phthalate for which we have adopted the value pH = 4.00 at 20° C or on the 0.05 molar sodium tetraborate solution for which we have adopted the value pH = 9.18 at 25° C. The possible liquid junction potentials by using a saturated calomel electrode are neglected [5]. In the second potentiometric method the liquid junction potentials have no significance. In the spectrophotometric method 8-quinolinol is dissolved in buffer solutions. The pH of these must be calculated from the values of dissociation constants and activity coefficients, or determined as in the first potentiometric method, but in addition there are still the spectrophotometric measurements. The significance of this method is that by it the measurements can be carried out in much more dilute solutions than by means of the potentiometric ones.

A comparison of the values obtained by the earlier authors is difficult since ionic strength and temperature are frequently not specified. The reader is referred in this respect to the paper of Irwing, Ewart and Wilson [1], who have given a detailed discussion. On the basis of their own studies the latter authors favour the values $pK_{1,0} = 5.00 \pm 0.10$ and $pK_{2,0} = 9.85 \pm 0.05$ at 20° C. Our values are seen to be within these limits.

The effect of additions of potassium chloride is illustrated in Fig. 2, in which $p(f_{H_2Ox^+} / f_{HOx})$ and $p(f_{Ox^-} / f_{HOx})$ are represented as functions of ionic

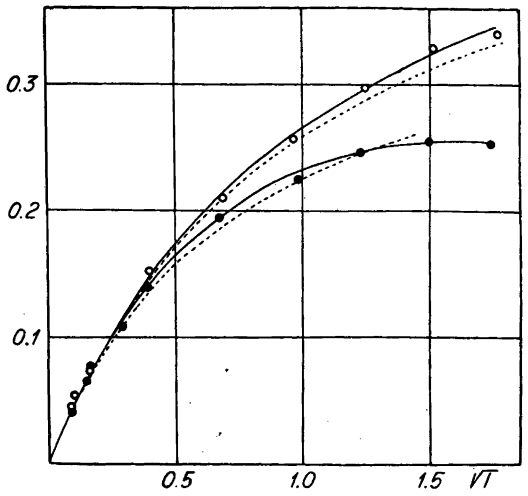

Fig. 2. Effect of ionic strength on ionization of 8-quinolinol. Upper curves, $p(f_{H_2Oz^+} / f_{HOz})$; lower curves $p(f_{Oz^-} / f_{HOz})$. Solid lines, potentiometric results; dotted lines, spectrophotometric results; ●, ○, experimental (potentiometric).

strength. The agreement between potentiometric and spectrophotometric measurements is seen to be very satisfactory. The values obtained for pf_{H^+} and pf_{OH^-} are in satisfactory agreement with the earlier determinations [6].

The former is obtained from equation (22) and for the latter we obtain from (20) and (23)

$$pf_{OH^-} = \frac{0.509\sqrt{I}}{1 + 1.018\sqrt{I}} - 0.083\ I \qquad (26)$$

The values obtained for the activity coefficients are naturally based on the pH scale described above.

SUMMARY

The ionization of 8-quinolinol in aqueous solutions of potassium chloride are investigated by three independent methods. The values obtained for the thermodynamic ionization constants with these three different methods are in very satisfactory agreement. The results concerning the effect of potassium chloride with a potentiometric and spectrophotometric method also agree very well. The activity coefficients of hydronium and hydroxyl ions were determined. These results are in good agreement with the earlier measurements. The ultra-violet absorption spectra of 8-quinolinol were determined at several pH values.

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Short Communications

Some Chemical Data Concerning The Epicuticle of Wool

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The wool fibre, as well as hairs from other animals, is enveloped by a thin membrane, the epicuticle [1], with a thickness varying from 50 to 250 Å.[2] Similar structures are found on feathers [3,4] and also in the surface layers of human skin [5].

There are two main methods for isolating the epicuticle from the wool. A common feature is, however, that the epicuticle, which amounts to 0.1—0.2 % of the scoured material, is always contaminated by material from the underlaying layers. — The one method is to dissolve the main part of the wool by treatment with dilute Na_2S_2 whereby the epicuticle is left as a residue [1,2]. The other is to treat the wool with chlorine or bromine for a short time, destroy the excess of halogen by treatment with dilute $NaHSO_3$ and finally shake off the epicuticle [1].

A sample of epicuticle from wool, isolated by the bromine method and containing approximately 30 % pure epicuticle, has been treated with 20 % HCl at 100° C for 10 hours. (The estimation of the percentage of pure epicuticle is based upon the fraction isolated from the original material and also on electron microscopical observation.) Part of the substance was dissolved during the hydrolysis. The hydrolysate was used for paper chromatography on sugars using a mixture of butanol, acetic acid and water as solvent and different types of developers in the different runs. The procedure is described by Werner and Odin [6]. In most cases rhamnose was used as a front sugar and other substances used for identification of the different spots were glucosamine, fucose, galactose and glucose. The method suggested by Novellie [7] using a developing agent containing β-naphtylamine and the method of Trevelyan, Procter and Harrison [8] using a solution of $AgNO_3$ in acetone and a spray of alcoholic NaOH gave similar results. In these cases spots indicating the presence of galactose or a sugar with almost the same R_f-value as galactose for this system were obtained. Moreover diffuse spots, or rather a band of diffuse spots, occurred in the test runs. This band indicates the presence of a reducing agent with an R_f-value between rhamnose and mannose and also that glucosamine may be present. The same results were obtained when the 20 % HCl in the hydrolysis was replaced by 36 % HCl. The original method of Partridge [9] utilizing ammonia + $AgNO_3$ gave diagrams with less distinct spots and the paper also had a tendency of blackening after some time. No paper chromatography on the amino acid composition of the sample was carried out as the contaminations most probably are constituted by amino acids more or less modified by the chemical treatment of the material. The carbohydrates are, however, probably constituents of the epicuticle. They probably form compounds with other

Table 1.

	Per cent epicuticle (approx.)	% O	% S	% N	% Br
Residue from Na_2S-treatment	10	22.3	3.5	14.3	
Sample obtained by brominating the wool	30	27.0	3.0	13.0	8.3
Untreated wool	0.1–0.2		2.8	14.7	

constituents such as proteins or possibly fatty acids. — The X-ray diagrams from samples isolated from wool give some interferences found neither in the α- nor in the β-diagram of wool [10], which are possible indications of structures of the above mentioned type.

As to the modification caused by the chemical treatment Table 1 should give an idea and at the same time give a few figures for the chemical composition of the epicuticle compared with ordinary wool. The elementary analysis has been carried out by the Microchemical Laboratory of the Medico-Chemical Institute of The University of Uppsala.

The nitrogen content of the epicuticle seems to be lower than that of the main part of the wool, the sulphur content is slightly higher. In the case of the Na_2S-treated sample some impurities may originate from polysulphides and sulphur containing products formed by the reagent and the original material. In the brominated sample it is evident that the bromine has reacted with the organic material. Moreover it is indicated that some oxidation has occurred as the oxygen content is higher than in the other samples. This oxidation may have led to the formation of carboxyl groups which may be an explanation to the fact that samples obtained in this way are to great extent soluble in dilute alkali.

This investigation, sponsored by a research fellowship from the International Wool Secretariat to one of us (G. L.), is beeing continued.

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Investigations in the Retene Field. IV. Nitration of Retene in the Presence of Boron Trifluoride

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Several attempts have been made to prepare nitro-derivatives of retene by direct nitration, the most successful being those reported by Fredriksen and Nielsen [1] (who also give further references on this subject). By nitration of retene under mild conditions these workers obtained a crude product from which they isolated 9-nitroretene in a yield of about 5 %. According to a private communication, they were also able to isolate 3-nitroretene but this

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compound was obtained in a very low yield. These mononitroretenes were separated from the reaction product by chromatography and molecular destillation. During the chromatography they observed several zones including a purple one and a yellow one, and it was from the latter that the two nitroretenes were isolated.

As it was of interest to establish what is actually formed when nitric acid reacts with retene we have repeated the nitration and examined the products rather more closely. Analyses showed that the purple zone on the chromatograms probably does not contain significant amounts of nitroretenes, whereas the yellow one consists chiefly of mononitroretenes, in amount corresponding to a yield of about 50 % of the theoretical.

In an attempt to obtain higher yields of mononitroretenes we have carried out some nitrations in the presence of boron trifluoride, a substance which is known to be a remarkable catalyst for many organic reactions including the nitration of aromatic hydrocarbons [2]. Using this catalyst it is often possible to nitrate organic compounds quickly and almost completely with stoichiometric amounts of nitric acid. In the presence of boron trifluoride, the nitration of retene was nearly complete after a few minutes at 80—90° C when only 1.05 moles of nitric acid/mole of retene were used. Chromatography of the reaction product revealed several zones, showing that many compounds are formed in the reaction. However, the mixed mononitroretenes accounted for the major portion, and were obtained in yields corresponding to 80—83 % of the theoretical. Some experiments were carried out in the absence of boron trifluoride and showed that, at this temperature, the mixture of mononitroretenes could be obtained in a yield of about 65 %, if 1.5 moles of nitric acid/mole of retene were used and the reaction time was prolonged to 15—25 minutes. With smaller amounts of nitric acid, much retene could be recovered unchanged.

This preliminary investigation of the nitration of retene indicates that mononitroretenes can be obtained in good yields by direct nitration of the hydrocarbon. The problem still to be solved is the separation of the different mononitroretenes. It may be mentioned that the mixture of mononitroretenes can be isolated in reasonably good yield by destillation of the crude reaction product under reduced pressure.

Experimental. Retene (5.0 g, m.p. 95°C) was dissolved in glacial acetic acid (50 ml) containing boron trifluoride (1.4 g), at 80—90 °C, and a solution of conc. (99—100 %) nitric acid (1.4 g) and boron trifluoride (1.4 g) in glacial acetic acid (10 ml) was added with stirring during 2—5 minutes. The mixture was poured into cold (0—5 °C) water immediately, and the precipitate which formed was filtered off, washed with cold water, and dissolved in benzene. The benzene solution was washed with dilute alkali and water and evaporated, and the residue, a viscous red-brown oil, was dissolved in a mixture of 10 vol. of petroleum ether (b. p. 40—60 °C) and 1 vol. of benzene and adsorbed on a column (2 × 25 cm) of alumina. The same solvent mixture was used to elute the yellow zone containing mononitroretenes. Evaporation of the solvents gave a yellow viscous oil (5.4 g) which contained some unchanged retene and other impurities, and was chromatographed again on a column (3.5 × 25 cm) of alumina. Retene was eluted with pure petroleum ether, then the mononitroretenes were eluted with the same solvent mixture as above. Evaporation of the solvents yielded the mononitroretenes as a yellow viscous oil, (4.9 g, 83 % of the theoretical yield).

$C_{18}H_{17}NO_2$ (279.3)

Calc.	C 77.4	H 6.14	N 5.0
Found	» 77.9	» 6.19	» 5.2

Titration with $TiCl_3$:

Found Molecular weight 281

1. Fredriksen, E., and Nielsen, E. J. *Acta Chem. Scand.* **1** (1947) 448.
2. Thomas, R. J., Anzilotti, W. F., and Hennion, G. F. *Ind. Eng. Chem.* **32** (1940) 408.

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Aspartic Acid as a Precursor for Ribonucleic Acid Pyrimidines

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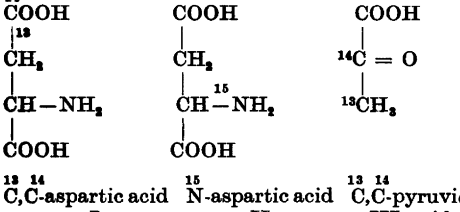
While the precursors of the carbon atoms of the purines seem to be well established [1], very little is known about the possible corresponding precursors of the pyrimidines.

Heinrich et al. [1] and Lagerkvist [2] have shown that carbon atom nr. 2 in uracil is derived from CO_2. As to carbon atoms 4—6 the work of Mitchell *et.al.* [3] and Wright *et.al.* [4] with microorganisms makes it appear possible that these are formed from some intermediate of the citric acid cycle. The present investigation was carried out both in order to test this possibility and in order to investigate the ability of aspartic acid as a whole molecule to act as precursor for pyrimidines. For this purpose slices from regenerating liver where used in the same way as described previously [5]. In three different experiments the tested precursors were added to the medium in 0.01 molar concentration. The precursors used were as follows:



In each experiment slices from 10—30 regenerating livers were incubated for 8 hours. Polynucleotides were prepared from the livers according to Hammarsten's [6] method. From pentose nucleic acid both pyrimidines were prepared as uracil by a combination of ion exchange chromatography [7] and starch chromatography [8]. Details of the method will be described later.

The results of the different experiments indicated that compounds II and III can hardly be direct precursors for pyrimidines, since in these experiments the dilution of the isotope from the precursor to uracil was about 500—1500 fold. Compound I, however, gave corresponding dilution factors of 100—400 for C^{14} and 30—70 for C^{13}. We believe that aspartic acid, after loss of the NH_2-group is relatively directly used for the synthesis of uracil. That this transformation is probably very extensive could be shown in the experiment with II, since at the end of the incubation large amounts of N^{15}-glutamic acid could be isolated from the medium, while practically no aspartic acid was left. *

In order to explain the difference in isotope incorporation from the carboxyl and methylene groups, and in order to get a clearer picture of the paths of their incorporation, we hope to degrade uracil from this experiment and to obtain the isotope content from each of its carbon atoms. A more detailed description of the experiments will be given in connection with publication of the degradations.

1. Heinrich, M. R., and Wilson, D. W. *J. Biol. Chem.* **186.** (1950) 447.
2. Lagerkvist, U. *Acta Chem. Scand.* **4** (1950) 1151.
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4. Wright, L. D., Miller, C. S., Skeggs, H. R., Huff, J. W., Weed, L. L. and Wilson, D. W. *J. Am. Chem. Soc.* **73,** (1951) 1898.
5. Reichard, P. and Bergström, S. *Acta Chem. Scand.* **5** (1951) 190.
6. Hammarsten, E. *Acta Med. Scand. Suppl.* **196,** (1947) 634.
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* We wish to thank Dr. S. Åqvist for the isolation of these amino acids.

A Synthesis of 2-Hydroxy-3-methyl-4,6-ω,-trimethoxyacetophenone and Some Related Compounds

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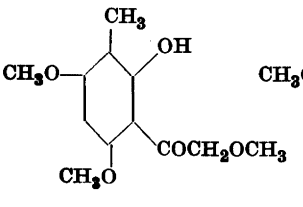
In connection with certain studies in the field of C-methylflavones, the previously not synthesised 2-hydroxy-3-methyl-4,6,-ω-trimethoxyacetophenone (I) was required as a starting product. This ketone has now been synthesised by two independent methods which are exactly analogous to those adopted for the synthesis of 2-hydroxy-3-methyl-4,6-dimethoxyacetophenone by Curd and Robertson [1]. The first and most convenient method involves methylation of ω-methoxyphloroacetophenone with methyl iodide and potassium carbonate in acetone, and the second consists of the condensation of 2-hydroxy-4,6-dimethoxytoluene (II) with methoxyacetonitrile by the Hoesch method. Both procedures yielded a colourless compound $C_{12}H_{16}O_5$, m. p. 148–149° *. The second method, starting from the phenol (II), can lead to two possible isomers, an *o*- and a *p*-hydroxyketone. On the other hand, the methylation of ω-methoxyphloroacetophenone must be expected to yield an *o*-hydroxyketone, since the hydroxyl groups in *o*-positions are less reactive because of hydrogen bonding to the carbonyl oxygen. Thus, (I) represents the only possible structure for the ketone synthesised.

On condensation with benzaldehyde, the ketone yielded 2′-hydroxy-3′-methyl-4′,6′, α-trimethoxychalkone (III), m. p. 117–119°. Selenium dioxide oxidation of this chalkone gave a colourless crystalline compound in 4 % yield. This had m. p. 154–155° and gave a pink colour on reduction with magnesium-hydrochloric acid. Since chalkones are generally oxidised to flavones by selenium dioxide [2], this product must be 3,5,7-trimethoxy-8-methylflavone (IV). To test the validity of this reaction for other α-methoxychalkones, we oxidised 2′-hydroxy-4′,6′, α-trimethoxychalkone and isolated the expected galangin trimethyl ether, although in very low yield.

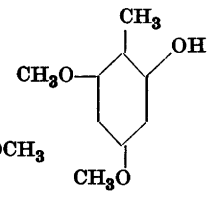
The ketone (I) is probably identical with "methoxymethylfisetol dimethyl ether", m.p. 148–149°, which was isolated after alkaline degradation of the product obtained by methylating quercetin with methyl iodide and alkali [3]. Assuming this to be so, the methylation product of quercetin should be 8-methylquercetin pentamethyl ether.

EXPERIMENTAL: Synthesis of the ketone (I), Method A: Dry ω-methoxyphloroacetophenone (10 g) was refluxed with methyl iodide (30 ml) and freshly ignited potassium carbonate (30 g) in acetone (100 ml) for three hours. The precipitate was filtered off and washed with hot acetone, and the yellow filtrate evaporated to dryness. The crystalline

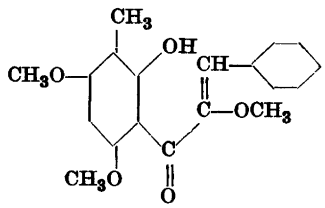
* All melting points uncorrected.



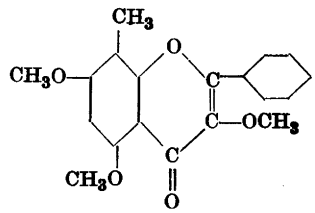
I



II



III



IV

residue was dissolved in ether, and the solution washed with sodium carbonate solution, dried over anhydrous sodium sulphate and filtered through aluminium oxide. The filtrate was concentrated, yielding a crystalline residue, which was recrystallised from ethanol, giving colourless needles, m.p. 143—144° (2.2 g). Further recrystallisation raised the m.p. to 148—149°. With ferric chloride in alcoholic solution, the substance gives a brownish-violet colour.

$C_{18}H_{16}O_5$ (240.3)					
Calc.	C 59.9	H 6.70	OCH_3 38.7		
Found	» 59.6	» 6.74	» 38.6		

Method B: 2-Hydroxy-4,6-dimethoxytoluene [4] (II) was condensed with methoxyacetonitrile by the Hoesch method, and the reaction product worked up as described by Curd and Robertson [1]. After recrystallisation from ethanol, the substance melted at 146—148° alone or in admixture with a sample prepared by method A.

Synthesis of 2'-hydroxy-3'-methyl-4',6',α-trimethoxychalkone (III): A suspension of 2-hydroxy-3-methyl-4,6,ω-trimethoxyacetophenone (0.5 g) in ethanol (8 ml) was added to a mixture of benzaldehyde (0.5 ml) and potassium hydroxide (2 g) in water (2 ml). The resulting red solution was left overnight in a stoppered flask and then acidified, giving a yellow crystalline precipitate. This material, after three recrystallisations from ethanol, formed pale yellow leaflets, m.p. 117—119° (0.6 g). Ferric chloride in alcoholic solution gives a strong brownish-violet colour.

$C_{19}H_{20}O_5$ (328.4)					
Calc.	C 69.5	H 6.14	OCH_3 28.3		
Found	» 70.0	» 6.19	» 28.2		

Synthesis of 3,5,7-trimethoxy-8-methylflavone (IV): The chalkone (1.0 g) was refluxed with selenium dioxide (1.2 g) in *iso*-amyl alcohol (10 ml) for 40 hours. The solvent was then removed by steam distillation and the residue was dissolved in chloroform. This solution was washed with 2 *N* sodium hydroxide and with water, dried and filtered through aluminium oxide to remove coloured impurities. The filtrate, on concentration, slowly deposited yellow crystals, which were recrystallised twice from methanol, yielding colourless needles (0.04 g), m.p. 154—155°. Colour reactions: ferric chloride, none; magnesium-hydrochloric acid, pink.

$C_{19}H_{18}O_5$ (326.3)					
Calc.	C 69.9	H 5.56	OCH_3 28.5		
Found	» 69.1	» 5.05	» 28.1		

Oxidation of 2'-hydroxy-4',6',α-trimethoxychalkone by the same method gave a 5 % yield of galangin trimethyl ether, m.p. and mixed m.p. 196—198°.

This investigation has been financially supported by *Fonden för Skoglig Forskning.*

1. Curd, F. H., and Robertson, A. *J. Chem. Soc.* **1933** 437.
2. Mahal, H. S., Rai, H. S., and Venkataraman, K. *J. Chem. Soc.* **1935** 866.
3. Perkin, A. G. *J. Chem. Soc.* **103** (1913) 1632.
4. Weidel, H. *Monatsh.* **19** (1898) 232.

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Intermolecular Free Lengths in Liquids in Relation to Compressibility, Surface Tension and Viscosity

BERTIL JACOBSON

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The intermolecular free length between molecules in the liquid state is an essential and characteristic feature. This is evident from the simplicity and generality of application obtained when empirical relations are sought between the intermolecular free length and the properties in liquids which are dependent on intermolecular processes. Those properties include compressibility, surface tension and viscosity. If these properties are designated j the following simple relation prevails:

$$j = k \cdot L^p \qquad (1)$$

where k and p assume for each property and each temperature certain constant values irrespective of composition, structure, size and shape of the molecules in the liquid or liquid mixture. The free intermolecular length is defined as

$$L = \frac{2(V_T - V_0)}{Y} \qquad (2)$$

residue was dissolved in ether, and the solution washed with sodium carbonate solution, dried over anhydrous sodium sulphate and filtered through aluminium oxide. The filtrate was concentrated, yielding a crystalline residue, which was recrystallised from ethanol, giving colourless needles, m.p. 143—144° (2.2 g). Further recrystallisation raised the m.p. to 148—149°. With ferric chloride in alcoholic solution, the substance gives a brownish-violet colour.

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$$j = k \cdot L^p \qquad (1)$$

where k and p assume for each property and each temperature certain constant values irrespective of composition, structure, size and shape of the molecules in the liquid or liquid mixture. The free intermolecular length is defined as

$$L = \frac{2(V_T - V_0)}{Y} \qquad (2)$$

where V_T is the molar volume at the temperature T. For the compressibility and surface tension, V_0 is the molar volume at absolute zero, according to Sugden [1]. For viscosity, V_0 is the molar volume at the temperature where the fluidity is zero, *i. e.*, the zero fluidity volume according to Friend [2]. $V_T - V_0$ is thus the available volume for the type of molecular motion that is decisive for the different properties. $Y = f(36\pi N V_0^2)^{\frac{1}{3}}$ is the molecule surface of one mol of the liquid where N is Avogadro's number and f is a form factor which gives the relation between the molecule's surface and the imagined spherical surface which encloses the same volume as the molecule's volume. For low molecular substances $f \approx 1$.

For the adiabatic compressibility, there apply at 20° C the empirical values $p = 2.082$ and $k = 1.25 \cdot 10^7$ c.g.s. For 53 non-associated liquids (hydrocarbons, hydrocarbon halogens, organic esters and certain nitrogen compounds), L has been computed both from equ. (1) from compressibility values given in the literature, and according to equ. (2) from molar volumes. The mean deviation in values thus computed for L is 3.3 %. The deviation is due in part but probably not wholly to the difficulty of determining V_0 exactly. This uncertainty is eliminated in liquid mixtures. Even if the error in V_0 for the different pure liquids forming the liquid mixture amounts to one or two per cent, nevertheless it is possible to compute very accurate values relatively for L with different concentrations. Thus values computed for L from equ. (2) for liquid mixtures have been compared with values for L according to equ. (1) computed from experimentally determined compressibility values in mixtures of the type: Methylene iodide — ethylether, toluene — pentane, diphenylmethane — hexane and pyridine — hexane. The mean deviation in L for a total of 16 mixtures is 0.8 %. The compressibility has been measured with an accuracy of 0.1 %. In Fig. 1 the compressibility has been plotted in logaritmic scale as a function of L for mixtures of diphenylmethane and hexane.

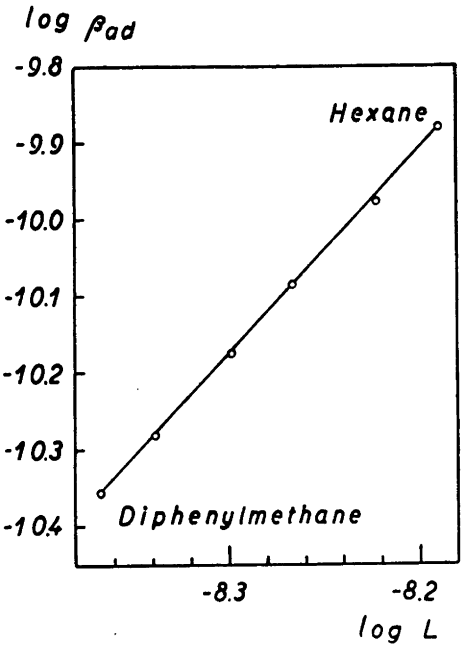


Fig. 1. Adiabatic compressibility (β_{ad}) plotted as a function of intermolecular free lengths (L) in mixtures of diphenylmethane and hexane at 30° C.

A trial of equ. (1) for surface tensions of 31 pure non-associated liquids shows that with certain preliminary values for k and p the mean deviation for L computed according to (1) and (2) is 3.4 %. Considering liquid mixtures the conditions are more involved due to the phase rule of Gibbs, still the relation should be useful in studying surface phenomena.

Equ. (1) is also valid for the viscosity of liquids. In view of the rather divergent definition of intermolecular free length as regards viscosity, this will here be called the intermolecular free fluidity length L_Φ. L_Φ has been computed from (1) with

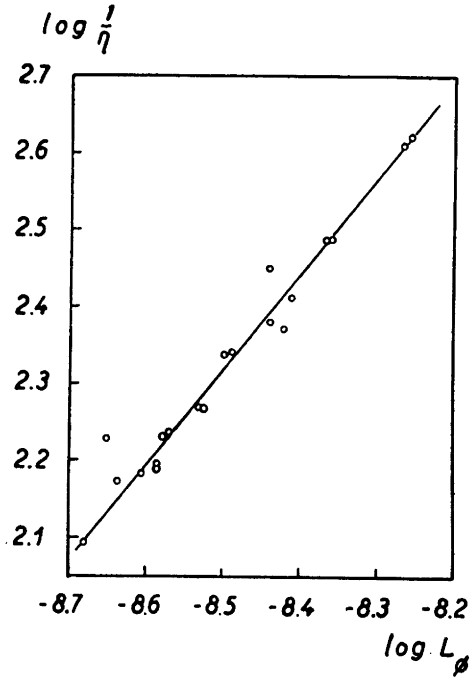


Fig. 2. Fluidities ($1/\eta$) plotted as a function of intermolecular free fluidity lengths ($L\Phi$) of 20 non-associated liquids at 20° C.

viscosity values given in the literature and from (2) by utilizing zero fluidity volumes according to Friend [2] for 20 non-associated liquids. The mean deviation in $L\Phi$ in this case is 4.3 %. Fig. 2 shows in logaritmic scale the fluidities for these 20 liquids as a function of $L\Phi$ according to (2). The largest deviation is observed with toluene (to the lower left in the figure). With a recalculated value of Friend's V_0 better agreement is obtained.

Owing to their simplicity these relations should be of value in the study of associated liquids. In order to arrive at the degree of association in a liquid there is first determined L or $L\Phi$ according to (1) from the values of compressibility or surface tension or viscosity. Then there can be determined from equ. (2) the number of monomers which are forming the associated complexes. For the lower fatty acids agreeing values are obtained for degrees of association computed from compressibility, surface tension and viscosity. They amount to about 2 and thus agree with the values obtainded by other methods.

Full data regarding these and for the constants k and p for the different properties at different temperatures will be published in this journal when a larger amount of material has been treated.

1. Sugden, S. *J. Chem. Soc.* **1927** (1780).
2. Friend, J. N. *Trans. Faraday Soc.* **3** (1938) 813.

Received October 15, 1951.

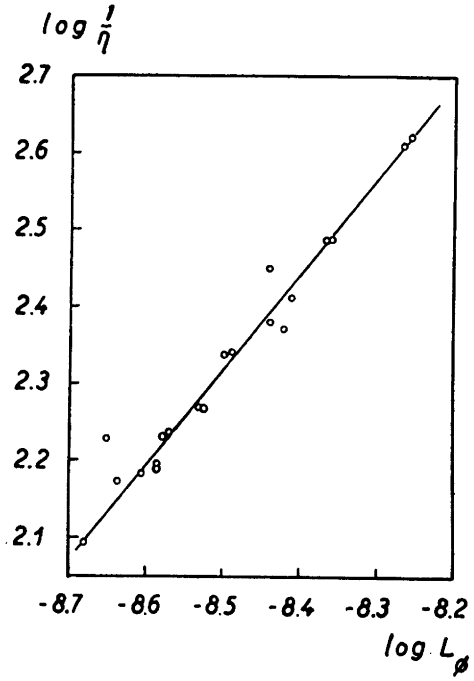
On the Phosphatase Activity of Low-phosphorus *Torulopsis utilis*

NIILO RAUTANEN and VEIKKO KARKKAINEN

Biochemical Laboratory of the University, Biochemical Institute, Helsinki, Finland

In a previous work Rautanen and Miikkulainen [1] have shown that the starvation of *Torulopsis utilis* yeast in respect to phosphorus is followed by a considerable decrease in various important phosphorus as well as nitrogen fractions of the cells. Concerning the physiological properties of the low-phosphorus yeast cells Wiame [2] has earlier shown with baker's yeast that these cells when placed in a medium containing phosphate have a peculiar ability to synthesize and accumulate inorganic metaphosphate in the cells. When investigating nearer this anomaly in the phosphorus metabolism of the low-phosphorus *Torulopsis* yeast cells we have observed very interesting changes in the activity of the acid phosphatase of the yeast cells of different phosphorus contents.

The cultivation of the normal, low-phosphorus and metaphosphate containing yeast cells was performed as reported earlier [1]. The phosphatase determinations were made both with fresh and dry yeast



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The cultivation of the normal, low-phosphorus and metaphosphate containing yeast cells was performed as reported earlier [1]. The phosphatase determinations were made both with fresh and dry yeast

preparations at pH 4.5 (acid phosphatase) and at pH 8.6 (alkaline phosphatase). Disodium phenylphosphate (Merck, p.a.) was employed as the substrate and the rate of the hydrolysis was followed by the determination of the liberated phenol with the Folin-Ciocalteau phenol reagent. The activity was calculated in Rae-Eastcott units (amount of phenol in mg liberated per 10 mg of the dry preparation from a 0.01 *M* solution in 20 min at 37° C).

As can be seen from the curves in Fig. 1 the decrease of the phosphorus content of the yeast cells during the starvation process is followed by a steady increase in the phosphatase activity of the cells. It is interesting to note that this activity of the normal yeast cells cultivated in excess of phosphate is practically zero. The activity increases during the starvation as long as the phosphorus content of the cells decreases.

The changes in the phosphorus content and in the phosphatase activity of the low-phosphorus yeast cells when placed again in a medium containing phosphate are illustrated in Fig. 2. The enormous

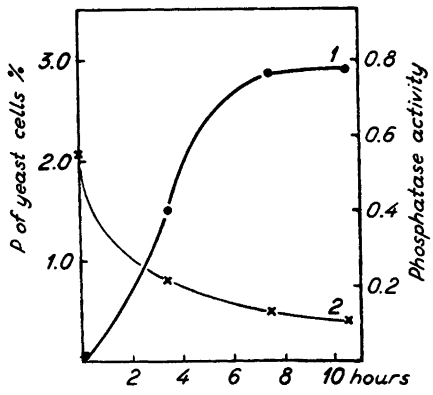

Fig. 1. Changes in phosphatase activity and total phosphorus content of yeast cells during the starvation in respect to phosphorus.

Curve 1: Phosphatase activity
Curve 2: Total P of yeast cells

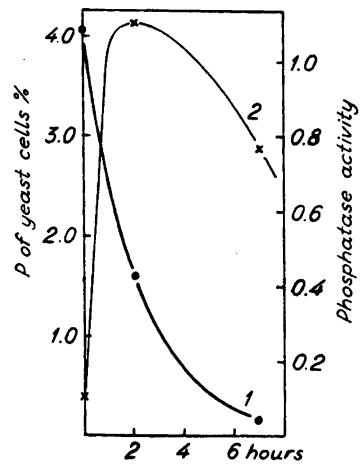

Fig. 2. Changes in phosphatase activity and total phosphorus content of yeast cells during the uptake of phosphate by low-phosphorus yeast.

Curve 1: Phosphatase activity
Curve 2: Total P of yeast cells

increase of the phosphorus content of the cells during the first two hours is due to the accumulation of inorganic metaphosphate in the cells. This metaphosphate disappears then continually and the phosphorus content of the cells falls toward the normal level. The phosphatase activity begins immediately to fall after the placing in the phosphate medium and this drop is most remarkable just during the accumulation of the metaphosphate in the cells.

The activities of the fresh and dry yeast preparations when calculated per dry matter were in every case practically the same.

When the phosphatase determinations were made at pH 8.6 (alkaline phosphatase) there could not be observed any activity in the normal or in the low-phosphorus yeast cells.

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On the Active Group in Rhodanese

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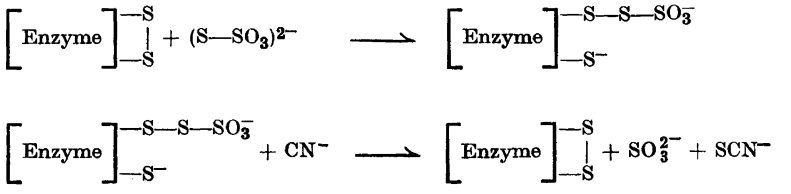
Saunders and Himwich [1] recently studied the effect of some inhibitors on rhodanese and found the enzyme to be inhibited by cysteine and other sulfhydryl compounds and in agreement with Lang [2] also by cyanide if added before the thiosulfate. They therefore suggested that the enzyme might contain a heavy metal as prosthetic group. On the other hand we [3] found no inhibition with cysteine, on the contrary it could protect the enzyme from the inhibition with cyanide. No inhibition was observed with other metal enzymes inhibitors, but sulfite in the absence of thiosulfate and strong oxidants were found to be potent inhibitors. Further investigations [4] showed that the cyanide inhibition was a comparatively slow reaction, 15 minutes being required for reaching 50 % inhibition in 0.001 *M* cyanide at pH 7.4. With purified preparations or aged homogenates the reaction was of the first order, but with fresh rat liver homogenates a much weaker inhibition was obtained and the reaction did not follow the first order curve. This was shown to be due to the presence of a sulfhydryl compound in the fresh homogenates, presumeably glutathione. Glutathione had a stronger protecting effect against cyanide inhibition than cysteine. The cyanide inhibition was not abolished by dialysis. These results seem to be incompatible with the assumption that rhodanese is a heavy metal enzyme.

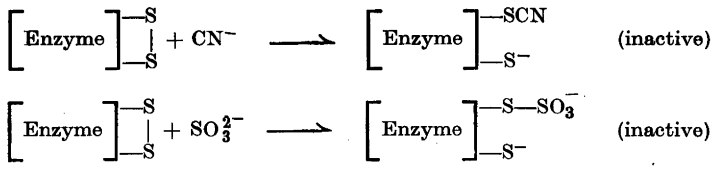
Sulfite was found to react more rapidly, 50 % inhibition being obtained in less than one minute in 0.001 *M* sulfite at pH 7.4.

The protecting effect of cysteine (and other sulfhydryl compounds) against cyanide can be explained if cysteine combines with the enzyme, giving a compound not attacked by cyanide. As no inhibition is obtained with cysteine alone, the cysteine must be easily displaced by thiosulfate. We thus have a case where thiosulfate, other sulfhydryl compounds, cyanide and sulfite react with the active group in the enzyme. This is easily explained if the active group is a disulfide linkage or a carbonyl group. The presence of an active carbonyl group in rhodanese has already been excluded [3] but all our findings are consistent with a disulfide linkage as the active group in the enzyme. The reaction mechanism could then be pictured as follows.

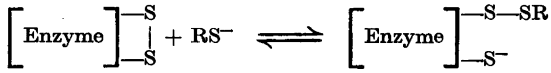
In the reaction scheme proposed, the enzymatic formation of thiocyanate from cyanide and thiosulfate thus consists of a hydrocyanolysis of one disulfide bond in the enzyme-substrate compound first formed from rhodanese and thiosulfate. Presumably sulfite is first split off, in analogy with similar reactions [5, 6] whereupon the labile enzyme-SCN compound breaks down, giving thiocyanate and regenerating the free enzyme. The inhibitions with cyanide or sulfite may be explained by the formulae:

The splitting of disulfide bonds with cyanide and sulfite has been studied by different authors, and the velocity, with





which the bond is attacked, is strongly influenced by other substituents in the molecule. Cystine and cyanide thus give thiocyanoalanine and cysteine [8], and cystine plus sulfite give a sulfinic acid and cysteine [9]. It is easy to understand that the corresponding reactions with the enzyme may destroy the activity of the latter. The protecting effect of sulfhydryl compounds (RS^-) may depend on the formation of a reversible compound.



The weak inhibition caused by *p*-chloromercuribenzoate, iodoacetate and iodosobenzoate [2,3] are also understandable, as sulfhydryl groups are released in the enzyme, when it combines with thiosulfate (eq. 1.).

Finally we found that rhodanese could not catalyse the reaction.

$$CNO^- + S_2O_3^{2-} \longrightarrow CNS^- + SO_4^{2-}$$

which could be predicted from the theory described above. No enzyme with a disulfide linkage as the active group has as yet to our knowledge been described, but their importance for the activity has been established in insulin and oxytocin. Gulland and Randall [10] studied the inactivation of the latter hormone with cyanide and sulfite, and obtained results similar with ours for the inactivation of rhodanese.

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7. Schöberl, A., and Ludwig, E. *Ber.* **70** (1937) 1422.
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New Books

Fulda-Ginsberg. *Tonerde und Aluminium.* I. Teil: *Die Tonerde.* Walter De Gruyter & Co., Berlin 1951.

Die Verfasser, von denen der erstgenannte während der Verfassung des Buches gestorben ist, berichten über die in der Praxis angewandten gegenseitig weit verschiedenartigen Verfahren zur Gewinnung der Tonerde mit Rücksicht auf ihre Anwendung in der Aluminiumindustrie. Die Verfasser besitzen eine ausgezeichnete Kenntnis aus erster Hand des Stoffes, weil sie in der betreffenden Industrie beschäftigt gewesen sind während der gewaltigen Entwicklung derselben durch die spätsten 30 Jahren. Es wird im Buche in klarer und wohlgeordneter Form ein Bericht gegeben über die hier gehörigen Erfahrungen und Probleme in so aufrichtiger und umfassender Weise, dass das Buch zum unschätzbaren Nutzen sein wird für jeden Techniker, der sich mit dem Projektieren von Anlagen der betreffenden Art beschäftigen muss. Die apparaturmässige, aber auch die ökonomische Seite der Sache wird eingehend behandelt. Nicht allein die Verfahren, die als Rohmaterial Bauxit verwenden, werden besprochen, sondern auch solche, die die verschiedenen Tonerdesilikate benutzen. Das Buch ist also allein anzusehen als ein Vorläufer für Band II, der die Herstellung des Aluminiummetalles behandelt. Die zahlreichen anderen Anwendungen des Oxydes und der Oxydhydrate des Aluminiums für feuerfeste Steine, chemische Apparatur, Schleifmittel, schneidende Werkzeuge und künstliche Edelsteine, fernerhin als Absorptionsmittel in der Färberei und in der Medizinalindustrie werden nicht behandelt.

A. H. M. Andreasen

ACTA CHEMICA SCANDINAVICA 5 (1951) 1221–1243

The Interaction of Polymetaphosphates with Hide Protein *

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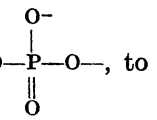
The protein-precipitating faculty of metaphosphoric acids has long been known, first observed by Berzelius in 1816; also the more pronounced effect of acidified solutions of polymetaphosphates (= PMP) was found far back. However, these reactions received serious attention first some 25 years ago. In the intervening years a number of investigations of various PMP protein systems are to be recorded. Since the chemistry of the condensed phosphate compounds, particularly that of the commonly employed "hexametaphosphate" (Graham's salt) is in itself exceedingly complicated, that being the case with polyacids generally, the inclusion of the elusive proteins in the system makes the problem still more difficult. The aqueous solutions of PMP, mostly applied after adjustment to pH values of 2—3, show continued alterations of the solute. In fact, very little is known about the composition and the molecular weight distribution of acidified PMP solutions.

The interaction of PMP and its acids with globular proteins has been investigated by Schofield [1], Perlmann [2], Briggs [3] and their coworkers. Thus, Perlmann and Hermann [4] showed that metaphosphoric acid combines stoichiometrically with the basic protein groups of egg albumin. Further, Perlmann applied this reaction for estimating the acid binding capacity of various proteins. Already in 1935, Schofield pointed out that the titration of ionizable amino groups of proteins would be considerably simplified by the use of an acid with its anion becoming firmly bound to the basic protein groups; suggesting metaphosphoric acid or its polymers as a suitable agent. The importance of the anion affinity for the interaction of acids with proteins has been convincingly proved by the fundamental researches of Steinhardt [27].

* The main findings of this paper were presented in a lecture given by the senior author at the meeting of the Nordic Leather Chemists' Association in Gothenburg, June 9, 1949.

The reactions of PMP are also of importance for fibrous proteins, particularly collagen. PMP of the type of Graham's salt, applied in acidic solution, possess marked affinity for hide protein, exerting a peculiar tanning action. Even at pH values corresponding to the maximum swelling of the hide protein by strong mineral acids, *i. e.*, pH 1.5—2.0, the PMP acids do not swell the hide structure, rather dehydrate it; the main part of the PMP taken up by collagen being irreversibly fixed. Hence it is not removed by prolonged extraction with water. This peculiar property of acidified solutions of PMP, discovered by Wilson [6], has already obtained important practical applications in pretreatment of neutral pelt (dehaired, alkali-treated ("limed") and further purified skins and hides) for the tanning processes. Wilson found that the PMP acids interacted with the basic protein groups. He conceived the reaction to be an electrovalent attachment of a chainlike PMP-molecule with regularly interspaced ionic groups,  , to the cationic protein groups of adjacent collagen chains.

The PMP-collagen system has further been studied by Riess [6], Schneider [7], Lindner [8] and Salo [9]. On the whole their results agree with the original findings and the conception of Wilson. Schneider has demonstrated the easy displacement of fixed PMP from its combination with collagen by means of sodium chloride of 2 *M* strength; a phenomenon earlier encountered with globular proteins [4]. Salo has shown that by deamination of collagen its binding capacity for PMP is lowered directly in proportion to the amount of amino groups removed; forming an additional proof of the stoichiometric nature of the reaction.

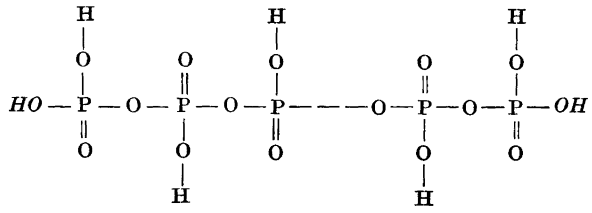
PMP is believed to be the best reagent known for evaluation of the number of cationic groups of proteins. In the light of the findings to be presented, it must be cautioned against uncritical application of this reaction, since steric factors enter in the reactions of the high-molecular acids of PMP with collagen, as the accessibility of the cationic protein groups to the numerous anionic loci of the large PMP molecule. This will make the reaction mechanism more complicated because a number of the anionic centers of the PMP structure are not able to react with the basic protein groups. Further, it will be shown that in systems of sodium polymetaphosphate, adjusted to pH values of 2.0—4.5 with hydrochloric acid, the anions removed by collagen contain not only —OH— groups but also —ONa— groups which latter do *not* interact with collagen. As evident from the literature, the question whether the acid as such is bound irreversibly by collagen or anions containing sodium in the form of —ONa links are taken up by collagen from solutions of acidified

sodium PMP, in the pH range 2.0—3.5 usually employed, is not settled. Riess [6] believed the latter type of anions to be involved in the fixation, whereas Schneider [7] takes the opposite view. No binding experimental evidences for any of the contrary views have been adduced by any of these workers; the data supplied by Riess being inadequate and inconclusive, as pointed out by Schneider. In the present paper the fixation of large PMP anions containing sodium has been proved experimentally by two independent methods.

SOME RELEVANT POINTS OF THE CHEMISTRY OF PMP

In the investigations of the PMP-protein systems reviewed, the Graham salt was assumed to be the hexa-compound and the corresponding acid or its split-up products (including the monomer) to be taken up by the protein. The literature of the condensed phosphates up to 1940 is reviewed in the monograph of Karbe and Jander [10] and the researches of the last decade in the clarifying papers of Van Wazer [11]. Only some main issues of importance for the understanding of the behaviour of PMP towards collagen will be accentuated in this connection.

As already mentioned, Wilson [5] was the first to conceive Graham's salt and the corresponding acid to consist of long chain-like molecules, interacting with the cationic groups of collagen by means of the numerous $-P-O^-$ groups of the PMP chain. Important experimental evidence for the high-molecular nature of some types of Graham's salt was offered by Samuelson [12] who pictures the acid of the PMP as mixtures of long chain polyphosphate:



The terminal OH-groups (in italics) are weak acid groups whereas the regularly interspaced OH-groups show strong acidic function. This difference in strength of the OH-groups forms the basis for the determination of the molecular weight of PMP by the end group method of Samuelson [12]; the weak acid groups titrating in the pH interval 5.5—8.5. An electrometric titration is shown in Fig. 1, curve I.

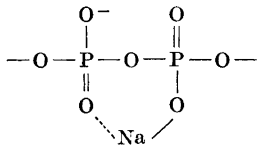
For a pure anhydrous Na-PMP, with no rings or orthophosphate present, the average chain length, n, is:

$$n = \frac{2 \text{ (equivalents of strong acid groups)}}{\text{equivalents of titrated weak acid groups}}$$

Titrating a solution of 1.02 g $(NaPO_3)_n$, as applied in the present investigation, and a ml 0.1 N NaOH being consumed in the end group titration, the molecular weight will be:

$$M = \frac{20400}{a}$$

since $M = 102\ n$ and further since $M = \frac{2 \times 102 \times 100}{a}$. The values of M obtained by means of this method are of the same order of magnitude as those derived from physico-chemical methods (diffusion [10] and ultracentrifugal [21]). Further, the fundamental researches of Van Wazer [11], particularly his findings of the distribution of M in acetone fractionated PMP, add strength to this conception of the structure of PMP and to the justification of the end group method. Van Wazer found that in solution of Graham's salt with large average chain length ($n = 193$) the fractionation follows the distribution curve derived for a random reorganization process. He also offered the first proof of the existence of phosphato-sodium complexes, probably chelate rings of the type:



Another cardinal point of the long-chain structure concept, relevant to the interaction of acidified solutions of Graham's salt with proteins, concerns the gradual displacement of the Na ions in the PMP chain by H ions with increasing H ion concentration of the system. This formation of chains with Na and H ions alternating as compensators of the regularly occurring —P—O⁻ groups, with the ratio of Na to H governed by the pH of the system, will explain some enigmatic findings of the variations of the equivalent weight of the PMP fixed by proteins. This is brought out by the data of this paper. Further, the average chain length of acidified PMP solutions as a function of time is a paramount issue as well as the molecular size of the PMP combined irreversibly with the protein, which problems are very difficult experimentally.

SCOPE OF THIS WORK

A number of problems pertaining to the reaction mechanism have been studied. Since the interaction of PMP with an insoluble protein like collagen in many respects offers better experimental possibilities than watersoluble globular proteins, the behaviour of collagen towards the PMP compounds should be expected to advance our knowledge of these complicated systems. The importance of theoretical fundamentals for rational technical application of PMP to various tanning processes is obvious.

In the present paper, the results from investigations of the fixation of PMP-compounds by collagen from solutions of sodium polymetaphosphate, adjusted to pH values in the range 1.4 to 5.8 by the addition of hydrochloric acid, are reported. Further, data on the interaction of ion-exchanged PMP-acids, free from foreign ions, with collagen at various pH-values are included as well as experimental findings pertaining to the chemical composition of the PMP fixed by collagen, its average molecular size and mode of binding, particularly its degree of stability towards sodium chloride. By measuring the changes in hydrothermal stability of collagen as a result of its fixation of PMP-compounds, a deeper insight into the mechanism of the reaction is to be expected.

The main themes to be discussed are the effect of the H-ion concentration on the irreversible fixation of PMP by collagen and the stability of the compounds formed. According to the literature, the maximum fixation of PMP from acidified solutions of the sodium salt of PMP (Graham's salt) occurs at an equilibrium pH value of 2.4. With further decrease of pH a very marked lowering of fixed P is generally reported. This is indeed an exceptional behaviour of a strong acid, with its anion exhibiting extraordinary affinity for the cationic protein groups. In previous known cases the fixation of such anion-combining acids shows a broad maximum zone in the pH range 1—3, whereas strong acids containing anions with little or no affinity for the basic protein groups and hence present in reversible combination with the proteins, show a steady increase of fixation until the pH corresponding to the maximum binding capacity of the protein for H-ions is reached. Since in the investigations of the workers mentioned the equivalents of PMP fixed by the protein have been obtained from determination of the P content of the treated proteins without due consideration of the existence of P groups of the PMP chains holding —ONa— -groups, non-reactive towards the protein, the data of the literature are misleading. In other words, the figures obtained from the P content of collagen containing anions of PMP taken up from the system: $(NaPO_3)$ + HCl show varying equivalent weights of P, from about 40 to 90

according to the pH of the system, the lower values recorded at 1 : 1 ratio of the components (pH 1.3) and the higher values for systems of high pH values, for instance 5. By means of indirect determination of the equivalent fixation of PMP (alkalimetric titration of the original and residual solutions of PMP), an evaluation of the degree of inactivation of the basic protein groups is possible. The uptake of ion-exchanged PMP-acid by collagen as a function of the final pH of the system shows the regular trend of the fixation of strong acids by proteins, *i. e.*, steady increased fixation by lowering the pH. This also applies to data from the direct P determination, since complications due to varying Na content and equivalent weight of P of the chains are excluded.

MATERIALS AND METHODS

American standard hide powder and iso-electric calf skin pelt (limed and subsequently delimed) served as substrates [sec 13]. The maximum binding capacity of these specimens for hydrochloric acid were 0.90 and 0.94 meq H^+ per g collagen (1.0 g collagen in 25.0 ml 0.1 *N* solution of HCl, containing 2 vol. % NaCl 24 h) and the N contents: 18.0 and 18.1, respectively. A number of sodium polymetaphosphates (Graham's salt) were investigated in the early stage of the work; the main series being carried out with products manufactured by *Reymersholms Gamla Industriaktiebolag*, Hälsingborg. The standard preparations contained 68.4 % P_2O_5. The Graham salt was dissolved in cold water to yield a 10.2 % solution of $NaPO_3$ (1 *N*). The pH of the 0.1 *N* solution was 5.6—5.8. The average molecular weight of the PMP:s used in the early work was 6800, determined by the end group method of Samuelson [12] whereas the product used in recent years showed an average value of 3600. The average molecular weights of the sodium polymetaphosphates employed are given in Table 1. No marked difference was found in the degree of fixation of the various preparations by hide powder from solutions with final pH values in the range 2.4—4.0, although the degree of stability of the fixed PMP generally decreased with lowering of the molecular weight. In solutions of final pH values below 2, the low molecular preparations showed lower degree of irreversible fixation, a marked tendency of instability upon prolonged washing of the PMP-collagen, lesser power of hydrothermal stabilization of collagen and in some instances, a marked tendency of swelling the protein.

Since in the most highly acidified solutions of PMP splitting of the larger units into smaller ones rapidly takes place, the time factor is of utmost importance [14]. In strongly acid systems brief periods of interaction of the PMP-acids with the protein (2—6 h) must be adhered to; rather two consecutive treatments with *freshly* prepared solutions for 2—4 h each time. Employing finely divided hide powder, in order to eliminate topochemical complications as far as possible, this time of interaction affords attainment of final equilibrium and complete inactivation of the basic protein groups. As a rule, about 90—95 % of the saturation value of the PMP fixation is reached upon two h violent shaking of the system. In reactions of solutions in the range of pH 2.4—3.5, the maximum fixation is generally obtained upon 12 h interaction in one operation.

Table 1. Average molecular weights of sodium polymetaphosphates. (Samuelson's end group method [12]*).*

No.	Manufacture	Molecular weight	Number of PO_3-units
1.	Calgon, Inc. Pittsburgh, Pa	1250	12
2.	Merck (1944)	2600	25
3.	Reymersholm (1945)	6800	66
4.	Reymersholm (1947)	3600	35
5.	Own preparation (fused at 800° C)	7600	75
6.	Own preparation from P_2O_5 in ether-water (cooled); subsequently neutralized by means of NaOH	1200	12

An approximation of the size distribution of the PMP used was obtained by gradual precipitation of aqueous solutions of sample no. 3 (average $n = 66$) by means of acetone, according to the method of Van Wazer (11). The end group determinations of n of the various fractions and their percentages of the total P gave: 120 and 25; 80 and 34; 35 and 29 and finally 15 units in the fraction containing 12 % of the total P. These figures are not corrected for the presence of rings (*e. g.*, trimeta), since the original preparation contained less than 2 % of the total P in this form (*Cf* (*11*)).

The determination of the molecular weight according to the end group method of Samuelson [12] was carried out by electrometric titration of 1.02 g of the sodium compound dissolved in 100 ml water at 20° C, noting the consumption of 0.1 *N* NaOH in the pH range from 5.6—5.8 to 8.5—8.6. Curve I of Fig. 1 shows the end group titration of specimen no. 3. Since in the present instance some idea of the average molecular weight of the specimens present in the acidified solutions was pertinent, the end group determinations were carried out on solutions of sodium polymetaphosphate adjusted to pH values of 2.0, 3.0 and 4.0, after various time of standing. The end group titrations of PMP no. 3, adjusted to pH 3.0 by means of HCl and kept at various periods at this pH, are graphically given in Fig. 1. Applying the method of Lowry and Lopez [15] to these solutions, simple phosphate ions were shown to be absent even after 24 h keeping. The curves of Fig. 2 show the general effect of decreasing pH of the system on the average molecular weights of the PMP (M = 6800). Acidifying by means of formic acid seems to give more stable solutions of PMP at pH > 2 than the HCl-adjusted solutions. Practical experience from pretreatment of skins in solutions of acidified PMP has also proved the superiority of formic acid as a pH-adjusting agent in regards to stability of the solutions of PMP.

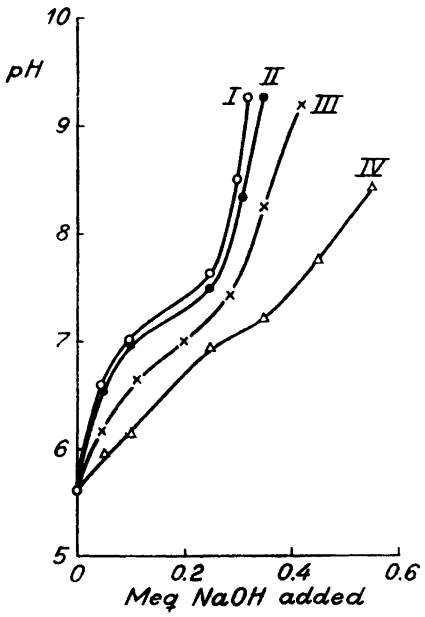


Fig. 1. End group titration of 1 g sodium polymetaphosphate in 100 ml water, adjusted to pH 3.0 (HCl), after keeping the solutions for various periods of time. Solutions kept:

I —○—○—○— · *0 h*
II —●—●—●— : *4 h*
III —×—×—×— : *24 h*
IV —△—△—△— : *72 h*

Polymetaphosphoric acid of 0.1 *N* strength was made by percolating 25.0 ml 10.2 per cent $NaPO_3$-solution, diluted with 75 ml water through a column of H-ion charged Dowex-50 (30 g with 165 meq capacity for Na^+) in 10 min. The filtrate and washings were made up to 250 ml. The solutions which were used immediately after preparation were exactly 0.100 N (methyl red).

The standard technique employed for the interaction of PMP with collagen was as follows: 2.0 g collagen in the form of hide powder was soaked in 20 ml water 2 h previous to the experiment. Portions of polymetaphosphate equal to 1 g $NaPO_3$ in total volume of 50 ml, adjusted to the desired pH values by means of 0.1 *N* hydrochloric acid, were added and the system shaken 4 h. At least duplicates were run. In the first the pH value of the residual solution was determined. The treated collagen was then washed, drummed and shaken out with water (2 l) in order to remove loosely attached PMP. The stock was sucked dry, dehydrated by means of acetone, dried and analyzed for P and collagen. In some instances the Na-content of the stock was determined by the Lundegårdh spectrometric method [16] *. The second sample was washed on a glass filter, the solutions and washings made up to 1000 ml. The hide powder was then acetone dehydrated, dried and analyzed like the first one. Aliquots of the collected effluents were titrated with 0.1 *N* NaOH (methyl red and phenolphthalein) and the former figure used in the "by difference" determination of the amount of removed acid (the total equivalent of acid of the original solution — the equivalents of acid present in the residual solution (methyl red figures).

* The kind cooperation of Dr. I. Ekdahl is gratefully acknowledged.

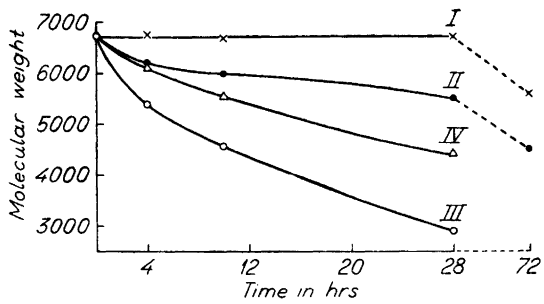


Fig. 2. The average molecular weight of sodium polymetaphosphate in 1 % solution, adjusted to pH values of: 2.0, 3.0 and 4.0 by means of HCl, kept at various periods of time, determined by end group titration.

I pH: 4.0
II pH: 3.0
III pH: 2.0
IV pH: 2.0 (adjusted by means of formic acid).

In some experiments, portions of hide powder equal to 1 g collagen were treated in 25.0 ml 1.0 % solution of PMP in order to increase the accuracy of the indirect method. Further, in the series with ion-exchanged acid of PMP (Fig. 3) 1 g collagen was treated in 50 ml portions of solutions of normalities 0.1—0.04 and in 100 ml portions of solutions of 0.03—0.01 normality, in order to obtain data in the higher pH range.

For the determination of the amount of combined PMP (determined and given as P), 0.2 g dry phosphated hide powder was digested for 20 min in a Kjeldahl flask with 15 ml sulphuric acid (1.84) and 5 g anhydrous sodium sulphate, using 50 mg selenium as catalyst. The digest was made up to 200 ml and 2.0 ml of this solution was used for P-determination. This was carried out in a Spekker photoelectric absorptiometer, employing the "molybdenum blue" method [17]. Particulars regarding special techniques and other details will be given in connection with the experimental data.

RESULTS

1. *The effect of pH upon the reaction.*

Fig. 3 shows the irreversible fixation of polymetaphosphoric acid, prepared by ion exchange, by collagen (hide powder) after 12 h interaction in the terms of meq acid fixed by 1 g collagen, determined indirectly from the difference in acid content of the original and residual solutions (methyl red) and directly by P-determination of the treated collagen preparations. The results of the two methods agree well. The steady increase of fixed P with increasing H-ion concentration is clearly brought out by the curves. Important to note is that such large amounts as 1.4 meq PMP acid per g collagen are recorded whereas

the amount of cationic protein groups is only about 0.94 meq. Since the maximum binding capacity of collagen was attained at the low pH values, the results indicate that only about two thirds of the anionic centers of PMP are discharged by the basic protein groups. In Fig. 4 the corresponding curves depicting the irreversible combination of PMP-anions from solutions of 2 % strength in $NaPO_3$ adjusted to pH values from 1.4 to 5.6 are given after 12 h interaction, leading to attainment of equilibrium. A marked divergency of the two curves is shown particularly in the higher pH range. Thus, for instance at a final pH value of 4.6, the amount of P in the phosphated collagen corresponds to 0.81 meq. PMP whereas the alkalimetric titration gives only 0.40 meq. On the other hand at a pH value of 1.3 with equivalent amounts of HCl and $NaPO_3$ present, the corresponding values are 1.38 and 1.30. The total Cl-content introduced with the hydrochloric acid was quantitatively accounted for in the analysis of the residual solutions. Hence, no hydrochloric acid is taken up by the protein under these conditions. Further, as will be shown by results from separate series given in the following, hide powder treated in solutions of pH values exceeding 2 contains considerable amounts of sodium.

Thus, PMP-hide powder was obtained by treating 25 g collagen as hide powder, hydrated in 200 ml water, in a final volume of 500 ml of 2 % sodium polymetaphosphate (M.W. 6800) for 6 h, adjusted to an initial pH of 2.4 and yielding a final pH of 3.4. The treated hide powder was then lightly washed and sucked to dryness. It was then immediately treated for 12 h in 400 ml of a 2 % $NaPO_3$ solution of pH 2.0. The final pH was 2.4. It was washed until the wash water gave negative P test (after acid hydrolysis), sucked dry, dehydrated in 200 ml acetone and airdried. It contained, figured on dry substance: 4.4 % P, 87.4 % collagen (N $\times$ 5.53) and hence 5.0 % P on collagen basis which corresponds to 1.61 meq. PMP per g collagen. Since the combining capacity of collagen for acids is equal to 0.94 meq per g collagen, the average combining weight of PMP expressed as P is 53. The sodium content was 1.56 % Na, based on collagen, which corresponds to 5 atoms of Na on 12 atoms of P, *i. e.*, an equivalent of P = 54 assuming complete interaction of the free $P-O^-$ groups with the cationic protein groups. The agreement between the equivalent weights figured from the data of the P content, on one hand, and from the value of sodium, on the other hand, is better than could be expected.

Finally some data on the uptake of the PMP-anions from solutions of sodium polymetaphosphate with constant ratio of $NaPO_3$:HCl at varying pH values are given in Table 2. The reactivity of solutions of HCl-adjusted polymetaphosphate towards hide powder at increasing PMP-concentration is illustrated by the data of Table 3. In both

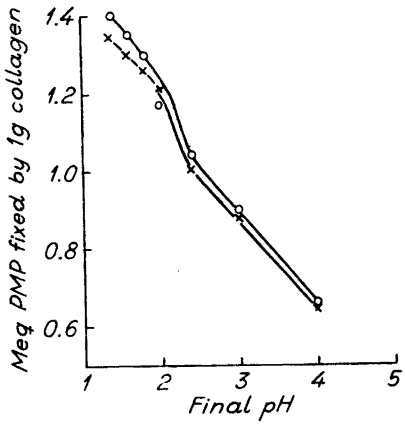


Fig. 3. The irreversible fixation of ion-exchanged polymetaphosphoric acid by hide powder, expressed in meq P fixed by 1 g collagen, as a function of equilibrium pH.

I —O—O—O— : *direct determination of P in substrate.*
II —×—×—×— : *indirect determination of equivalents PMP fixed by 1 g collagen from alkalimetric titration of initial and residual solutions.*

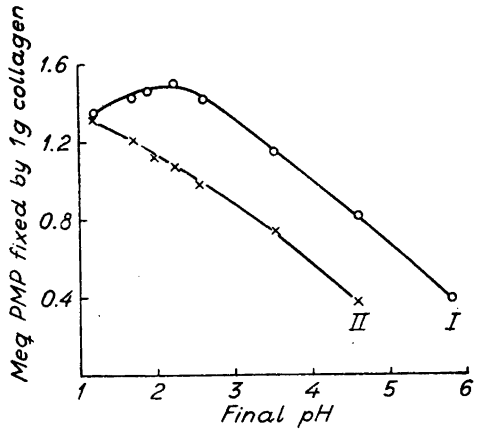


Fig. 4. The irreversible fixation of PMP by hide powder from acidified solutions of sodium polymetaphosphate expressed in meq P fixed by 1 g collagen, as a function of equilibrium pH.

I —O—O—O— : *direct determination of P in substrate*
II —×—×—×— : *indirect determination of equivalents PMP fixed by 1 g collagen, from alkalimetric titration of initial and residual solutions.*

series portions of hide powder = 1 g collagen were shaken for 8 h with 25 ml of the solution. The equivalents of PMP fixed were determined directly by P-determination on the washed specimens and indirectly by the difference in the acid content of the original and residual solutions.

Table 2. Fixation of PMP-acid from solutions of constant ratio $NaPO_3$:HCl (5 : 2).

No.	Normality of solution in P	Final pH	meq P fixed by 1 g collagen (P-determination)	meq P fixed by 1 g collagen (alkalimetric) indirectly
1.	0.1	1.8	1.46	1.02
2.	0.2	1.5	1.65	1.20
3.	0.4	1.2	1.68	1.18
4.	0.6	1.0	1.72	1.20

Table 3. Fixation of PMP-acid from solutions of constant Cl^--concentration at varying PMP-content.

No.	Normality of solution in P	in Cl	Final pH	meq P fixed by 1 g collagen (P-determination)	meq P fixed by 1 g collagen (alkalimetric) indirectly
1.	0.1	0.06	2.05	1.43	1.09
2.	0.2	0.06	2.0	1.65	1.05
3.	0.4	0.06	2.0	1.68	1.00
4.	0.6	0.06	1.95	1.75	0.96

Evidently the uptake of PMP by collagen from solutions of $(NaPO_3)_n$ with varying P-content and H-ion concentration is practically the same in P-concentrations from 0.2 to 0.6 *N* and in the pH range 1.0—1.5. This is also the case in the system with constant Cl-ion concentration (added HCl) and equilibrium pH, varying the concentration of the PMP. The large difference of the bound PMP found by the two methods is noteworthy; anew demonstrating the erroneous results obtained in figuring the degree of inactivation of the basic groups of the protein by means of equivalents of P (from data of the P content of the PMP-protein).

2. *The effect of hydrochloric acid on PMP-collagen.*

As an illustration of the application of other reactions to the PMP-collagen, its behaviour to solutions of 0.1 *N* HCl is of interest. Generally, the degree of inactivation of the basic groups of collagen by an anionic agent present in stable combination with collagen is conveniently evaluated by assessing the meq of HCl removed from a 0.1 *N* solution in contact with the collagen with partly inactivated cationic groups. This method works well for polysulphonic acid dyestuffs and high-molecular lignosulphonic acids [18]. In the case of PMP-treated collagen, particularly specimens prepared in strongly acidic solutions, many complications enter; in the latter instance particularly the exchange of PMP-anions for Cl-ions besides the removal of H ions by any carboxyl ions of collagen remaining free. Although the displacement reaction does not enter so markedly in the HCl-treatment of PMP-collagen prepared from acidified PMP solutions of pH 2.4—3.5, the sodium atoms contained in the PMP chains introduce an additional error.

In the present instance the aforementioned specimen containing 1.61 meq P in combination with collagen, in an amount equal to 1 g collagen, was shaken for 6 h in 20 ml 0.1 *N* HCL-solution. The amounts of H- and Cl-ions of the residual solution were determined. It was 0.1 *N* in regards to Cl-ions and further a quantity of 9 mg P was found. Hence, 0.29 meq P was displaced;

that is, 18 per cent of the total P content of the substrate had gone into solution. Since the concentration of Cl-ions of the solution remained the same, no free basic protein groups were present. On the other hand, 0.46 meq H-ion was removed from the solution, probably displacing the greatest part of the sodium atoms of the fixed PMP. Evidently the large PMP anions are split into smaller fragments by means of the H-ions; no direct action of Cl-ions being apparent.

Similar experiments were run with hide powder equilibrated with solutions of acidified PMP at final pH values of 2.0 and 1.4.

The following figures are worthy of notice. The specimen treated at pH 2.0 contained 4.5 % P and 0.95 % Na, figured on collagen. The meq of PMP fixed were 1.45 (P-determination) and 1.00 (alkalimetric titration). Further, this PMP-hide powder (= 1 g collagen) removed 0.21 meq H^+ and 0.10 meq Cl^- from solutions of 0.1 *N* HCl, loosing 0.37 meq P, indicating displacement of Na- for H-ions as well as PMP-anions for Cl-ions and also a minor removal of H and Cl ions by the free anionic and cationic protein groups. This example is cited to show the difficulties encountered in the interpretation of such experimental data.

Finally, the hide powder reacted with PMP in a solution containing equivalent amounts of $NaPO_3$ and HCl, at a final pH value of 1.4, gave the following figures: Its P content was 4.05 on the weight of collagen, corresponding to 1.31 meq P and the titrometrically determined content of P was 1.26 meq P. The sodium content was only 0.08 meq Na per g collagen. By treating 1 g collagen in the form of the PMP-collagen in 25 ml 0.1 *N* HCl for 6 h, no H-ions were taken up. An amount of Cl-ions equal to 0.24 meq was removed by the substrate. Alkalimetrically the residual solution was 0.108 *N* containing displaced PMP, which amounted to 0.41 meq P. This example shows anew that good agreement of the equivalent weights of P of the PMP fixed at low pH values is obtained by P-determination on the substrate and by alkalimetric titration of the system since formation of complexes containing sodium is practically eliminated. It is of interest to note that the PMP fixed by collagen at low pH values is much more easily displaced by HCl and may be gradually removed upon prolonged washing than is the case with specimens equilibrated with PMP at moderate pH values (about 3). Evidently the breaking up of the large molecules of PMP in solutions of the low pH range is largely responsible for the instability of the phosphate-collagen compound. This is still more evident from the behaviour of collagen treated in pure PMP acid solution and furthermore accentuated in specimens which have been brought to react with such solutions of pH 1.3 – 1.4 standing 24 – 48 h before application. Anew, it is proved that the molecular size of the fixed PMP is a main factor determining the degree of stability of the PMP-protein compound. This problem is under further investigation.

3. *The effect of sodium chloride on PMP-collagen.*

Another unique type of displacement of the irreversibly fixed PMP from the collagen lattice is effected by short treatment of PMP-collagen in solutions of sodium chloride, a 2 *M* NaCl solution being effectively employed, first noted by Schneider [26]. Taking as an example the specimen equilibrated with

PMP solutions of final pH 2.4, the following data are of interest. A portion of 3 g of dry PMP-hide powder was shaken for 24 h in 100 ml 2 *M* solution of NaCl. The treated powder was then washed until the wash water became free from Cl-ions. The percentage of combined P, figured on the weight of collagen, was then reduced from 5.0 to 2.3 and the amount of sodium from 1.56 % Na to 0.06 %. Hence, it appears as the PMP-chains containing sodium are preferentially removed in the salt treatment. The identical treatment of PMP-hide powder equilibrated in 0.1 *N* solution of pure PMP acid, showed the following figures. The original specimen contained 3.2 % P or 1.03 meq PMP acid per g collagen. After treatment in 2 *M* NaCl-solution and subsequent washing, it analyzed 0.3 % P on collagen basis, showing 94 % displacement of the fixed PMP acids. Also in this instance it is clear that the PMP-collagen compound obtained from the solution of PMP of low pH values is easier hydrolyzed than the PMP-compound of collagen introduced from solutions of higher pH values. Finally, the nature of the displacing effect of sodium chloride on the PMP fixed by collagen will be exemplified.

Hide powder equilibrated with ion-exchanged polymetaphosphoric acid at pH 1.4 containing 1.23 meq P (by P-determination) and 1.28 meq P (by alkalimetric titration) per g collagen was treated in 2 g portions in 80 ml 2 *M* NaCl-solution for 24 h. The residual solution and the wash water were made up to 100 ml. The filtrate contained 0.78 meq H^+ and 2.32 meq P (on 2 g collagen). Analysis of the treated hide powder gave 0.06 meq P per g collagen. Accordingly 95 % of the fixed PMP was removed in the treatment. Further, the treated stock contained 0.84 meq H^+ ions per g collagen (1.23—0.39) in the form of hydrochloric acid. Thus, the main reaction is an exchange of anions, Cl ions displacing PMP anions in the collagen lattice. It is simply a mass action effect.

The average molecular weight of the displaced polymetaphosphoric acid was determined by end group titration. It showed an average molecular weight of 2000—2500. The method was checked on solutions of ion-exchanged polymetaphosphoric acid of various concentrations and in the presence of sodium chloride. The data obtained in Table 4 show that the presence of 2 mol NaCl/l of the solution has no effect on the end group titration. Similar titrations on PMP displaced by NaCl from PMP-collagen equilibrated with acidified sodium PMP of pH 3.0 gave molecular weights of 3000—3500.

The decrease of the pH value of no 3 from 2.4 to 1.8 by making the solution 2 *M* in regards to NaCl is due to the hydrochloric acid formed. The decreasing effect of neutral chlorides, particularly sodium chloride, upon the fixation of PMP by collagen has been proved by Riess, Schneider and Salo, who determined the total amount of polymetaphosphate taken up by collagen and not

Table 4. End group titration of solutions of polymetaphosphoric acid.

No.	Solutions	Initial pH	Total ml 0.1 *N* NaOH consumed (to pH 5.6)	ml 0.1 *N* NaOH consumed from pH 5.6 to 8.6	Average mol. weight
1.	0.1 *N* PMP-acid (ion exchanged)	1.35	52.8	3.8	2800
2.	0.02 *N* » » » »	2.1	10.8	0.88	2500
3.	0.01 *N* » » » »	2.4	5.76	0.60	2000
4.	0.01 *N* » » » » containing 2 M/1 NaCl	1.8	—	0.60	2000

the total amount of acid equivalents removed from the solutions with added sodium chloride. With increasing concentrations of Cl ions of the solution of acidified PMP or free polymetaphosphoric acid, pelt will take up more and more acid in the form of hydrochloric acid; the final effect being to keep the equivalents of total acid fixed constant or in some instances to increase it, compared to the equivalent fixation of the PMP-acid. Hence, it is also evident that in the system of acidified PMP, the increasing amounts of sodium chloride formed with increased acidity will have a slight lowering effect on the fixation of the PMP-compound. However, as previously mentioned no chloride ions are taken up by hide powder from the system $NaPO_3$-HCl of initial pH values 1.3—5.0.

As an example of the preferential fixation of Cl-ions in the presence of large excess of salt, the following data are interesting. Portions of 1.0 g collagen as hide powder were treated for 6 h in 50 ml 0.05 *N* ion-exchanged polymetaphosphoric acid and further in the same solution made 2 *M* in NaCl. The pH of the residual solution from the pure acid treatment was 2.0, that of the 2 *M* NaCl exhaust 1.3. The milliequivalents fixed PMP per g collagen were 1.15 and 0.24 respectively; the latter specimen containing an additional amount of acid (HCl) equal to 0.75 meq per g collagen. In both instances, the maximum acid binding capacity of collagen was satisfied.

4. The effect of PMP fixed by collagen on its hydrothermal stability.

The irreversible fixation of PMP by collagen even at pH values as low as 1.5, does not produce swelling of the hide substrate, rather dehydration of the isoelectric protein is effected. Thus, hide powder completely hydrated in water at pH 5.8 contained after removal of superfluous water by suction on a glass filter under standardized conditions, 2.3 g water per g collagen, whereas

hide powder equilibrated with 0.1 *N* ion-exchanged polymetaphosphoric acid of pH 1.5 contained 2.0 g water per g collagen, determined under identical experimental conditions. Hide powder treated with the 0.1 *N* polymetaphosphoric acid solution after heating it to 95 °C for 5 min., swelled considerably and turned glue-like, probably due to the formation of scission products reacting unifunctionally with collagen. The treated hide powder contained 6.2 g water per g collagen. Similar experiments were run on iso-electric calf skin pelt, which after 24 h treatment in water and following pressing under standardized conditions contained 1.7 g water per g collagen. By pretreatment in 0.1 *N* polymetaphosphoric acid, the value of 1.5 was recorded, whereas the corresponding treatment in the solution boiled before use was 8.5 g water per g collagen. These simple experiments show that the nonswelling action and the dehydrating effect of PMP-compounds are intimately bound up with the polyfunctional interaction of large molecules of PMP. The split products produced upon keeping the solutions for long time at room temperature or upon brief heating are not irreversibly fixed and offer conditions of attachment to collagen favourable for the establishment of Donnan effects (swelling) which require completely reversible systems and low affinity of the reacting anion for the cationic protein groups. In this connection, the effect of storing of hide powder originally in irreversible combination with ion-exchanged polymetaphosphoric acid in slightly moist state (20 % moisture) for 12 months on its stability is of interest. Treating the aged specimen (1 g in 200 ml water) two consecutive times, each 24 h, 42 per cent of the originally fixed PMP was washed out, indicating a gradual formation of scission products from the condensed phosphoric acid originally fixed by collagen.

The postulate of the polyfunctional attachment of PMP on collagen and the resulting stabilization of the protein lattice by the rivetting together of adjacent protein chains by one large chain of PMP is given additional support by the data on the effect of the fixed PMP on the hydrothermal stability of collagen. Portions of strips of isoelectric calf skin pelt in duplicates (20.0 g pressed weight = 6.0 g collagen) were shaken for 20 h in 100 ml of a 2 % solution of sodium polymetaphosphate (M.W. 6800) of various pH values, adjusted by means of hydrochloric acid (initial pH: 1.3—5.8). The residual solution was drawn off and the pH determined. The treated pelt was then washed, alternating with shaking in water in several runs. The residual solution and the wash water of the duplicate was made up to 1000 ml for alkalimetric determination of the PMP remaining in the solution. As a measure of the hydrothermal stability the shrinkage temperature (T_s) is generally used. The T_s was determined both on the freely suspended strips and on strips with both ends fastened in a shrinkage tester, the latter mode of determination

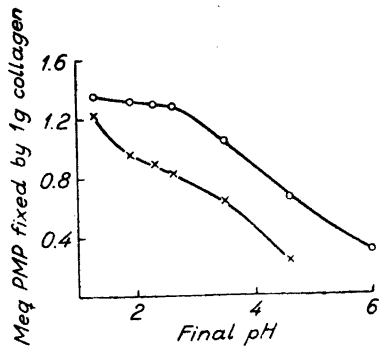


Fig. 5. The irreversible fixation of PMP by calf skin pelt from acidified solutions of sodium polymetaphosphate, expressed in meq P fixed by 1 g collagen, as a function of equilibrium pH.

I —O—O—O— : *direct determination of P in substrate*

II —×—×—×— : *indirect determination of equivalents PMP fixed by 1 g collagen, from alkalimetric titration of initial and residual solutions.*

giving 2—3 °C higher values. However, the difference recorded in shrinkage temperature, $\triangle T_s$, of the various specimens by the two methods showed good agreement (0.5° C). The results of these series are given in Table 5.

Table 5. The interaction of acidified sodium polymetaphosphate with calf skin pelt.

No.	Final pH	meq P fixed by 1 g collagen (indirectly by alkalimetric titration of solutions)	meq P fixed by 1 g collagen (from direct P determination of treated pelt)	meq Na per g collagen	ΔT_s
1.	1.3	1.24	1.36	0.04	+ 5
2.	1.9	0.96	1.32	—	+ 4
3.	2.3	0.90	1.30	—	+ 4
4.	2.6	0.82	1.29	—	+ 3.5
5.	3.5	0.64	1.05	0.31	+ 3
6.	4.6	0.32	0.67	—	0
7.	6.0	—	0.30	—	— 7
8.	1.5 (ion exchanged PMP-acid)	1.32	1.26	0.00	0

Also in these series the direct P-determination and the indirect alkalimetric estimation of the fixation of PMP, expressed in meq per g collagen, shows large differences in systems of pH values greater than 1.3 as evident from the graphs of Fig. 5. Regarding the main theme, the effect of the PMP treatment on the T_s of the skin, it is evident that the PMP fixed by collagen from systems of low pH values is most effective in raising the T_s, which in this instance

probably partly reflects the fact of the more complete inactivation of the basic protein groups at the highest H-ion concentration. Thus, the potential of anionic centers of the PMP-chains is more fully made use of and hence the protein chains are rivetted together more extensively. Of particular importance is the considerable lowering of the T_s of collagen induced by the unadjusted PMP-solution (pH 5.5—6.0) which actually has a hydrotropic effect, also shown by the swelling of the pelt [19]. This swelling is to a large extent permanent and apparently belongs to the lyotropic class of ion and molecular effects, shown by strong solutions of urea, thiocyanates and halogen salts, particularly of bivalent metals. Then, illustrations are given on the stabilizing effect of a polyfunctional, irreversibly fixed large anion on the collagen lattice, on one hand, and the opposite behaviour of an unifunctional anion, which by its fixation on collagen weakens the intermolecular cohesion of the native protein by breaking crosslinks originally present, on the other hand. This problem and the behaviour of related compounds will be further discussed in next section.

By keeping pelt no. 1 in toluene-saturated water for 6 months at 4° C, it showed marked swelling and peptization, probably due to the formation of low-molecular acids (including phosphoric acid). Hence, PMP-treated collagen should be immediately dehydrated (acetone) and stored in this conditions in order to eliminate secondary splitting of the large PMP-molecules in the protein lattice.

It has erroneously been claimed [9, 20] that free PMP-acid swells hide. That is not the case if the solutions of ion-exchanged acid (from sodium salts of molecular weights 3000—8000) are used *immediately* after preparation, limiting the time of interaction with collagen to 24—48 h. By the formation of hydrolysis products of the acids, occurring gradually upon storing the solutions or rapidly by heating, swelling of hide is promoted, as shown earlier.

COMMENTS

Restating facts, the main finding of this investigation is that in the reaction of collagen and acidified solutions of sodium polymetaphosphate molecular chains of PMP of varying length and proportions of H and Na ions associated with the PO^--groups, governed by time, pH and other factors, are taken up and irreversibly fixed by collagen. The degree of inactivation of the basic protein groups measured in equivalents fixed PMP (alkalimetrically) by collagen shows steady increase with decreasing pH of the reacting systems, being complete at final pH values about 1.5. The generally accepted view of an optimum range of reactivity in the vicinity of pH 2.4 of the equilibrated

PMP solution is needy of revision, being a measure of the total fixation of PMP; overlooking the numerous PO^--units which cannot function as anionic centers for the interaction with cationic protein groups on account of the association of sodium ions with the former groups and unfavourable steric environment of the reactive PO^- groups for discharge of electropositive groups of collagen. Thus, such high values of fixed P as 1.6—1.7 meq per g collagen obtained upon direct P determination of the substrate are explained. It is also obvious that any simple stoichiometric relationship of the PMP compounds fixed is not discernible since, for instance, in a chain of 30 units the proportion of Na to H will vary continuously according to the proportion of hydrochloric acid added to Na-PMP. Hence, it is only a coincidence that the composition of the compound fixed at pH 4, in one case found, corresponds most closely to the formula: $(Na_4(PO_3)_6)_x$. The marked affinity of sodium for the P—O-ion is responsible for the fact that from solutions of pH values 2—1.5, with nearly one equivalent of HCl added per equivalent $NaPO_3$ in the last instance, small amounts of Na is present in the anions fixed by collagen.

Regarding the average molecular weight of the PMP attached to collagen, no definite figures are available. However, in treatment of collagen in solutions adjusted to pH 3—4 directly after preparation and limiting the time of interaction to a few hours, the average molecular weight of the original PMP is only slightly lowered and large molecules probably are interacting with collagen. In solutions of pure polymetaphosphoric acid, working in the pH range of 2.0—2.5, hydrolysis of the large molecules evidently takes a rapid course although practically no PO_4 ions are formed. Since gelatin still precipitates these solutions after 4—8 h standing, *i. e.*, the time of interaction with the protein generally employed and also the benzidine test is positive, part of the PMP combining with collagen probably remains as rather large molecules. This is also brought out by the marked stabilization effect of PMP, reacting with hide substance in this pH range, as evident by the T_s figures and also by their dehydrating effect.

A comparison with other anionic agents is illuminating. A strong mineral acid, such as HCl, forming a reversible system with collagen, swells hide and lowers its T_s 30—40° C. The strong unifunctional acid, β-naphthalene sulphonic acid, reacting at pH 1.4, possesses affinity for the cationic protein groups solely, being partly irreversibly fixed. It does not swell hide. On the other hand, complete inactivation of basic protein groups by means of this acid (breaking completely the ionic crosslinks of collagen) decreases the T_s 16—18° C; accounted for by the discharge of the carboxyl ions of collagen and the inactivation of the basic protein groups by the anion of the acid[22]. A bifunctional acid is formed from the naphthalene sulphonic acid by condensing it

by means of formaldehyde, dinaphthalene methylene disulphonic acid, which does not swell collagen and which only exerts a slight T_s-decrease. By further condensation polynaphthalene compounds of disulphonic acids are formed which stabilize collagen to a minor degree, judging from the improved hydrothermal stability.

This effect is also shown by the highmolecular fractions of lignosulphonic acids. Their interaction with collagen is remarkable since the large number of sulphonic acid groups interspaced in a large molecule practically quantitatively react with the basic protein groups. Thus, preparations of average molecular weights of 8000—10000, containing from 16 to 20 sulphonic acid groups in a single molecule, show an average combining weight to collagen equal to the equivalent weight of the original acid [23]. Hence, the experimental findings from various sources do not disclaim the possibility of a large chain-like molecule with numerous, active anionic groups being introduced into the collagen lattice. The binding of lignosulphonic acid by collagen appears to be an ionic reaction in the initial stage. However, it is indicated that the electrovalent links formed are rapidly converted into a more stable binding, probably co-ordinate-covalent which resists the action of water, acids and neutral salts [24]. The linking of the PMP anion to the cationic protein groups apparently is of the electrovalent type and all evidence points to it remaining so. The anchorage of the long chain of PMP with its numerous anionic centers discharging adjacent basic protein groups should be expected to result in crosslinking of adjacent protein chains and hence will explain the irreversible nature of the reaction towards water, the improved hydrothermal stability and the lowered degree of water-binding imparted to the protein by the PMP-anion introduced into the collagen lattice. Also the easy replacement of the PMP anion in the collagen lattice by other anions, for instance, by means of treatment of PMP-collagen in concentrated solutions of neutral salts, conforms to the maintenance of the electrovalent linking of the original compound formed. It is a question of *anion exchange*.

From steric considerations of the availability of reactive protein groups to a single molecule of great length, certain objections may be raised against the concept of large multifunctional chains of PMP being the actual stabilizing agent. However, the presence of an —O— bridge for each P atom of the chain would be expected to impart a high degree of flexibility to the chain and hence adaptability to the cationic protein groups. Furthermore, the data obtained for the complete inactivation of cationic protein groups by means of poly-

metaphosphoric acids prove that only about two thirds of the —O—P(O⁻)(=O)— groups

groups can find their oppositely charged partners of the protein (1.4 meq P of $(HPO_3)_x$ being required, whereas the theoretical figure is 0.9 meq). This is undoubtely a case of steric hindrance and what is more, it constitutes evidence for the existence of long-chain condensed phosphates in combination with collagen.

On the whole, the concept of introduction of polyfunctional PMP-chains into the collagen lattice is in harmony with our present knowledge of the properties of PMP-collagen. The view of Salo [9], also subscribed to by Bear and his coworkers [25] in a recent paper, assuming monomeric HPO_3-units as the ultimate form of attachment of PMP on collagen has no experimental support and is improbable not only from the point of view of the existence of such a monomer being problematic but above all from the labilizing and swelling effect of simple phosphoric acid structures on collagen, in sharp contrast to the marked stabilization afforded by the PMP fixed.

In forthcoming papers the fixation of PMP by vegetable tanned collagen and by hide protein of various pretreatments will be reported as well as the important problem of the interaction of vegetable tannins with collagen in native state and with its basic groups completely inactivated by means of PMP. Further, investigations of the diffusion into heavy hide and fixation of different fractions of PMP, obtained by acetone fractionation according to Van Wazer are in progress.

SUMMARY

The interaction of sodium polymetaphosphate of average molecular weights of 3000—7000 with collagen, in aqueous solutions of PMP adjusted to pH values from 5 to 1.3 by means of hydrochloric acid as well as the corresponding reaction of ion-exchanged polymetaphosphoric acid have been investigated. The main findings are:

1. From solutions of acidified Graham's salt in the pH range 2.0—6.0 collagen fixes irreversibly PMP anions of varying composition which contain sodium, the relative amount of which increases with increasing pH of the reacting solution. The generally accepted view of maximum PMP interaction with collagen being reached at a final pH of 2.4 is incorrect. It is shown that the maximum degree of inactivation of the cationic groups of collagen requires pH values of 1.3—1.5 although the maximum amount of PMP fixed by hide powder, determined as P, is at equilibrium pH of 2.4, explained by the greater combining weights of the PMP of higher pH values. The interaction of polymetaphosphoric acid, free from foreign ions shows a steady increase of total amounts of P fixed by collagen as well as of the equivalent fixation with decreasing pH of the system.

2. The average molecular weights of the PMP present in acidified solutions of the sodium salt have been determined at pH 5.6, 4, 3 and 2 by means of end group titration. The molecular weights of PMP in solutions of the natural pH of 5.6, were constant upon keeping; values of 3600 and 6800 being recorded for the two PMP preparations investigated. Only a slight lowering of the molecular weight was shown at pH 4.0 upon time. PMP in solutions of pH 3 is gradually broken down to smaller units, the change being insignificant upon 4 h keeping of the solution. On the other hand, in solutions adjusted to pH 2 or less, the formation of splitting products is very marked, particularly for solutions of ion-exchanged polymetaphosphoric acid. By displacing the fixed PMP from its combination with collagen by means of concentrated solutions of sodium chloride, the PMP gone into solution showed average molecular weights of 2000—2500.

3. The displacement of irreversibly fixed PMP from its combination with collagen by means of sodium chloride and the retarding effect of sodium chloride on the uptake of PMP by collagen is shown in the latter instance to be caused by the preferential fixation of Cl ions by collagen, a Donnan effect, and in the former instance to be a case of anion exchange, both reactions governed by the law of mass action. The easy removal of fixed PMP by electrolytes points to the initial electrovalent binding of PMP by collagen being retained in the final product.

PMP applied in solutions of pH < 4.5 exerts a dehydrating effect on hide protein. Free PMP acid interacting with collagen directly after preparation does not swell collagen. On the contrary, it has a dehydrating action. The shrinkage temperature of hide is increased, up to 5° C, by incorporation of PMP from solution of acidified PMP of final pH 1.3—4.0, with the optimum reached in the lower pH range. On the other hand, solutions of sodium polymetaphosphate of pH values 5—6, have a hydrotropic effect on collagen, inducing permanent swelling of hide and decreasing its shrinkage temperature 7—8° C. The hydrothermal stabilization of hide protein by irreversible fixation of PMP indicates polyfunctional reactivity of the PMP and a probable crosslinking of adjacent protein chains.

4. The maximum acid binding equivalent of collagen; 0.9 meq H ions per g collagen, is markedly exceeded in the fixation of polymetaphosphoric acid, saturation values of 1.2 to 1.3 meq being recorded. This shows that not all of the available anionic centers of the PMP chains are able to interact with the cationic collagen groups. The reason is probably unfavourable steric conditions in the collagen lattice. The P fixed by collagen from solutions of $NaPO_3$—HCl of pH 2.5—3.5 corresponds to 1.6—1.7 meq per g collagen. Besides the factor of steric hindrance, this high value can be attributed to the

uptake of PMP anions containing Na and hence of anions of higher combining weight than that of polymetaphosphoric acid.

The incitation to the present investigation which has been carried out during the period 1943—1951 was given by the senior author's friend, the late Dr. John Arthur Wilson, New York, N. Y., the discoverer of the versatility of hexametaphosphates in tanning processes. This contribution is dedicated to his memory.

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Studies Related to Naturally Occurring Acetylene Compounds. VII. The Synthesis of two Stereoisomers of Methyl *n*-Decadiene-2,8-diyn-4,6-oates; the Configuration of Matricaria Ester

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A year ago Bruun, Haug and Sörensen described in this journal[1] the synthesis of methyl *n*-decene-2-diyn-4,6-oate, the *trans* form of the lachnophyllum ester, isolated in 1935 from the composité plant *Lachnophyllum gossypinum* Bge [2]. The synthesis was accomplished through oxidative coupling according to Glaser [3] of pentyne-1 with penten-3-yn-1-ol-5 [4] to the alcohol decene-2-diyn-4,6-ol-1 which was oxidized with chromium trioxide to the corresponding acid. This synthesis was carried through as a model experiment for the synthesis of matricaria ester, methyl *n*-decadiene-2,8-diyn-4,6-oate (I), which two of us [5] isolated in 1941 from scentless mayweed (*Matricaria inodora* L.)

For a comparable synthesis of matricaria ester the starting materials must be the same penten-3-yn-1-ol-5 (IV) and penten-3-yne-1 (V). When our synthetic work was initiated some years ago there was no reliable method of preparation for this simple hydrocarbon. A short note by Henne and Greenlee [6] indicated that, in the reaction of dibromo-1,3-propane with acetylene to prepare heptadiyne-1,6, a small forerun was obtained (about 15 %) which was tentatively regarded as penten-3-yne-1.

In agreement with Henne and Greenlee we obtained a fore-run in this reaction, but the amounts were quite insufficient for determination of structure and for preparative purposes.

Since ethylidenacetone is a readily available substanc, we tried to convert this ketone into 2,2-dichloropentene-3 and then to split off 2 moles of hydrogen

chloride. The reaction of ethylidenacetone with PCl_5 was vigorous; the reaction product distilled over a broad temperature range and was obviously not homogeneous. Chlorine analysis revealed that it mainly consisted of 2-chloropentadiene with varying amounts of 2,2-dichloro-pentene-3. As the loss of one mole hydrogen chloride at this stage was inconsequential, we used the crude chlorination product as such. We tried several different methods for the removal of the second mole of hydrogen chloride, but invariably met with bad yields. However, since the crude pentenyne (boiling range 50—60° C) in test experiments gave a readily crystallizing decadienediynol on oxidative coupling with penten-3-yn-1-ol-5, large quantities of the starting materials were prepared for a synthesis along these lines.

At that time Dr. B. C. L. Weedon of Imperial College (London) who, through one of us (T. B.), was aware of our experiments, made available to us an (at that time) unpublished method for the preparation of penten-3-yne-1 (V), worked out by Eglinton and Whiting in the laboratories of Professor E. R. H. Jones at Manchester and subsequently published. Pentyn-4-ol-2 is esterified with p-toluene-sulphonyl chloride and the ester decomposed with hot concentrated potassium hydroxide. This elegant synthesis gives very good yields (>90 % of theory based on pentyn-4-ol-2).

The b.p. of penten-3-yne-1 prepared according to Eglinton and Whiting, is however 46—48° C. This discrepancy gave rise to a more detailed investigation of the pentenynes obtained by these two different methods. Both are conjugated enynes since both exhibit a maximum in the U.V. at 2 240 Å ($\varepsilon = 16\,000$), the two absorption curves being practically identical. The spectrum of the pentenyne prepared according to Henne and Greenlee is also identical (*cf.* Fig. 1).

The physical properties of the preparations of pentenyne differ remarkably (Table 1).

Table 1.

Pentenyne according to	d_4^{20}	n_D^{20}	M_D	B. p. °C
Henne & Greenlee [6]	0.7415 [6]	1.4491 [6]	23.9	59.2
	0.738 *	1.4501 *	24.1 *	58.5—59 *
From ethylidenacetone		1.4480 *		57—60 *
Eglinton & Whiting [7]	—	1.4356 at 19°		46—48
	0.7288 *	1.43532 *	23.7 *	47.5—48 *

* This paper.

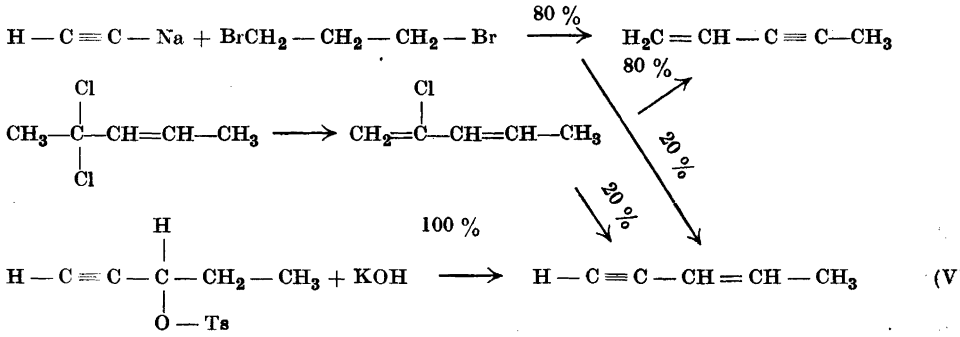
The refraction, and dispersion too (cfr. experimental part), reveal the conjugation of the double and triple bond. The exaltation for sodium light being 1.2 for the pentenyne prepared according to Henne and Greenlee, 0.9 for the pentenyne according to Eglinton and Whiting.

All three preparations of the crude pentenynes gave precipitates with silver nitrate, and all three reacted in the oxidative coupling according to Glaser.

Refractionation and determination of acetylenic hydrogen with methyl magnesium iodide showed that only the preparation according to Eglinton and Whiting is pure penten-3-yne-1 (V). The purest fraction, b.p. 58.5° of the pentenyne from ethylidenacetone gave no active hydrogen. The crude preparations contained about 20 % of monosubstituted acetylene, which gave rise to the silver reaction and the Glaser coupling. The preparations b.p. 58—59° then must be penten-1-yne-3; the physical properties are in excellent agreement with data given by

Carothers and Jacobson[8]: b.p. 59.2° $d_4^{20} = 0.7401$, $n_D^{20} = 1.4496$, $M_D = 23.94$ and by Schlubach and Wolf [9]: b.p. 59.2—60.1 $n_D^{20} = 1.4492$ for this compound.

As will be seen from the reaction scheme, unexpected rearrangements occur in the two first reactions. These rearrangements follow the same lines as do some of the reactions studied by Schlubach and Wolf [9].



Penten-3-yne-1 may occur in a *cis*- and a *trans* modification. Eglinton and Whiting supposed their preparation to be homogeneous (*trans*), since reaction with *cyclo*hexanone gave a good yield of a crystalline reaction product. Our experiences with their penten-3-yne-1 indicate strongly that it must be a mixture of both stereisomeric modifications. In accordance with our synthesis of lachnophyllum ester, penten-3-yne-1 (V) was first condensed with pent-2-en-4-yn-1-ol (IV). This oxydative coupling gave rise to two isomeric decadienediynols (III). Since pent-2-en-4-yn-1-ol (IV) has been used frequently in synthetic work without reports of resulting stereoisomers, the origin of our

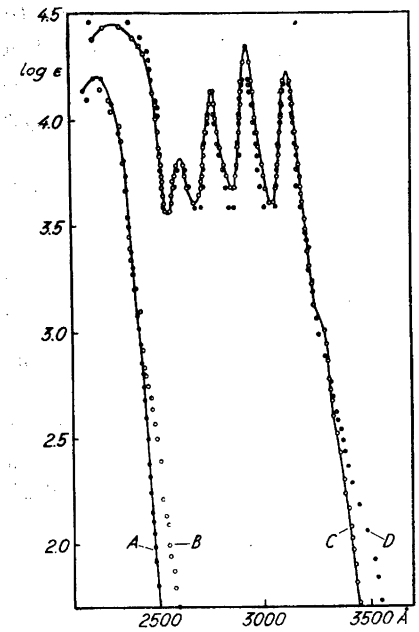


Fig. 1. Ultraviolet absorption in hexane of:
A: —●—●— $CH_3-CH=CH-C\equiv CH$[7]
B: ○○○○○ $CH_3-C\equiv C-CH=CH_2$
C: —○—○— *Matricarianol m. p. 104°*
D: ◑◑◑◑◑ *Matricarianol, liquid*
($\varepsilon = \frac{\log I_0/I}{c \cdot d}$, *c in mol/l, d in cm)*

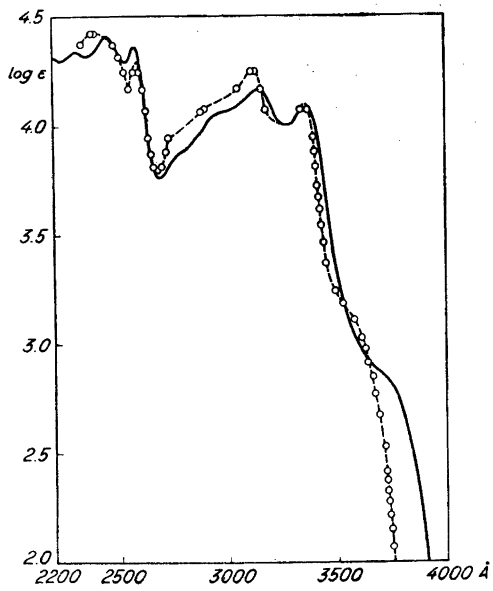


Fig. 2. Ultraviolet absorption in hexane of:
cis, cis-Matricaria ester
——— *R. T. Holman and N. A. Sørensen*
--○---○-- *trans, trans-Matricariaester synthetic, this paper*

isomeric decadienediynols must be sought in the pentenyne moiety of the reaction components. In accordance with this view the other products of the oxidative coupling were the decadiene-2,8-diyne-4,6-diol-1,10 m.p. 153—155° of Heilbron, Jones and Sondheimer[4] and at least two decadiene-2,8-diynes-4,6, one of them being a readily crystallizing compound, m.p. 96.5—97.5°. Of these two decadiene-2,8-diyn-4,6-ols-1 (= "matricarianols") (III) one was a solid, m.p. 104—105°, the other a liquid; their U.V.-spectra, *cf.* Fig. 1, were almost identical. They were characterized through their 3,5-dinitrobenzoates. The solid matricarianol gave a 3,5-dinitrobenzoate of m.p. 126—128°, the corresponding ester from the liquid form one of m.p. 82—84°. Both matricarianols were hydrogenated to *n*-decanol, characterized again as the 3,5-dinitrobenzoate, m.p. and mixed m.p. 54—55°. The difference between the two synthetic matricarianols must therefore be configurational.

Oxydation of the two matricarianols with chromic acid according to Bowden *et.al.*[10] gave, only in the case of the matricarianol of m.p. 104—105°, a decadiene-2,8-diyn-4,6-oic acid or "matricaria acid" (II). The liquid matricarianol suffered oxidative destruction. The acid was isolated as the methyl ester $C_{11}H_{10}O_2$, m.p. 60—61°, that is some 26° higher than the matricaria ester isolated from the essential oil of scentless mayweed. That this synthetic ester, m.p. 60—61°, had the constitution of methyl *n*-decadiene-2,8-diyn-4,6-oate (I) was proved by its U.V.-spectrum *cf.* Fig. 2 which was very similar to that of the natural stereoisomer and further through catalytic hydrogenation to methyl caprate which was saponified to capric acid, converted to the acid chloride and thence to the amide, m.p. 96°, alone and in 1:1 mixture with an authentic specimen.

From the pure methyl ester, m.p. 60—61°, the corresponding acid (II) was prepared by saponification under nitrogen. The free acid crystallized in yellow needles which show no definite m.p. but decomposed at about 173°. Re-esterification with diazo-methane gave back the 61°-matricaria ester.

The stereoisomeric difference between the synthetic 61° and the naturally occurring matricaria ester made it very desirable to realize the synthesis of some of the remaining three stereoisomers of methyl *n*-decadiene-2,8-diyn-4,6-oate. Our failure in the oxidation of the liquid isomer of matricarianol was overcome through the analogous Glaser coupling of penten-3-yne-1 (V) with methyl pent-2-en-4-yn-oate (VI). This ester was synthesized in 1947 by Heilbron *et. al.*[4] from pent-2-en-4-yn-ol-1.

Oxidative coupling of penten-3-yne-1 (prepared according to Eglinton and Whiting) with methyl pent-2-en-4-yn-oate gave rise to a mixture of the 61° matricaria ester mentioned above with a liquid isomer. To obtain a satisfactory enrichment in both stereoisomers the penten-3-yne-1 was fractionated in a spinning band column according to Björkman-Olavi. This column is not constructed for b.ps. below 55—60° and we did not succeed in a complete separation into *cis* and *trans* penten-3-yne-1. Between 46—46.8° we obtained, however, fractions with $n_D^{20} = 1.4230—1.4340$; the higher boiling part 47°—48.9° C had n_D^{20} about 1.4400.

The lowest boiling fractions of penten-3-yne-1 on coupling with methyl pent-2-en-4-yn-oate gave a liquid matricaria ester mixture from which only about 4 % could be separated as the 61°-ester. The residual liquid after distillation in a high vacuum showed $n_D^{20} = 1.6236$ and m.p., 2° C.

The high boiling pentenyne fractions when submitted to the same coupling reaction gave a solid product in good yield, m.p. 60.5° after one crystallisation, no depression with the above mentioned 61°-matricaria ester.

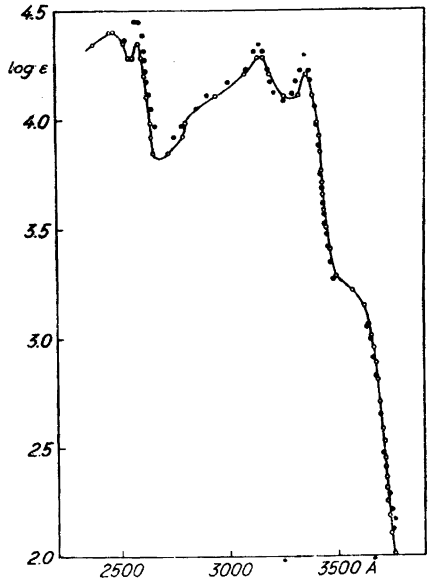


Fig. 3. Ultraviolet absorption in hexane of 2trans, 8cis-matricaria ester
—○—○— *synthetic*
● ● ● ● ● *photoisomer of the naturally occurring cis, cis-isomer (= "trans"-matricaria ester)*

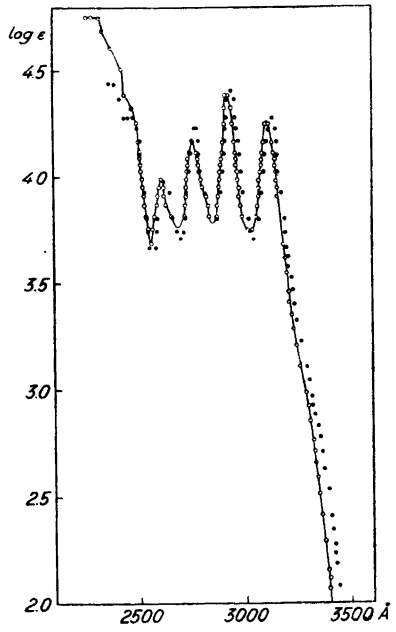


Fig. 4. Ultraviolet absorption in hexane of:
—○—○— *Deca-2,8-dien-4,6-diyn m.p. 97.5*
● ● ● ● ● *Deca-2,8-dien-4,6-diyn-1,10-diol*

Both these synthetic matricaria esters were saponified to furnish the free acids. The 60.5°-ester gave slightly yellow needles which decomposed at about 172°. The 2°-ester afforded colourless leaflets which melted unsharply at 82.5—91°. Obviously, none of the synthetic matricaria acids or esters is identical with the stereoisomer found in nature. From Table 2 it will be seen that the low melting synthetic stereoisomer has physical properties very close to the socalled "*trans*-matricaria ester" prepared by two of us [5] in 1941 through isomerisation of the naturally occurring matricaria ester with U.V.-light. This photoisomer was provisionally given the prefix "*trans*" because its molecular refraction and refractive dispersion were somewhat higher than those for the naturally occurring matricaria ester.

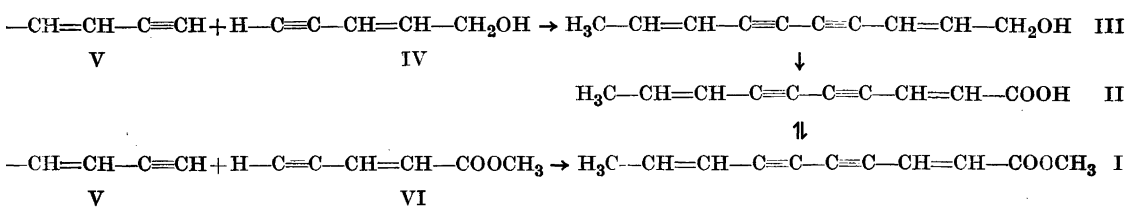
Table 2. Physical constants of the stereoisomeric methyl decadiene-2,8-diyn-4,6-oates ("matricaria esters").

	Synthetic I	Synthetic II	Photoisomer "*trans*"	Naturally occurring
M. p. of methyl ester	61°	+ 2°	− 2 − + 1°	+ 37°
n_D^{20} » » »	—	1.6236	1.6247	—
M. p. » free acid	Decomp. 173°	82.5 − 91°	83 − 91°	98 − 99°

The U.V. spectrum of the synthetic isomer II (methyl ester) is, within the limits of experimental error, identical with that of the "*trans*"-matricaria ester, *cf.* Fig. 3. The U.V. spectra of the stereoisomers differ very little, as will be seen from a comparison of Fig. 2 and Fig. 3. The reason for this similarity may be the rod-shaped diacetylene grouping which separates the end groups sufficiently to allow a planar conformation to be adopted by all stereoisomers.

To strenghten the identity of the liquid synthetic isomer (II) with the "*trans*" matricaria ester, this photoisomer was prepared once more and saponified to give the free acid, the m.p. of which was found to be 84.5 − 91.5° in agreement with the original statement. The mixed m.p. with the synthetic acid was 83.5 − 91° C. Chem.eng. H. Sörum took Debye-Scherrer diagrams of both acids and thus definitely established their identity. We are greatly indebted to Mr. Sörum for his valuable assistance.

The synthetic work described above-summarized in the reaction scheme — confirms beyond doubt the constitution I given to matricaria ester in 1941 on the basis of degradation with alkali.



At the same time these syntheses render valuable hints as to the stereochemistry of the matricaria esters. With the starting materials used here in the oxydative coupling the synthetic isomers must be the 2-*trans*-8-*trans* and the 2-*trans*, 8-*cis* isomers. In all probability the high melting decadienediynol and the corresponding high melting matricaria acid and its methyl ester are the 2-*trans*, 8-*trans* modifications. The low melting synthetic series where the

methyl ester is identical with the photoisomer "*trans*" matricaria ester thus must be the 2-*trans*-8-*cis* isomers. The crystalline isomer found in the essential oil of scentless mayweed then must be the 2-*cis*-8-*cis* isomer. Only with this configuration is the photoisomerisation a simple process, involving a change from *cis* to *trans* at the 2.3-double bond. Were the matricaria ester the fourth isomer, 2-*cis*, 8-*trans*, the photoisomerisation would have to invert the 2.3-double bond to *trans* and the 8.9-double bond to *cis*, which is rather unlikely.

The 2-*cis*, 8-*cis* configuration for the naturally occurring solid matricaria ester implies interesting stereochemical relations for some of the other highly unsaturated derivatives of methyl caprate. The main component of the essential oil from the root of European mugwort (*Artemisia vulgaris* L.) is a dehydro-matricaria ester [11]. The position of the third acetylenic bond and the configuration at the double bond are so far unknown, but in all probability the matricaria ester m.p. 37°, which is widely distributed amongst some subgroups of the plant family of the Compositae is its dihydro-derivative.

Formally the hydrogenation of the 8.9-double bond in the 2-*cis*-8-*cis* matricaria ester gives the lachnophyllum ester [2] for which the Russian discoverers established the *cis*-configuration of the 2.3-double bond. The lachnophyllum ester has recently [12] been shown to occur in some fleabane oils (*Erigeron acre* L. and *E. uniflorum* L.) Its occurrence in some further composite oils will be described in a forthcoming paper. Some other, botanically closely related, fleabanes contain exclusively the *cis*, *cis*-matricaria ester.

It is worthy of note that three isobutylamides of different unsaturated capric acids have so far been found in nature, *viz.* affinin = N-isobutyl-deca-trien-2,6,8-amide [13], spilanthol = N-isobutyl-decadien-4,6-amide [14] and pellitorine = N-isobutyl-decadien-2,6-amide [15]. The configuration at the double bonds in these interesting isobutyl-amides are not exactly known. In all of them there must occur at least one *cis*-bond, since the synthetic all *trans* isomers are different from the naturally occurring compounds. These three isobutylamides of unsaturated normal C_{10}-acids have been isolated from species belonging to the plant family of the Compositae, which so far has furnished all the known acetylenic C_{10}-acids (as methyl esters).

EXPERIMENTAL

Penten-1-yne-3 according to Henne and Greenlee [6]

In a series of experiments carried out with only small variations of the instructions given in the original paper [6], only a very few experiments gave a small fore-run of pentenyne. In the best experiment (on a four mole scale) the crude product gave 28 g boiling below 100° at ordinary pressure. This fraction was dried and distilled again,

affording 8 g boiling below 90°. Slow refractionation under nitrogen gave 4 g b. p. 57—61°, n_D^{20} = 1.4530. Fractions corresponding to this one from two experiments were combined and refractionated slowly, giving I 2.2 g, b. p. 57.5—58.5 and II, 3 g b. p. 58.5—60°. U. V. absorption in hexane, *cf.* Fig. 1 curve B, I, λ_{max} = 2 245, ε = 14 100 II, λ_{max} = 2 230, ε = 12 500.

Table 3. Dispersion of penten-1-yne-3 prepared according to Henne and Greenlee.

I	d_4^{20} = 0.7378	λ_0 = 1 228 Å	$R_{\lambda=\infty}$ = 23.02	$R_{D\ theory}$ = 22.82
II	» = 0.7388	» = 1 225 »	» = 23.00	» = 22.82

	I			II		
λ	n_λ^{20}	$R_{\lambda,\ obs.}$	$R_{\lambda,\ calc.}$	n_λ^{20}	$R_{\lambda,\ obs.}$	$R_{\lambda,\ calc.}$
6678.1	1.44502	23.83	23.83	1.44526	23.81	23.80
5895.9	1.45001	24.06	24.06	1.45024	24.04	24.04
5875.7	1.45022	24.07	24.07	1.45046	24.05	24.05
5790.7	1.45090	24.10	24.10	1.45109	24.08	24.08
5460.7	1.45386	24.24	24.25	1.45404	24.21	24.22
5015.6	1.45924	24.48	24.49	1.45945	24.46	24.46
4471.5	1.46835	24.90	24.89	1.46848	24.87	24.87
4358.3	1.47082	25.01	25.01	1.47085	24.98	24.97

Penten-1-yne-3 from ethylidenacetone. The ethylidenacetone was prepared according to [16].

2,2-Dichloropentene-3. To 250 g PCl_5 suspended in 150 ml carbon disulfide and cooled with ice, 90.7 g ethylidenacetone was added slowly. After half an hour the temperature was raised slowly to 55—58° C and maintained there for 40 minutes. The reaction mixture was then cooled to 5° C and poured on 100 g ice. The carbon disulphide phase was washed successively with water, twice with dilute sodium bicarbonate solution, once again with water and then dried over $CaCl_2$. The carbon disulphide was distilled off at ordinary pressure, and then the reaction mixture was distilled at reduced pressure (110 mm). Between 65—80° there passed fractions with a chlorine content of 38—44 %; between 80—90° fractions with the correct composition $Cl_{calc.}$ = 51.0 %, Cl_{found} = 51.3 %, n_D^{20} = 1.4667. The dichloride readily looses hydrogen chloride. For conversion into *2-chloro-pentadiene-1,3* it was found to be sufficient to distill the crude reaction solution with steam. The pale yellow carbondisulphide phase was removed from this distillate, dried over $CaCl_2$ and distilled. The main fraction passed over at 130° 760 mm, n_D^{20} = 1.4558, $Cl_{calc.}$ = 34.57 %, $Cl_{found.}$ = 35.2 %.

Many attempts were made to remove all the chlorine from 2,2-dichloro-pentene-3 and 2-chloropentadiene-1,3. Sodium or sodium amide in liquid ammonia or in liquid paraffin, with temperatures from ÷ 40—160°, gave only traces of hydrocarbon. Similar results were obtained with most of the experiments with potassium hydroxide. The following procedure was more successful. 35 g potassium hydroxide were dissolved in

180 g diethyleneglycol monomethyl ether ("Carbitol") and the solution heated to 150—155° in a stream of pure nitrogen. 51.5 g 2-chloropentadiene1.3 was then added through a capillary terminating below the "carbitol" surface. The condensate (17 g) was distilled in a stream of nitrogen, crude fraction was collected below 86° (8.4 g). Refractionation gave 4.1 g (12 % of theory) b-range 57—60°, $n_D^{20} = 1.4480$.

Penten-3-yne-1 (V) prepared according to Eglinton and Whiting

The procedure kindly placed at our disposal by professor E. R. H. Jones was practically identical with that given in [7]. The yields of pentyn-4-ol-2-tosylate and pent-3-en-1-yne were excellent with very good reproducibility. U. V. absorption, *cf.* Fig. 1, curve A, $\lambda_{max} = 2\,240$, $\varepsilon_{max} = 16\,200$.

Table 4. Dispersion of penten-3-yne-1.

$d_4^{20} = 0.7288$ $\lambda_0 = 1\,208$ Å $R_{\lambda=\infty} = 22.67$

λ	n_λ^{20}	$R_{\lambda,\,obs.}$	$R_{\lambda,\,calc.}$
6678.1	1.43056	23.44	23.44
5895.9	1.43532	23.66	23.66
5875.7	1.43544	23.67	23.67
5790.7	1.43610	23.70	23.70
5460.7	1.43883	23.83	23.84
5015.6	1.44381	24.06	24.07
4471.5	1.45217	24.46	24.46
4358.3	1.45451	24.57	24.56

Decadiene-2,8-diyn-4,6-ol-1 (III)

Penten-3-yn-1-ol-5 was prepared according to [17] and coupled with penten-3-yne-1 according to [1] *. The mixture of condensation products was taken up in ether and further treated thus: After evaporation of the ether the residue was partially distilled at 0.001 mm and 40° in the air bath. The hydrocarbons produced (the decadienediynes) then distilled into a trap cooled to −80°. To the solid left was added 5 ml chloroform; the suspension was heated in a water bath to 60—70°, and then filtered rapidly with suction. The decadiene-2,8-diyne-4,6-diol-1,10 [4], which invariably was a by-product, was left mainly undissolved. The filtrate was evaporated to dryness in a vacuum. The residue was heated with portions of petroleum (b.r. 60—80°) and filtered. On cooling mainly the solid form of the matricarianol was precipitated, whilst the liquid one mainly remained in solution. After evaporation of the petroleum the carbinol was distilled at 70° (bath)/0.001 mm. The best yields, obtained from 5.0 g penten-3-yne-1 (V) and 6.2 g penten-3-yn-1-ol-5 (IV), were: 1.2 g hydrocarbons, 1.5 g crude, solid matricarianol, 0.7 g of distilled liquid matricarianol and 4.2 g decadiene-2,8-diyne-4,6-diol-1,10.

* Owing to a clerical error the amount of cuprous chloride was not given. It should be 72.5 g.

Matricarianol m.p. 104—105° (= *trans, trans* decadiene-2,8-diyn-4,6-ol-1.)

Crystallisation of the crude, solid carbinol (above) from petroleum (b.r. 60—80°) gave a slightly yellow substance, crystallizing in plates, m.p. 104—105° U.V.-absorption in petroleum ether, *cf.* Fig. 1.

ε_{max}	16 000	21 000	14 000	6 500	27 500	
λ_{max}	3 122	2 930	2 765	2 615	2 300	Å
$\nu_{max.10^{-12}}$	960.9	1 023.9	1 085.0	1 147.2	1 304.4	cm^{-1}
$\Delta\nu$ »	63.0	61.1	62.2			cm^{-1}

3,5-Dinitrobenzoate of matricarianol, m.p. 104—105°

To a solution of 96 mg of the matricarianol m.p. 104—105° in dry pyridine was added a pyridine solution of the calculated amount (150 mg) of 3,5-dinitrobenzoyl chloride. The reaction mixture was left under nitrogen for 24 hours and worked up in the usual way. Crystallisation from petroleum afforded faintly yellow plates, m.p. 126—128°. U.V.-absorption in ethanol:

ε_{max}	17 500	20 500	16 500
λ_{max}	3 146	2 945	2 755

$C_{17}H_{12}N_2O_6$ (340.3) Calc. C 60.0 H 3.55
Found » 59.7 » 3.80

n-Decanol from matricarianol m.p. 104—105°

73.3 mg matricarianol was hydrogenated with 150 mg palladium on $BaSO_4$ catalyst (2 % Pd). Hydrogen consumption (760 mm, 0°) calc. 67.5 ml, found 63.4 ml, which corresponds to $5.64\sqrt{=}$. The liquid hydrogenation product was esterified with 3,5-dinitrobenzoyl chloride in pyridine. Crystallization from petroleum (b.r. 60—80°) gave *n*-decanol 3,5-dinitrobenzoate, m.p. 54—55°, undepressed in m.p. on admixture with an authentic specimen (m.p. 56—57°)[18].

Matricarianol, liquid isomer (2-trans, 8-cis-Decadiene-2,8-diyn-4,6-ol-1)

The fraction which distilled at 70° (bath)/0.001 mm (above) showed U.V.-absorption in petroleum ether very similar to that of the matricarianol of m.p. 104—105°.

ε_{max}	16 000	22 000	13 000	6 500	28 000	
λ_{max}	3 132	2 938	2 755	2 612	2 320	Å
$\nu_{max\ 10^{-12}}$	957.9	1 021.1	1 098.9	1 148.5	1 293.1	cm^{-1}
$\Delta\nu$ »	63.2	67.8	59.6			cm^{-1}

3,5-Dinitrobenzoate of the liquid matricarianol

202 mg of the liquid matricarianol was esterified as above with 320 mg 3,5-dinitrobenzoyl chloride. The crude ester was an oil, but a petroleum solution deposited yellow plates, m.p. about 80°, which after one more crystallization from petroleum (b.r. 60—80°) melted constantly at 83—84.5°.

U.V.-absorption in alcohol:

ε_{max}	17 500	20 000	17 000
λ_{max}	3 140	2 945	2 766

$C_{17}H_{12}N_2O_6$ (340.3)	Calc.	C 60.0	H 3.55
	Found	» 59.8	» 3.40

n-Decanol from the liquid matricarianol

70.2 mg was hydrogenated with $Pd/BaSO_4$ as above. Hydrogen consumption: calc. 64.7 ml (0°, 760 mm), found 61.2 ml corresponding to $5.67/\overline{=}$. The liquid hydrogenation product was esterified with 3,5-dinitrobenzoyl chloride as above. M. p. of the 3,5-dinitrobenzoate 54—55°, mixed m. p. with authentic ester 54—57°.

Decadiene-2,8-diynes-4,6. In the trap two different substances were distinctly visible. The one precipitated on the upper parts of the wall was solid at room temperature, whilst the other, precipitated on the lower parts liquified at room temperature.

Decadiene-2,8-diyne-4,6, solid form. The precipitated solid was dissolved in ether in which it was relatively sparingly soluble. Recrystallization from this solvent produced colourless, flexible blades, m. p. 97.5°.

$C_{10}H_{10}$ (130.2)	Calc.	C 92.3	H 7.75
	Found	» 92.4	» 7.85

U. V.-absorption in petroleum ether, *cf.* Fig. 4.

ε_{max}	18 200	25 100	15 000	9 700	
λ_{max}	3 100	2 915	2 755	2 605	Å
$\nu_{max}\ 10^{-12}$	967.7	1029.2	1088.9	1151.6	cm^{-1}
$\Delta\nu_{max}$ »	59.5	59.7	62.7		»

Hydrogenation. 30.2 mg decadienediyne was hydrogenated with $Pd/BaSO_4 \cdot C_{10}H_{10}$ $2/\overline{=}$, $2/\equiv$ calc. 31.2 ml (0°, 760 mm), found 30.4 ml corresponding to $5.85/\overline{=}$.

Decadiene-2,8-diyne-4,6 (liquid form)

The liquid in the trap was redistilled slowly at 0.1 mm and 40° C.

U. V.-absorption in petroleum ether:

ε_{max}	20 500	23 500	16 000	7 000	31 500	
λ_{max}	3 120	2 930	2 758	2 618	2 320	Å
$\nu_{max}\ 10^{-12}$	961.5	1023.9	1087.7	1145.9	1293.1	cm^{-1}
$\Delta\nu_{max}$ »	62.4	63.8	58.2			»

Table 5. Dispersion of liquid decadiene-2,8-diyne-4,6.

$d_4^{20} = 0.8696$	$\lambda_0 = 1565$ Å		$R_{\lambda=\infty} = 47.95$
λ	n_λ^{20}	R_λ obs.	R_λ calc.
6678.1	1.59392	50.76	50.74
5895.9	1.60585	51.59	51.59
5875.7	1.60631	51.62	51.62
5790.7	1.60763	51.71	51.73
5460.7	1.61564	52.26	52.25
5015.6	1.62926	53.18	53.13
4046.6	1.67675	56.33	56.39

The U. V.-absorption, the refraction and the dispersion of decadienediyne are in good agreement with those for its 3,7-dimethyl homologue [19]. Exaltation for the sodium line for $C_{10}H_{10}$ = 8.2, for $C_{12}H_{14}$ = 7.5.

Matricaria ester, m. p. 61°. (= Methyl decadiene-2 *trans*-8 *trans*-diyn-4,6-oate) from the matricarianol of m. p. 104—105°.

The oxidation of matricarianol with CrO_3 to the corresponding acid and conversion to the methyl ester was carried out in the same way as the conversion of lachnophyllol to lachnophyllum acid methyl ester [1]. The matricarianol m. p. 104—105° was recrystallized immediately before the oxidation was carried out. From 3.2 g of crystallized carbinol there was obtained from the acid fraction 420 mg of methyl ester b. p. up to 66° (bath)/ 0.001 mm, which, after crystallisation from petroleum ether, gave 350 mg of faintly yellow crystalls, m. p. 60—61°.

U. V.-absorption in petroleum ether (cf. Fig. 2).

ε_{max}	18 000	19 000	14 000	21 000	25 000	
λ_{max}	3 345	3 145	2 950 *	2 570	2 450	Å

Methyl caprate from synthetic matricaria ester, m. p. 61°

83.2 mg of the synthetic matricaria ester m. p. 61° was hydrogenated with $Pd/BaSO_4$ catalyst. $C_{11}H_{10}O_2$ $2/\overline{=}$, $2/\overline{=}$ calc. 64.4, found 61.5 ml hydrogen. The liquid perhydro compound had the characteristic strawberry odour of methyl caprate. It was saponified to capric acid, which was converted to its acid chloride and then to capric amide, m. p. 96°, m. p. on admixture with an authentic specimen m. p. 96°.

Matricaria acid, m. p. 173° = decadiene-2 *trans*, 8 *trans*-diyn-4,6-oic-1 acid

To 51 mg of the synthetic matricaria ester m. p. 61° dissolved in 4 ml methanol was added 96 mg of potassium hydroxide in 4.5 ml water. The solution, which was prepared under pure nitrogen, was shaken under pure nitrogen in a bath of ice-water for 5 hours

* Inflexion.

until all the ester had dissolved. The soap solution was diluted with water and extracted with ether. The alkaline solution was then acidified with very dilute, cold sulphuric acid and the liberated acid extracted with ether. The ether solution was dried and the ether evaporated. The matricaria acid was crystallized from a mixture of very little acetone with much petroleum ether as yellow needles decomposing at 169—173°.

U. V.-absorption: (the acid was dissolved in a few drops of chloroform and quickly diluted with petroleum ether).

ε_{max}	13 000	16 500	21 000	29 500	
λ_{max}	3 310	3 090	2 585	2 435	Å

Re-esterification of the acid with diazomethane produced the original ester, m. p. 59°, no depression on admixture with the starting material.

Methyl decadiene-2,8-diyn-4,6-oate-1 from methyl penten-2-yn-4-oate-1

Methyl penten-2-yn-4-oate-1 was prepared according to [4], m. p. 18—19° C.

U. V.-absorption in hexane

λ_{max} = 2 400 Å with inflexion at 2 510 Å
ε_{max} = 30 000 ε = 24 000

Heilbron *et. al.*[4] give for alcoholic solutions

λ_{max} = 2 440 Å with inflexion at 2 510 Å
ε_{max} = 18 000 ε = 15 500

Penten-3-yne-1 prepared according to [7] was refractionated with a spinning band column (Björkman-Olavi). The oxidative coupling was carried out as in [1] with omission of free ammonia to avoid saponification.

I. 7.3 g methyl penten-2-yn-4-oate-1 was coupled with 6 ml penten-3-yne-1 b. p. 46.1—46.8°. The reaction mixture was worked up as above; distillation at 0.001 mm gave 522 mg condensate boiling at < 75°. Of this crude distillate 20 mg could be separated as crystalls, solid at room temperature. The rest after redistillation showed m. p. + 2° n_D^{20} = 1.6236.

$C_{11}H_{10}O_2$ (174.2)	Calc.	C	75.84	H	5.79
	Found	»	75.16	»	5.62

U. V.-absorption in hexane; *cf.* Fig. 3.

ε_{max}	1 600	15 800	19 500	11 200	22 400	25 100	
λ_{max}	3 580 *	3 355	3 140	2 850 *	2 582	2 460	Å

* Inflexion.

Hydrogenation: 36.0 mg matricaria ester, m. p. + 2°, was hydrogenated with 2 % $Pd/BaSO_4$. Calc. H_2 = 27.9 ml, found 27.0 ml (0°, 760 mm) corresponding to 5.81/=. The methyl caprate generated was isolated and the saponification value determined:

$$M_{calc.} = 186.0 \qquad M_{found} = 188.5$$

Free matricaria acid from methyl decadiene-2,8-diyn-4,6-oate-1 m. p. + 2° C

The saponification was carried out under pure nitrogen. The only difference to the procedure given above was the application of acetone-water as a solvent. The free acid was recrystallized from ether-petroleum ether, as leaflets with unsharp m. p. The melting starts at about 82.5° and is completed at 91°. This melting interval, previously described for "*trans*"-matricaria acid by Sörensen and Stene [5], has in the meantime been confirmed by J. Stene in connection with unpublished work (rearrangement at 47—48° when the acid is heated, then the substance remains quite solid until melting starts at 83°, completed at 89.5°) and was confirmed again with "*trans*"-matricaria acid prepared once according to the original procedure: melting starts at 84.5, completed at 91.5°. A 1 : 1 mixture of this synthetic matricaria acid with "*trans*"-matricaria acid starts melting at 83.5, completed at 91°.

Since it is obvious that some phase transformation takes place when this stereoisomer of matricaria acid is heated, Debye-Scherrer diagrams were taken. These definitely established the identity of the synthetic acid with the photoisomer of the naturally occurring acid.

II. Methyl penten-2-yn-4-oate-1 was coupled as above with 2 ml penten-3-yne-1, b. p. 47—48.9°. Yield of crude distillate boiling at < 75° at 0.001 mm was 840 mg ester. After one crystallization from petroleum ether slightly yellowish needles were obtained m. p. 60.5°.

$C_{11}H_{10}O_2$ (174.2)	Calc.	C	75.84	H	5.79
	Found	»	75.56	»	5.67

U. V.-absorption in hexane, *cf.* Fig. 2.

ε_{max}	1 260*	12 600	17 800	12 600*	19 500	26 900	
λ_{max}	3 600*	3 355	3 125	2 900*	2 585	2 390	Å

Hydrogenation: 10.1 mg ester, m. p. 60.5°, was hydrogenated with 2 % $Pd/BaSO_4$. Calc. 7.8 ml H_2, Found 8.1 ml (0°, 760 mm), corresponding to 6.2/=.

Free matricaria acid from methyl decadiene-2,8-diyn-4,6-oate-1 m. p. 60.5°

The saponification was carried out as above for the liquid ester. The crude acid showed decomposition from 167° C, after recrystallization from ether-petroleum-ether the decomposition starts at 172°. The acid does not melt, but decomposition takes place giving dark solids before melting is completed.

* Inflexion.

SUMMARY

Two stereoisomers of decadiene-2,8-diyn-4,6-ol-1 and the corresponding acids and their methyl esters have been synthesized through oxydative coupling of acetylenic precursors according to the method of Glaser. Depending on the route of synthesis the two series must represent the 2-*trans*, 8-*trans* and the 2-*trans*, 8-*cis* isomers. The methyl decadiene-2*trans*, 8-*cis*-diyn-4,6-oate-1 was shown to be identical with the photoisomeric „*trans*"-matricaria ester, prepared in 1941 through the action of U.V.-light on the naturally occurring matricaria ester. The configuration of the latter must be 2-*cis*, 8-*cis*.

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A New Type of Ion-exchange Column for the Separation of Radioactive Bromine or Chlorine

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The radioactive isotopes of chlorine and bromine — ^{36}Cl and ^{82}Br — usually are prepared through (n, γ)-reactions in the uranium reactor. In order to increase the specific activity the use of a Szilard-Chalmers reaction is desirable. As the target material for the irradiation in such a case is a halate and the radioactive isotope appears as halide, the chemical problem associated with the Szilard-Chalmers reaction is the separation of halide from halate. This problem has been treated before by the present author [1] but the methods used cannot be applied to preparations, the radioactivity of which is so high, as to make necessary remote control technique.

The purpose of this investigation is to develop a suitable method which may be used for the separation of highly radioactive bromide or chloride, from bromate respectively chlorate. The best method is now considered to consist in the use of an ion-exchange column, since such an apparatus may be easily run by remote control.

THE CHOICE OF ION-EXCHANGER

The separation of halide and halate must take place in neutral or alkaline solution since an isotopic exchange takes place in acid solution. The only existing ion-exchange resin which may well be used for such solutions is Amberlite IRA-400. Some preliminary experiments showed, however, that it was very difficult to get this product absolutely free from chloride. Thus a small column was treated for several days with 20 % sodium hydroxide with a flow rate of about 0.3 mls per minute per cm^2 without getting the eluate

* Operated under the auspices of the Swedish Atomic Commission.

free from chloride. Since it is essential to produce a pure product, the possibility of the preparation of a new type of ion-exchanger was considered.

The following considerations led to the development of the material. If a silver compound, the solubility of which is larger than that of silverbromide, is treated with a solution containing bromide, part of the anion in the compound is replaced by bromide:

$$AgY + Br^- \rightleftharpoons AgBr + Y^- \tag{1}$$

where Y is some anion. The equilibrium between the solution and the solid phase is determined by

$$\frac{\{Y^-\} \cdot \{AgBr\}}{\{AgY\} \cdot \{Br^-\}} = K \tag{2}$$

where $\{\ \}$ denotes activities. Furthermore it is true that

$$\frac{\{Ag^+\} \cdot Br^-\}}{\{AgBr\}} = K_1 \tag{3}$$

and

$$\frac{\{Ag^+\} \cdot \{Y^-\}}{\{AgY\}} = K_2 \tag{4}$$

If AgBr and AgY form ideal solid solutions, K_1 and K_2 equals the solubility products L_{AgBr} and L_{AgY}. Hence

$$K = \frac{L_{AgY}}{L_{AgBr}} \tag{5}$$

If AgY and AgBr do not form solid solutions, AgY is completely converted into AgBr if Br^- is present in excess, and the solubility product of AgY is larger than that of AgBr. Accordingly an adsorption column containing a suitable silver compound could be used to remove bromide from a solution. It is desirable to use a compound which does not form solid solutions with silverbromide. If the solution contains bromate the solubility product of the compound used must be smaller than that of silverbromate. The best silver compound was considered to be silver oxide. In Table 1 is listed the concentration of silverion in saturated solutions of some silver compounds [2]. It is seen from the table that a column containing silver oxide will absorbe chloride and bromide but not bromate. Furthermore it is improbable that silver oxide will form mixed crystals with either of the other compounds.

Table 1. Silverion concentration in saturated solutions of different silver compounds.

Compound	$[Ag^+]$
$AgBrO_3$	$6.7 \cdot 10^{-3}$
Ag_2O	$1.85 \cdot 10^{-4}$
AgCl	$1.05 \cdot 10^{-5}$
AgBr	$4.7 \cdot 10^{-7}$

If silver oxide is present as large aggregates the establishment of equilibrium between the solution and the solid phase must be expected to be a very slow process. Probably only the surface will react at once and after absorption of bromide during a longer time when perhaps deeper layers are converted to bromide it would be difficult to desorbe the bromide. It should thus be convenient to have the silver oxide present in very thin layers adsorbed on some inert material having a large surface area. Diatomaceous earth was believed to be a suitable adsorbens for the silver oxide.

Accordingly silver oxide adsorbed as a thin layer on diatomaceous earth constitutes an ion-exchanger capable of exchanging chloride or bromide against hydroxyl ions. Once the chloride or bromide is adsorbed on a column containing the ion-exchanger and thus separated from the bulk of the solution it may be eluted with a solution of sodium iodide.

EXPERIMENTAL

Preparation of ion-exchanger: 5 ml of 0.1 *M* silver nitrate solution were added to different amounts of diatomaceous earth suspended in water. Then a slight excess of 0.1 *M* NaOH was slowly added during vigorous stirring. It was found that 5 g of diatomaceous earth had to be used to completely adsorbe the silver oxide formed. If smaller amounts were used two solid phases appeared, one consisting of pure silver oxide not adsorbed. If, however, 5 g or more per 5 ml of 0.1 *M* silver nitrate were used, a homogeneous, fine, brownish suspension was obtained, apparently consisting of silver oxide adsorbed as a very thin layer on the diatomaceous earth. The substance was washed by decanting with water containing a little sodium hydroxide.

Separation experiments: A column shown in Fig. 1 was used. The ion-exchanger was allowed to settle in the column filled with water. The solution to be treated was allowed to flow with a rate of 0.2—0.3 ml per minute.

Experiment 1: 5 ml of a 0.02 *M* solution of sodium bromide was poured into the column. It was found that a very sharp band of silver bromide about 5 cm long was formed. Then a 0.02 *M* solution of sodium iodide was poured into the column. It was found that the bromide band migrated down the column followed by an iodide band. The boundary between bromide and iodide was very sharp. However after some ml of sodium iodide

solution had flowed, part of the silver iodide in the top of the column was colloidally dissolved and appeared in the eluate.

Experiment 2: The peptization of silver iodide ought to be prevented if the solution contains a positive ion of higher valence. Hence in this experiment the bromide and iodide solutions used were made 0.01 *M* in Ba^{2+}. It was found that in this case it was possible completely to elute the bromide with sodium iodide without the appearance of any colloidal silver iodide. As soon as the bromide band had reached the bottom of the column samples of 1—5 ml volume were taken from the eluate and bromide and iodide determined by potentiometric titration with silver nitrate. The results are shown in Fig. 2.

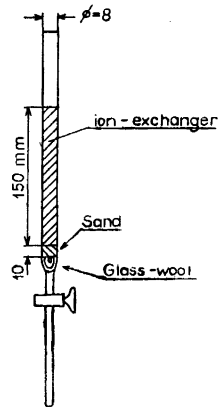

Fig. 1. Ion-exchange column.

Experiment 3: 5 ml of a solution containing 50 g of potassium bromate per litre and being 0.02 *M* in sodium bromide were allowed to flow through the column. The bromide was eluted with a solution 0.01 *M* in sodium iodide and 0.01 *M* in Ba^{2+}. In the eluate bromate was determined by reduction to Br_2 with Br^- followed by colometric determination of Br_2. Bromide and iodide were determined by potentiometric titration with silver nitrate. The results are shown in Fig. 3.

Experiment 4: The bromide preparations obtained from the column contain Ba^{2+} and Ag^+. In order to get a pure solution a 4 cm layer of a cation exchanger — Amberlite IR-120 —in hydrogen form was placed under the anion exchanger in the column. This experiment was performed in order to study the boundary between iodide and bromide in this case. In order to simplify the analytical determinations radioactive bromine — ^{82}Br- and iodine — ^{131}J — were used. The experiment was performed according to experiment 3, the only difference being that bromide and iodine were determined by radioactivity measurements and that 9 ml of bromide solutions were used. Thus samples from the eluate were prepared by neutralizing with sodium hydroxide followed by evaporation of 0.05 ml on glass disks. The radioactivity of the samples was measured with a scale-of-64 amplifier using an end-window counter. Two measurements were carried out, one of which was performed 14 days after the end of the experiment when ^{82}Br had decayed completely. From the activity at the last measurement the iodine concentration could be

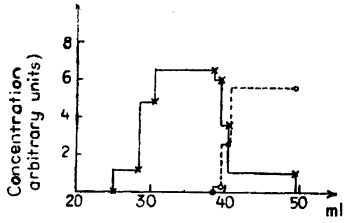

Fig. 2. Experiment 2. Elution curves.
× *bromide* ○ *iodide*

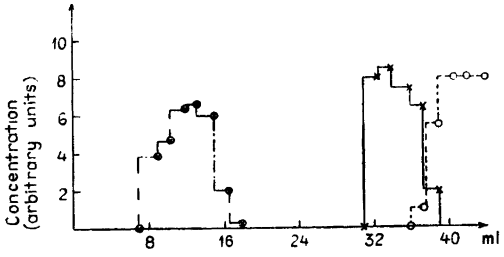

Fig. 3. Experiment 3. Elution curves.
⊕ *bromate* × *bromide* ○ *iodide*

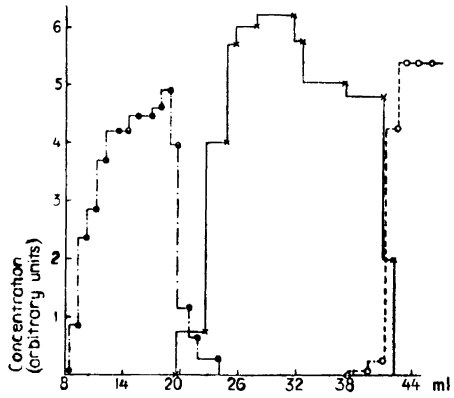

Fig. 4. Experiment 5. Elution curves.
⊕ *bromate* × *bromide* ○ *iodide*

determined and from the first measurement the sum of iodide and bromide concentrations could be obtained. The results are shown in Fig. 4.

Experiment 5: This experiment was performed in order to make sure that separation of perchlorate and chloride could be carried out with the ion-exchanger used. 5 ml of a solution 0.1 *M* in sodium perchlorate and 0.02 *M* in sodium chloride were used. The elution was carried out with a solution 0.02 *M* in sodium iodide. Both of the solutions were 0.01 *M* in Ba^{2+}. It was found that the column behaved exactly as in the cases with bromide and bromate.

DISCUSSION

The experiments have shown that the ion-exchanger prepared may be successfully used to separate small amounts of bromide or chloride from alkaline solutions containing ions which prevent the formation of colloidal silver halide solutions. From the elution curves shown it is seen that the boundary between the bromide and the iodide is not absolutely sharp. The yield of bromide free from iodide was about 90 %. This surely is due to the fact that the solution has to move a rather long distance after the ion-exchanger. For preparative purposes it is desirable to increase the yield. This ought to be possible through a better shaping of the column. A following paper on the preparation of ^{82}Br will deal more closely with this problem.

The capacity of the ion-exchanger has been found to be $4 \cdot 10^{-5}$ equivalents per cm^3 bed wolume. Since the calculated capacity of dry substance is 10^{-4} equivalents per gram the liquid content of the column is approximately 60 vol-% if the density is set equal to one. Some simple batch experiments have shown that the establishment of equilibrium between solution and ion-exchanger is practically instantaneous. Hence the column may be easily calculated for the solution to be separated. In order to get only a small part of the bromide in

the iodide containing fraction of the eluate, the diameter of the column has to be such that the bromide band will be at least 5 cms long. As mentioned above in this case 90 % of the bromide may be obtained free from iodide.

For the calculation of the column the following notations are used:

Volume of solution = V cm^3
Capacity of ion-exchanger = C mequiv./cm^3
Volume fraction of liquid in the column = α
Area of the column = A cm^2
Depth of ion-exchanger = l cm
The area of the column is obtained from the equation:

$$V \cdot [Br^-] \geqq 5A \ (c + \alpha[I^-]) \tag{6}$$

The depth of the ion-exchanger is determined by the length of the bromide band and the iodide concentration of the solution used for the elution. The rest of the original solution which is in the column when the addition of iodide solution is started must be displaced before the bromide band reaches the lower end of the exchanger bed. This gives the equation:

$$\frac{V \cdot [Br^-]}{A(c + \alpha[I^-])} + \frac{\alpha \cdot A \cdot l \cdot [I^-]}{A(c + \alpha[I^-])} = l \tag{7}$$

Hence

$$l = \frac{V \cdot [Br^-]}{C \cdot A} \tag{8}$$

As the bromate concentration owing to diffusion in the solution does not fall sharply, it is advisable to make the depth about double the value obtained from equation (8).

Using the above mentioned values for α and C, the values for A and l may be calculated from (6) and (8).

SUMMARY

An anion-exchanger consisting of silveroxide adsorbed on diatomaceous earth has been prepared.

It has been demonstrated that this substance may be used to separate small amounts of bromide or chloride for preparative purposes.

Equations for the calculation of the adsorption column have been derived.

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Separation of Bile Acids with Reversed Phase Partition Chromatography

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In connection with an investigation of the intermediate metabolism of bile acids a method was needed to separate the bile acids quantitatively on a small scale. In earlier work chromatography of different types of esters of bile acids on alumina have been used [1–4].

The excellent method of Howard and Martin [5] for the separation of higher fatty acids by "reversed-phase partition chromatography" prompted us to try to find a solvent system suitable for the separation of the bile acids with this technic. The hydroxylated bile acids are too insoluble in the stationary phases that gave the best results with the higher fatty acids.

The best results have so far been obtained with chloroform containing ten per cent heptane as the stationary phase and aqueous methanol as the descending phase. The addition of heptane increased the stability of the columns, which can be used for several consecutive runs.

The columns contained 4.5 g hydrophobic Hyflo-Supercel to which 4 ml of the stationary phase was added. The material was then slurried in the aqueous phase giving a column of the approximate dimensions 12 × 110 mm.

A typical separation of a mixture of cholic, deoxycholic and lithocholic acid in 58 per cent methanol is shown in figure 1. The acids appear in the effluent in the order mentioned and at the same place as when each acid was run alone.

Decreasing the methanol concentration from 58 to 55 per cent with an unchanged stationary phase delayed the appearance of the band of deoxycholic acid from 38 ml to approximately 50 ml (*cf.* Figs. 1 and 2).

Increasing the proportion of heptane in the stationary phase or increasing the strength of the aqueous methanol resulted in an earlier appearance of the acids.

The effect of varying the amounts of bile acids used on the same column is exemplified in Figure 2. The cholic acid band appeared at the same place

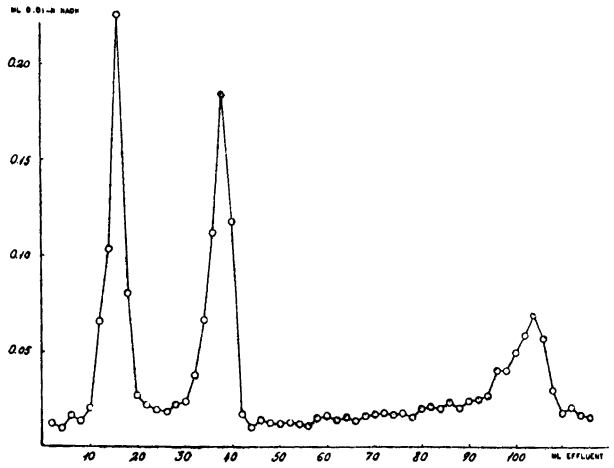

Fig. 1. The separation of a mixture of cholic acid (2.53 mg), deoxycholic acid (3.01 mg) and lithocholic acid (2.56 mg). Stationary phase: 4 ml chloroform-heptane (9 + 1) supported on 4.5 g hydrophobic kiselguhr. Moving phase: 58 % (%$_v$) aqueous methanol.

(15—20 ml) when varying the amount between 2 and 20 mg and the deoxycholic acid appeared at approximately the same place irrespective of this variation in the amount of cholic acid.

This method will thus be easily adaptable for many preparative problems when it is desirable to separate and identify the different components of a mixture of bile acids. As the recoveries are generally about 70—90 per cent the method can also be used to determine the composition of mixtures in a semiquantitative way.

The separation of other bile acids is being studied.

EXPERIMENTAL

The columns were prepared essentially as described by Howard and Martin. The kiselguhr (Hyflo-Supercel, Johns Manville Co.) was made hydrophobic by storage in a dessicator containing dimethyl-dichlorosilane or a lowboiling mixture of chlorosilanes (British Thomson-Houston Export Co., Ltd., Rugby). It was then washed with methanol, dried at 100° and stored in a closed vessel. The two phases were saturated with one another by shaking in a separatory funnel. — All work was done at room temperature (18—20°).

For analytic runs 4 ml of the stationary phase was added to 4.5 g of hydrophobic kiselguhr in a 50 ml Erlenmeyer flask and worked with a spatula until

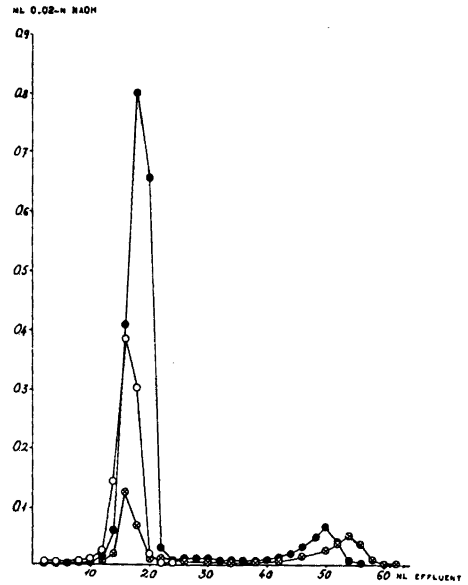

Fig. 2. Chromatography of mixtures of cholic and deoxycholic acid.

1. × — × *cholic acid 2.69 mg, deoxycholic acid 2.95 mg.*
2. ○ — ○ » » *9.50* » » » — »
3. ● — ● » » *20.0* » » » *2.30* »

Stationary phase: see fig. 1. Moving phase: 55 % (%v) aqueous methanol.

homogeneous. Enough of the other phase was then added to make a slurry that could be poured into the chromatographic tube, i.d. 12 mm, height about 400 mm with a narrow tube with a stop cock at the lower end. The slurry was homogenized with a plunger and then allowed to settle under weak suction until the height was about 10—12 cm.

When the column was almost dry at the top, the suction was disconnected and the substance to be tested, dissolved in 2—4 ml of the moving phase, was carefully added. When the solution had disappeared into the column the wall of the tube was washed twice with one ml of the solvent.

The moving phase was then added to a height of about 20 cm above the top of the column and maintained there with a separatory funnel with the tip of the stem at this height.

The rate was then regulated to about 2 ml per 10 minutes with the stopcock of the column.

The effluent was either collected and titrated as described by Howard and Martin [5] or alternatively collected in test tubes with an automatic fraction collector. After addition of bromthymol blue the contents of the tubes were then titrated in the tubes with a Rehberg microburette with a slow stream of nitrogen bubbling through the solution. With appropriate illumination the latter simple and time saving method was found to give as good results as the former one.

SUMMARY

A method for the separation of cholic, deoxycholic and lithocholic acids by reversed phase partition chromatography has been described.

We are greatly indebted to professor Erik Larsson, Lund and to General Electric Co., U.S.A., for samples of dimethyldichlorosilane.

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On the Complex Chemistry of the Uranyl Ion

VI.* The Complexity of Uranyl Chloride, Bromide and Nitrate

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Several authors have discussed the complexity of uranyl chloride and uranyl nitrate, whereas that of uranyl bromide does not yet appear to have been treated.

From his early conductometric and cryoscopic investigations Dittrich [6] concluded that both uranyl chloride and uranyl nitrate are weakly complex. As these measurements were performed at a time when the necessity of activity corrections was not yet realized, the result is uncertain in so far that it is not actually possible to decide if any complexity exists at all. It is however certain that it cannot be strong. From Suttons [7] discovery of discrepancies between the course of hydrolysis of uranyl chloride and uranyl perchlorate, a conclusion of a weak chloride complexity may, however, be drawn. The same conclusion has been drawn by Nelson and Kraus [8] on account of measurements on the equilibrium $2\ UO_2^+ + 4\ H_3O^+ \rightleftharpoons UO_2^{2+} + U^{4+} + 6\ H_2O$. The equilibrium constant is influenced by an exchange of perchlorate for chloride, which is interpreted as a consequence of a chloride complexing of UO_2^{2+} and U^{4+}. Introducing a number of simplifying assumptions, the authors calculate for UO_2Cl^+ a value of the thermodynamic complex constant $\beta_1^0 = 2.4\ C^{-1}$ (25° C). Also for uranyl nitrate, a number of experimental results speak in favour of a slight, but definite complexity (for an exhausting survey, see Glueckauf and McKay [9]). A value of $\beta_1 = 0.21\ C^{-1}$, valid at $I = 5.38$ C and the temperature 25° C, is found extinctiometrically by Betts and Michels [10]. However, objections must be raised against their method of measurement (*cf.* V p. 1151).

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* The preceding papers of this series (Ahrland [1–5]) are in the following referred to as I–V. The symbols of the present paper refer to the same quantities as those of I–V.

Owing to the lack of a suitable measurement method, no quantitative investigation of the complexity of the systems in question has thus been performed which is reasonably free from objections. But in the potentiometric method of ligand displacement, developed by Fronaeus [11], a method is now available which fairly well meets the demands of the problem.

It sometimes proves possible to confirm the potentiometric results by extinction measurements. This has been done for the uranyl chloride system.

As in I—V, all measurements have been performed at $I = 1$ with $NaClO_4$ as neutral salt, and at the temperature 20° C.

CHEMICALS USED

The *sodium chloride*, Kahlbaum *p. a.* and *sodium bromide*, Bakers analyzed, have been ignited. Then 1.00 C stock solutions are prepared by weighing. The concentration of the bromide solution is checked by titration according to Mohr.

The *sodium nitrate*, Bakers analyzed, is dried at 130° C. By weighing a 1.00 C stock solution is prepared, the concentration of which is checked by ion exchange analysis (*cf.* V p. 1152).

The *other chemicals* used are of the same preparations as in I and IV.

THE POTENTIAL MEASUREMENTS

In V p. 1152, the way is described of calculating complexity constants according to the method of ligand displacement. The only quantities necessary to know are the free ligand concentration, [B], and the ligand number $\bar{n}_B$ of the displacing ligand in solutions of known stoichiometric composition. As in V, the acetate ion is used as a displacing ligand, and [B] and $\bar{n}_B$ are therefore calculated in the same manner as there, *viz.* from measurements of $[H^+]$ of an acetate buffer, simply and accurately performed by quinhydrone electrode. To avoid uranyl hydrolysis, the buffer used has $\delta = 5.0$ (*cf.* IV p. 207). As Cl^- Br^- and NO_3^- do not combine with H^+, the calculations are in the present case simplified so that ϑ and ϑ' of V p. 1154 are transformed into $[H^+]$ and $[H^+]'$. Thus the following formulas of [B] and $\bar{n}_B$ are obtained:

$$[B] = \frac{[H^+]'}{[H^+]} \left(C'_B + 0.2 \left(C^0_H - [H^+]\right) + 1.2\ [H^+]'\right) \tag{1}$$

$$\bar{n}_B = \frac{C'_B - \left(C^0_H - [H^+]\right) - [B]}{C_M} \tag{2}$$

Table 1. Determination of E' as a function of C'_B for given values of the parameter C_A ($A = Cl^-$, Br^- or NO_3^-). The correction term $[H^+]'$, calculated with $[H^+]_0 = 10.19$ mC.

A →	Cl^-		Br^-		NO_3^-		
C_A → mC	200	600	200	600	200	600	0
C'_B mC	E' mV						
10.37	108.7	104.0	109.1	104.9	109.8	107.3	110.7
20.20	108.2	103.4	108.6	104.2	109.5	107.1	110.5
29.55	107.9	103.1	108.4	104.0	109.4	107.0	110.3
38.4	107.8	102.9	108.3	103.8	109.3	106.9	110.2
46.9	107.7	102.7	108.2	103.6	109.2	106.8	110.1
66.5	107.6	102.6	108.1	103.4	109.1	106.7	110.1
99.7	107.4	102.2	107.9	103.1	109.0	106.6	110.1
138.9	107.3	101.8	107.7	102.7	109.0	106.5	110.1
177.3	107.2	101.6	107.7	102.4	109.0	106.5	110.1
$[H^+]'$ mC	0.14	0.17	0.14	0.17	0.13	0.15	0.13

The complex solutions are prepared analogously to those of V (p. 1155). Two different values of C_A (= C'_A for the present ions, *cf.* V p. 1159) are used *viz.* 200 and 600 mC. C'_M is 40 or (at the nitrate titrations) 50 mC; besides, titrations with $C_M = 0$ are performed (*cf.* V p. 1154). As the complexity proves to be very weak for all the systems investigated here, it is not necessary to use more than a single value of C'_M if this is chosen not too high. In such a case, one may always put $[A]_n = C_A$ with sufficient accuracy. RE has in the present measurements $[H^+]_0 = 10.19$ mC.

The titrations with $C_M = 0$ are collected in Table 1. For the sake of comparison, the values of E' for $C_A = 0$ are also given. They are obtained by interpolation from IV Table 1, and recalculated with the present value of $[H^+]_0 = 10.19$ mC. It is seen from the courses of E' that an exchange of perchlorate for any of the anions here used causes a pronounced change of the ionic medium, increasing in the sequence $NO_3^- < Br^- < Cl^-$. It is not surprising that NO_3^- brings about the smallest change, as this ion is the one which resembles ClO_4^- most. The effect of Br^- and especially Cl^- is very considerable, the latter ion causing almost the same change as an equal concentration of SO_4^{2-} (V Table 1), in spite of the fact that SO_4^{2-} is divalent and thus contributes four times as much to I than does Cl^-.

Table 2. A measurement of Table 1 repeated with approximately the same ionic medium throughout the whole cell. $A = Cl^-$, $C_A = 600$ mC, $[H^+]_0 = 10.19$ mC. — Calculation of K_c in the medium used.

C'_B mC	E'' mV	$[H^+]'$ mC	$K_c \cdot 10^5$ C
10.37	107.8	0.143	2.90
20.20	107.2	0.146	2.94
29.55	107.0	0.147	2.95
38.4	106.9	0.148	2.97
46.9	106.7	0.149	2.99
66.5	106.5	0.150	3.00
99.7	106.3	0.151	3.02
138.9	105.9	0.154	3.08
177.3	105.6	0.155	3.10

Of course the great medium changes makes the calculation of $[H^+]'$ according to (16) of II somewhat dubious as this formula postulates equal conditions of activity in both the half-cells. But as $[H^+]'$ is only a small correction term in (1), the apparent changes of its value, given in Table 1, are of no importance. It may be emphasized here that the ratio $[H^+]'/[H^+]$ is obtained according to (18) of II, *i. e.* from the emf of an element with the same C_A and C'_B in both halfcells. This factor is thus independent of the fact that the activity conditions of RE are no longer the same as in the halfcell containing buffer (*cf.* II p. 794).

Strictly speaking, the course of E' by the exchange of ClO_4^- for the ions A only implies an increase of the hydrogen ion activity of the buffer solution. One may assume that this in turn implies an increase of $[H^+]'$, and thus an increase of $K_c \cdot$ A quantitative determination cannot be made, however, before the activity conditions of the two half-cells have been made approximately equal, *i. e.* the whole cell must contain approximately the same ionic medium. Such a determination is now performed with the series of the greatest medium change measured, *viz.* the Cl^--series of $C_A = 600$ mC, using an RE with $[H]_0 = 10.19$ mC, $C_A = 600$ mC and $NaClO_4$ to $I = 1$. This RE thus has a composition equal to that of the other half-cell, the buffer concentration then being left out of consideration. The measured emf, E'' as well as the calculated $[H^+]'$ and K_c are found in Table 2. K_c is not a constant, evidently the exchange of perchlorate for buffer also causes perceptible change of the medium in the present case. In the beginning however, where C'_B is low, K_c may be considered as the value valid for the medium 600 mC NaCl, 400 mC $NaClO_4$. This value is approximately $K_c = 2.9 \cdot 10^{-5}$ C, which implies an increase of K_c with > 10 % in relation to 1 C $NaClO_4$. It is clearly seen however that the value of $[H^+]'$ calculated here does not differ so much from the corresponding one of Table 1 that an error of [B] can arise for that reason.

The results of the main titrations are found in Table 3 and Fig. 1, where $\bar{n}_B/[B]$ is given as a function of [B] for all the series measured. Moreover, the

Table 3. Determination of corresponding values of $\bar{n}_B/[B]$ and $[B]$ at the two C_A used.
Table 3 A. Uranyl chloride system.

C_A mC		200				600			
C_M mC	C'_B mC	E_B mV	C^0_H-[H$^+$] mC	[B] mC	$n_B/[B]$ C^{-1}	E_B mV	C^0_H-[H$^+$] mC	[B] mC	$\bar{n}_B/[B]$ C^{-1}
38.95	10.37	58.6	0.46	1.045	218	55.7	0.45	1.175	191
38.0	20.20	56.3	0.51	2.20	209	53.5	0.49	2.465	184.5
37.05	29.55	54.8	0.53	3.40	203.5	52.3	0.49	3.76	181.5
36.15	38.4	53.6	0.5	4.63	199	51.0	0.5	5.14	176.5
35.3	46.9	52.4	0.6	5.92	193.5	49.7	»	6.59	171
34.5	55.1	51.0	»	7.36	185.5	48.3	»	8.18	164.5
33.35	66.5	48.2	»	9.91	169.5	46.0	0.6	10.82	153
31.6	84.0	42.4	0.7	15.75	136	40.6	0.7	16.9	124.5
30.0	99.7	35.2	0.8	24.8	99.5	34.1	0.8	25.9	94
28.6	114.0	28.5	0.9	37.0	72	27.9	0.9	37.8	69.5
27.3	127.0	23.3	»	50.6	54.5	23.2	»	50.8	54.5
26.1	138.9	19.5	»	64.4	44	19.3	»	64.8	43.5
24.0	159.6	14.4	»	90.4	31.5	14.8	»	89.1	32.5

Table 3 B. Uranyl bromide system.

38.95	10.37	59.3	0.46	1.016	225	57.4	0.47	1.096	206
38.0	20.20	57.0	0.51	2.15	215	55.1	0.51	2.32	197
37.05	29.55	55.7	0.53	3.29	211	53.9	0.52	3.53	195
36.15	38.4	54.6	0.5	4.46	207	52.8	0.5	4.79	191
35.3	46.9	53.4	»	5.69	202.5	51.7	»	6.09	187.5
34.5	55.1	52.0	0.6	7.08	194.5	50.3	»	7.57	180
33.35	66.5	49.2	»	9.52	177.5	47.7	0.6	10.09	166
31.6	84.0	43.1	0.7	15.3	141	42.0	0.7	16.0	133
30.0	99.7	35.7	0.9	24.3	102	35.0	0.9	25.0	98.5
28.6	114.0	28.8	»	36.5	73.5	28.2	1.0	37.4	70.5
27.3	127.0	23.4	1.0	50.4	55	23.2	»	50.8	54
26.1	138.9					19.4	»	64.6	43.5
24.0	159.6					14.6	0.9	89.7	32

value of the function for $C_A = 0$, as determined in IV, is introduced. All the $\bar{n}_B/[B]$-functions are extrapolated to $[B] = 0$.

Considering the high values of C_A used, the displacements of the found $\bar{n}_B/[B]$-curves in relation to the curve of $C_A = 0$ are really very slight. Hence it

Table 3 C. Uranyl nitrate system.

$C_A \rightarrow$ mC		200				600			
C_M mC	C'_B mC	E_B mV	C^0_H-[H$^+$] mC	[B] mC	$\bar{n}_B$/[B] C^{-1}	E_B mV	C^0_H-[H$^+$] mC	[B] mC	$\bar{n}_B$/[B] C^{-1}
48.7	10.37	64.8	0.50	0.818	227.5	62.7	0.52	0.889	207
47.5	20.20	62.6	0.57	1.715	220.5	60.6	0.58	1.86	201
46.3	29.55	61.4	0.59	2.63	216	59.3	0.61	2.86	197
45.2	38.4	60.3	0.6	3.56	212.5	58.2	0.6	3.87	194
44.1	46.9	59.2	»	4.54	209	57.2	»	4.90	190
43.1	55.1	58.0	»	5.57	203.5	56.1	»	6.01	187
41.7	66.5	56.0	0.7	7.28	193	54.0	0.7	7.87	176.5
39.5	84.0	51.7	0.8	10.89	168	49.8	0.8	11.75	154
37.5	99.7	46.0	0.9	16.2	136	44.6	0.9	17.1	127.5
35.7	114.0	39.2	1.0	24.3	102.5	38.2	1.0	25.2	97.5
34.1	127.0	32.6	1.1	35.6	76	31.9	1.1	36.0	73
32.6	138.9	27.1	»	47.6	58	27.0	»	47.9	57.5

is clear that all the systems in question are only very weakly complex. A variation of C_A of, say, 50 mC causes a displacement of the $\bar{n}_B$/[B]-curve which is not greater than the experimental error of the measurement. Thus it is certainly allowed to neglect that part of C_A in relation to the rest which has formed complexes with uranyl ions, *i.e.* one can put $[A]_n = C_A$. The found curves thus represent the functions $(\bar{n}_B/[B])_{C_M=0}$ ultimately required. The values of [B] (= b) where these curves coincide with the one of $C_A = 0$ are now determined. Then the graphical integrations according to (1) of V can be performed. From the $X([A])$-functions hence obtained the $X_1([A])$-functions are calculated (Table 4). Within the random error, which may be estimated to $\pm$ 0.2 C^{-1}, these functions are constant for all the measured systems, and thus $= \beta_1$. Any further complexes between M and A thus cannot be proved, nor is it possible to calculate any significant values of the mixed constants $\beta_{1,1}$ (*cf.* V p. 1153).

With the found values of β_1, $\bar{n}_A$ is calculated ((2) of II) and also given in Table 4. It can be seen that the amount of C_A which combines with M ($= \bar{n}_B \cdot C_M$) does not exceed 15 mC at a $C_A = 600$ mC of that ligand which forms the strongest complexes, *viz.* the chloride ion. Such a quantity cannot perceptibly displace the $\bar{n}_B$/[B]-curve. The approximation $[A]_n = C_A$ is thus correct.

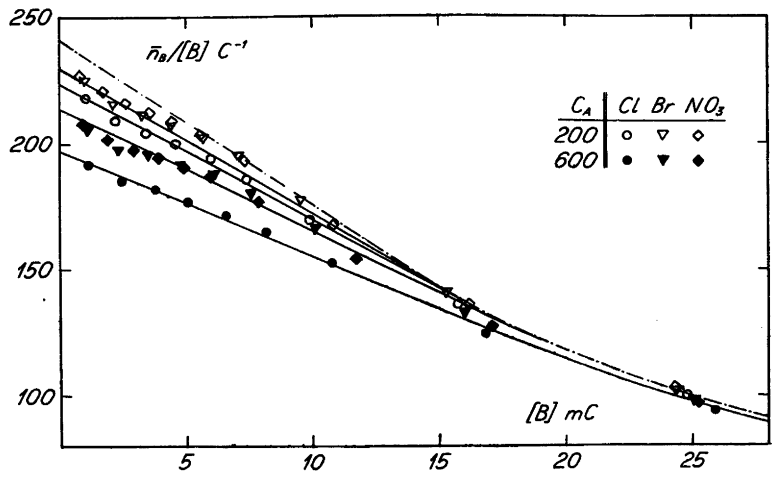

Fig. 1. $\bar{n}_B/[B]$ *as a function of* [B]. *The curve of dots and dashes refers to pure acetate complex formation (IV Fig. 2). The signs refer to the different experimental series according to the scheme on the fig. The fulldrawn curves are drawn in connection with the experimental points,* Br^- *and* NO_3^- *then giving the same curves.*

Of course, the $\bar{n}_B/[B]$-function may be displaced also by the change of the medium, which has been stated above to follow the exchange of ClO_4^- for Cl^-, Br^- or NO_3^-. One expects however that such a change would displace the whole curve, not merely its first part. On the contrary, the actually observed behaviour of the curve is just that which would be expected as the consequence of a complex formation (*cf.* V p. 1152). Then it seems most likely, that a complex formation occurs, but an activity change may also be involved. If it is assumed that the dissociation of the first uranyl acetate complex MB is affected in the same manner as the dissociation of the acetic acid. its dissociation increases (*cf.* p. 1274) *i.e.* β_1 decreases and $\bar{n}_B/[B]$ decreases. If such an activity effect is present here, the displacements owing to complex formation have been overestimated. The found values of β_1 have therefore a systematic error; probably they are too high.

THE EXTINCTION MEASUREMENTS

The extinctiometric method of calculating complexity constants used in II—V imports the determination of the ligand number $\bar{n}_A$. This is not possible here however, owing to the slight complexity of the present systems. At all C_M of reasonable size, the difference $C_A - [A]$ would be too small compared with the experimental error of [A]. The complexity of uranyl thiocyanate, measured in

III, may be said to represent about the lowest order of magnitude of complexity which can be determined by the method previously used.

But even if a complete extinctiometric determination is impossible, one might sometimes test the potentiometrically obtained result that only the first complex would exist in the investigated systems. In such a case (22) of II is simplified:

$$\varepsilon_M - \varepsilon_0 = \frac{(\varepsilon_1 - \varepsilon_0)\,\beta_1\,[\mathrm{A}]}{1 + \beta_1\,[\mathrm{A}]} \tag{3}$$

If ε_M is determined at two different values of C_A, denoted $'$ and $''$, the ratio q between the found quantities $\varepsilon'_M - \varepsilon_0$ and $\varepsilon''_M - \varepsilon_0$ then is

$$q = \frac{\varepsilon'_M - \varepsilon_0}{\varepsilon''_M - \varepsilon_0} = \frac{[\mathrm{A}]'}{[\mathrm{A}]''} \cdot \frac{1 + \beta_1\,[\mathrm{A}]''}{1 + \beta_1\,[\mathrm{A}]'} \tag{4}$$

If only the first complex exists, q is thus independent of the molar extinctions ε_0 and ε_1, *i.e.* q is independent of that wave-length λ at which ε_M is measured. Indeed, this is generally valid if a system contains only one complex. On the other hand, if more than one complex exists q will always depend on the molar extinctions, or, rather, on the ratios between them, as is easily deduced from (22) of II.

Thus, if extinction curves are measured for two solutions of a complex system and the ratio q formed shows a good constancy, that conclusion may be drawn that only one complex exists. It is however a necessary condition that the measurements cover a rather wide λ-range. Only then it can be taken

Table 4. The X-functions of the investigated systems, as obtained by graphical integration according to (1) of V. The ligand numbers as calculated from the found complexity constants.

A	$[\mathrm{A}]_n$ mC	b mC	ln $X([\mathrm{A}])$	$X([\mathrm{A}])$	$X_1([\mathrm{A}])$ C^{-1}	β_1 C^{-1}	$\bar{n}_A$
Cl^-	200	16	0.140	1.151	0.76	0.8	0.14
	600	40	0.427	1.534	0.89		0.32
Br^- & NO_3^-	200	14	0.0825	1.085	0.43	0.5	0.09
	600	19	0.263	1.300	0.50		0.23

for granted, that the ratios between the molar extinctions must have really changed. The conclusion may be further strengthened by measuring more solutions and forming new ratios q which have all to be independent of λ.

If only one complex exists in the present systems it is no doubt the first one, potentiometrically proved above. Then (4) is valid and hence β_1 can be calculated if the usual approximation $[A] = C_A$ is introduced:

$$\beta_1 = \frac{q \cdot C''_A - C'_A}{C''_A \cdot C'_A \ (1 - q)} \tag{5}$$

If more than one q has been calculated, of course all of them have to give the same β_1 according to (5).

The extinction curves of the following solutions have been measured:

chloride system:	bromide system:	nitrate system:
33 mC $UO_2(ClO_4)_2$	33 mC $UO_2(ClO_4)_2$	15 mC $UO_2(ClO_4)_2$
250 mC $HClO_4$	250 mC $HClO_4$	100 mC $HClO_4$
250, 500 or 750 mC NaCl	750 mC NaBr	750 mC $NaNO_3$
$I = 1$ (by $NaClO_4$)	$I = 1$ (by $NaClO_4$)	$I = 1$ (by $NaClO_4$)

All solutions are so acid, that no hydrolysis can exist.

The curves of the chloride and bromide solutions are obtained by a Hilger Medium Spectrograph, combined with a Spekker Photometer (*cf.* I p. 375), whereas that of the nitrate solution is obtained by a Beckman Quartz Spectrophotometer (*cf.* IV p. 212). The reproducibility of the spectrographically determined values are considerably lower than that of the spectrophotometrically measured ones. This circumstance has however little importance here, as that error always predominates which arises from the fact that the compared ε_M are not measured at the same E. Because of that, the error of ε_M must be estimated to ≈ 3 %, which is the normal error of absolute extinction measurements (*cf. e. g.* Kortüm and v. Halban [12], or Olerup [13] pp. 46—56.

The general rule that the blank cell has to contain all the components of the solution except the uranyl ions is especially important for the nitrate system, as the nitrate ion has a considerable extinction in UV (v. Halban and Eisenbrand [14]). Owing to the slight complexity of the nitrate system, [A] of the two absorption cells may be considered to be equal and thus ε_A completely compensated. A disadvantage of the nitrate absorption is however that very wide slits have to be used in the Beckman, causing a bad monochromaticity of the light and thus an especially great systematic error of ε_M. The absorption of the chloride and bromide ions may be neglected in the UV range used (Fromherz and Menschick [15]).

All the curves of the measured solutions are found in Fig. 2, together with that of UO_2^{2+} from I p. 377. In the same Fig., the curve of NO_3^- from [14] as well as those of Cl^- and Br^- from [15] are also introduced.

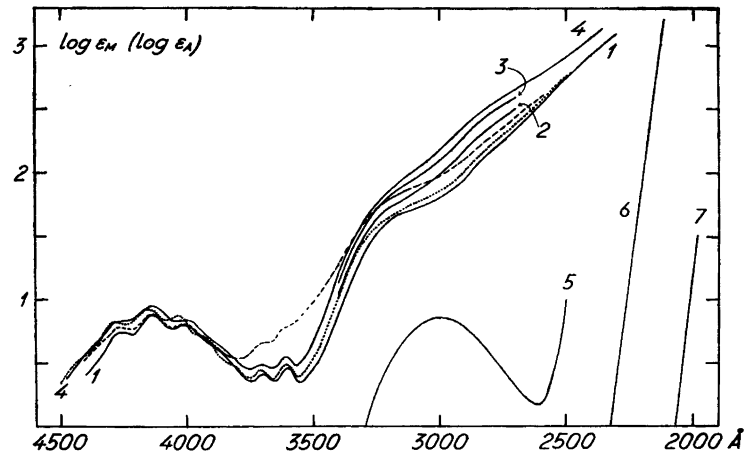

Fig. 2. Extinction curves of a) uranyl ion (fulldrawn, 1) b) uranyl chloride solutions with C_M = 33 mC, C_A = 250, 500 and 750 mC (fulldrawn, 2, 3 and 4), c) uranyl bromide solution with C_M = 33 mC, C_A = 750 mC (dashed), d) uranyl nitrate solution with C_M = 15 mC, C_A = 750 mC (dotted) and e) nitrate, bromide and chloride ions (fulldrawn to the right, 5, 6 and 7 respectively).

An addition of Cl^-, Br^- or NO_3^- evidently causes a not very large but nevertheless definite displacement of the UO_2^{2+}-curve. This agrees very well with the conception of a weak complex formation in all the systems. A quantitative examination of the potentiometrically found complexity according to (4) and (5) can however be performed only for the chloride system. In this case only

Table 5. The constant values of q_1 and q_2 of the uranyl chloride system, as obtained from the interpolated values of log ε_M of Fig. 2.

λ Å	log ε_M'''	log ε_M''	log ε_M'	log ε_0	q_1	q_2
2700	2.655	2.595	2.505	2.345	0.43	0.75
2800	2.535	2.47	2.355	2.165	0.41	0.76
2900	2.37	2.29	2.18	1.95	0.43	0.73
3000	2.175	2.095	1.98	1.815	0.36	0.69
3100	2.015	1.95	1.84	1.705	0.35	0.72
3200	1.855	1.81	1.725	1.615	0.39	0.77
3300	1.62	1.565	1.49	1.345	0.45	0.75
3400	1.195	1.14	1.045	0.89	0.42	0.76
				Mean	0.41	0.74

the addition of ligand causes within a wide λ-range a displacement of the UO_2^{2+}-curve which is large enough to permit a sufficiently accurate determination of $\varepsilon_M - \varepsilon_0$. For the bromide system, this displacement is large enough only in such a narrow λ-range that no certain conclusion can be drawn about the constancy of q, and for the nitrate system it is very small in the whole observed λ-range.

For the chloride system, the molar extinctions of $C_A = 250$, 500 and 750 mC are indicated by ε'_M, ε''_M and ε'''_M respectively. Hence the two ratios $q_1 = (\varepsilon'_M - \varepsilon_0)/(\varepsilon'''_M - \varepsilon_0)$ and $q_2 = (\varepsilon''_M - \varepsilon_0)/(\varepsilon'''_M - \varepsilon_0)$ are formed. In Table 5, the values of log ε_M for every 100 Å are given as obtained by extrapolation from Fig. 2. The values of q_1 and q_2 hence calculated are in the same Table. Within the experimental errors, they are constant throughout the whole λ-range measured. Thus only the first complex seems to exist, and it is allowed to apply (5), which gives $\beta_1 = 0.5\ C^{-1}$ for both q_1 and q_2. From the spreading of the values of q_1 and q_2, the random error of β_1 may be estimated to $\pm 0.3\ C^{-1}$, thus being of the same order of magnitude as in the potential measurements.

Olerup [13 p. 60] has very fully investigated the consequences of an unallowed simplification of (22) of II into (3). He finds that the existence of not recognized higher complexes causes too low values of β_1 when calculated according to (5) or an equivalent formula. Thus, if such higher complexes exist in the present system but are not discovered owing to the great random errors of q_1 and q_2, the value of β_1 calculated here is too low.

The extinction measurements are of course also influenced by the activity changes occurring when ClO_4^- is exchanged for Cl^- as the law of mass action is used at the deduction of (3). Nothing can be surely said however about the consequence upon β_1 of this influence.

Considering the random errors, the extinctiometric investigation may be said to confirm the potentiometric one completely. The difference between the values may, however, also have systematic reasons, as the potentiometric method is likely to give a too high value, whereas the extinctiometric one possibly gives a figure which is too low.

SUMMARY

The complexity of the uranyl chloride, bromide and nitrate systems are potentiometrically determined according to the method of ligand displacement. As in V, the acetate ion is used as the displacing ligand. The experiments are therefore also carried out in quite the same manner as those of V.

All the systems investigated form very weak complexes. Only the first complex can be proved, and its constant β_1 is calculated to be 0.8, 0.5 and 0.5 C^{-1}

respectively, with an estimated experimental error of $\pm$ 0.2 C^{-1}. Owing to systematic errors, these values are probably too high.

Concerning the chloride system the result that only one complex exist is confirmed in an extinctiometric way, which gives $\beta_1 = 0.5 \pm 0.3$ C^{-1} in good agreement with the potentiometrically determined value If the existence of higher complexes is overlooked, which to some slight extent may be the case, the extinctiometric value of β_1 may be too low.

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Pyrazole Studies

IV. Potentiometrical Titrations of 1-Arylpyrazolones *

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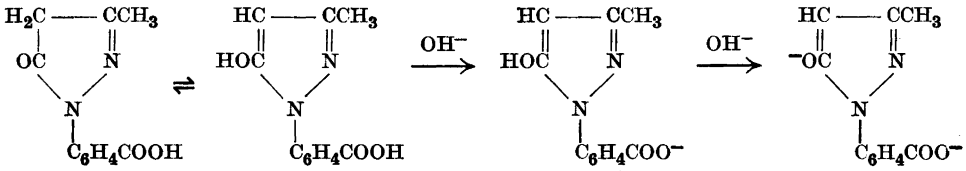
In a previous paper[1] it was shown that 1-*p*-carboxyphenyl-3-methyl-pyrazolone-5 by potentiometrical titration behaved as a dibasic acid, claiming 2 equivalents of base to complete neutralisation. The titration curve does only show an inflexion point when 1 equivalent of base has been added, whereas a real jump of potential is observed after the addition of two equivalents of base.

This means that during the titration complete enolisation of the pyrazolone takes place and that not only the carboxyl group but the hydroxyl group too will give off a proton at the pH at which the jump of potential is observed.



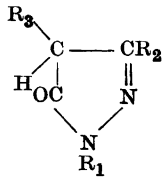
As the titration is carried out in ethanolic solution a definitive pH-value corresponding to the end-point can hardly be indicated without further investigation, but it seems to be about pH 10. The carboxyl group is without doubt more ready to give off a proton than the hydroxyl group, but from the shape of the titration curve it may be concluded that the difference between the dissociation constants cannot be very great.

As the presence of a carboxyl group in the 1-phenyl-group cannot increase the tendency of enolisation of the pyrazolone it is obvious that 1-aryl-3-alkyl-

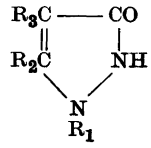
* Preliminary note: *Sjätte nordiska kemistmötet i Lund* (1948) p. 287 *Proc. XI. Intern. Congress Pure and Applied Chemistry. London* (1950) Vol. 2 p. 329.

pyrazolones-5 in general may be titrated in ethanolic solution. The substituents at 1 and 3 in the pyrazolone-nucleus may, however, be able to modify slightly the tendency of enolisation of the pyrazolone group and thus to some extent cause modifications in the shape and position of the titration curves, and to a still greater extent this might be valid for substituents at position 4, whereas substituents at position 2 will prevent the enolisation of the pyrazolone and thus make it untitratable.

In order to investigate the effect of different substituents we prepared a series of 5-pyrazolones (I) with R_1 = phenyl or o-tolyl, R_2 = methyl or



I

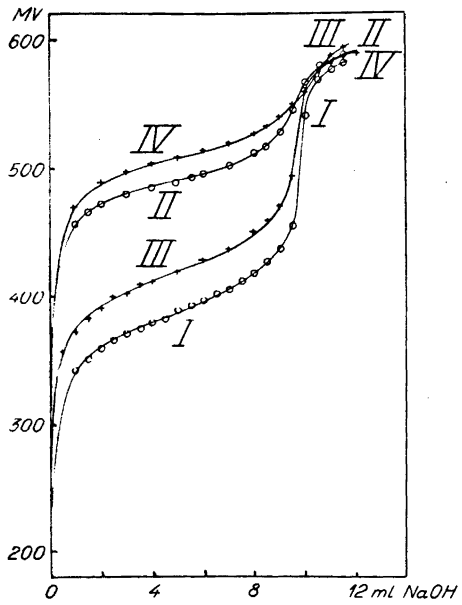


II

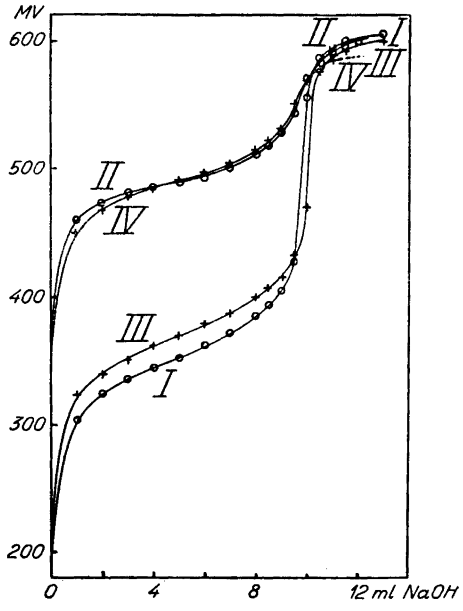
phenyl and R_3 = hydrogen or ethyl and determined the titration curves. As expected, the general shape of the curves is the same but an influence of the nature of the substituents is evident and most pronounced for R_3 = ethyl.

In the 5-pyrazolones the enolisation is of the usual type, $-CO-CH_2- \rightleftharpoons -C(OH) = CH-$. For the isomeric 3-pyrazolones (II), on the other hand, there is a possibility of an enolisation of a different type, $-CO-NH- \rightleftharpoons -C(OH) = N-$, but it is not possible to predict whether the tendency of enolisation is great enough to allow the titration of these pyrazolones or not. We therefore prepared a series of 3-pyrazolones isomeric with the 5-pyrazolones investigated. Electrometrical titration of these substances in ethanolic solution with 0.1 n aqueous sodium hydroxide showed that the tendency of enolisation is lesser than for the 5-pyrazolones, but even for the 3-pyrazolones a jump of potential may be observed.

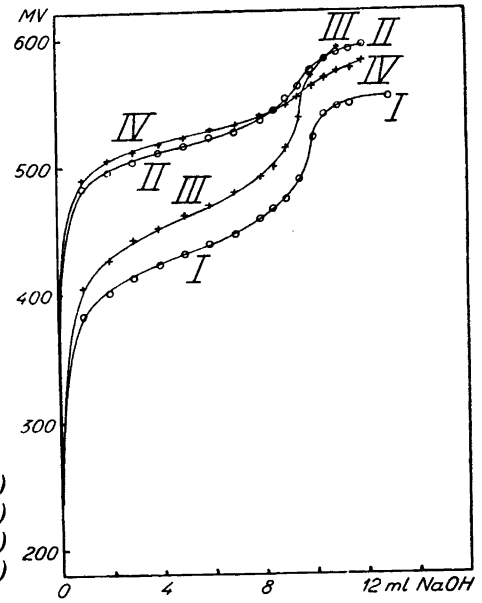
The jump of potential is not great enough to allow an exact determination of the molecular weight of the pyrazolones, the inexactitude being some 3—4 %. But in another way the titration curves seem to be useful. In Figs. 1—3 the titration curves for the pyrazolones examined may be seen, and it is obvious that all curves for 5-pyrazolone are of one type, all curves for the 3-pyrazolones of another type. This means that for an unknown pyrazolone it may be disclosed if it belongs to the 5- or the 3-series simply by determining its titration curve. Till now it has been necessary to establish the structural formula of the substance either by somewhat complicated chemical methods



I 1-Phenyl-3-methyl-pyrazolone-(5)
II 1-Phenyl-5-methyl-pyrazolone-(3)
III 1-o-Tolyl-3-methyl-pyrazolone-(5)
IV 1-o-Tolyl-5-methyl-pyrazolone-(3)



I 1,3-Diphenyl-pyrazolone-(5)
II 1,5-Diphenyl-pyrazolone-(3)
III 1-o-Tolyl-3-phenyl-pyrazolone(5)
IV 1-o-Tolyl-5-phenyl-pyrazolone(3)



I 1-Phenyl-3-methyl-4-ethyl-pyrazolone-(5)
II 1-Phenyl-5-methyl-4-ethyl-pyrazolone-(3)
III 1-o-Tolyl-3-methyl-4-ethyl-pyrazolone-(5)
IV 1-o-Tolyl-5-methyl-4-ethyl-pyrazolone-(3)

Fig. 1—3. Titration curves for different 3- and 5-pyrazolones.

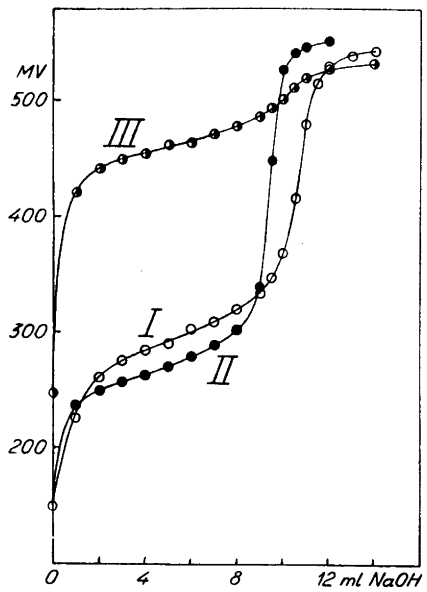


Fig. 4. Titration curves for o-carboxyphenylhydrazine (I), acet-o-carboxyphenylhydrazide (II) and benzopyrazolone (III).

or by determining the ultraviolet absorption spectrum. Biquard and Grammaticakis [2,3] have shown that the absorption spectra of 5- and 3-pyrazolones are sufficiently different to be used for the structural determination, but that the interpretation of the spectra of at all events the 5-pyrazolones is complicated by the enolisation, the absorption of the enolised form being different from that of the ketoform.

Also as a practical means of structural determination the potentiometrical titration is by far more convenient than the determination of the absorption spectrum, and besides it makes possible the calculation of the molecular weight of the substance.

In Fig. 4 is shown that not only alkyl- or aryl-substituted pyrazolones may be titrated, but that condensed systems as *e. g.* benzopyrazolone-3 as well may be titrated [4]. As this substance may often contaminate preparations of o-carboxyphenylhydrazine or acet-o-carboxyphenylhydrazide it is very convenient to be able to control the purity of the hydrazine derivative by a simple titration method. Fig. 4 gives the titration curves for all three substances. The difference between the three curves is, as might be expected, very great.

EXPERIMENTAL PART

1-Phenyl-3-methyl-pyrazolone-5, 1,3-diphenyl-pyrazolone-5, 1-phenyl-3-methyl-4-ethyl-pyrazolone-5, 1-o-tolyl-3-methyl-pyrazolone-5, 1-phenyl-5-methyl-pyrazolone-3, 1,5-diphenyl-pyrazolone-3, 1-phenyl-4-ethyl-5-methyl-pyrazolone-3 and 1-o-tolyl-5-

methyl-pyrazolone-3 were prepared by current methods and showed the melting points indicated in the litterature.

1-o-Tolyl-3-phenyl-pyrazolone-5 was prepared essentially as indicated by Knorr and Klotz [5] for its lower homologue. It may be recrystallised from 10 parts (by weight) of ethanol. Colourless crystalpowder with m.p. 187°.

$C_{16}H_{14}N_2O$ (250.3) N calc. 11.20, found 11.30 % * M found by titration 251.7

1-o-Tolyl-3-methyl-4-ethyl-pyrazolone-5 was prepared by analogy to lower homologues by heating 14.3 g o-tolylhydrazine (1 mol) with 18.5 g ethyl ethylacetoacetate (1 mol) to 145° for 2 hours. The mixture was then vacuum-distilled. At 222—225° (12 mm) a fraction weighing 12 g was collected. It solidified to a yellowish resinous mass which could not be brought to crystallisation. By titration the equivalent weight was found to 215.2, calc. 216.3.

$C_{13}H_{16}N_2O$ (216.3) N calc. 12.96, found 12.91, 13.05 %

The substance may thus be regarded as pure even if it has not been obtained in the crystalline state. Curve III in Fig. 3 is obtained using this preparation. We are, however, indebted to Mr. Knud Eggersen, M. Sc., for repeating the preparation. He obtained sensibly the same results, but on distilling the substance once more in a high vacuo (< 1 mm Hg) and keeping the distillate for 48 hours in a dry ice acetone bath germs of crystals were formed, which eventually caused crystallisation of the resin after it had been mollified by treatment with ether.

The pyrazolone forms colourless crystals with m.p. 82.5—83.5°. N calc. 12.96, found 12.75 %. M found by titration 215.1. The titration curve obtained with the crystalline compound was identical with the one shown in Fig. 3.

1-o-Tolyl-5-phenyl-pyrazolone-3 was prepared analogously to the method of Michaelis and Willert [6] for the preparation of 1,5-diphenylpyrazolone-3, but with a very poor yield. Recrystallised from ethanol it forms colourless crystals with m.p. 207—208°.

$C_{16}H_{14}N_2O$ (250.3) N calc. 11.20, found 11.12, 11.17 %. M found by titration 250—253.

The shape of the titration curve proves that the substance is a 3-pyrazolone, not a 5-pyrazolone.

1-o-Tolyl-4-ethyl-5-methyl-pyrazolone-3 was prepared analogously from acet-*o*-tolylhydrazide, ethyl ethylacetoacetate and phosphorous oxychloride with slightly better yield than the above mentioned pyrazolone. It forms colourless crystals, m.p. 161.5°.

$C_{13}H_{16}N_2O$ (216.3) N calc. 12.96, found 13.01, 13.07 %. M found by titration 216—220.

Here, too, the shape of the curve was that of a 3-pyrazolone.

Benzopyrazolone was obtained as by-product from the preparation of *acet-o-carboxyphenylhydrazide* (refluxing *o*-carboxyphenylhydrazine with glacial acetic acid for 2 hours). It forms faint yellow crystals with m.p. 247°. The same m.p. is found for both *o*-carboxy-

* All microanalyses by Mr. O. Rosenlund, M. Sc. or by Mr. A. Grossmann. Department of Organic Chemistry, University of Copenhagen.

phenylhydrazine and acet-o-carboxyphenylhydrazide, ring-closure evidently taking place during the heating, comp. Pfannstiel and Janecke [7].

The titrations were carried out potentiometrically, using a glass electrode and a Radiometer-potentiometer, type PHM 3f, measuring the potential between the glass electrode and a calomel electrode with saturated potassium chloride as liquid junction. 0.01 mol substance was dissolved in 50 ml 96 % ethanol. This solution was titrated with 0.1 *N* aqueous NaOH.

SUMMARY

The potentiometric titration of some 1-aryl-5- or 3-pyrazolones with NaOH has been investigated. Two types of titration curves were obtained. One, belonging to the 5-pyrazolones, corresponds to a distinct acidic character of the substance titrated, the other, belonging to the 3-pyrazolones, corresponds to substances, the acidic character of which may only just be observed.

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The Occurrence of Sedoheptulose in the Dried Root of *Primula elatior* (L) Hill

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For some time we have been engaged in investigating the distribution of heptoses in the plant kingdom [1–6] and have carried out screening tests on aqueous extracts of about 100 plants belonging to 60 different families. Initially we utilised the Bial reaction [7] but subsequently also made use of a new spraying reaction for ketoheptoses [5], and the results obtained encouraged us to continue the investigation.

For more detailed examination we chose several Primula species, as previous workers had demonstrated that the heptahydric alcohol volemitol occurs in several Primulas [8] and we felt it would be of interest to determine whether the corresponding sugars — sedoheptulose and mannoheptulose — are present in the same material. V. Ettel and L. Liebster [9], and others have shown that volemitol can be converted into these two sugars.

We have now examined the constitutents of aqueous extracts of dried root stock of *Primula elatior* (L) Hill and have identified sedoheptulose, fructose, glucose, saccharose and volemitol, and obtained evidence for the presence of mannoheptulose. All the carbohydrates were identified by paper chromatography, authentic samples being run simultaneously and the spots being detected by spraying with the reagent mentioned above which gives colours with fructose and saccharose as well as with ketoheptoses. Aldoses, on the other hand, do not react with this reagent, and were detected with *m*-phenylenediamine hydrochloride.

In addition, the sedoheptulose was isolated by partition chromatography on a column of cellulose, and identified by conversion into the dibenzylidene derivative of sedoheptulosan [10].

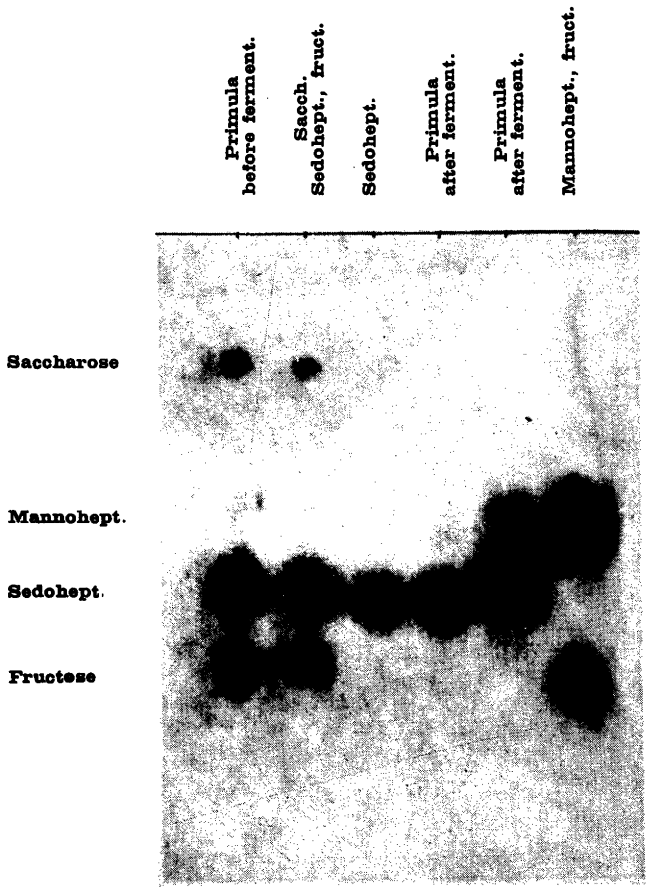

Fig. 1. The chromatogram has been run for 48 hours with ethyl acetate — acetic acid — water, then sprayed with orcinol — trichloroacetic acid reagent and heated at 100° for 10 min. The colours of the spots were:

Saccharose and fructose	*yellowish green*
Mannoheptulose	*green*
Sedoheptulose	*bluish green*

EXPERIMENTAL

The dried root (2.3 kg of commercial material shown by microscopical examination to consist mainly of *Primula elatior*) was extracted twice with a total of ten times its weight of water and the residue was pressed to remove as much of the liquid as possible. The extract was treated with a slight excess of basic lead acetate, the excess lead being removed with H_2S. The resulting solution was concentrated under reduced pressure (12—15 mm) to a weight of approximately 300 g, then set aside in the refrigerator. Most of the volemitol crystallised out and was collected. The mother liquor was precipitated

with alcohol, the insoluble material filtered off, and the alcoholic solution evaporated under reduced pressure. Paper chromatography of the resulting syrup indicated the presence of a considerable amount of sedoheptulose.

Attempts to prepare the dibenzylidene derivative of sedoheptulosan from this syrup were unsuccessful. The volemitol present reacted to form a tribenzylidene derivative [11], which inhibited the crystallisation of the derivative of the anhydro sugar. It was found necessary to remove the volemitol before successful preparation of the dibenzylidene sedoheptulosan.

Pure sedoheptulose was isolated from the syrup as follows: A part of the syrup (4 g) was dissolved in water (30 ml) and the resulting solution was freed from organic acids and salts by passage through "Zeokarb 215" and "Amberlite IR-4B" ion-exchangers. One drop of dilute sulphuric acid and a little phosphate were added to the resulting neutral solution, and the mixture was fermented with yeast until gas formation ceased (4 hours). The solution obtained was filtered, boiled, and evaporated on a water bath to yield a syrup (ca. 0.5 g) Chromatography of the syrup before and after fermentation, using ethyl acetate — acetic acid — water (3 : 1 : 3) as solvent, Whatman No. 1 paper, and orcinol — trichloroacetic acid for detection, gave the results shown in Fig. 1. Water saturated phenol as solvent gave similar results.

The volemitol present could be identified by paper chromatography using water-saturated *n*-butanol as solvent and ammoniacal silver nitrate (5 % $AgNO_3$ in ca. 90 % alcohol with excess ammonia) as developing agent.

The sedoheptulose and volemitol were separated by partition chromatography on a column of powdered cellulose (Whatman ashless tablets) using water-saturated *n*-butanol [12]. The fractions were tested for sedoheptulose, mannoheptulose, and volemitol with the reagents mentioned above. A trace of unfermented fructose passed through the column first, and was followed by the fractions containing the sedoheptulose.

The latter fractions were combined and evaporated under reduced pressure, and the residue (ca. 50 mg) was treated with conc. sulphuric acid (one drop) and benzaldehyde (one drop) with shaking for two hours, during which time crystallisation took place. The mixture was set aside in the refrigerator for 24 hours, and the solid was collected, washed with alcohol, water, and hot alcohol, and recrystallised from acetic anhydride: m.p. (micro) 242—245°.

This material consisted of small rods exhibiting parallel extinction, some prismatic with refractive indices 1 614, 1 624 and some acicular with a higher refractive index, 1 640 — in agreement with the data found previously for dibenzylidene sedoheptulosan [6].

SUMMARY

Partition chromatographic methods have demonstrated the presence of sedoheptulose, fructose, glucose, and saccharose, and the probable presence of mannoheptulose in dried root stock of *Primula elatior* (L) Hill. This is the first occasion on which sedoheptulose has been identified in a plant which is not a member of the family *Crassulaceae*.*

* Since this paper was submitted for publication we have noticed the appearance of a paper by A. A. Benson, J. A. Bassham, and M. Calvin (*J. Am. Chem. Soc.* **73** (1951) 2970). Studying the compounds formed during short periods of photosynthesis in $C^{14}O_2$– atmosphere, they isolated small amounts of labelled sedoheptulose monophosphate from several plants.

The authors are indebted to Dr. R. Galbis, Marianano, Havana, for a supply of Avocado pears for the isolation of mannoheptulose, to Amanuensis Dr. A. Wickström, Pharmaceutical Institute of the University, Oslo for the crystallographic measurements, and to *Norsk Varekrigsforsikringsfond* for a grant.

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ACTA CHEMICA SCANDINAVICA 5 (1951) 1293–1300

Potentiometric and Spectrophotometric Studies on 8-Quinolinol and Its Derivatives. II. 8-Quinolinol Chelate of Calcium in Aqueous Solution

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The equilibria of 8-quinolinol chelates of divalent metals in aqueous solution can obviously be represented as follows: [1]

$$MOx_2(\text{solid}) \rightleftharpoons MOx_2(\text{solution}) \quad (1)$$

$$MOx_n^{(n-2)-} \rightleftharpoons MOx_{n-1}^{(n-3)-} + Ox^- \quad (n = 1,2,\ldots) \quad 2)$$

$$Ox^- + H_3O^+ \rightleftharpoons HOx + H_2O \quad (3)$$

$$HOx + H_3O^+ \rightleftharpoons H_2Ox^+ + H_2O \quad (4)$$

The law of mass action gives

$$[M^{++}][Ox^-]^2 = S \quad (5)$$

$$[MOx_n^{(n-2)-}]/[M^{++}][Ox^-]^n = k_n \quad (n = 1,2,\ldots) \quad (6)$$

$$(H^+)[Ox^-]/[HOx] = K_2' \quad (7)$$

$$(H^+)[HOx]/[H_2Ox^+] = K_1' \quad (8)$$

In the case of chelates which are soluble already in slightly acidic media, it is more convenient to use the equations:

$$[M^{++}][HOx]^2/(H^+)^2 = S/K_2'^2 = K_S \quad (9)$$

$$[MOx_n^{(n-2)-}](H^+)^n/[M^{++}][HOx]^n = k_n K_2'^n = k_n' \quad (10)$$

which are obtained by combining (7) with the other equations.

When alkali hydroxide is added to a solution of 8-quinolinol, the pH of the solution is changed in a way represented in Fig. 1 by the highest curve. If the solution of 8-quinolinol contains calcium salt the course of the titration curve is changed in the way represented by the other curves in Fig. 1. This effect of the calcium salt is obviously due to the formation of $CaOx^+$ and to some extent to the formation of $CaOx_2$. From the measured pH values and stoichiometric concentrations it is possible to calculate the equilibrium constants k_1' and k_2'. For this purpose the following equations are required. From the stoichiometric equations

$$c_M = [M^{++}] + [MOx^+] + [MOx_2] \tag{11}$$

$$c_{Ox} = [Ox^{--}] + [H_2Ox^+] + [HOx] + [MOx^+] + 2[MOx_2] \tag{12}$$

where c_M and c_{Ox} are stoichiometric concentrations, the former that of metal salt, the latter that of 8-quinolinol, and from equations (7), (8), (10) we obtain

$$k_1' + 2([HOx]/(H^+))\ k_2' - c_{Ox}'\ (H^+)/[M^{++}][HOx] = 0 \tag{13}$$

where

$$c_{Ox}' = c_{Ox} - [HOx](1 + K_2'/(H^+) + (H^+)/K_1') \tag{14}$$

The electroneutrality gives

$$2[M^{++}] + [MOx^+] + [H_2Ox^+] + c_B = [OH^-] + [Ox^{--}] + 2\ c_M + c_A \tag{15}$$

where c_B and c_A are stoichiometric concentrations, the former that of sodium hydroxide and the latter that of a strong acid. From equations (7), (8), (11), (12) and (15) we obtain

$$[HOx] = (c_{Ox} + c_A + [OH^-] - c_B)/(1 + 2(H^+)/K_1') \tag{16}$$

and from equations (10) and (11)

$$[M^{++}] = c_M / \left\{ (1 + k_1'[HOx]/(H^+) + k_2'[HOx]^2/(H^+)^2 \right\} \tag{17}$$

By means of equations (13), (14), (16) and (17) we obtain on the basis of the titration curves of Fig. 1 the constants k_1' and k_2'. Each curve refers to one ionic stregth. The results are recorded in Table 1. Each value of k_1' is calculated by the

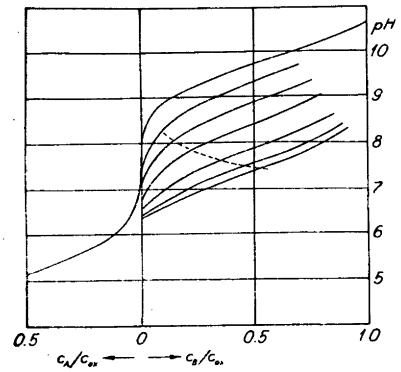


Fig. 1. Effect of calcium chloride on the neutralization curve of 8-quinolinol. $c_{Ox} = 3.38 \cdot 10^{-3} - 3.66 \cdot 10^{-3}$. *From the highest curve downwards:* $c_{Ca} = 0, 0.0027, 0.0087, 0.35, 0.173, 0.46, 0.81$.

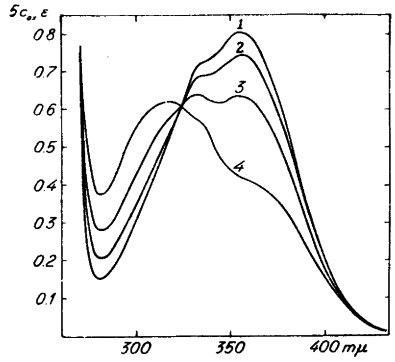


Fig. 2. Ultraviolet absorption spectra of a mixed solution of 8-quinolinol and calcium chloride at some pH values. $c_{Ox} = 6.67 \cdot 10^{-5}$. $c_{Ca} = 5.03 \cdot 10^{-2} \cdot \sqrt{I} = 0.4$. *Curve 1, pH = 9.65. Curve 2, pH = 9.04. Curve 3, pH = 8.69. Curve 4, pH = 8.12.*

Table 1. Potentiometric determination of the constant k_1' *in calcium chloride solutions at 20° C.*

$c_{Ox} \cdot 10^3$	$c_{Ca} \cdot 10^2$	$\sqrt{I}$	pk_1'
3.61—3.66	0.273—0.276	0.109	6.775
3.40—3.45	0.855—0.865	0.171	6.842
3.39—3.44	3.41 —3.47	0.328	6.955
3.38—3.45	17.0 —17.3	0.720	7.135
3.40—3.45	45.5 —46.3	1.18	7.260
3.40—3.44	79.6 —81.0	1.55	7.335

method of least squares from six points of the respective titration curve. For k_2' only an approximate value ($k_2' = 10^{-14}$) was obtained. The results can be presented by the Debye-Hückel equation

$$pk_1' = pk_{1,0}' + \frac{1.514\sqrt{I}}{1 + a\sqrt{I}} \qquad (18)$$

The method of least squares gave for the parameters the values:

$$pk_{1,0}' = 6.63 \text{ and } a = 1.54 \ (20° \text{ C})$$

Using the value $pK_{2,0}' = 9.90$ of Näsänen, Lumme and Mukula [1] we obtain:

$$\log k_{1,0} = pK_{2,0}' - pk_{1,0}' = 3.27 \ (20° \text{ C})$$

Similarly we obtain

$$\log k_{2,0} = 2\ pK'_{2,0} - pK'_{2,0} \sim 6$$

The complex $CaOx^+$ was also investigated spectrophotometrically. In Fig. 2 the spectrum (270—430 mμ) of an 8-quinolinol solution containing calcium chloride is presented at certain pH's. Ammonia-hydrochloric acid buffer solutions were used. The constant k'_1 can be calculated from the values of the molar extinction coefficient ε. For the calculation the equation

$$k'_1\varepsilon_{CaOx^+} - \varepsilon k'_1 - \left\{(\varepsilon - \varepsilon_{HOx^-})(H^+) + (\varepsilon - \varepsilon_{Ox^{--}})K'_2\right\} / [Ca^{++}] = 0 \qquad (19)$$

was derived. In this equation the ε's are molar extinction coefficients as indicated. The values of ε_{HOx} and $\varepsilon_{Ox^{--}}$ were obtained from the paper of Näsänen, Lumme and Mukula [1], the value of K'_2 from the equation [1]

$$pK'_2 = 9.90 - \frac{0.509\sqrt{I}}{1 + 1.018\sqrt{I}} + 0.022I \qquad (20)$$

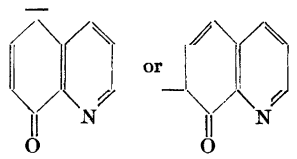
The results are recorded in Table 2. For the calculation of k'_1 the range 340—385 mμ was used. In this case the concentration of 8-quinolinol is so small that the complex $CaOx_2$ can be neglected. The results agree very well with the

Table 2. Spectrophotometric determination of the constant k'_1 at 20° C. $\sqrt{I} = 0.40$ · $c_{Ox} = 6.67 \cdot 10^{-5} \cdot c_{Ca} = 5.03 \cdot 10^{-2}$. $K'_2 = 1.77 \cdot 10^{-10}$.

λ	$\varepsilon \cdot 10^{-3}$	pH	pk'_1
340	1.86	8.69	7.01
	2.08	9.04	
345	1.87	8.69	7.03
	2.13	9.04	
350	1.90	8.69	7.02
	2.20	9.04	
355	1.91	8.69	7.00
	2.23	9.04	
360	1.88	8.69	7.03
	2.22	9.04	
365	1.81	8.69	7.00
	2.13	9.04	
370	1.69	8.69	6.99
	1.98	9.04	
375	1.53	8.69	7.00
	1.79	9.04	
380	1.33	8.69	7.01
	1.56	9.04	
385	1.11	8.69	6.99
	1.30	9.04	mean value 7.01

potentiometric results, as is seen from Fig. 3 where pk_1' is represented as a function of ionic strength.

By means of equation (19) the molar extinction coefficient of $CaOx^+$ can also be calculated. In Fig. 4 this quantity as well as these of HOx and Ox^- are compared at the range 280—400 mμ. The spectra of $CaOx^+$ and Ox^- show a certain similarity but both differ distinctly from that of HOx. The following structure for Ox^- has been suggested [2]



The peaks at 335 mμ and 355 mμ may be assigned to these 7,8 and 5,8 keto-tautomers. The peak at 355 mμ also appears in the spectrum of $CaOx^+$. The maximum at 335 mμ appears in the spectrum of $CaOx^+$ as a shoulder. This resemblance of the spectra of $CaOx^+$ and Ox^- seems to point to a certain similarity of structures.

The results obtained for the solubility equilibria are recorded in Table 3. In Fig. 1 the broken line represents the pH at which precipitation should begin. This system has however a very great tendency to supersaturation and therefore the parts of the curves in Fig. 1 above this limits correspond to a homogenous equilibrium. Before long, however, precipitation begins and the pH suddenly falls. Generally the solubility equilibrium is reached four hours after the beginning of precipitation. However, an interval of about twenty hours was allowed to elapse before the pH was measured.

Table 3. Solubility equilibria of calcium chelate in potassium chloride solutions at 20° C.

$\sqrt{I}$	$c_{Ox} \cdot 10^3$	$c_{Ca} \cdot 10^3$	$c_A \cdot 10^3$	$c_B \cdot 10^3$	pH	log K_S
0.085	3.41	1.61	3.41	5.50	8.94	8.90
0.092	3.84	1.94	3.84	6.27	8.87	8.91
0.117	6.55	3.09	6.55	11.20	8.74	8.93
0.124	3.41	2.73	3.41	5.50	8.61	8.97
0.178	3.41	3.43	3.41	5.55	8.71	9.00
0.269	3.41	3.43	3.41	5.55	8.75	9.08
0.461	3.41	3.43	3.41	5.55	8.78	9.17
0.782	3.41	3.43	3.41	5.55	8.81	9.21
0.952	3.41	3.43	3.41	5.55	8.80	9.20
1.004	3.41	3.43	3.41	5.55	8.78	9.16
1.180	3.41	3.43	3.41	5.55	8.77	9.13

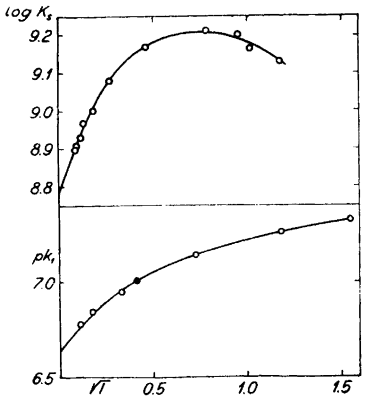


Fig. 3. Constant k_1' in calcium chloride solutions and constant K_S in potassium chloride solutions as a function of ionic strength. ● spectrophotometric, ○ potentiometric method.

For the calculation of K_S the following equations were required. The stoichiometric equations are in this case

$$c_{Ca} = [Ca^{++}] + [CaOx^+] + [CaOx_2] + x \tag{21}$$

$$c_{Ox} = [Ox^{--}] + [HOx] + [H_2Ox^+] + [CaOx^+] + 2[CaOx_2] + 2\,x \tag{22}$$

where x is the decrease in total calcium concentration owing to precipitation. Combining these equations with the electroneutrality equation (15) we obtain also in this case equation (16). From equations (7), (8), (10), (21) and (22) we obtain

$$[Ca^{++}] = \frac{2\,c_{Ca} - c_{Ox} + [HOx](1 + (H^+)\,/\,(K_1' + K_2'\,/\,(H^+))}{2 + k_1'\,[HOx]\,/\,H^+)} \tag{23}$$

The results can be presented by the Debye-Hückel equation

$$\log K_S = \log K_{S,0} + \frac{2.018\sqrt{I}}{1 + a\sqrt{I}} - BI \tag{24}$$

The calculation by the method of least squares gave

$$\log K_{S,0} = 8.76, \quad a = 2.24 \text{ and } B = 0.203 \ (20^\circ\,C)$$

For the solubility product of calcium chelate we thus obtain (*cf.* equations (5) and (9))

$$pS_0 = 2\ pK_{2,0} - \log K_{S,0} = 11.12 \ (20^\circ\,C)$$

In Fig. 3 $\log K_S$ is represented as a function of ionic strength.

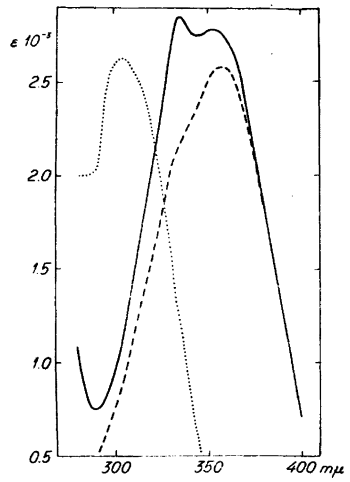


Fig. 4. Molar extinction coefficients of Ox^-, HOx and $CaOx^+$ at the range 280—400 mμ.

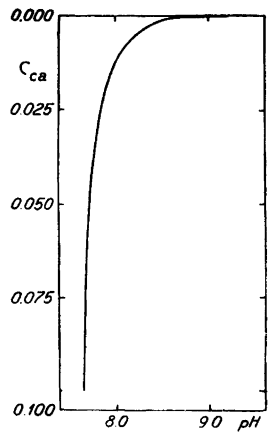


Fig. 5. Beginning of precipitation. $c_{Ox} = 3.78 \cdot 10^{-3}$. $\sqrt{I} = 0.4$.

By means of the values of the equilibrium constants it is now possible to calculate the pH at which the precipitation begins. This value is not constant but is dependent on the calcium and 8-quinolinol concentration (and to a lesser extent also on ionic strength). Neglecting complex formation and H_2Ox^+, which have a relatively slight effect, we obtain from equations (7), (9) and (12)

$$(H^+) = c_{Ox}\sqrt{c_{Ca} / K_S} - K_2' \tag{25}$$

The exact calculation is possible by means of the following equations:

$$(H^+) = [HOx]\sqrt{[Ca^{++}] / K_S} \tag{26}$$

$$[HOx] = c_{Ox} / \left\{1 + K_2' / (H^+) + (H^+) / K_1' + k_1'[Ca^{++}] / (H^+) + 2k_2'[HOx][Ca^{++}] / (H^+)^2\right\} \tag{27}$$

$$[Ca^{++}] = c_{Ca} / \left\{1 + k_1'[HOx] / (H^+) + k_2'[HOx]^2 / (H^+)^2\right\} \tag{28}$$

When $K_2' > (H^+)$ all terms in the denominators of (27) and (28) except the first and second terms can generally be neglected. In this case we obtain

$$(H^+) = c_{Ox}\sqrt{[Ca^{++}] / K_S} - K_2' \tag{29}$$

and

$$[Ca^{++}] = c_{Ca}^2 / (2\ c_{Ca} + k_1'^2 K_S - [Ca^{++}]) \tag{30}$$

Using the values obtained above for the equilibrium constants, we have calculated for some values of c_{Ox} and c_{Ca} the pH values at which precipitation begins. These results are represented in Fig. 5. According to Irving, Ewart and Wilson [3] the solubility of 8-quinolinol in pure water is 3.78 g-mol/l at 20° C. When precipitation begins near the isoelectric point c_{Ox} cannot be higher than this value. From Fig. 5 we see that when $c_{Ox} = 3.78 \cdot 10^{-3}$ and $c_{Ca} = 0.1$ precipitation begins at pH = 7.7. When $c_{Ca} = 0.01$ and the 8-quinolinol concentration is the same, precipitation begins at pH = 8.1. The same curve indicates also how complete precipitation is. For instance, the calculation gives that the total calcium concentration of the solution is $1 .10^{-4}$ when the excess of 8-quinolinol is $3.78.10^{-3}$ and the pH is 9.2. With the same excess of 8-quinolinol the total calcium concentration is $1 \cdot 10^{-5}$ if the pH is 11.2. In analytical practice the excess of 8-quinolinol may in these higher pH's, be somewhat greater than in the case treated, because a part of the 8-quinolinol exists as Ox^-.

Concerning experimental details the reader is referred to the paper of Näsänen, Lumme and Mukula [1].

SUMMARY

The equilibria of 8-quinolinol chelate of calcium in aqueous solution has been studied. The complexity constant of the first complex between calcium and 8-quinolinol ions has been determined potentiometrically and spectrophotometrically as a function of ionic strength. For the thermodynamic constant the value $k_{1,0} = 5.4.10^3$ at 20° C was obtained. For the complexity constant of the second complex the approximate value $k_2 \sim 10^6$ was obtained. The ultra-violet spectrum of the first complex has been determined. It shows a similarity to that of the 8-quinolinol ion, pointing to a certain resemblance of structures. The solubility product of calcium chelate has been determined in solutions of potassium chloride as a function of ionic strength. For the thermodynamic solubility product of calcium chelate the value $S_0 = 6.0 \cdot 10^{-12}$ (20° C) was obtained.

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A Method for Production of High-Intensity, Multi-Fringe Rayleigh Interference Patterns

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The Rayleigh interferometer is characterized by an aperture-splitting device for the production of two coherent beams of light. This is contrary to the Michelson and Jamin types of interferometers, which are amplitude-splitting. This brings about certain characteristic differences in the functioning and applicability between the two types of interferometers.

The Rayleigh interference fringes are situated within the illuminated portions of the diffraction pattern formed when only one of the two aperture slits is in action. Already the first order diffraction bands on either side of the central one, however, are so weak that interference fringes situated there become underexposed when the fringes in the central band are properly exposed. The lateral extension of the interferogram is thus determined by the width of the central diffraction band. It is true that this can be increased at will by decreasing the width of the aperture slits, but only at the cost of light intensity.

The number of interference fringes formed within the central diffraction band depends upon the ratio of the distance between the two aperture slits and their width. The larger this ratio, the greater the number of interference fringes within the central diffraction band. The reader who is not familiar with these facts is referred to the very instructive p. 136 in Jenkins' and White's [1] book.

It may sometimes be advantageous to produce a greater number of interference fringes than is possible by decreasing the width of the two aperture slits or by increasing the distance between them. In the former case, a limit is set by the rapidly decreasing light intensity. Too long exposure times are not practicable in cases where the refractive index course in the object (*e.g.* diffusion, electrophoresis, or ultracentrifuge cells) changes with time. In the

latter case, the fringes will come too close together, and a limit defined by the resolving power of the photographic material is soon reached.

The author has been studying the application of interferometry to electrophoresis and diffusion, and the cells available had both an internal dimension of 3 mm, and 3 mm thick walls. The simplest way of producing Rayleigh interference fringes with these cells was, therefore, to use a single aperture slit 9 mm wide and to allow the cell wall to subdivide this aperture into two vertical slits, each 3 mm wide, and with a distance from centre to centre of 6 mm. A new type of electrophoresis cells with the same dimensions but with the optical walls extending 3 mm beyond the side walls have been constructed for use in interferometry, *cf.* Svensson [2], p. 400, Figs. 1-2. This is in order to give essentially the same optical path length to the rays passing through the cell and to those passing beside the cell.

With these aperture constants, an interferogram is obtained which has three interference fringes within the central diffraction band. The number of fringes can now be increased almost indefinitely in the following way, without altering the spacing between them, and without reducing the light intensity. On the contrary, the resulting multi-fringe pattern is more intense than the three-fringe interferogram in the original arrangement.

This is achieved by replacing the single light source slit by a raster with a certain specified constant. It is easily realized that each slit in the raster will produce a three-fringe interferogram, and that this interferogram will be situated around the optical image of the slit in question. Provided that the lines in the raster are sufficiently close together, the individual interferograms will overlap considerably. Consequently a coincidence criterion for the individual fringes in the different component interferograms will have to be satisfied. On the other hand, if this problem is solved, the resulting multi-fringe interferogram will become much brighter than that resulting from a single slit, and, moreover, all fringes will be equally intense.

The coincidence requirement mentioned above can be formulated in the following way. The raster constant must have such a value that intensity maxima resulting from any component slit in the raster coincide with intensity maxima resulting from the neighbouring component slits. The greatest possible light intensity is gained by using a raster with the smallest constant satisfying this condition.

It remains to derive a mathematical expression for the coincidence condition. The distance between two consecutive fringes resulting from one slit is given by the equation:

$$\delta = \frac{D\,\lambda}{d} \tag{1}$$

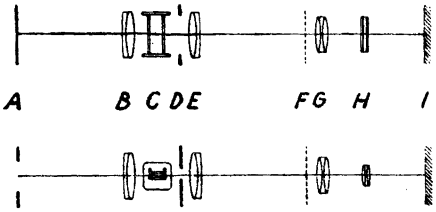

Fig. 1. Optical arrangement. Upper figure: elevation. Lower figure: plan. A light source slit or slits. B, E, and G spherical lenses. C cell. D aperture slit. F image plane of A, site of diagonal edge or slit. H cylindrical lens with a vertical axis. I photographic plate. In elevation, A and F, and C and I, are corresponding image planes. In plan, A, F, and I are corresponding image planes.

where D = the optical distance between the double slit and the photographic plate, λ = the wave-length of the light used, and d = the distance (from centre to centre) between the two aperture slits. We must then demand that the raster constant possesses a value that gives the separation δ between the optical images of neighbouring lines in the raster. We can also transform the coincidence condition to the plane of the raster and state that the raster constant e must have the value δ divided by the magnification factor G of the imagery from raster to plate:

$$e = \frac{D\,\lambda}{G\,d} \tag{2}$$

In the actual optical system used by the author, which is shown and explained in Fig. 1, there is a lens system between the aperture slit and the plate. In addition, the cell wall which subdivides the aperture slit into two equal halves, does not lie in the same plane as the aperture. Consequently, the meaning of the optical distance D between the aperture double-slit and the photographic plate has to be elucidated. A generalized conception of optical distance (there called active distance) was defined by the author in an earlier publication (Svensson [3]). According to that definition, the optical distance between two planes on either side of a lens system is identical with the optical distance between one of the planes and the optical image of the other plane given by the lens system, divided by the magnification factor of the imagery. It was shown there that this definition gives the same result regardless of what plane is brought into focus by the intervening lens system. As a special case of this definition, one recognizes that the optical distance between two corresponding image planes is nil. Another useful corollary is that the optical distance between the focal plane of a lens and a plane on the other side of the lens is equal to the focal length of the lens, regardless of the position of the other plane. It also follows from this generalization that optical distances are not additive.

The optical distance D in equation (2) between the aperture double-slit and the photographic plate would, according to this definition, be identical with the optical distance between the aperture slit and the raster, divided by the magnification factor of the imagery from photographic plate to raster, since these are corresponding image planes. The magnification factor mentioned is, however, $1/G$, hence G disappears in equation (2). Finally, the optical distance between the aperture slit and the raster is equal to the focal length of the lens B, Fig. 1, since the raster is situated in the focal plane of that lens. In addition, the difficulty of the subdividing cell wall and the aperture slit not lying in the same plane disappears, since the optical distance from the raster to the cell is the same as that to the aperture slit. Our final equation thus becomes:

$$e = \frac{f \lambda}{d} \tag{3}$$

where f is the focal length of the lens B, Fig. 1. With numerical figures characteristic of the author's arrangement, $f = 1235$ mm, $\lambda = 5461$ Å, and $d = 6$ mm, one gets a raster constant of $e = 0.1124$ mm. The coincidence condition is satisfied by this value and by every integer multiple of it.

Such a raster has been produced by photographing, with a suitable reduction, a thin thread wound round two identical, mutually parallel screws.* The photographic negative can be used directly as a light source raster.

If the overlapping between the diffraction bands resulting from each individual slit in the raster is slight, the condition (3) is not very critical. The raster constant ought to be exactly known, however, because it defines, rather than equation (1), the spacing between the fringes in the image plane.

If the subdividing wall of the aperture slit is removed, the interference phenomenon of course disappears, but the picture in the image plane does not change. This is easily understood if one remembers that the optical images of the component slits in the raster and the interference fringes coincide with each other.

When a vertical raster of the above specification was used in an electrophoresis experiment, the pattern in Fig. 2 was obtained. The chief advantage of such a pattern in comparison with a three-fringe interferogram obtainable by a single slit is, in addition to the increased light intensity, that one and the same fringe can be followed throughout the whole cell. The counting of

* The author is indepted to Mr. K. E. Larsson for this suggestion and for the mechanical construction.

fringes and the measurement of their fractions can be replaced by a single measurement of distance. Another advantage is as follows.

As described in reference 2, the optical system shown in Fig. 1 is capable of giving records of the refractive index throughout the cell (by interferometry) as well as of its derivative (by the diagonal slit method or other modification of the *Schlieren* method). The reasons why such a combined recording is especially valuable were given in an earlier publication (Svensson [4]). It is thus interesting to know if the multi-fringe Rayleigh patterns described here can be combined with the derivative record.

First, it should be mentioned that the same procedure as was adopted in reference 2 might be employed here too. That would mean that the illumination device should be composed of a horizontal slit and a vertical raster side by side, and the resulting picture would be composed of a derivative pattern and a multi-fringe pattern side by side. However, both these patterns would require an appreciable lateral extension, and the field of view would have to be rather large. Moreover, the interference pattern would have a much greater light intensity, which would cause difficulties when trying to get both patterns in the same exposure. Some other possibilities of producing combined records will be described. The author cannot maintain that these methods are superior in daily routine use, but they are certainly more interesting from an optical point of view.

The results of these procedures are shown in Figs. 3—5. It is seen that we have to do here with an intimate incorporation of the derivative contour into the interference pattern, or vice versa, and no longer with two separate patterns placed side by side. The principle leading to these results is to use mechanical constructions in the plane of the inclined slit (F, Fig. 1) which extinguish the conditions for interference on one side of the derivative contour (Fig. 3), within the derivative contour and the base-line (Fig. 4), and everywhere except in the derivative contour. (Fig. 5).

To obtain these results, it is necessary to use a one-dimensional raster, *i. e.* a number of point sources arranged in a straight horizontal line, with the spacing between the points satisfying the coincidence condition (3). Such a light source can be obtained by placing the vertical raster close behind the edges of a horizontal slit. The author has also used a special slit, one jaw of which was shaped as a saw with about 200 teeth along its 20 mm length. The tips of the teeth were cut off a little in order to make sure completely dark portions between the bright spots in the resulting slit.

Such a slit, composed of a great number of equidistant bright spots, acts essentially, as far as the derivative pattern is concerned, as an ordinary horizontal slit, and it is not possible to see any difference between derivative pat-

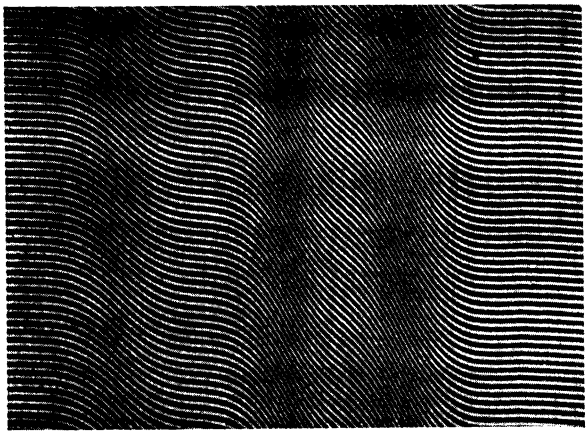

Fig. 2. Part of multi-fringe Rayleigh interference pattern obtained in an electrophoresis experiment with three amino acids. Cell axis horizontal.

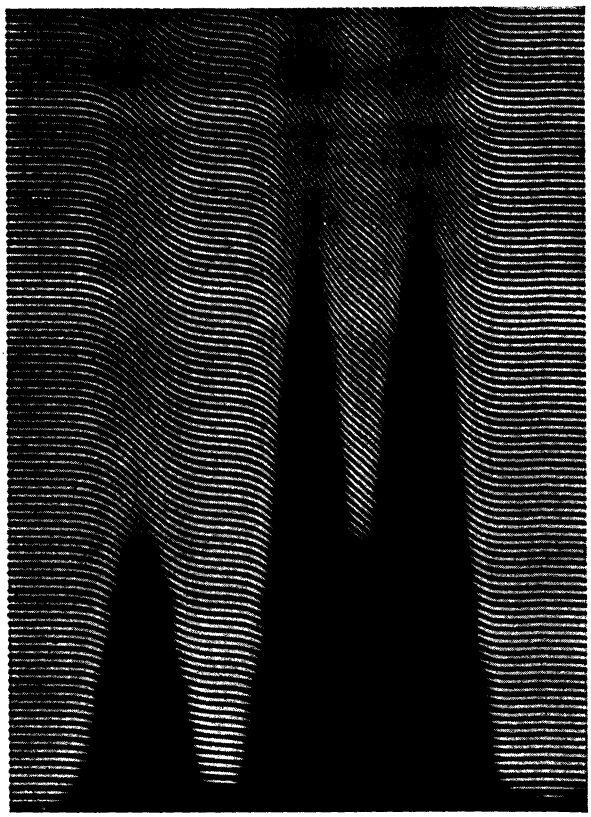

Fig. 3. Combined interference and Schlieren shadow pattern from the same experiment.

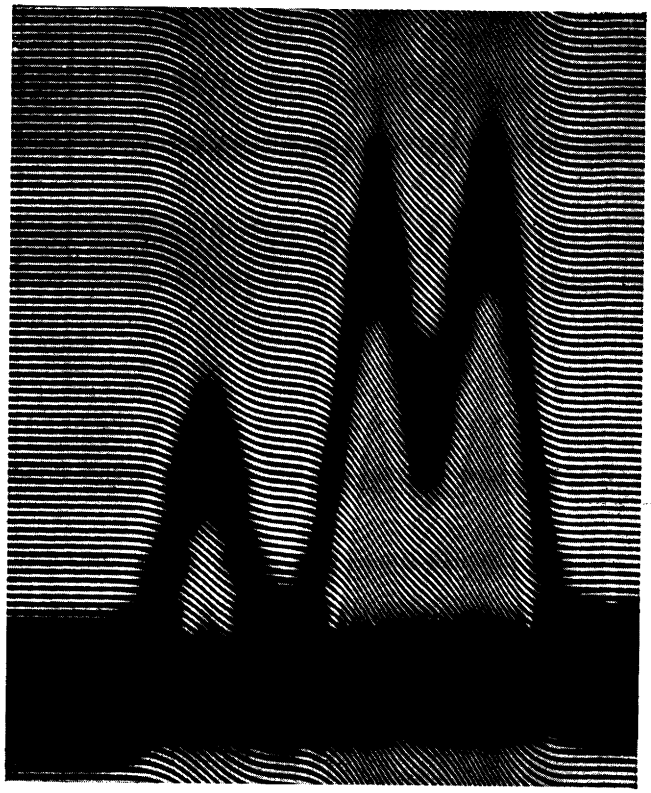

Fig. 4. Combined interterference and diagonal thread pattern from the same experiment. Baseline tilting because of maladjustment of the thread along the optic axis.

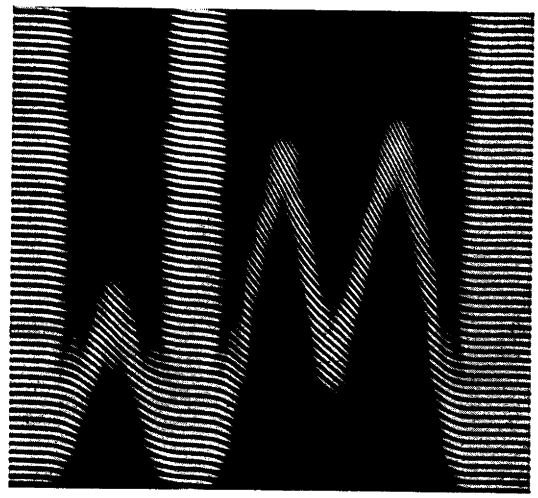

Fig. 5. Combined interference and diagonal slit pattern.

terns obtained by the two types of slits. As the light source also acts as a vertical raster interference fringes will, however, appear in addition in some parts of the picture where the conditions for interference are fulfilled. The mechanical arrangements in the plane of the diagonal slit determine which parts of the derivative pattern that satisfy these conditions.

Let us first consider the case of a diagonal edge in the plane F, Fig. 1. It is known that such an edge will give rise to a *Schlieren* shadow in the image plane, the outline of which is the gradient curve. The field on one side of this contour, therefore, does not receive any light from the cell, while the field on the other side does. The coherent light passing beside the cell is not deflected at all, and will thus illuminate the whole field of view. Interference can only take place in the field that receives light from both the coherent pencils, hence the interference fringes will be extinguished when they reach the derivative contour. The field on the other side of it will only get a weak general illumination of the light from beside the cell (Fig. 3).

Fig. 4 has been obtained by using a diagonal thread in the plane F, Fig. 1. Such an arrangement leads to extinguishing of the interference fringes within the derivative curve and within the base-line, but they are still present on both sides of the curve, and even below the base-line.

Fig. 5, finally, has been obtained by means of one horizontal and one inclined slit in the plane F, Fig. 1 (both slits being in contact with one another). The undeflected light passes through the horizontal slit and will thus illuminate the whole field of view. Light coming from the cell and having thus suffered deflection will, however, only be able to pass through the diagonal slit, and this light is known to form the derivative curve. Hence the conditions for interference will be satisfied only within the derivative curve itself, because this is the only area that receives light from both the coherent pencils. An exception is formed by those portions of the cell which are free from refractive index gradients. Light through these portions is not deflected and passes through the horizontal slit, just as does the light passing outside the cell. As is clear from Fig. 5, this results in the interference fringes extending laterally over the whole field of view in the gradient-free portions of the cell.

The pictures of the type shown in Fig. 3 require the simplest mechanical construction in the plane F, Fig. 1. A thread with a variable angle of inclination, giving pictures like Fig. 4, is also easy to arrange, but different gradient patterns would require different thicknesses of the thread to give the best possible picture. Fig. 5, finally, requires the most complicated mechanical construction, and the result is, moreover, not very useful. No fringe

can be followed throughout the pattern, nor can the fringes be counted along the direction of the cell axis. Fig. 5 is just a peculiarity.

Interferograms where the interference fringes are produced within, on one side of, or on both sides of the derivative contour can also be produced by means of the amplitude-splitting interferometers. This has been described in a patent [5] and will possibly by described in a more accessible place in the future. The amplitude-splitting interferometers correspond to the limiting case of $d = 0$ in the Rayleigh interferometer. This, if it could be realized, would give rise to an infinite lateral distance between the Rayleigh fringes, whereas the distance along the cell axis is dictated by the refractive index course as before. Consequently these fringes will all be perpendicular to the cell axis, and the interferograms corresponding to Fig. 5 would be more than a peculiarity. They would be as useful in practise as the other two types of pictures.

In the author's opinion, however, the curved Rayleigh fringes are superior to Jamin or Michelson fringes because they give detailed information even of fractions of wave-lengths.

SUMMARY

By replacing the single-slit source of light in the Rayleigh interferometer by a raster, interference patterns have been produced possessing a practically unlimited number of fringes of mutually equal and of higher intensity than those given by the original interferometer. The raster constant must satisfy the condition that the optical images of the lines of the raster must coincide with the interference fringes; this condition has been worked out mathematically, and a directly applicable equation for the raster constant has been given. The increased light intensity is supposed to become important in the measurement of rapid diffusion processes and in centrifuge cells.

By placing the vertical raster behind a horizontal slit, a recti-linear row of point sources is obtained. If derivative patterns are produced by the aid of this light source and suitable mechanical constructions in its image plane, interference fringes are formed within the derivative contour, on one side of it, or on both sides. Such patterns are useful in the optical analysis of multi-component boundary systems such as those appearing in electrophoresis, sedimentation, and adsorption analysis.

The possibilities of obtaining similar combined derivative and integral patterns by means of Jamin and Michelson interferometers have been discussed.

This investigation is part of a research program for the development of improved methods of optical analysis of stationary and flowing liquids, which program is generously supported by the Swedish Technical Research Council. Laboratory facilities and additional financial aid has been given by *LKB-Produkter Fabriksaktiebolag*, Stockholm, which is also gratefully acknowledged. The pictures Figs. 2—5 were made by Mr. Karl Odengrim.

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Relationship between Concentration and Sedimentation in Different Preparations of Bovine CO-Hemoglobin

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It has been customary to regard different preparations of the same protein as being generally identical, provided that the preparation methods are more or less the same. It appears probable, however, that due to difficulties in repeating exactly the same preparative procedure from time to time, as well as possible variations in the biological starting material, one may obtain certain variations from preparation to preparation.

Two years ago Jacobsen [1] published an extensive investigation of different preparations of β-lactoglobulin, in which comparisons were made of different preparative methods, as well as of different preparations made at different times but according to the same method. Jacobsen showed that there are appreciable variations among preparations made at different times and from different materials, but was not able to prove any definite differences for preparations from the same material but obtained by different methods.

The present paper concerns itself with comparable ultracentrifugations of six preparations of bovine CO-hemoglobin, all obtained by the same method. Unlike β-lactoglobulin the results here reported seem to indicate, that hemoglobin is affected by handling during the preparative work. This can be explained however by the fact that Jacobsen studied certain chemical properties, whereas the present investigation is concerned only with the relationship between concentration (c) and rate of sedimentation (s), *i.e.* with a function solely dependent on size and shape of the molecules.

It is generally accepted that there is a linear relationship between s and c, providing that one deals with a well defined single component system with approximately spherical molecules. Under these conditions it is then permissible to use the method of least squares to calculate this relationship, which was done here in every case.

The linear equations were set up according to the form

$$s_{20} = s^0_{20} - K \cdot c$$

or according to the formulation used by Enoksson [2],

$$s_{20} = s^0_{20} \ (1 - c \cdot \Phi)$$

where s^0_{20} is the s_{20} value extrapolated to zero concentration, c is the concentration, K the slope of the line and Φ the specific sedimentation volume, or a factor directly proportional to this.

As the differences here obtained are comparatively small, a great difficulty in the present investigation is the estimation of the magnitude of the experimental error. In the appendix there is given an approximate calculation of the accidental error for each run, as well as the method of calculation of the mean error given in the table. As can be seen from the respective formulae, the mean error is a good measure as to how well the points fit a certain line, and will therefore together with the values of Max $\triangle$ (see Table I), give a relative measure of the accuracy.

EXPERIMENTAL

For the preparation of CO-hemoglobin a slightly modified method by Adair and Adair [3] (Method 1) was used. The only difference consisted in increasing the number of washings of the blood curpuscle with 1.5 % sodium chloride from 4 to 6 (seven washings were used for preparation IX). All preparations were made in the cold room at a temperature of about 5° C.

For preparation VI, three different determinations, designated a, b and c, were made at different times and different pH. (See Fig. 1 b, c and d.)

In all subsequent experiments the pH was kept around the isoelectric point in order to avoid too large variations of pH with different hemoglobin concentrations. The buffer then used contained 0.0588-M Na_2HPO_4 + 0.0236-M NaH_2PO_4.

For the last two preparations the blood was immediately divided into two parts, designated A and B, and worked up separately but simultaneously, in order to insure the greatest possible uniformity of conditions.

Part of preparation IX B; designated as IX B (F) was freezedried and the concentration dependence determined.

The experimental solutions, prepared at most two days before the run, were prepared by diluting a concentrated mother liquid with CO-saturated buffer. The concentration of the mother liquid was obtained by dry substance determinations. For prep. V and VI this was done by drying at 40° C to constant weight. For the other preparations the dry weight was obtained from an average of one freeze-drying to constant weight and one drying in vacuo over phosphorus pentoxide at 20° C.

All runs were performed with the same centrifuge (A) and with the same rotor, which was not removed between September 15, 1949 and April 14, 1950. The optical system used has been Lamm's scale method. Hydrogen pressure was 15 mm Hg. The number of revolutions has been determined by means of a stroboscope and a vibrating-reed frequency meter for preps. V and VI and with a LKB-frequency meter for the other preparations.

RESULTS AND DISCUSSION

The results obtained are shown in Table 1. Two different calculations (1) and (2) were made for preparation V. In the first instance, all points were taken into consideration whereas in the second only those between concentra-

*Table 1.**

Prep. No.	Date of prep.	s^0_{20}	$K \cdot 10^{-2}$	$\Phi \cdot 10^{-2}$	Max. Δ	Notes
V (1)	30.10.48	4.60 ± 0.05	17.1 ± 1.6	3.72	0.23	All points taken
V (2)	»	4.78 ± 0.05	21.0 ± 1.2	4.39	0.07	Conc. from 2.0 to 7.0 % Hb
VI, a	24. 1.49	4.59 ± 0.05	18.0 ± 1.4	3.92	0.17**	Run 31.1—16.2 pH ca. 7.0
VI, b	»	4.60 ± 0.06	17.7 ± 1.4	3.85	0.08	Run 17—19.2 pH ca. 5.4—6.2
VI, c	»	4.54 ± 0.02	16.9 ± 0.5	3.72	0.03	Run 21—24.3 pH ca. 7.1
VIII A	6.10.49	4.48 ± 0.02	16.4 ± 0.5	3.66	0.04	Run 20—29.10
VIII B	»	4.50 ± 0.01	15.8 ± 0.3	3.51	0.02	Run 3—5.11
IX A	16. 2.50	4.53 ± 0.02	15.4 ± 0.6	3.40	0.05	Run 8—13.3
IX B	»	4.57 ± 0.03	17.8 ± 0.7	3.89	0.06	Run 16—20.3
IX B (F)	»	4.57 ± 0.03	16.5 ± 0.8	3.61	0.06	Run 1.—12.4

s^0_{20} and Max. Δ are given in Svedberg units.
Max. Δ is the greatest deviation in s_{20} from the corresponding line, shown by any point (see also appendix).

* Note added in proof (9. 12. 51): Recent meltingpoint experiments by Dr Pedersen indicate that the real temperature of the cell was higher than the one recorded by the thermo-element. That means that the absolute values of s^0_{20} given here may be of the order of 5 % too high. It does not however affect the relative temperature nor the discussion here.

** The next highest value of Δ is 0.09.

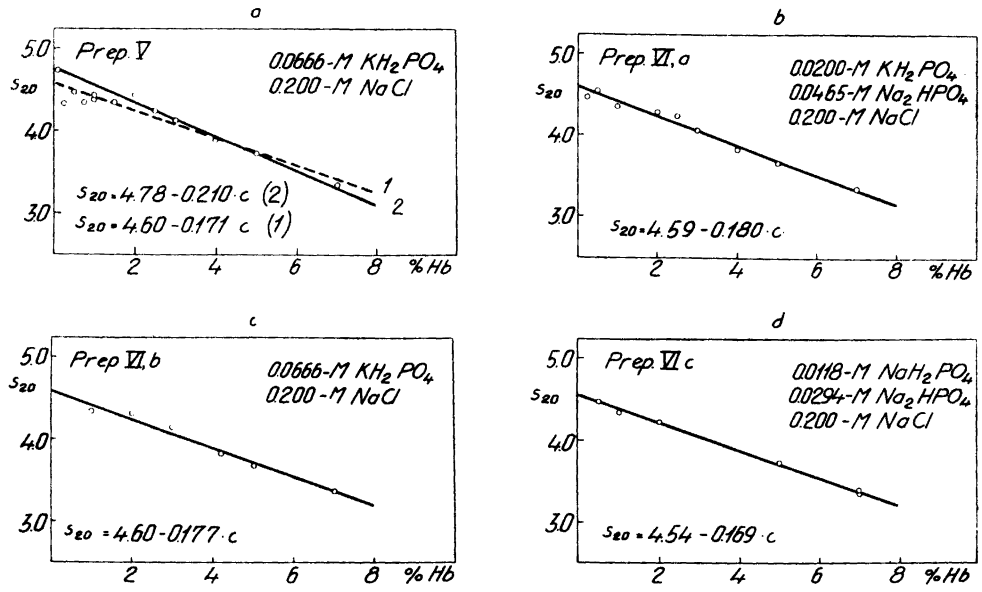

Fig. 1. Relationship between concentration and sedimentation for preparations V and VI. For prep. V two different calculations have been made, in (1) all points are taken into consideration, in (2) only those between concentrations of 2.0 and 7.0 % hemoglobin.

tions of 2.0 and 7.0 % hemoglobin were taken. The reason for this is a noticeable tendency of the points corresponding to the lower concentrations to deviate from the line, as can be seen from Fig. 1a. This may be due either to accidental experimental errors, or to some alteration in form or size of the molecules in the more deluted solutions, as for example, incipient dissociation.

Corresponding deviations could not, however, be observed with the other preparations. Since there is no cause to assume that the conditions for the runs of the preparations V and VI were different from one another the only possible explanation for the unusually large error for V (1) (see appendix) is that there is no linearity when the low hemoglobin concentrations are taken into account.

With the buffer system used in V and IV b, (0.0666 *M* KH_2PO_4 + 0.200 *M* NaCl) the pH of the solutions varies approximately linearly with the concentration of hemoglobin (Fig. 2). If one then assumes that the deviation discussed above is due to incipient dissociation, one could explain it by the fact, that at low protein concentrations, the pH is at the limit of the stability range for the hemoglobin.

On the other hand, however, a comparison between VI a and VI b (see Fig. 1 b and c) indicates that variations in pH such as the ones that comes into

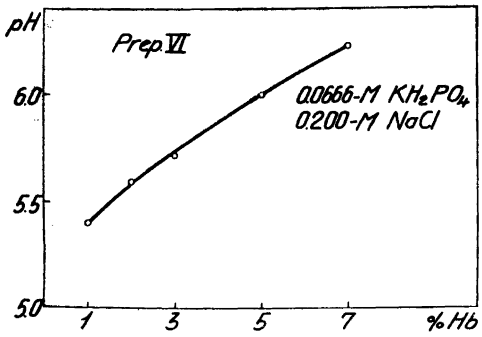

Fig. 2. The variations of the pH with the concentration of hemoglobin for the buffer-system 0.0666-M KH_2PO_4 and 0.200-M NaCl.

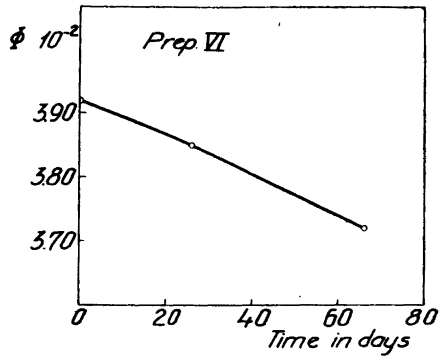

Fig. 3. The specific sedimentation volume Φ plotted against the time elapsed between the preparation and the respective runs.

consideration here do not affect the sedimentation. Further, since V and VI b were run with the same buffer concentrations, the large deviations of V from the rest of the material could be explained only by pH influence if it is assumed that the pH stability varies somewhat from one preparation to the next.

By comparing VI a, b and c, one finds that the value for K has a noticeable tendency to fall. Since the error areas partly overlap, this observation is naturally somewhat uncertain. By plotting, the specific sedimentation volume Φ against the time elapsed between the preparation and the respective runs, one obtains three points on a somewhat curved line (Fig. 3).

Although these observations are also subject to some uncertainty, they were thought to be of some interest, due to the comparatively short time (2 months) in which the alterations took place. During this time the preparation was kept at about 3° C in the presence of toluol.

But even if there should not be any changes according to time, these three determinations for prep. VI will give an idea of the reproducibility of the relationship between concentration and sedimentation for one preparation.

The duplicate preparations (VIII A — B and IX A — B) were made in order to ascertain whether the preparation work as such had any influence on the sedimentation. By comparing the values for s^0_{20} one sees that they agree rather well two by two. There is, however, a difference between the K values of IX A and IX B and possibly also between those for VIII A and VIII B. Since variations in K are due to variations in Φ, they indicate that the preparation procedure can affect the specific sedimentation volume.

Finally, considering all comparable determinations, *i.e.* preparations V (2), VI a, VIII A and B and IX A and B, the following can be concluded. Even after excluding prep. V which evidently is different from the others, the values of the other determinations of s^0_{20} and K are spread over so wide an area, that it seems hardly possible to attribute these variations exclusively to experimental error, especially in view of the relatively small mean errors.

Preparation IX B and the freeze-dried prep. IX B (F) give also the same s^0_{20} but show slightly deviating K-values.

If one assumes that the specific volume of hemoglobin is constant and that the shape of the molecules is approximately the same, the variations in Φ could be explained by assuming that the layer of hydration around the hemoglobin molecules is not constant. Whether these variations depend only on the preparative procedure or if there are also some biological variations to be taken into consideration cannot be answered at the present time. A much larger number of preparations would be necessary to determine the magnitude of the preparative variations.

APPENDIX

Accidental errors in the calculation of s^0_{20} values are due to the following three factors:

1. The number of revolutions as calculated by means of the stroboscope and vibrating-reed frequency meter can be given with an accurasy of $\pm$ 1.7 r.p.s. With a frequency meter the error depends only on the calibration, which in the LKB instrument is done with a tuning fork with 150.0 $\pm$ 0.1 vibrations/sec. Consequently the error in the speed of the ultracentrifuge will be less than $\pm$ 0.5 r.p.s. The corresponding error in s_{20} will then be $\pm$ 0.33 % for the stroboscope and $\pm$ 0.1 % with the frequency meter.

2. The *relative* temperature in the cell. It is difficult to determine how accurately this is recorded by the thermo-element but if no sudden changes in the temperature are registered it is assumed that the value recorded by the instrument and the real temperature value correspond within $\pm$ 0.3° C. To this has to be added an error of about $\pm$ 0.1 °C in reading of the instrument. The total error in the determination of the temperature is therefore $\pm$ 0.4 °C which corresponds to $\pm$ 1.0 % error in s_{20}.

3. Determination of the x-values. Taking into consideration that the great majority of all calculated exposures — with exception of a small minority — give narrow and symmetrical peaks, it can be assumed that the x-values in a diagram can be given to $\pm$ 0.5 mm. Since the scale is 1 : 20 the position in the cell should be determined to within $\pm$ 0.0025 cm, which agrees with the accuracy given by Cecil and Ogston [4] for their diagonal schlieren system according to Philpot. The corresponding error in s_{20} should then be less than $\pm$ 0.5 %. The total error in s_{20} thus should not exceed $\pm$ 1.9 % if the speed is determined by means of the stroboscope or $\pm$ 1.6 % if it is determined by means of a frequency meter.

The error in the determination of the concentration is due partly to the error in the determination of the concentration of the mother liquid and partly to an error of dilution. Estimating each separately with $\pm$ 0.5 %, the total error is $\pm$ 1.0 %. Especially for the later determinations, however, with more experience in the technique, this error is probably too high.

As can be seen from the column "Max $\triangle$" in Table 1, all values from runs performed with a frequency meter lie within the limits of error. For prep. VI a two runs are outside the limits. If for prep. V the concentrations between 2.0 and 7.0 % only are taken into consideration (alternative 2) all runs lie within the limits of error (Max $\triangle$ 0.07). If all points from prep. V are taken into the calculation (alternative 1) as many as four points deviate from the limite (Max $\triangle$ 0.23).

Assuming that the only errors are the deviations from a linear relationship which the separate points show, one obtains the following expression for the mean errors [5]:

$$E_{s_{20}^0} = \pm \sqrt{\frac{\bar{s} \cdot \Sigma \triangle^2}{n-2}}$$

$$E_K = \pm \sqrt{\frac{\bar{K} \cdot \Sigma \triangle^2}{n-2}}$$

where $\bar{s} = \frac{\Sigma c^2}{n \cdot \Sigma c^2 - (\Sigma c)^2}$; $\bar{K} = \frac{n}{n \cdot \Sigma c^2 - (\Sigma c)^2}$

$\triangle_i = s_{20}^0 - K \cdot \gamma_i - \xi_i$; ($\gamma_i$; ξ_i coordinates for a point) and n the number of points. The errors shown in Table 1 have all been calculated according to the above formulas.

SUMMARY

The linear relationship between concentration and sedimentation has been studied for six preparations of bovine CO-hemoglobin, all obtained by the same method. The differences between the slopes of the lines seem to be greater than could be expected from the experimental errors. This indicates that hemoglobin is affected by handling during the preparative work. Further it is shown that there is a small alteration upon storage during the time of two months. A possible explanation in both cases is that the layer of hydration is not constant.

The author wishes to express his thanks to Dr Kai O. Pedersen for suggesting the present problem and for his kind interest and valuable advise. Further thanks are given to Prof. Stig Claesson for his interest and helpful criticism, and to Mr. Evald Hellman and Miss Annalisa Norling for assistance with the calculations.

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Cyclic Compounds from Acetylpyruvic Esters. II

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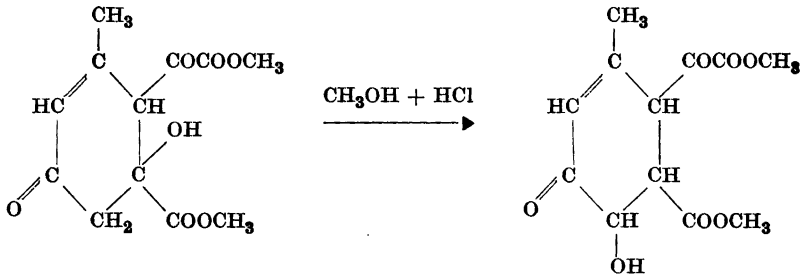
HYDROGENATION AND SUBSEQUENT DEGRADATION OF THE HCl-PRODUCT

In part I of this series Berner and Laland [1] described an interesting intermolecular rearrangement obviously involving the migration of a hydroxyl group in a hydroaromatic ring under the influence of methanolic hydrogen chloride. The reaction was assumed to proceed in the following way:

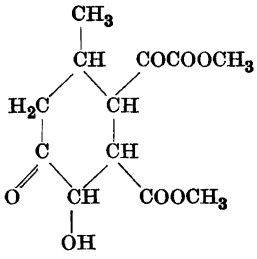


I II

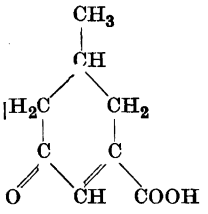
The resulting product II (designated as HCl-product) could be titrated as a monobasic acid while the original dimethyl ester (I) was found to be neutral. The acidity was assumed to be due to a free hydroxyl group in ortho position to a carbonyl group.

When the HCl-product was hydrogenated with PtO_2 as a catalyst in 98 to 99 per cent methanol under a pressure of about 1200 mm it took up one mole of hydrogen. The reaction product could, however, only be obtained as a highly viscous syrupy oil, from light to deep yellow in colour. It did not crystallise on standing for several weeks in a refrigerator but hardened into a glassy mass when kept above phosphorus pentoxide in a desiccator. Obviously it contained free carboxyl groups because of its acidic properties and low methoxyl content.

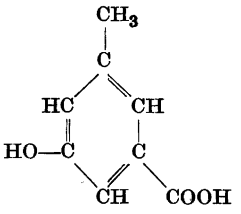
When the hydrogenation was undertaken in anhydrous methanol also one mole of hydrogen was taken up, but the product consisted then of about 65 per cent crystalline material besides the above-mentioned oil. The crystalline product had the composition $C_{12}H_{16}O_7$ and was evidently the normal hydrogenated HCl-product III.



III



IV

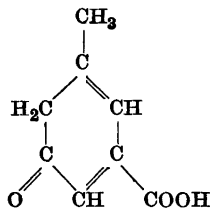


V

It should be mentioned that this compound was very easily hydrolysed. If not absolutely pure it deliquesced in moist air giving a syrupy liquid which could not be brought to crystallisation again and whose methoxyl content was very low. A similar syrupy liquid was obtained if recrystallisation was attempted in methanol that contained from 1 to 2 per cent of water.

On heating with aqueous barium hydroxide the compound III was decomposed into oxalic acid and a substance $C_8H_{10}O_3$ which was assumed to have the formula IV. The same compound could also be obtained from the above-mentioned syrupy hydrogenation product by heating it with aqueous barium hydroxide.

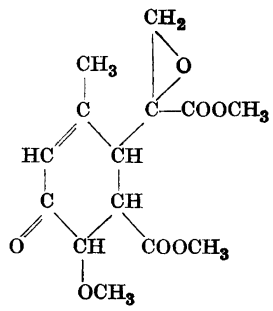
The hydroaromatic compound IV could be dehydrogenated in one operation with N-bromosuccinimide giving an acid $C_8H_8O_3$ which was identified as 3-methyl-5-hydroxybenzoic acid (V). Ziegler [2] writes that the presence of free carboxyl groups interferes with the bromination by means of N-bromosuccinimide. The low yield of our dehydrogenation (about 5 per cent) may be in accordance with this fact. Consistent with the same fact the yield was increased to about 30 per cent when the methyl ester of IV was dehydrogenated. According to Ziegler [3] conjugated dienes such as 1,3-cyclohexadiene are very resistant to N-bromosuccinimide. In our case the bromination most likely will take place with the keto form of IV giving the compound VI which then enolises into the aromatic acid V.



VI

INTERACTION BETWEEN THE HCl-PRODUCT AND DIAZOMETHANE

When treated with diazomethane the HCl-product $C_{12}H_{14}O_7$ gave a compound $C_{14}H_{18}O_7$ to which Berner and Laland [1] assigned the formula VII assuming that a ketomethylation had taken place. It was now found that such a compound was not formed directly and that a nitrogen-containing intermediate could first be isolated. On heating this intermediate easily split off one mole of nitrogen giving the compound $C_{14}H_{18}O_7$. The pronounced thermolability makes it probable that the intermediate is a pyrazoline derivative. From the works of Buchner [4], von Pechmann [5] and others pyrazolines are known to be formed by the action of diazomethane (or ethyl diazoacetate) on unsaturated carbon-carbon double bonds. The author has found no case of ketomethylation described in the literature in which any nitrogen-containing intermediate was isolated.



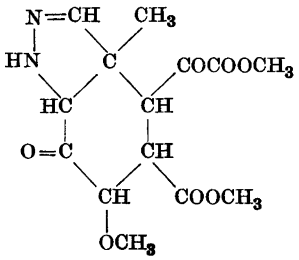
VII

On attempting a catalytic hydrogenation of $C_{14}H_{18}O_7$ no hydrogen was taken up and a carbon-carbon double bond is therefore hardly present in the molecule.

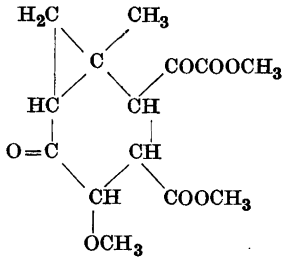
When heated with aqueous barium hydroxide $C_{14}H_{18}O_7$ split off oxalic acid to an extent of 33 per cent based on the assumption that one mole of oxalic acid has been split off from each mole of $C_{14}H_{18}O_7$. This reaction indicates the presence of an α-keto acid in the side chain. It ought to be added that the precipitate obtained on heating with barium hydroxide besides oxalate also contained barium carbonate. Thus a semi-quantitative experiment performed in a nitrogen atmosphere gave a precipitate containing about 30 per cent barium carbonate. The degradation of $C_{14}H_{18}O_7$ must therefore at least have followed two different courses. From the reaction mixture an acid $C_{10}H_{10}O_4$ could be isolated which reduced Tollens reagent, easily gave a precipitate with dinitrophenylhydrazine and contained no methoxyl groups. The elucidation of its structure will, however, make further investigations necessary.

Based upon the above facts the structure VIII is proposed for the nitrogen-containing intermediate $C_{14}H_{18}N_2O_7$.

As the splitting off of nitrogen from a compound containing a pyrazoline ring is known to result in the formation of a cyclo-propane derivative the new formula IX is proposed for the final product from the action of diazomethane on the HCl-product.



VIII



IX

One fact remains still to be explained, namely that $C_{14}H_{18}O_7$ only gives a mono-dinitrophenylhydrazone. This has also been observed by Berner and Laland [1] and is in accordance with their formula (VII). The author hopes later on to be able to give a satisfactory explanation of this apparent discrepancy.

EXPERIMENTAL PART

Hydrogenation of HCl-product

a) In 98–99 per cent methanol:

A solution and dispersion of HCl-product (10 g) in "AnalaR" methanol (150 ml) to which platinum oxide (0.3 g) had been added took up 565 ml hydrogen (1 200 mm Hg) in 110 minutes; calc. for one mole hydrogen 554 ml. After filtering the methanol was evaporated above calcium chloride in a vacuum desiccator.

The syrupy residue was placed in a refrigerator for five weeks with occasional scratching but did not crystallise. Placed in a vacuum desiccator above phosphorus pentoxide for one week it became hard and brittle but weakened again after being exposed to the air for a couple of days.

The product evolved carbon dioxide from a solution of sodium carbonate (2N) and evolved hydrogen chloride and sulfur dioxide from thionyl chloride.

Methoxyl determinations after Zeisel-Fanto:

OCH_3 4.21, 4.22

Other preparations showed different but still low methoxyl contents.

b) In anhydrous methanol:

The commercial methanol (AnalaR) was dehydrated with magnesium methoxide. A solution and dispersion of HCl-product (5.0 g) in methanol (150 ml) to which platinum oxide (0.1 g) had been added took up 290 ml hydrogen (1 200 mm Hg) in one hour; calculated for one mole hydrogen 277 ml. After filtering the methanol was evaporated as before.

The residue crystallised into a hard cake which was broken up and washed thoroughly with dry ether giving a completely white and dry crystalline powder (3.3 g).

The raw product was exposed to moist air for four days and was then completely deliquesced. The oil was placed in a desiccator above phosphorus pentoxide but did not crystallise again. It evolved carbon dioxide from a solution of sodium carbonate (2N).

Methoxyl content 6.91

Another part of the raw product was dissolved in anhydrous methanol (50 ml) and then water (one ml) was added. The solution was placed as usual in a vacuum desiccator above calcium chloride. The residue consisted of a small part of crystalline material besides an oil with strong acidic properties.

The raw product from the hydrogenation was recrystallised twice from acetone-petrol ether. M. p. 126—129°. With alkali the substance gave only a faint yellow colour.

$C_{12}H_{16}O_7$	Calc.	C	52.94	H	5.93	OCH_3	22.81
	Found	»	53.11	»	5.99	»	22.70, 22.95

Degradation of hydrogenated product

a) Crystalline product:

The hydrogenated product (20.0 g) was heated with barium hydroxide (40 g) and water (200 ml) for fifty minutes on a water-bath. During the reaction a salt separated which was found to be barium oxalate. It was dissolved in dilute hydrochloric acid and again precipitated by adding ammonia until pH about 6. The dry salt weighed 16.03 g which is 96.8 per cent of the amount (16.56 g) calculated on the assumption that one mole of oxalic acid had been split off each mole of the hydrogenated product. The oxalic acid was isolated by extracting the solution of the salt in hydrochloric acid several times with ether. A crystalline substance was obtained which had m. p. 101° and gave a crystalline precipitate with calcium ions.

The filtrate from the barium oxalate was acidified with hydrochloric acid and extracted four times with 150 ml ether. Evaporation of the combined and dried extracts gave 3.7 g of a crystalline substance which on recrystallising twice from benzene-carbon tetrachloride gave colourless tables with m. p. 95—96°.

0.1179 g subst. required 7.58 ml 0.1013 N NaOH

$C_8H_{10}O_3$	Calc.	C	62.33	H	6.52	M	154.2
	Found	»	62.33	»	6.56	»	153.4

b) Oily product:

The hydrogenated product (7.7 g) was heated with barium hydroxide (10 g) and water (75 ml) for forty-five minutes on a water-bath. A salt precipitated immediately and was treated and identified as barium oxalate as in the preceeding paragraph. 5.36 g which is 84 per cent of the theoretical amount (6.37 g).

The filtrate from the barium oxalate was acidified with hydrochloric acid and extracted four times with 75 ml ether. Evaporation of the combined and dried extracts gave an oily residue which did not crystallise on scratching, inoculation or after having been kept in a refrigerator for two days. It was therefore dissolved in 96 per cent alcohol and boiled with some charcoal for a few minutes. After evaporation of the alcohol the residue

crystallised partially on scratching giving 0.6 g of a substance which when recrystallised twice from benzene-carbon tetrachloride had m. p. 95—96° and showed no depression in mixed melting point with the substance $C_8H_{10}O_3$ from the preceeding paragraph.

Dehydrogenation of $C_8H_{10}O_3$

$C_8H_{10}O_3$ (1.0 g), N-bromosuccinimide (2.5 g) benzoyl peroxide (ca 5 mg) and carbon tetrachloride (25 ml) was refluxed for six hours with occasional stirring. During the reaction the solution became yellowish brown. After cooling the carbon tetrachloride layer was decanted and the residue extracted with ether. The ether was evaporated very slowly to a small volume and then decanted into another evaporating dish, the separated crystals of succinimide sticking to the first dish. The ethereal solution was then evaporated to dryness and the residue extracted again with a small volume of ether. Again the ether was evaporated and the residue was sublimed in a vacuum giving a white substance with m. p. 206—208° (50 mg). Mixed melting point with 3-methyl-5-hydroxybenzoic acid from another source showed no depression.

Dehydrogenation of methyl ester of $C_8H_{10}O_3$

The ester was prepared by aid of diazomethane. To an ethereal suspension of $C_8H_{10}O_3$ (2 g) was added the calculated amount of ethereal diazomethane (CH_2N_2-content determined by the benzoic acid method). The ester was obtained as an oil which could not be brought to crystallisation.

Methyl ester (2 g), N-bromosuccinimide (5 g), benzoyl peroxide (ca 5 mg) and carbon tetrachloride (25 ml) was refluxed for six hours with occasional stirring. During the reaction hydrogen bromide was evolved. After cooling the carbon tetrachloride layer was decanted and the residue extracted several times with the same solvent. The combined extracts were shaken with some sodium thiosulphate solution and dried over sodium sulphate. The carbon tetrachloride was evaporated on a steam bath whereby more hydrogen bromide was evolved. The brownish, oily residue was distilled at ordinary pressure. At about 200° a clear liquid passed over and crystallised in the receiver. The crystalline product (0.58 g) was recrystallised from dilute alcohol. M. p. 96—97°. Mixed melting point with an authentic sample of methyl 3-methyl-5-hydroxybenzoate showed no depression.

HCl-product and diazomethane

Diazomethane was prepared from nitrosomethylurea (5 g). The clear ethereal solution was cooled in ice and HCl-product (4 g) was added slowly. After the brisk evolution of nitrogen had ceased the solution was kept at room temperature for 17 hours. The separated crystals was filtered and washed with dry ether. Yield 3.0 g.

Thermolabile nitrogen:

$C_{14}H_{18}O_7N_2$	Calc.	8.58
	Found	8.34, 9.02

The nitrogen-containing compound (3.0 g) was recrystallised from methanol. During the recrystallisation nitrogen was evolved. On cooling and scratching colourless crystals

separated. Washed with 1 N sodium carbonate in order to remove unchanged HCl-product and recrystallised again from methanol they had m. p. 135—137° (yield 2.5 g). Berner and Laland [1] found the same m. p.

Attempted hydrogenation of $C_{14}H_{18}O_7$

A solution of $C_{14}H_{18}O_7$ (1.0 g) in methanol (30 ml) to which platinum oxide (0.1 g) had been added was exposed to hydrogen of a pressure of 1 200 mm Hg at 20° for 3 hours, but no uptake of hydrogen could be observed. After filtering and evaporation of the methanol $C_{14}H_{18}O_7$ could be recovered unchanged.

Degradation of $C_{14}H_{18}O_7$

$C_{14}H_{18}O_7$ (2.0 g) was heated with barium hydroxide (4 g) and water (50 ml) for 135 min. During the heating a white precipitate was formed and the solution became gradually red-orange. The precipitate was dissolved in hot dilute hydrochloric acid. During the dissolution a violent evolution of carbon dioxide was observed. The dissolved salt was precipitated again by adding ammonia until pH about 6. The dry salt weighed 0.50 g which is 33 per cent of theory. Oxalic acid was isolated by extracting the solution of the salt in hydrochloric acid several times with ether. A crystalline substance was obtained which under the microscope underwent some change at about 100° and melted at 188—189°; with calcium ions it gave a crystalline precipitate in dilute acetic acid.

The red-orange filtrate from the barium oxalate was acidified with hydrochloric acid. After a couple of hours a crystalline dirty-red substance precipitated. The substance (50 mg) was recrystallised twice from acetone-petrol ether and was still faintly brownish red. M. p. 156° dec.

$C_{10}H_{10}O_4$	Calc.	C	61.80	H	5.20	
	Found	»	62.00	»	5.12	no OCH_3

It evolved carbon dioxide from a solution of sodium carbonate and gave easily a precipitate with dinitrophenylhydrazine in 1 N hydrochloric acid.

A similar experiment with 1.0 g $C_{14}H_{18}O_7$ and 2 g barium hydroxide was performed with a stream of nitrogen bubbling through the reaction mixture. When dissolved in hot dilute hydrochloric acid the precipitate (0.38 g) briskly evolved carbon dioxide. After reprecipitation as before it weighed 0.25 g.

$C_{14}H_{18}O_7$ and dinitrophenylhydrazine

a) Dinitrophenylhydrazine (0.6 g) was dissolved in 2 N hydrochloric acid (50 ml) by heating. The solution was filtered and cooled to 30—40° and $C_{14}H_{18}O_7$ (0.3 g) dissolved in methanol (25 ml) was added. Next day the precipitate was collected and recrystallised from chloroform-gasoline. M. p. 178—179°.

$C_{22}H_{22}O_{10}N_4$	Calc.	N 11.71	Found	N 11.66

b) Dinitrophenylhydrazine (0.3 g) was dissolved in alcohol (200 ml) to which glacial acetic acid (3 drops) had been added. To the filtered solution $C_{14}H_{18}O_7$ (0.19 g) dissolved in alcohol (10 ml) was added. The solution was heated for one hour on a steam-bath and

left by room temperature for 36 hours. Then about 100 ml of the ethyl alcohol was distilled off, the rest being diluted with some water and left in an evaporating dish. The first fraction of crystals which consisted of dark red and light orange ones was discarded. The next fraction in which the light orange crystals were preponderant was recrystallised from chloroform-gazoline. M. p. 177°. Mixed melting point with the preparate from a): 178°.

SUMMARY

Hydrogenation and subsequent degradation of the hydroaromatic so-called HCl-product gave a cyclohexene derivative, which could be transformed into an aromatic compound by means of N-bromosuccinimide.

The reaction between the HCl-product and diazomethane is investigated and a new formula for the resulting product is proposed.

The author wishes to express his gratitude to professor Dr. E. Berner for his kind interest in the work and for many valuable discussions.

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Über die Umsetzung von Formaldehyd mit Allylcarbinol,* ^{3}-Dihydropyran und Butadien.** Synthetische Versuche in der Reihe des Patulins und verwandter Verbindungen. Synthese der Dihydro-desoxy-patulinsäure

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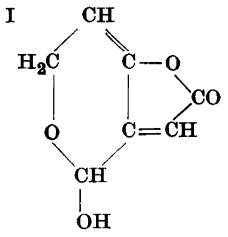
Die Frage der Konstitution des Patulins, die eine Reihe namhafter Forscher [1–7] beschäftigt hat, wurde kürzlich durch Woodward und Singh [8] in Verbindung mit einer Totalsynthese des Antibiotikums im Sinne der Formel I entschieden.

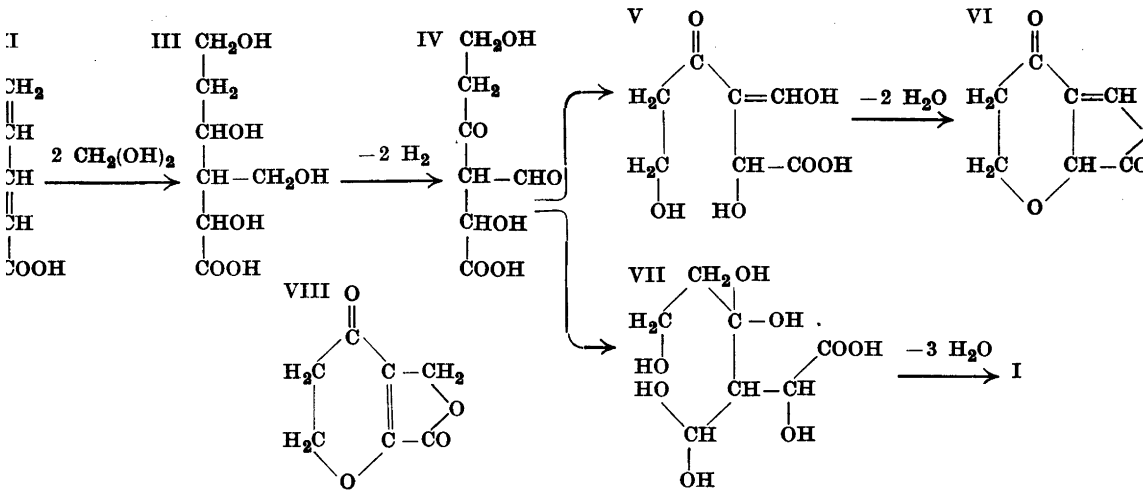


Die Synthese, die vom Tetrahydro-γ-pyron ausgeht, ist zwar ausbeutemässig betrachtet wenig befriedigend, aber durch sie wurden die strukturellen Seiten des Problems überzeugend beleuchtet und somit neue Versuche von anderer Seite mit dem gleichen Ziel überflüssig gemacht — dieses umsomehr, als der therapeutische Wert des Antibiotikums wegen der toxischen Nebenwirkungen umstritten ist.

Die unmittelbare Veranlassung für uns, dennoch eine neue Untersuchung auf diesem Gebiete vorzunehmen, war in erster Linie die, dass es uns nach Sicherstellung der Konstitution besonders reizvoll erschien, die synthetische Leistungsfähigkeit der *Formaldehyd-Olefin-Reaktion* gerade an diesem Naturstoff zu prüfen, nachdem dessen Aufbau auf Grund der von uns erkannten Bauprinzipien dieser Reaktion [9–18] durch Formaldehyd-Olefin-Aufbau an der β-Vinyl-acrylsäure (II) *theoretisch* durchaus möglich erschien. Nimmt man — wie bereits früher angedeutet [19] — an, dass bei der doppelten »Glykolreaktion»

* 3. Mitteilung.

** 10. Mitteilung über Formaldehyd-Olefin-Reaktionen.



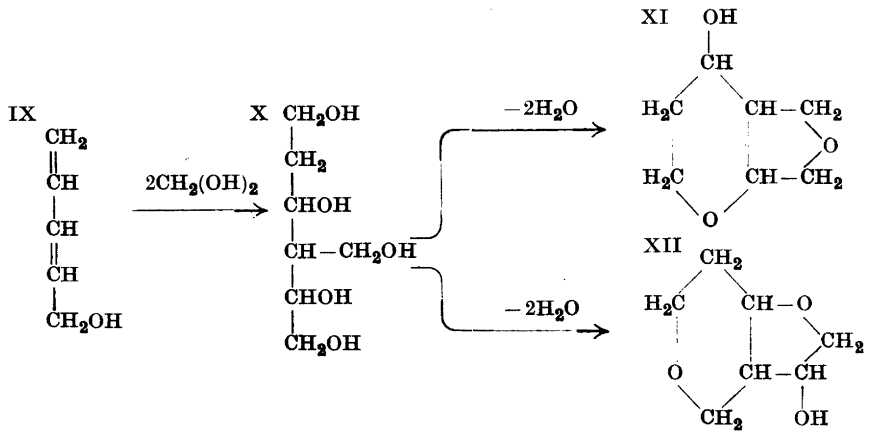
die Carbinolgruppe jeweils die der Carboxylgruppe entferntere Stellung einnimmt, so ergibt sich die Formel einer $\alpha,\gamma,\varepsilon$,-Trioxy-$\beta$-oxymethyl-capronsäure (III). Bei deren Oxydation wäre die Bildung der Ketoaldehyd-Verbindung (IV) und von dieser aus zwei Ringschlussmöglichkeiten denkbar gemäss den Formelübergängen V *→ VI bzw. VII *→ I. Die Formel I entspricht der Patulinformel von Woodward und Singh, die Formel VI der früher von Birkinshaw, Michael, Bracken und Raistrick aufgestellten.

Diese Formeln, insbesondere die Zurückführung der Isomere I und VI auf die Ketoaldehyd-Verbindung IV, vermögen die besonderen Schwierigkeiten bei der Deutung des von den verschiedenen an der Lösung des Konstitutionsproblems beteiligten Forscherkreisen erbrachten experimentellen Materials sinnfällig zu demonstrieren. Gleichzeitig aber legen die Formeln die Frage nahe, ob die Verbindung VI wegen ihrer nahen Verwandtschaft zum Patulin nicht auch in der Natur vorkommt und welche antibiotischen Eigenschaften diese hat, nachdem das Isomere VIII nach Puetzer, Nield und Barry [3] nur eine schwach bakteriostatische Wirkung besitzt.

Die Übertragung der Formaldehyd-Olefin-Reaktion auf Olefin- und Diolefin*säuren* [20] hat nun bisher kein eindeutiges Ergebnis gebracht; nach unseren Erfahrungen scheinen Säuren, wenn überhaupt, sehr viel träger zu reagieren als die ihnen entsprechenden ungesättigten Alkohole. Wir haben daher vorerst weitere Untersuchungen an Olefin- und Diolefin-*alkoholen* in Angriff genommen,

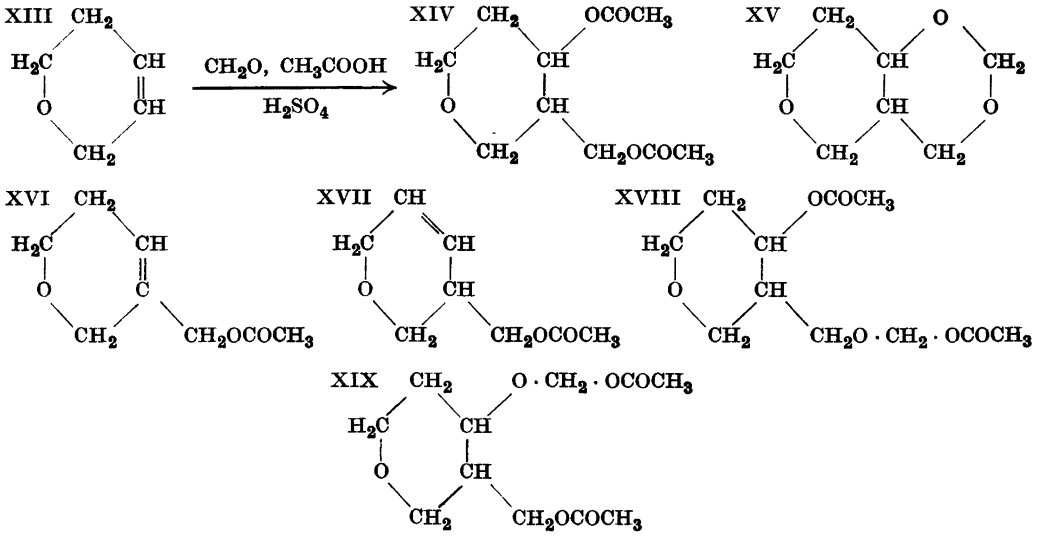
* In den Formeln V und VII wurden die Carbonylgruppen teilweise oder ganz in der Enol- oder Hydratform geschrieben.

über die später berichtet werden soll. Besonders wichtig in diesem Zusammenhange erschien uns das Pentadienol-(1) (IX), dessen Umsetzung mit Formaldehyd zu der Hoffnung berechtigt, im Sinne der Formelreihe IX → X → XI bzw. XII auf direktem Wege zu reduzierten Vorstufen des Patulins zu gelangen:



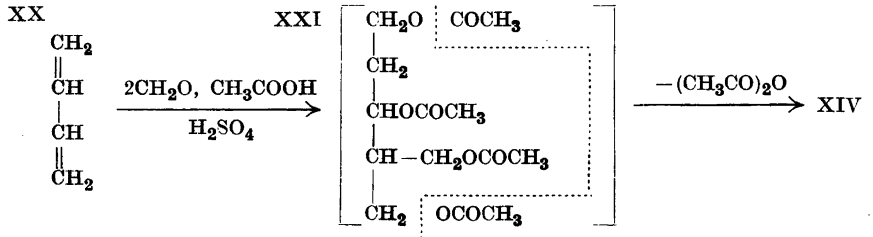
Ein anderer Weg zur Synthese reduktiver Vorstufen der Patulinreihe — und von diesem soll in der vorliegenden Mitteilung die Rede sein — besteht in einer Fortsetzung des von uns beim Äthylen begonnenen und bisher zur Fünfkohlenstoffkette durchgeführten stufenweisen Formaldehyd-Olefin-Aufbaues. Es ist uns nämlich gelungen das *Δ^3-Dihydropyran* (XIII), über dessen Darstellung wir kürzlich berichteten [18], durch Umsetzung mit Formaldehyd in Eisessig-Schwefelsäure zu dem bisher nicht bekannten *3-Acetoxymethyl-4-acetoxy-tetrahydropyran* (XIV) aufzubauen und so den Übergang zur Sechskohlenstoff-Verbindung zu verwirklichen.

Bei der Reaktion entstanden gleichzeitig der *Methylenäther des 3-Oxymethyl-4-oxy-tetrahydropyrans* (XV) und ein *ungesättigtes Acetat*, dem wahrscheinlich die Formel eines *3-Acetoxymethyl-dihydropyrans* mit vorläufig unbekannter Lage der Doppelbindung (XVI bzw. XVII) zuzuschreiben ist. Letzteres ist auch aus dem Diacetat (XIV) durch Destillation mit *p*-Toluolsulfonsäure unter vermindertem Druck erhältlich. Überraschend war die Beobachtung, dass bei der Umsetzung des *Butadiens* mit Formaldehyd in Eisessig-Schwefelsäure im wesentlichen die gleichen Verbindungen wie aus Dihydropyran, nämlich das *Diacetat* (XIV), der *Methylenäther* (XV) und das *ungesättigte Acetat* (XVI bzw. XVII) entstehen:



Beim Kochen des Methylenäthers mit Essigsäureanhydrid-Schwefelsäure enstand neben 3-Acetoxymethyl-4-acetoxy-tetrahydropyran eine gesättigte Verbindung vom Sdp.$_8$ 161—163°, die auf Grund ihrer Eigenschaften wahrscheinlich als ein »*Methylenäther-diacetat*» anzusehen ist und für das vorläufig die isomeren Formeln XVIII und XIX zur Wahl stehen.

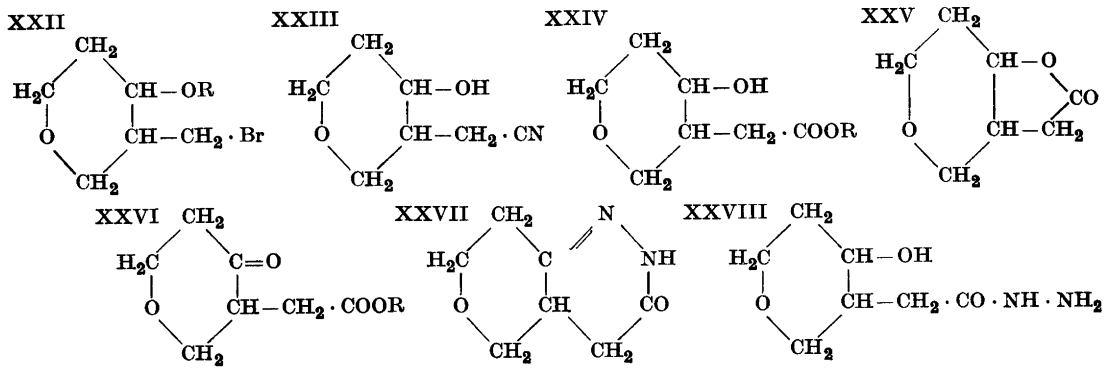
Gresham und Steadman [21] haben gezeigt, dass das 2-Methylpentadien-(1,3) mit Formaldehyd im Sinne einer Diensynthese unter Bildung von 2,4-Dimethyl-Δ^3-dihydropyran reagiert. Beim Butadienversuch musste man folglich auch an eine Dienreaktion unter primärer Entstehung von Δ^3-Dihydropyran denken, jedoch war es uns nicht möglich, Butadien und Formaldehyd im Druckgefäss bei ca. 150° zur Reaktion zu bringen. Wir fassen daher die Butadien-Formaldehyd-Umsetzung bezüglich der Bildung von 3-Acetoxymethyl-4-acetoxy-tetrahydropyran vorläufig als eine doppelte Glykolreaktion auf, wobei der Ausbau der Methylolgruppen im Sinne der Formeln XX→XXI →XIV an den Kohlenstoffatomen 1 und 3 erfolgt, da sonst die Entstehung der erwähnten Reaktionsprodukte nicht möglich wäre. Die Formel XXI bezeichnet ein hypothetisches Intermediärprodukt:



Unter diesen Gesichtspunkten bildet die Tatsache, dass aus dem Dihydropyran- und dem Butadien-Versuch die gleichen Umsetzungsprodukte hervorgehen, bereits ein gewichtiges Argument für die Richtigkeit der dem Diacetat zuerteilten Formel XIV. Der endgültige Konstitutionsbeweis ergibt sich jedoch erst aus dessen weiter unten beschriebenen Umsetzungen.

Wir haben schon mehrfach darauf hingewiesen, dass bei der Umsetzung eines Olefins oder eines geeigneten Olefinderivates mit Formaldehyd nicht allein das um *ein* Kohlenstoffatom reichere glykolische Aufbauprodukt entsteht. Aus diesem kann, wie wir gezeigt haben, während der Reaktion durch Essigsäureabspaltung ein ungesättigtes Acetat hervorgehen, das seinerseits erneut mit Formaldehyd reagiert und so *simultan* den Aufbau des Moleküles um ein *weiteres* Kohlenstoffatom vermittelt. Auf diese Weise müsste man bei der Umsetzung des Allylcarbin-acetates mit Formaldehyd unter den Reaktionsprodukten bereits das 3-Acetoxymethyl-4-acetoxytetrahydropyran vermuten. Nachdem dessen Eigenschaften durch die nun erfolgte unabhängige Synthese hinreichend bekannt waren, konnten wir dieses Diacetat tatsächlich aus den Umsetzungsprodukten des Allylcarbin-acetat-Versuchs isolieren und sicher identifizieren.

Es liegt zu Tage, dass Verbindungen vom Typus des Diacetates (XIV) und des »ungesättigten Acetates» (XVI bzw. XVII) in hervorragendem Masse als Ausgangsverbindungen für den Aufbau des Patulinskelettes oder isomerer Anordnungen in Betracht kommen. Das Diacetat oder das daraus durch Umesterung gewonnene freie *3-Oxymethyl-4-oxy-tetrahydropyran* liessen sich mittels Bromwasserstoffs in das *3-Brommethyl-4-acetoxy-tetrahydropyran* (XXII, R = CH_3CO) bzw. das *3-Brommethyl-4-oxy-tetrahydropyran* (XXII, R = H) überführen:

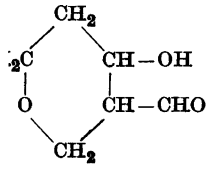


Beide Verbindungen, die nicht rein dargestellt wurden, gaben bei der Umsetzung mit Kaliumcyanid in Alkohol das rohe *Oxynitril* (XXIII) das sich durch

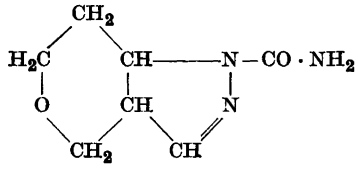
alkoholisches Kali langsam zu der bisher unbekannten *4-Oxy-tetrahydropyranyl-(3)-essigsäure* (XXIV, R = H) vom Schmp. 110—115° bzw. deren *Lacton* (XXV) verseifen liess. Die freie Säure und das Lacton lieferten beim Behandeln mit Diazomethan den *Methylester* (XXIV, R = CH_3) und bei der Oxydation mit Chromsäure die nicht krystallisierende *Dihydro-desoxy-patulinsäure* (XXVI, R = H), die erstmalig von Bergel, Morrison, Ross und Rinderknecht aus Patulin erhalten, von diesen Autoren irrtümlich als eine Methyltetrahydrocomansäure angesehen und deren richtige Konstitution erst durch die späteren Untersuchungen des Züricher [5] und Cambridger/Mass. [10] Arbeitskreises erkannt wurde. Zwecks Identifizierung wurde die Dihydro-desoxy-patulinsäure in das *2,4-Dinitrophenylhydrazon*, den *2,4-Dinitrophenylhydrazon-methylester* und das *Pyridazinonderivat* (XXVII) übergeführt. Die Oxysäure (XXIV, R = H) oder deren Methylester (XXIV, R = CH_3) reagieren mit Hydrazinhydrat unter Bildung des *Hydrazides* (XXVIII). Die weitere Untersuchung der Oxysäure (XXIV, R = H) behalten wir uns vor.

Versuche, das 3-Oxymethyl-4-oxy-tetrahydropyran vorsichtig zu oxydieren, haben bisher nur in schlechter Ausbeute zu einer unreinen Carbonylverbindung geführt, die mit Semicarbazid und 2,4-Dinitro-phenylhydrazin krystallisierte Derivate gibt. Bei der Carbonylverbindung handelt es sich vermutlich um den *4-Oxy-tetrahydropyranyl-(3)-aldehyd* (XXIX):

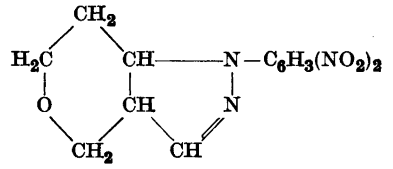
XIX



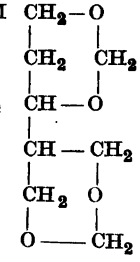
XXX



XXXI



XXXII



Die Analysen von dessen *Semicarbazid-* bzw. *2,4-Dinitro-phenylhydrazin-Derivat* stimmen auf die *Pyrazolinverbindungen* der Formeln XXX bzw. XXXI.

EXPERIMENTELLER TEIL

(Unter Mitarbeit von Gunnar Aksnes und Eirik Hidle.)

Isolierung des 3-Acetoxymethyl-4-acetoxy-tetrahydropyrans (XIV) aus dem Reaktionsgemisch des Allylcarbinacetat-Formaldehyd-Ansatzes. Durch erneute sorgfältige Fraktionierung der höhersiedenden Anteile des früher [17] beschrieben Allylcarbinacetat-Formaldehyd-Ansatzes erhielt man 25,1 g einer beim $Sdp._8$ 130—131° übergehenden farblosen Flüssigkeit, V. Z. 506,6, J. Z. 4,0, n_D^{19} = 1,4552. Bei dieser Fraktion handelt es sich um

3-Acetoxymethyl-4-acetoxy-tetrahydropyran. Durch Umesterung gewann man daraus: *3-Oxymethyl-4-oxytetrahydropyran*, $Sdp._1$ 140—141°. Dessen *Bisphenylurethan*, Schmp. 145°, gab mit einem authentischen Präparat keine Schmelzpunktsdepression.

Überführung des 1,3-Methylenäthers des Pentantriol-(1,3,5)-monoacetates in Pentantriol-(1,3,5)-triacetat. 50 g »Methylenäther-acetat» wurden nach Olsen und Padberg[9] mit 50 g Essigsäure und 7 ml konz. Schwefelsäure 3 Stden. unter Rückflusskühlung gekocht. Man erhielt 34 g Pentantriol-(1,3,5)-triacetat, das zum Pentantriol-(1,3,5) umgeestert wurde. Das daraus gewonnene *Trisphenylurethan* schmolz mit einem Vergleichspräparat unverändert bei 154°.

Umsetzung des Δ^3-Dihydropyrans mit Formaldehyd. 102,5 g Paraformaldehyd wurden in 440 g Eisessig unter Zusatz von 10 ml konzentrierter Schwefelsäure warm gelöst. Zu der warmen Lösung fügte man 205 g Δ^3-Dihydropyran und erwärmte allmählich zum Sieden. Nach 5-stündigem Kochen unter Rückflusskühlung wurde das braunschwarze Reaktionsgemisch zur Neutralisation der Schwefelsäure mit 20 g wasserfreier Soda versetzt und zur Entfernung nicht umgesetzten Dihydropyrans und des grössten Teiles überschüssiger Essigsäure zunächst bei gewöhnlichem Druck destilliert. Hierbei wurden 27,6 g Dihydropyran zurückerhalten. Durch dreimalige Destillation des Rückstandes an der Widmerspirale bei 10 mm Druck gewann man folgende Fraktionen:

			V. Z.	*J. Z.*	
1) Sdp.	69— 79°	4,7 g	316.0	4,6	farbl. Flüss.
2)	79— 85°	9,9 g	107,5	9,8	» »
3)	85— 90°	10,8 g	135,8	28,8	farbl. Flüss., aus der sich Krystalle abschieden
4)	90—100°	1,7 g	202,4	52,8	farbl. Flüss.
5)	100—138°	4,3 g	354,2	23,2	» »
6)	138—141°	160,1 g	432,3	0,5	» »
7)	141—145°	14,0 g	375,9	0,5	» »
8) Rückstand		16,3 g	—	—	gelbbraun. Öl

Bei erneuter Destillation der Fraktion 3) ($Sdp._{10}$ 85—90°) krystallisierte eine farblose Substanz (V. Z. O; J. Z. O), die, aus Essigester umkrystallisiert, bei 54—55° schmolz und beim Erwärmen mit Säure Formaldehyd abspaltet: *Methylenäther des 3-Oxymethyl-4-oxy-tetrahydropyrans* (XV)

$C_7H_{12}O_3$ (144,2)	Ber.	C	58,30	H	8,39
	Gef.	»	58,37	»	8,43

Der Methylenäther liess sich durch Behandeln mit Eisessig-Schwefelsäure glatt in *3-Acetoxymethyl-4-acetoxy-tetrahydropyran* (XIV) überführen. Durch Kochen des Methylenäthers mit Essigsäureanhydrid-Schwefelsäure erhielt man ausser dem Diacetat (XIV) eine *gesättigte Verbindung* vom $Sdp._8$ 161—163°, V. Z. 525. Diese spaltet beim längeren Stehen oder beim Erwärmen mit Säuren oder Alkalien Formaldehyd ab. Bei der Umsetzung mit methylalkoholischer Salzsäure erhielt man *3-Oxymethyl-4-oxy-tetrahydropyran.*

Bei der gesättigten Verbindung vom $Sdp._8$ 161—163° handelt es sich daher wahrscheinlich um eines der beiden isomeren »*Methylenäther-diacetate*» (XVIII bzw. XIX)

$C_{11}H_{18}O_6$ (246,3)	Ber.	C	53,65	H	7,37
	Gef.	»	53,51	»	7,56

In der *Fraktion 4)* ($Sdp._{ca.\ 10}$ 90—100°) ist gemäss dem Siedepunkt und der ermittelten Jodzahl wahrscheinlich das *ungesättigte Acetat* (XVI bzw. XVII) enthalten.
Die *Fraktion 6)* ($Sdp._{ca.\ 10}$ 138—141°) wurde zur Analyse erneut destilliert: $Sdp._{14}$ 147°: *3-Acetoxymethyl-4-acetoxy-tetrahydropyran* (XIV)

$C_{10}H_{16}O_7$ (216,2)	Ber.	C	55,54	H	7,46
	Gef.	»	55,49	»	7,50

Durch Umesterung mit methylalkoholischer Salzsäure erhielt man daraus das *3-Oxymethyl-4-oxy-tetrahydropyran*, $Sdp._1$ 140—143°, $Sdp._{12}$ 158—160°, als dickflüssiges Öl, $n_D^{22.5} = 1{,}4840$.

Bisphenylurethan Schmp. 145°

$C_{20}H_{22}O_5N_2$ (370,4)	Ber.	C	64,85	H	5,99	N	7,56
	Gef.	»	64,85	»	6,04	»	7,56

Umsetzung des Butadiens mit Formaldehyd. 138 g Paraformaldehyd wurden in einer Mischung von 550 g Eisessig und 15 ml konzentrierter Schwefelsäure warm gelöst. In die im siedenden Wasserbad erhitzte Lösung leitete man unter Rückflusskühlung Butadiengas (dargestellt nach Olsen [15] aus Butandiol-(2,4)-diacetat) bis zu einer Gewichtszunahme von 93,3 g ein. Im Anschluss hieran wurde 1/2 Stunde zum Sieden erhitzt, die Schwefelsäure durch Zusatz von 30 g wasserfreier Soda neutralisiert und die Hauptmenge überschüssiger Essigsäure abdestilliert. Der Rest wurde unter vermindertem Druck vom Salz abdestilliert. Man erhielt 239,2 g Destillat vom $Sdp._{10}$ 42—134°, das nach dreimaliger Fraktionierung folgende Fraktionen gab:

				V. Z.	*J. Z.*	
1)	$Sdp._{10}$	80— 84°	59,1 g	73,0	0,5	Farbl. Flüssigkeit, die bald krystallisierte
2)	»	84— 91°	1,4 g	—	—	—»—
3)	»	91—137°	2,0 g	—	—	Farbl. Flüssigkeit, die Krystalle enthielt
4)	»	137—139°	44,9 g	410,7	0,7	Farbl. Flüssigkeit
5)	Rückstand		12,2 g	—	—	Gelbbraun. Öl

Die aus der *Fraktion 1)* ($Sdp._{10}$ 80—84°) durch Absaugen erhaltene krystalline Substanz wurde zweimal aus Äther umkrystallisiert. Schmelzpunkt und Mischschmelzpunkt mit dem aus Dihydropyran erhaltenen Methylenäther 54—55°, V. Z. 0; J. Z. 0: *Methylenäther des 3-Oxymethyl-4-oxy-tetrahydropyrans (XV)*

$C_7H_{12}O_3$ (144.2)	Ber.	C	58,30	H	8,39
	Gef.	»	58,10	»	8,21

Durch Kochen des Methylenäthers mit 10 %iger Salzsäure wurde das *3-Oxymethyl-4-oxy-tetrahydropyran* erhalten und dieses als *Bisphenylurethan* durch Schmelzpunkt und Mischschmelzpunkt identifiziert:

$C_{20}H_{22}O_5N_2$ (370,4)	Ber.	C	64,85	H	5,99	N	7,56
	Gef.	»	64,92	»	6,02	»	7,45

Die aus der *Fraktion 3)* ($Sdp._{10}$ 91—137°) erhaltene krystalline Substanz wurde mehrfach aus Äther umkrystallisiert. Nadeln. Schmp. 147—148°; V. Z. O: Vermutlich *Bismethylenäther* (XXXII)

$C_8H_{14}O_4$ (174,2)	Ber.	C	55,15	H	8,10
	Gef.	»	55,20	»	8,15

Die *Fraktion 4)* ($Sdp._{10}$ 137—139°) besteht im wesentlichen aus *3-Acetoxymethyl-4-acetoxy-tetrahydropyran* (XIV), das auf oben beschriebene Weise charakterisiert wurde.

Abspaltung von Essigsäure aus 3-Acetoxymethyl-4-acetoxy-tetrahydropyran. 100 g 3-Acetoxymethyl-4-acetoxy-tetrahydropyran wurden mit 60 g p-Toluolsulfonsäure an einer 50 cm langen Raschigkolonne bei etwa 8—10 mm Druck destilliert. Badtemperatur 180—226°. Destillationstemperatur etwa 75°. Man erhielt 63,3 g Destillat, das in Äther aufgenommen und durch Waschen mit gesättigter Sodalösung von Essigsäure befreit wurde. Nach dem Trocknen der Ätherlösung über wasserfreiem Natriumsulfat und Verjagen des Äthers erhielt man nach dreimaliger Destillation ca 12 g einer farblosen Flüssigkeit vom $Sdp._8$ 90—93° von charakteristischem, esterartigem Geruch, V. Z. 310,2, J. Z. 139,0. Bei dieser Verbindung handelt es sich wahrscheinlich um *3-Acetoxymethyl-dihydropyran* (XVI bzw. XVII) (Ber. V. Z. 360, J. Z. 162,7)

$C_8H_{12}O_3$ (156,2)	Ber.	C	61,51	H	7,75
	Gef.	»	61,00	H	7,81

Oxydation des 3-Oxymethyl-4-oxy-tetrahydropyrans. 9,5 g des Diols, in 30 ml Wasser gelöst, wurden portionsweise mit einer Lösung aus 14,2 g Kaliumdichromat, 65 ml Wasser und 10 ml konzentrierter Schwefelsäure versetzt, wobei erhebliche Wärmeentwicklung erfolgte. Das Oxydationsgemisch wurde mehrfach mit Äther ausgeschüttelt. Nach dem Waschen und Trocknen der gesammelten Ätherauszüge erhielt man bei der Destillation 0,5 g eines gelben Öles vom $Sdp._{12}$ 79—90° (Hauptmenge destillierte zwischen 83—85°), das Fehlingsche Lösung reduziert: *4-Oxy-tetrahydropyranyl-(3)-aldehyd* (XXIX) (?). Durch Umsetzung der Substanz mit *Semicarbazid* erhielt man eine farblose Verbindung vom Schmp. 208° (Zers.): *Pyrazolinderivat der Formel XXX* (?)

$C_7H_{11}O_2N_3$ (169,2)	Ber.	C	49,69	H	6,55	N	24,84
	Gef.	»	49,72	»	6,30	»	24,74

Durch Umsetzung der Substanz mit *2,4-Dinitrophenylhydrazin* erhielt man eine Verbindung vom Schmp. 234°: *Pyrazolinderivat der Formel XXXI* (?)

$C_{12}H_{12}O_5N_4$ (292,2)	Ber.	C	49,31	H	4,14	N	19,19
	Gef.	»	49,57	»	4,04	»	18,80

Umsetzung des 3-Oxymethyl-4-oxy-tetrahydropyrans mit Bromwasserstoff. In 46,1 g des Glykols (Sdp.$_{12}$ 158—160°) leitete man unter gleichzeitigem Erwärmen im Wasserbad auf ca 100° trockenen Bromwasserstoff bis zu einer Gewichtszunahme vom 53,5 g ein. Durch mehrmalige Destillation des braunen Reaktionsgemisches an der Widmerspirale erhielt man neben anderen Fraktionen 27,5 g Öl vom Sdp.$_{10}$ 131—134°, das im wesentlichen das *3-Brommethyl-4-oxy-tetrahydropyran* (XXII, R = H) enthalten dürfte. Die Substanz ist unrein

$C_6H_{11}O_2Br$ (195,0)	Ber.	C	36,92	H	5,69	Br	40,98
	Gef.	»	34,75	»	5,29	»	45,05

Umsetzung des 3-Brommethyl-4-oxy-tetrahydropyrans mit Kalimcyanid. 27,5 g des vorstehend beschriebenen Öles vom Sdp.$_{10}$ 131—134° wurden mit 18,3 g Kaliumcyanid und 40 ml abs. Alkohol 5 Stden. gekocht. Die alkoholische Lösung wurde von Salzen abfiltriert und nach Entfernen des Lösungsmittels unter vermindertem Druck destilliert: 1) Sdp.$_{10}$ 115—128°, 3,6 g; 2) Sdp.$_{10}$ 128—135°, 12 g; 3) Sdp.$_{10}$ 135—159°, 5,7 g. Die Fraktionen 2.) und 3.) enthalten das *Oxynitril* (XXIII).

4-Oxy-tetrahydropyranyl-(3)-essigsäure und deren Lacton. Vorstehend beschriebene Fraktionen 1.)—3.) wurden wieder vereinigt und einige Tage mit überschüssiger alkoholischer Kalilauge gekocht, wobei kräftige Ammoniak-Entwicklung zu beobachten war. Das Verseifungsgemisch wurde mit verd. Salzsäure kongosauer gemacht und auf dem Wasserbade weitmöglichst eingedampft. Den etwas klebrigen Eindampfrückstand extrahierte man im Soxhlet-Apparat erschöpfend mit Äther. Schon während der Extraktion schied sich eine krystalline Substanz ab, die abgesaugt wurde (3 g) und bei 108—110° schmolz. Filtrat *A*. Durch mehrmaliges Umkrystallisieren aus Aceton-Benzol erhielt man die reine *4-Oxy-tetrahydropyranyl-(3)-essigsäure* (XXIV, R = H) vom Schmp. 110—115°.

$C_7H_{12}O_4$ (160,2)	Ber.	C	52,49	H	7,55
	Gef.	»	52,46	»	7,29

Durch Eindampfem des *Filtrates A* gewann man ein Öl, das nicht krystallisierte und im wesentlichen aus dem *Lacton der 4-Oxy-tetrahydropyranyl-(3)-essigsäure* (XXV) besteht. Bei einem analogen, etwas grösseren Ansatz wurde dieses Öl destilliert. Man erhielt dabei 18 g eines fast farblosen Öles vom Sdp.$_{0,4}$ 137—142°, V. Z. 357 (ber. 395).

4-Oxy-tetrahydropyranyl-(3)-essigsäure-methylester (XXIV, R = CH_3). 4,2 g des vorstehend genannten Lactons wurden in Methanol gelöst und mit einer ätherischen Diazomethanlösung in üblicher Weise methyliert. Bei der Destillation erhielt man 2,6 g einer farblosen Flüssigkeit vom Sdp.$_{11}$ 135—137°, V. Z. 368 (ber. 322). Der Methylester wurde auch durch Methylierung der freien Säure (XXIV, R = H) hergestellt.

4-Oxy-tetrahydropyranyl-(3)-essigsäure-hydrazid (XXVIII). Durch 1-stündiges Erwärmen des Methylesters mit überschüssigem Hydrazinhydrat in Methanol erhielt man farblose Krystalle vom Schmp. 162—163°

$C_7H_{14}O_3N_2$ (174,2)	Ber.	C	48,30	H	8,10	N	16,08
	Gef.	»	47,89	»	7,98	»	15,98

Dihydro-desoxy-patulinsäure (XXVI, R = H). 1 g 4-Oxy-tetrahydropyranyl-(3)-essigsäure wurde in 6 ml Wasser gelöst und die Lösung portionsweise mit einer warmen Lösung aus 0,448 g Chromsäureanhydrid, 5 ml Wasser und 1,5 ml konz. Schwefelsäure versetzt. Die Mischung blieb über Nacht sich selbst überlassen. Die blaugrüne Flüssigkeit schüttelte man erschöpfend mit Äther aus. Die vereinigten Ätherauszüge wurden über wasserfreiem Natriumsulfat getrocknet und durch Eindampfen vom Äther befreit. Es hinterblieb 0,81 g helles Öl *A*, das im wesentlichen *Dihydro-desoxy-patulinsäure* enthält. Das gleiche Produkt wurde durch Oxydation des Lactons (XXV) erhalten.

2,4-Dinitro-phenylhydrazon Schmp. 187—190°

$C_{13}H_{14}O_7N_4$ (338,3)	Ber.	C	46,16	H	4,17	N	16,56
	Gef.	»	45,87	»	4,16	»	16,86

Dihydro-desoxy-patulinsäure-methylester (XXVI, R = CH_3). Ca 0,6 g des vorstehend beschriebenen Öles A wurde in Methanol mit ätherischer Diazomethan-Lösung methyliert. Das Lösungsmittel wurde verdampft und der Rückstand in 10 ml abs. Methanol aufgenommen: *Lösung B.*

2,4-Dinitro-phenylhydrazon des Methylesters. 5 ml der vorstehend genannten Methanollösung B wurden mit einer warmen Lösung von 2,4-Dinitro-phenylhydrazin in Methanol-Schwefelsäure (Brady's Reagenz) versetzt. Beim Erkalten schieden sich schöne hellgelbe Nadeln aus, die — mehrfach aus Methanol umkrystallisiert — bei 150—151° schmolzen

$C_{14}H_{16}O_7N_4$ (352,3)	Ber.	C	47,73	H	4,58	N	15,94
	Gef.	»	47,94	»	4,73	»	15,80

Pyridazinon-Derivat (XXVII). Die restlichen 5 ml der Methanollösung B versetzte man mit 0,3 ml einer 85 %igen Hydrazinhydrat-Lösung und erwärmte 1 Stde auf etwa 70°. Nach dem Einengen und Erkalten krystallisierte eine farblose Substanz, die durch mehrfaches Umkrystallisieren aus Methanol nicht ganz rein erhalten wurde. Schmp. 156—160°

$C_7H_{10}O_2N_2$ (154,2)	Ber.	C	54,53	H	6,54	N	18,17
	Gef.	»	53,40	»	7,17	»	—

Umsetzung des 3-Acetoxymethyl-4-acetoxy-tetrahydropyrans mit Bromwasserstoff. In 100 g Diacetat leitete man bei Zimmertemperatur trockenen Bromwasserstoff ein bis die Gewichtszunahme 82 g betrug. Nach dreimaliger Destillation an der Widmerspirale erhielt man 62,2 g eines hellen Öles vom $Sdp._8$ 126°, V. Z. 464 (ber. für *3-Brommethyl-4-acetoxy-tetrahydropyran* (XXII, R = CH_3CO) V. Z. 473). Die Substanz ist nicht rein

$C_8H_{13}O_3Br$ (237,1)	Ber.	C	40,52	H	5,53	Br	33,71
	Gef.	»	42,41	»	5,77	»	29,22

Durch Umsetzung dieser Substanz mit Kaliumcyanid in Alkohol und anschiessende alkalische Verseifung des Nitriles wurde *4-Oxy-tetrahydropyranyl-(3)-essigsäure* (XXIV R = H) erhalten.

ZUSAMMENFASSUNG

In Ergänzung früherer Befunde wird gezeigt, dass Olefinalkohole bzw. deren Ester, Olefinäther und Diolefine einem stufenweisen Molekülaufbau durch Formaldehyd in *saurem* Medium zugänglich sind. Es wird erneut bewiesen, dass die Reaktion nicht nach dem Aufbau um *ein* Kohlenstoffatom zum Stillstand kommt; vielmehr kann während der Reaktion aus dem entstandenen Aufbauprodukt durch Essigsäureabspaltung wieder eine Olefin-Verbindung hervorgehen, die ihrerseits *simultan* mit Formaldehyd reagiert und so das ursprüngliche Molekül um ein *weiteres* Kohlenstoffatom vergrössert. — In dieser Weise liefert das Allylcarbinol (C_4) neben 4-Acetoxy-tetrahydropyran (C_5) und Pentantriol-(1,3,5)-triacetat (C_5) das 3-Acetoxymethyl-4-acetoxy-tetrahydropyran (C_6). — Butadien (C_4) und Δ^3-Dihydropyran (C_5) geben bei der Umsetzung mit Formaldehyd die gleichen Reaktionsprodukte. Vorläufig wurden nur die »normalen» Aufbauprodukte (Aufbau um *ein* Kohlenstoffatom pro Doppelbindung), nämlich die bisher unbekannten Verbindungen das 3-Acetoxymethyl-4-acetoxy-tetrahydropyran, der Methylenäther des 3-Oxymethyl-4-oxy-tetrahydropyrans und das 3-Acetoxymethyl-dihydropyran isoliert.

Die früheren und die vorliegenden Ergebnisse zusammenfassend, darf es als erwiesen angesehen werden, dass der Formaldehyd befähigt ist, sich nach dem dargelegten Aufbauprinzip auch in *saurem Medium* zu Polyoxyverbindungen mit längeren Kohlenstoffketten zu kondensieren. Zum Unterschied von der langbekannten Selbstkondensation des Formaldehyds in schwach alkalischer Lösung ist in saurem Milieu zur Einleitung der Reaktion ein geeignetes Olefin oder Olefinderivat erforderlich.

Die synthetische Leistungsfähigkeit der Formaldehyd-Olefin-Reaktion erhellt aus der Natur der Reaktionsprodukte. Im vorliegenden Falle wurde das 3-Acetoxymethyl-4-acetoxy-tetrahydropyran über die bisher unbekannte 4-Oxy-tetrahydropyranyl-(3)-essigsäure bzw. deren Lacton in die Dihydrodesoxy-patulinsäure übergeführt.

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Über die Umsetzung von Formaldehyd mit Erythrol. * Eine neue Synthese des 3,4-Dioxy-tetrahydropyrans

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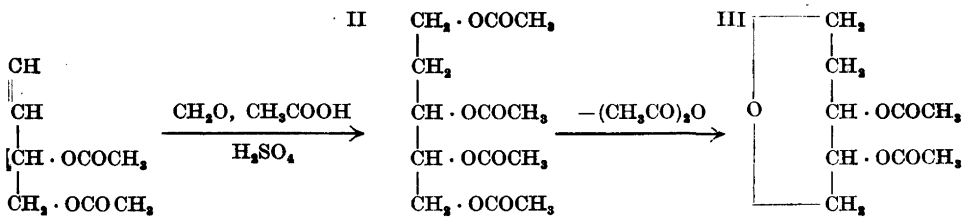
Vor einiger Zeit konnte gezeigt werden, dass das durch Formaldehyd-Olefin-Aufbau aus Allylalkohol leicht zugängliche *Butantriol- 1,2,4* über dessen Formiat oder Acetat in drei verschiedene Olefinderivate der C_4-Reihe überführbar ist, nämlich in *Allylcarbinol, Crotonaldehyd* und *Erythrol* [1]. Dieser Befund erscheint in sofern bemerkenswert, als jede dieser Olefinverbindungen *grundsätzlich* wieder als die Muttersubstanz einer ganz bestimmten durch Formaldehyd bewirkten Aufbaureihe anzusehen ist. Über die Tauglichkeit ungesättigter *Carbonylverbindungen* und *Säuren* als Reaktionspartner der aufbauenden Formaldehyd-Olefin-Reaktion kann gegenwärtig ein abschliessendes Urteil noch nicht gefällt werden. Als zweifelsfrei erwiesen darf es aber gelten, dass Olefin-*alkohole*, *-glykole* und *-ätherverbindungen* dem »Glykolaufbau» durch Formaldehyd ohne weiteres zugänglich sind. Die entstehenden Reaktionsprodukte sind ihrerseits wieder mehrwertige *Alkohole* oder deren innere *Anhydride* (Furan- bzw. Pyranderivate), wobei man hier vernachlässigen kann, dass sich der Aufbau unter den vorliegenden Versuchsbedingungen über die Esterstufe vollzieht.

Während, wie kürzlich berichtet wurde, der Formaldehyd-Aufbau des Allylcarbinols (C_4)[2] über die Zwischenstufen Δ^3-Dihydropyran (C_5)[3], 3-Oxymethyl-4-oxy-tetrahydropyran (C_6) und schliesslich durch den »Kunstgriff» der Nitrilierung in die Patulinreihe (C_7)[4] führte, gelangte man durch Formaldehyd-Aufbau des Erythrols unmittelbar zu einem reduktiven Anhydrid der 2-Desoxypentose-Reihe, nämlich zum *3,4-Dioxy-tetrahydropyran.*

Das *Erythrol* (Buten-(1)-diol-(3,4)) wurde auf dem früher angegebenen Wege aus 3,4-Epoxybuten-(1) durch Hydrolyse mittels verdünnter Salzsäure dar-

* 11. Mitteilung über Formaldehyd-Olefin-Reaktionen.



gestellt. Als Nebenprodukt entstand dabei das von Prévost [5] beschriebene Isomere *Buten-(2)-diol-(1,4), das* als *Bisphenylurethan* und als *2,3-Dibrom-butandiol-(1,4)* charakterisiert wurde. — Bei der Umsetzung des Erythrol-diacetates (I) mit Formaldehyd in Eisessig-Schwefelsäure entstand infolge der Neigung des als Intermediärprodukt anzunehmenden Tetraacetates (II) zur Ringbildung als Hauptprodukt das *3,4-Diacetoxy-tetrahydropyran* (III). Das daraus durch Umesterung gewonnene *3,4-Dioxy-tetrahydropyran* erwies sich in allen Eigenschaften identisch mit dem früher [3] durch Hydroxylierung des Δ^3-Dihydropyrans erhaltenen Glykol. Wie aus den Kennzahlen der im experimentellen Teil wiedergegebenen Destillationstabelle ersichtlich ist, entstanden bei der Erythrol-Umsetzung eine Reihe anderer Reaktionsprodukte, auch ungesättigter Natur, auf deren Isolierung jedoch wegen der geringen Mengen verzichtet wurde.

EXPERIMENTELLER TEIL

(Bearbeitet von Gunnar Aksnes)

Darstellung des Erythrols (Buten-(1)-diol-(3,4)) und des Buten-(2)-diol-(1,4). 130 g 3,4-Epoxybuten-(1) ($Sdp._{760}$ 67°) wurden, wie früher beschrieben, mit stark verdünnter Salzsäure hydrolysiert und aufgearbeitet. Bei der Destillation bei 14 mm Druck erhielt man folgende Fraktionen: 1.) 94—96°, 92 g, $n_D^{20} = 1{,}4595$, $D_4^{20} = 1{,}0500$: *Erythrol;* 2.) 96—130°, 15,1 g: *Zwischenlauf;* 3.) 136—137°, 20,1 g, $n_D^{20} = 1{,}4680$, $D_4^{20} = 1{,}0557$, J. Z. 263,2: *Buten-(2)-diol-(1,4)**; Rückstand 12,1 g braunes Harz.

Bisphenylurethan des Buten-(2)-diol-(1,4) aus Alkohol Schmp. 148—150°.

$C_{18}H_{18}O_4N_2$ (327,3)	Ber.	C	66,28	H	5,56	N 8,59
	Gef.	»	66,25	»	5,53	» 8,61

Bei der Bromierung des Buten-(2)-diol-(1,4) in Chloroform erhielt man farblose Krystalle vom Schmp. 133—135°: *2,3-Dibrom-butandiol-(1,4)* *

$C_4H_8O_2Br_2$ (247,9)	Ber.	C	19,37	H	3,25	Br	64,46
	Gef.	»	19,64	»	3,17	»	64,91

* Prévost gibt folgende Konstanten an für *Buten-(2)-diol-(1,4):* $Sdp._{12}$ 131,5, $n_D^{20} = 1{,}4772$, $D^{20} = 1{,}0687$; für *2,3-Dibrom-butandiol-(1,4):* Schmp. 132,5—133° (korr.).

Umsetzung des Erythrol-diacetates mit Formaldehyd. 92 g Erythrol (vorstehend beschriebenes Präparat) wurden mit 214 g Essigsäureanhydrid in üblicher Weise acetyliert. Das Acetylierungsgemisch vermischte man nach dem Abkühlen ohne weiteres mit einer warmen Lösung von 47,1 g Paraformaldehyd und 5 ml konzentrierter Schwefelsäure in 126 g Eisessig. Die Mischung erhitzte man vorsichtig auf 70°, bei welcher Temperatur Reaktion unter Selbsterwärmung (Temperatur stieg bis 123°) und Verfärbung eintrat. Nach dem Abklingen der exothermen Reaktion wurde ca. 3 Stunden gekocht. Nach Neutralisieren der Schwefelsäure mit 10 g wasserfreier Soda wurde das Reaktionsgemisch dreimal destilliert. Der Destillationsverlauf und die chemischen Kennzahlen der einzelnen Fraktionen sind aus der nachstehenden Tabelle ersichtlich:

Destillations-Tabelle.

Nr.	$Sdp._{0.7}$°C	Ausbeute in g	V. Z.	J. Z.	
1.)	40— 70	3,5	585,3	79,0	
2.)	70— 93	2,4	592,0	98,1	
3.)	93— 95	60,8	524,5	4,9	3,4-Diacetoxy-tetrahydropyran. V. Z. (ber.) 554,5
4.)	95—111	7,5	454,9	12,7	
5.)	111—135	1,1	339,1	14,4	
6.)	135—164	3,2	412,7	15,0	
7.)	164—170	5,6	401,3	3,8	
8.)	170—188	1,5	342,7	6,4	
9.)	Rückstand	3,8	—	—	Harz

Die Hauptfraktion 3) ($Sdp._{0.7}$ 93—95°) besteht aus *3,4-Diacetoxy-tetrahydropyran* (III). Sie wurde erneut zur Analyse destilliert: $Sdp._2$ 105°

$C_9H_{14}O_5$ (202,2) Ber. C 53,46 H 6,98
Gef. » 53,14 » 7,04

Durch Umesterung mit methylalkoholischer Salzsäure wurde daraus eine Fraktion vom $Sdp._{4.5}$ 127—128° erhalten, die im wesentlichen aus *3,4-Dioxy-tetrahydropyran* besteht. *Bisphenylurethan* aus Alkohol Schmp. und Mischschmp. mit authentischem Präparat 204—206°

$C_{19}H_{20}O_5N_2$ (356,4) Ber. C 64,03 H 5,66 N 7,86
Gef. » 64,13 » 5,60 » 7,90

ZUSAMMENFASSUNG

Bei der Umsetzung von Formaldehyd mit Erythrol-diacetat in Eisessig-Schwefelsäure entstand neben anderen, nicht näher untersuchten Verbindungen als Hauptprodukt *3,4-Diacetoxy-tetrahydropyran.*

Dem *Fridtjof Nansen-Fond* danke ich für finanzielle Unterstützung. Frau Birgit Haugen bin ich für die Ausführung der chemischen Kennzahl-Analysen verbunden.

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A Desalting Apparatus for Amino Acids

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The desalting apparatus described by Consden, Gordon and Martin [1] has probably been modified in several laboratories; the apparatus described here has been developed during the last two years, and was demonstrated recently [2]. The original apparatus of Consden, Gordon and Martin may be used with only minor alterations for treating small volumes of amino acid mixtures. We have tried, however, to design an apparatus which could be used for small amounts of fluid, and at the same time had a high desalting capacity without overheating the amino acid solution. It should further be able to remove other salts than chlorides and to prevent any back-diffusion of dissolved chlorine. The apparatus in its final form appears in Fig. 1. The essential modifications introduced concern the construction of the cathode vessel and the anode holder.

Sulphuric acid (0.1 *M*) is passed continuously through the anode compartment during the electrolysis in order to remove the heat evolved by passage of the current. The acid may be cooled first by passing through a glass coil placed in an icebath, but this is not always necessary. The anode compartment is constructed in a manner which allows the sulphuric acid to flow immediately over the dialysis membrane (a piece of Visking casing, size 36/32, which is renewed at least once a day), before it enters an inner tube containing the anode. The anode is a coil of platinum wire reaching to about 1 cm from the end of the inner glass tube. In this manner the flowing H_2SO_4 will carry away all the ions passing through the dialysis membrane into the anode compartment. It will at the same time remove all chlorine produced at the electrode, thus avoiding any back-diffusion of dissolved uncharged chlorine molecules into the compartment containing the amino acid mixture. It is possible to remove ions, such as phosphate and acetate, from the solutions and thus avoid the time consuming conversion into salts of sulphuric acid used by Consden *et al* [1]. The sulphuric acid on its flow from the anode passes a small thermo-

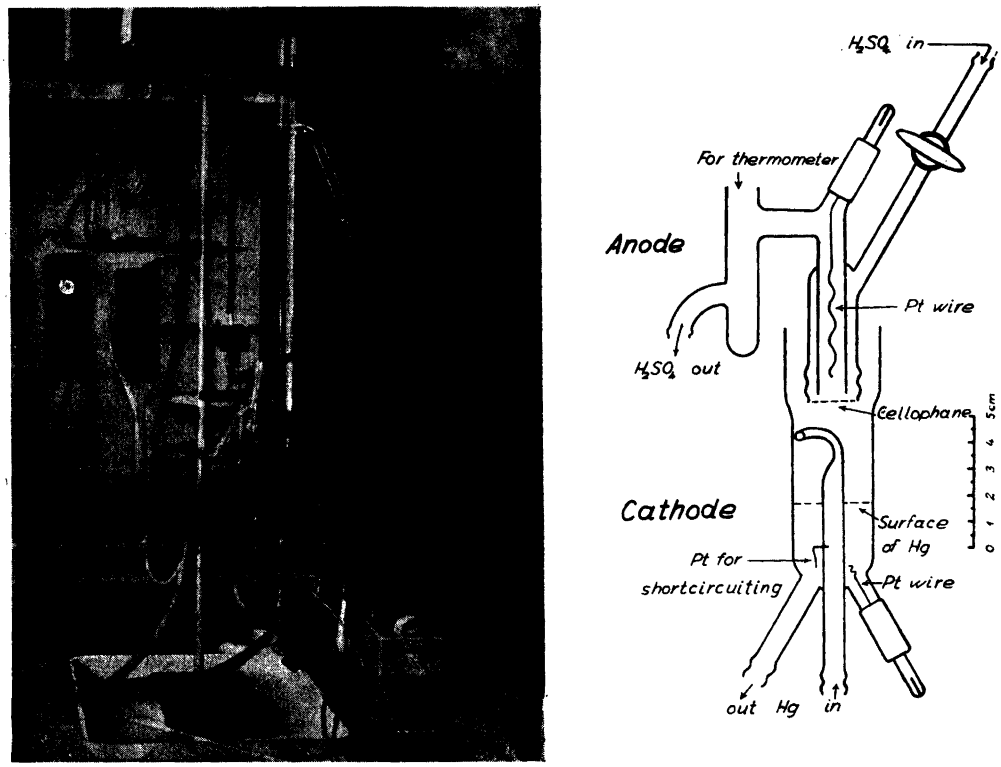

Fig. 1. Construction of electrode compartments of the desalting apparatus and the assembled apparatus with ammeter and buzzer to the right.

meter pocket for controlling its temperature. From there the acid runs to the sink.

The mercury is circulated through the cathode vessel by means of the water lifting pump introduced for this purpose by Consden *et al* [1]. It is, however, not passed directly into the cathode vessel but is introduced through an inner glass tube, which protrudes up into the amino acid solution. The tip of the glass tube is narrowed and bent so that mercury leaves it tangentially to the wall of the vessel and appears as a jet introduced into the solution. In this way the amino acid solution undergoing desalting is given a rotational movement, which helps to mix it, and at the same time is efficiently cooled by the cold, washed mercury. The stream of mercury falls down on the mercury surface and thus continuously furnishes a fresh electrode surface to the solution. In this manner the ions taken up by the Hg during electrolysis are rapidly removed. In order to form an electrical connection between the Hg in the

bottom of the cathode vessel and the Hg introduced into the inner tube a platinum wire is inserted through the walls of the latter. In this manner the Hg system is short-circuited and the appearance of sparks during or at the beginning of the desalting process is avoided. The capacity of the cathode vessel depends upon the amount of Hg present. When the vessel is filled with amino acid solution to about 3 cm above the tip of the inner tube, it contains 10—20 ml solution. By increasing the amount of the mercury in the bottom smaller volumes may be handled. From the cathode vessel the mercury passes in the usual manner to the water lifting pump, from which it flows to a wide vertical glass tube where it settles after being washed by the water from the pump, and finally into the cathode vessel again. The mercury can be removed from the system by means of a side tube at the lowest point of the connection between the vertical settling tube and the inner tube of the cathode vessel. This system contains a much smaller amount of Hg than the apparatus described by Consden *et al.* and may need renewal more often. The water appearing from the settling tube passes through a sedimenting flask in the usual manner in order to prevent Hg escaping from the settling tube into the sink. In order to avoid breckage of the tip of the inner tube and the platinum wires the fluid is emptied and the vessel washed out by means of a pipette provided with a short rubber tubing (5 cm) on its tip.

Solutions of 0.9 per cent NaCl (10 ml) are desalted in 15—20 minutes starting with a current of about 0.7 amps. and ending with about 0.1 amps. Such a desalted solution gives practically no reaction with silver nitrate. Because the time used for carrying out the desalting is only a fraction of that needed in the original apparatus it is convenient to have a buzzer to indicate the endpoint. It can be regulated to indicate this endpoint at any strength of current wanted. The use of an automatic current breaker is not recommended, as the back-diffusion will set in as soon as the electrical potential is removed.

TESTING THE APPARATUS

The desalting capacity of the apparatus was tested with solutions of NaCl, Na-acetate and Na_2HPO_4. Table 1 presents the results of such an experiment.

It is seen that even solutions containing high concentrations of phosphate or acetate may be desalted with reasonable speed in the apparatus here described. This is of importance when the solutions, as is often the case, have been prepared with other salts than chlorides. Paper chromatograms prepared after desalting amino acid mixtures in 1 *M* phosphate or acetate are however usually slightly inferior to those prepared from solutions containing 1 *M* NaCl.

Table 1. Desalting of 5 ml 1 M salt solution diluted to about 10 ml with H_2O.

Salt	Time mins.	Amps.		Resistance (arbitrary in ohms)		H_2SO_4 solution used
		Start	Stop	Start	Stop	liters
NaCl	55	1.6	0.3	30	900	8
Na-acetate	120	1.2	0.3	35	800	15
Na_2HPO_4	120	1.5	0.3	35	1000	15

No measurable loss in amino acid content was observed when a solution was washed with the circulating mercury alone for 2 hours (measured as mg N per ml solution by mikro-Kjeldahl).

The amount of uncharged compounds able to pass through the membrane during a desalting period was determined by measuring the amount of amino acid removed from the solution, when the mercury and the sulphuric acid were circulated in the usual manner but with no potential applied to the electrodes. Table 2 shows such an experiment.

Table 2. 10 ml glycine solution (530 mg in 100 ml 0.9 per cent NaCl). The solution was removed from the vessel, diluted with wash water to an appropriate vol. and an aliquot removed for analysis. No electrical potential applied.

Time	mg N/ml	Diffusion loss	H_2SO_4 used in liters
0	0.980	—	—
$^1/_4$ h	0.920	0.060 mg = 6.1 %	3
$^1/_2$ h	0.855	0.125 mg = 12.8 %	6.5
1 h	0.805	0.175 mg = 17.8 %	9.4
2 h	0.675	0.305 mg = 31.1 %	23.0

The desalting procedure was tried out on selected neutral, acid and basic amino acids. The acids were dissolved in 0.9 per cent NaCl in an amount corresponding to about 0.5 mg N per ml. 10 ml was desalted in about 20 minutes.

The total solution was removed from the desalting apparatus and diluted to 25 ml. 5 ml was used for N determination (Kjeldahl). A control was analyzed similarly. Table 3 presents such an experiment. Aminoethylphosphoric ester was prepared according to Outhouse [3]. The other acids were commercial samples (Hoffmann-La Roche).

Table 3. Loss of nitrogen during desalting.

Amino acid	mg N/2 ml		Loss in %
	before	after	
Glycine	0.90	0.90	0
Aspartic acid	0.64	0.64	0
Arginine	1.00	0.93	7
Taurine	0.96	0.89	7
Aminoethyl phosphoric ester	0.91	0.91	0

Arginine was included in these investigations at the suggestion of Dr Stanford Moore, because he had observed a conversion of arginine into ornithine during desalting in the original apparatus of Consden *et al.* (Stein and Stanford Moore [4]).

Paper partition chromatograms using phenol in NH_3-atmosphere were run on the concentrated desalted solutions and compared with controls. All the acids mentioned, except arginine, showed normal chromatograms. While the recovery of most acids is almost quantitative, it is supposed that the loss of taurine is caused by diffusion of this strong acid against the sulphuric acid in the anode compartment. Arginine behaved otherwise. Here a spot for ornithine was always found, so that the total loss of arginine amounted to more than the observed loss of 7 % in nitrogen. Another basic amino acid, lysine, was tried in other experiments (as dihydrochloride). Here only an insignificant loss was observed.

In a number of experiments the conversion of arginine to ornithine during the desalting was followed by means of the colorimetric method of Chinard [5], which method was kindly made available to us by Dr Moore. To the salt solution used by Dr Moore, containing in 200 ml H_2O: Na_2SO_4 (200 mg), NaCl

(900 mg), K_2HPO_4 (275 mg), $CaCl_2$ (20 mg) and $MgSO_4$ (50 mg), was added 20 mg arginine. 5 ml was diluted to about 10 ml with H_2O and desalted in 15 mins, starting with 0.6 amp. and ending with 0.1 amp. The conversion of arginine into ornithine amounted to 40—50 %. When instead of a solution of different salts only NaCl was used the desalting took about 11 mins and the loss of arginine amounted to 20—25 %. These losses, although large, are considerably lower than those observed by Stein and Moore for the original Consden apparatus. Several experiments have been performed in order to reduce the loss, but so far without much success. The reactions occurring have been found more complicated than expected and the results will be described in a subsequent communication.

SUMMARY

A modified desalting apparatus for amino acids is described *. Some of its advantages are: Small volumes may be used. High desalting capacity without heating the solution. No back-diffusion of chlorine. Anions other than chloride may be removed, *e.g.* phosphates and acetates. Small Hg-surface with reduction of secondary processes (arginine).

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* Messrs. *Buch & Holm, Copenhagen*, deliver an apparatus according to this description.

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Crystal Structure of the 1α3α5 -Triaminocyclohexane Dihydrate and of the Dihydrate and Diammoniate of α Phloroglucitol

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The crystal structure of α phloroglucitol dihydrate was determined by the present authors three years ago [1] with the aim of deciding whether the substance contains the *cis* or *trans* form of 1,3,5-*cyclo*hexanetriol. It was found that α phloroglucitol is indeed the *cis* form of the triol and that it has the αααα configuration (Fig. 1) in the solid dihydrate.

As anticipated hydrogen bonds between oxygen atoms play a dominating role in the structure of the crystal. It was found, however, that such bonds are not formed between alcoholic oxygen atoms, but between such oxygen atoms and water oxygens and between oxygen atoms belonging to neighbouring water molecules. In the structure thus obtained the oxygen atom of each water molecule forms four hydrogen bonds: one to a neighbouring water oxygen atom and three additional bonds to hydroxy oxygen atoms belonging to three *different* phloroglucitol molecules. The arrangement of hydrogen bonds around each water oxygen atom, has trigonal symmetry, it is not very different from that observed in the ice structure, and the bond lengths are of the same magnitude (about 2.76 Å). The mutual positions of the two water molecules and the additional six hydroxy groups linking together six molecules of phloroglucitol is shown in Fig. 2.

The subsequent finding [2] that an isomorphous crystalline diammoniate may be obtained by evaporating the solvent from a solution of α phloroglucitol in liquid anhydrous ammonia made it seem possible that a symmetrical *cis*-1,3,5-triamino*cyclo*hexane might form a dihydrate having the same crystal structure. An attempt to prepare the triamine turned out to be succesfull [3], and X-ray powder photographs indicated that a dihydrate with the expected structure is indeed formed.

Fig. 1. The configuration of a phloroglucitol in crystals of the dihydrate.

When studying this new compound we had a rather limited amount of material at our disposal and all our work had to be carried out in closed vessels in order to protect the substance against the carbon dioxide of the air. We were able to state, however, that the dihydrate gives off its water of crystallisation at 65° C and that the anhydrous amine melts at 148° C. We succeeded also in preparing single crystals of the dihydrate which gave very good rotation and Weissenberg diagrams although the crystals showed poor external development, and had to be kept in sealed capillary tubes, circumstances which made the orientation in the X-ray cameras difficult. We did not succeed in orientating the crystal for rotation about the principal axis, but the diagrams obtained by rotation about the edge of the (face-centered) rhombohedron, the edge of the primitive rhombohedron and the shortest identity period in the basis plane were very satisfactory, the corresponding identity periods being: 12.40 Å, 8.91 Å and 8.62 Å respectively. From these values the identity period along the principal axis could be evaluated and was found equal to 22.23 Å.

The recorded absences of reflexions coincide with those observed for α phloroglucitol dihydrate and the space group is therefore R3c.

The intensities of reflexions are rather similar in both cases indicating that the two substances are indeed isomorphous. The angle φ between the shortest identity period in the basis plane and the projections of the C-O bonds was found to be zero or very nearly zero in the trihydroxy compound, (comp. Fig. 3), a very reasonable finding because in this case only the two sets of O_3-triangles present in the structure (compare Fig. 2) will have the same size. The same argument seems to hold in the case of the triamine structure where a very satisfactory agreement between observed and calculated intensities is obtained with the value $\varphi = 0$ and a model of the triammine molecule having strictly

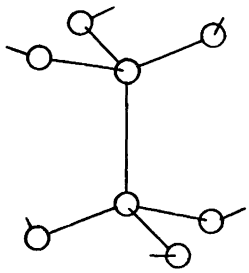

Fig. 2. The assembly of two water oxygens (middle) and six hydroxy oxygens linked together by hydrogen bonds in the α phloroglucitol dihydrate structure.

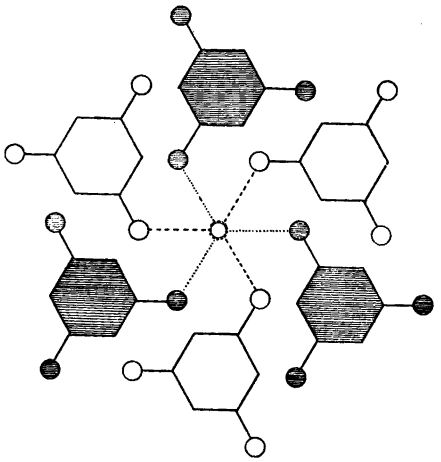

Fig. 3. Projection along the trigonal axis of part of the a phloroglucitol dihydrate structure.

tetrahedral valency angles, a C-C-distance of 1.54 Å and a C-N distance of 1.47 Å.

The only parameters still necessary for the calculation of structure factors are those of the water molecules along the C-axis of the crystal. We have made the assumption that the two water molecules directly connected by hydrogen bonds are symmetrically arranged with respect to the centers of the two nearest cyclohexane rings situated on the same trigonal axis and that the distance of the two oxygen atoms is the same as in the phloroglucitol dihydrate (2.76 Å). This corresponds to a N-H-O bond distanee of 2.93 Å which seems reasonable.

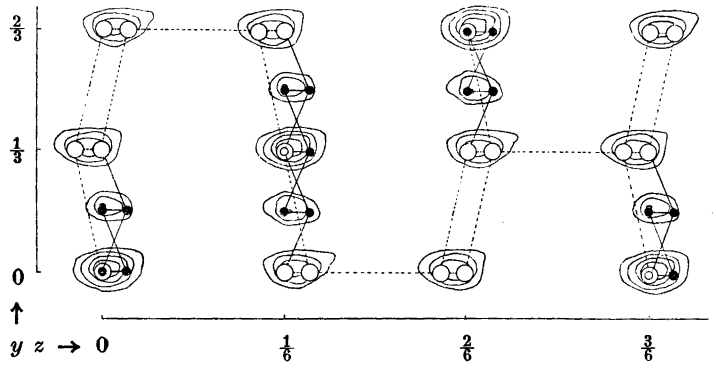

Fig. 4. Map showing the electron density in the triaminocyclohexane dihydrate structure projected along the hexagonal a-axis.

Table 1. Observed and calculated F-values for the triaminocyclohexane dihydrate crystals.

$h\ k\ i\ l$	$\lvert F_{calc.}\rvert$	$\lvert F_{obs}\rvert$	$\lvert F_{obs}\rvert e^{3,33\left(\frac{\sin\vartheta}{\lambda}\right)^2}$	$h\ k\ i\ l$	$\lvert F_{calc.}\rvert$	$\lvert F_{obs}\rvert$	$\lvert F_{obs}\rvert e^{3,33\left(\frac{\sin\vartheta}{\lambda}\right)^2}$
0 0 0 6	225.0	190.8	203.0	0 2 $\bar{2}$ $\bar{10}$	36.7	25.7	32.0
0 0 0 12	126.2	86.4	109.4	0 2 $\bar{2}$ $\bar{16}$	35.6	18.5	30.2
0 0 0 18	27.8	34.4	59.3	0 2 $\bar{2}$ $\bar{22}$	16.9	<11.0	<24.0
0 0 0 24	13.9	10.2	26.7	0 3 $\bar{3}$ 6	82.2	57.2	69.8
0 1 $\bar{1}$ 4	99.3	99.8	104.0	0 3 $\bar{3}$ $\bar{12}$	13.7	17.4	34.0
0 1 $\bar{1}$ 10	84.7	55.9	66.5	0 3 $\bar{3}$ $\bar{24}$	8.7	<9.3	<27.3
0 1 $\bar{1}$ 16	50.9	25.7	40.1	0 4 $\bar{4}$ $\bar{2}$	31.8	26.1	34.4
0 2 $\bar{2}$ 2	58.1	64.3	69.1	0 4 $\bar{4}$ $\bar{8}$	41.2	25.1	35.4
0 2 $\bar{2}$ 8	60.6	52.5	62.5	0 4 $\bar{4}$ $\bar{14}$	21.6	8.1	14.2
0 2 $\bar{2}$ 14	29.1	19.8	29.1	0 5 $\bar{5}$ $\bar{4}$	46.0	38.3	57.1
0 3 $\bar{3}$ 0	102.8	98.5	113.1	0 5 $\bar{5}$ $\bar{10}$	57.2	29.0	50.0
0 3 $\bar{3}$ 6	82.2	57.2	69.8	0 5 $\bar{5}$ $\bar{16}$	36.6	16.1	35.8
0 3 $\bar{3}$ 12	37.7	19.8	28.7	0 6 $\bar{6}$ $\bar{6}$	119.5	61.7	112.2
0 3 $\bar{3}$ 18	13.7	17.4	34.0	0 6 $\bar{6}$ $\bar{12}$	71.4	28.1	61.0
0 4 $\bar{4}$ 4	2.2	<8.6	<11.1	0 6 $\bar{6}$ $\bar{18}$	24.7	9.6	28.3
0 4 $\bar{4}$ 10	28.7	14.0	20.8	0 7 $\bar{7}$ $\bar{2}$	21.1	11.4	23.9
0 4 $\bar{4}$ 16	31.4	8.1	15.8	0 7 $\bar{7}$ $\bar{8}$	18.0	<11.3	<26.2
0 5 $\bar{5}$ 2	18.6	22.9	33.7	0 8 $\bar{8}$ $\bar{4}$	4.0	7.2	20.6
0 5 $\bar{5}$ 8	20.6	19.8	32.0	0 8 $\bar{8}$ $\bar{10}$	13.1	<9.0	<28.2
0 5 $\bar{5}$ 14	20.0	11.4	22.9	0 8 $\bar{8}$ $\bar{16}$	18.0	<5.4	<21.6
0 6 $\bar{6}$ 0	143.5	81.3	140.1	0 9 $\bar{9}$ $\bar{6}$	29.4	<7.5	<26.6
0 6 $\bar{6}$ 6	119.5	61.7	112.2	0 7 $\bar{7}$ $\bar{14}$	14.8	<9.7	<27.8
0 6 $\bar{6}$ 12	71.4	28.1	61.0	0 7 $\bar{7}$ $\bar{20}$	8.9	<4.5	<18.0
0 6 $\bar{6}$ 18	24.7	9.6	28.4	0 5 $\bar{5}$ $\bar{22}$	15.0	<8.4	<27.0
0 7 $\bar{7}$ 4	39.0	16.1	34.3	0 4 $\bar{4}$ $\bar{20}$	2.4	<10.9	<27.2
0 7 $\bar{7}$ 10	46.8	10.9	26.6	0 4 $\bar{4}$ $\bar{26}$	17.3	<5.1	<19.8
0 7 $\bar{7}$ 16	38.8	<8.7	<28.2	0 2 $\bar{2}$ $\bar{28}$	2.3	<5.1	<20.5
0 8 $\bar{8}$ 2	22.0	10.9	28.6	0 1 $\bar{1}$ $\bar{26}$	22.3	<8.6	<26.7
0 8 $\bar{8}$ 8	26.9	7.2	20.6	0 1 $\bar{1}$ 22	17.8	<11.3	<25.6
0 8 $\bar{8}$ 14	16.7	<7.1	<25.4	0 1 $\bar{1}$ 28	13.9	<5.9	<22.0
0 9 $\bar{9}$ 0	32.0	<8.3	<27.6	0 2 $\bar{2}$ 20	1.2	<11.4	<23.3
0 9 $\bar{9}$ 6	29.4	<7.5	<26.6	0 2 $\bar{2}$ 26	15.9	<8.1	<26.1
0 1 $\bar{1}$ $\bar{2}$	57.5	62.7	64.0	0 3 $\bar{3}$ 24	8.7	<9.3	<27.3
0 1 $\bar{1}$ $\bar{8}$	33.2	44.3	49.7	0 4 $\bar{4}$ 22	16.3	<9.7	<27.3
0 1 $\bar{1}$ $\bar{14}$	25.0	29.6	41.7	0 5 $\bar{5}$ 20	2.7	<9.7	<27.8
0 1 $\bar{1}$ $\bar{20}$	6.2	<11.4	<22.4				
0 2 $\bar{2}$ $\bar{4}$	1.3	10.2	11.1				

The intensities were estimated from Weissenberg diagrams using CuK-radiation. Two films were exposed simultaneously inserting a thin aluminium foil between the films. The blackening standard scale was prepared using the

strongest reflexion of the crystal itself as an X-ray source. Varying the exposure time and considering both α and β reflexions the agreement between the results obtained from different pairs of films was very satisfactory.

The agreement between observed intensities of reflexions (o k $\overline{k}$ l) and those calculated from the model described above may be judged from Table 1. The calculated values were obtained using Robertsons f-values, thus disregardening the positions of the hydrogen atoms. The value of the reliability factor is 0.19. The structure factors were also calculated with the aid of James and Brindley's f-curves and introducing a temperature factor equal to $e^{3.33(\frac{\sin\vartheta}{\lambda})^2}$. The reliability factor thus obtained was nearly the same as that obtained in the first case (0.20).

The usefulness of a further examination of the structure using Fourirer methods seemed rather doubtful because the structure does not contain centers of symmetry, but one projection was nevertheless worked out, namely along the hexagonal a-axis. The resulting electron density map is reproduced in Fig. 4 in which the atomic position on which the structure factor calculations were based are indicated by filled and open circles. The general positions of the peaks thus agree very well with the structure described above, but cannot give more detailed informations regarding the value of the parameters. The height of the peaks are not given on an absolute scale.

DISCUSSION OF THE STRUCTURES

According to our findings the structure of the triamino*cyclo*hexane dihydrate corresponds very closely to that of the α phloroglucitol dihydrate and the separation of the two water molecules linked together by a hydrogen bond seems to be nearly the same in both cases (about 2.75 Å). It then follows that the distance between one of those water oxygen atoms and the plane containing the three nitrogen atom linked to it by hydrogen bonds is 0.47 Å and the N-O hydrogen bond distance 2.93 Å. The four hydrogen bonds reaching each water oxygen atom are tetrahedrally distributed in space, but the angles between them are markedly different from the "tetrahedral" angle (109°,5). The angle between the O-O and each of the O-N bonds is only 99°, the angle between two O-N bonds 117°.

Each amino nitrogen atom forms *two* hydrogen bonds to water oxygen atoms. It is obvious, therefore, that the number of hydrogen atoms available for hydrogen bond formation is greater than that necessary in order to establish the number of hydrogen bonds actually present in the structure. It would indeed seem probable that one of the hydrogen atoms of each amino group

Table 2. Lattice constants (hexagonal a and c axes) and hydrogen bond lengths in the three substances.

Substances	a in Å	c in Å	O-H-O bond in Å	O-H-N bond in Å
α phloroglucitol dihydrate	8.22	21.96	2.76	
1,3,5-triamino cyclohexane dihydrate	8.62	22.23	2.76	2.93
α phloroglucitol diammoniate	8.60	22.00		2.91

does *not* participate in hydrogen bond formation. In this respect the structure differs from that of the α phloroglucitol dihydrate in which all available hydrogen atoms are required for hydrogen bond formation. It is easily seen that the fraction of available hydrogen atoms *not* used for the formation of hydrogen bonds is $^3/_{10}$ in the triaminocyclohexane dihydrate structure, zero in the structure of α phloroglucitol dihydrate and $^2/_9$ in the α phloroglucitol diammoniate structure. The corresponding fraction in a hypothetical triaminocyclohexane diammoniate crystal having a structure of the same type would be $^5/_{12}$. Here, however, a serious difficulty would arise: the surplus hydrogen atoms could not remain attached to nitrogen atoms without raising the coordination number of some of the nitrogen atoms above *four*. Without having so far tried to prepare an ammoniate of triaminocyclohexane we therefore think it justified to conclude that a diammoniate having a structure corresponding to those under discussion does *not* exist.

The determination of the structure of the diammoniate of α phloroglucitol has proved more difficult than originally expected. The development of the (very unstable) single crystals is rather poor, and it has therefore not been possible to determine the intensities of reflexions with a degree of accuracy comparable to that obtained for the two other substances. However, the lattice constants derived from layer line photographs permit some conclusions to be drawn concerning the hydrogen bond distances in this case also. (Compare Table 2.) The position of the ammonia molecules on the trigonal axis may be varied within rather wide limits without seriously altering the O-H-N bond distance which cannot differ much from 2.91 Å. The N-H-N bond distance, however, could not be determined in a similar way with any degree of accuracy.

SUMMARY

Single crystals of 1*α* 3*α* 5*α*-triamino*cyclo*hexane dihydrate have been prepared and examined by X-ray crystallographic methods. The structure corresponds to that of *α* phloroglucitol dihydrate previously determined [1]. The distance between the two water molecules linked together by a hydrogen bond is the same (2.76 Å) in both crystals and the N-H-O hydrogen bond length is 2.93 Å in the triamino compound. The single crystals obtained of *α* phloroglucitol diammoniate were too poor to deliver intensity values on which a detailed structure determination could be based. It may safely be concluded, however, that the crystals are isomorphous with those of the two dihydrates mentioned above, and from the lattice constants the O-H-N hydrogen bond distance may then be calculated. The value thus obtained is close to 2.91 Å.

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The Constitution of "Cryptopinone"

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In 1947 two of us [1] reported the isolation of a carbonyl compound, $C_{20}H_{30}O$, from the twig roots and resinified trunks of Scots fir (*Pinus sylvestris* L.). This compound was given the name "cryptopinone" and tentatively regarded as a ketone. Shortly afterwards Harris and Sanderson [2] described the isolation of a neutral compound, $C_{20}H_{30}O$, from commercial wood and gum rosins (from *P. palustris* and *P. caribaea*). A comparison of physical constants (see Table 1) led Harris and Sanderson to suggest that these two substances were identical.*

Table 1.

Compound	Sörensen and Bruun [1] and this paper	Harris and Sanderson [2]
Free aldehyde ("cryptopinone")	m. p. 50– 52°	m. p. 50– 52°
Semicarbazone	m. p. 223–224°	m. p. 223–225°
2,4-Dinitrophenylhydrazone	m. p. 195–196°	m. p. 192–194°

* Since this paper was submitted for publication Dr. G. C. Harris has kindly carried out a direct comparison of the 2,4-dinitrophenylhydrazones of his carbonyl compound and of "cryptopinone". Dr. Harris writes:

The melting point of the 2,4-dinitrophenylhydrazone of cryptopinone in our apparatus was found to be 195–196.5° C. The melting point of the corresponding derivative of isodextropimarinal is 192–194° C. A mixed melting point of the two showed no depression at 193–195° C. Further evidence of the identity of the two substances was their identical x-ray diffraction patterns.

On these bases it may be said that cryptopinone and isodextropimarinal are the same compound.

We are very greateful to Dr. Harris for confirming this identity.

Harris and Sanderson [2] concluded that their compound was *isodextro*-pimarinal on the basis of dehydrogenation evidence and because chromic acid oxidation under mild conditions furnished *isodextro*-pimaric acid [3], m.p. 158—161°. The identity of the latter was said to be established by mixed m.p. and by its X-ray diagram.

We have now converted *dextro*-pimaric acid to the known *dextro*-pimarinol [4] and oxidised the latter under mild conditions to *dextro*-pimarinal. This aldehyde was shown to be identical with "cryptopinone" by comparison of the corresponding 2,4-dinitrophenylhydrazones. The identity was established by crystal form, m.p., mixed m.p., optical rotation and absorption spectrum. We conclude, therefore, that "*cryptopinone*" *is, in fact, dextro-pimarinal.* This conclusion is in agreement with the evidence of Harris and Sanderson *except* so far as the oxidation to *isodextro*-pimaric acid is concerned. In an effort to explain the discrepancy authentic *isodextro*-pimaric acid was reduced by lithium aluminium hydride to *isodextro*-pimarinol and the latter oxidised under mild conditions to *isodextro*-pimarinal, characterised as the 2,4-dinitrophenylhydrazone. The latter was entirely different from "cryptopinone" (*dextro*-pimarinal) 2,4-dinitrophenylhydrazone. Furthermore it melted at 180—182° and did not correspond, therefore, to the 2,4-dinitrophenylhydrazone, m.p. 192—194°, obtained by Harris and Sanderson [2] from their naturally occurring carbonyl compound. We are forced to conclude that, in spite of the reported evidence to the contrary, the acid obtained by Harris and Sanderson by oxidation was really *dextro*-pimaric acid *not isodextro*-pimaric acid.

EXPERIMENTAL

M.ps. are uncorrected. Rotations were determined, unless stated to the contrary, in chloroform solution at room temperature, which varied from 15 to 20°. Values of $[\alpha]_D$ have been approximated to the nearest degree. Absorption spectra were taken in chloroform solution using a Unicam S. P. 500 Spectrophotometer.

"*Cryptopinone*" *semicarbazone.* The previously described preparation was recrystallised as long needles from warm (but not hot) ethyl acetate. It had m. p. 228—230° decomp. (taken using a paraffin bath, but 223—224° decomp. taken, as before, using a concentrated sulphuric acid bath), $[a]_D$ + 57° (c, 1.91).

"*Cryptopinone*" *2,4-dinitrophenylhydrazone.* "Cryptopinone" regenerated from the semicarbazone as reported previously [1], was treated with a methanolic hydrochloric acid solution of 2,4-dinitrophenylhydrazine in the usual way. The crystalline orange ppt. was filtered and purified by filtration through alumina in benzene followed by crystallisation from chloroform-methanol. "Cryptopinone" 2,4-dinitrophenylhydrazone crystallised in orange needles, m. p. 195—196° decomp., $[\alpha]_D$ — 26° (c, 2.09), λ max. 360 mμ, ε max 26,000 (Found: C, 67.0, 67.4; H, 6.9, 7.4; N, 12.2. $C_{26}H_{34}N_4O_4$ requires C, 66.95; H, 7.35; N, 12.0 %).

dextro-*Pimarinal 2,4-dinitrophenylhydrazone. dextro*-Pimarinol [4] (350 mg), prepared by lithium aluminium hydride reduction of a specimen of *dextro*-pimaric acid kindly supplied by the U. S. Dept. of Agriculture, was dissolved in 10 ml of "Analar" acetic acid and oxidised by the addition of 100 mg of chromium trioxide dissolved in a few drops of water. The homogeneous solution was left overnight at room temperature. Addition of water and working up in the usual way gave oily *dextro*-pimarinal. For characterisation this was converted to the 2,4-dinitrophenylhydrazone in the same way as for "cryptopinone". Filtration through alumina and recrystallisation from chloroform-methanol afforded fine orange needles of *dextro*-pimarinal 2,4-dinitrophenylhydrazone, m. p. 195—196° decomp., $[\alpha]_D$ — 27° (c, 3.15), λ max 360 mμ, ε max 27 800 (Found: C, 66.75; H, 7.3; N, 12.0. $C_{26}H_{34}N_4O_4$ requires C, 66.95; H, 7.35; N, 12.0 %). Admixture of this 2,4-dinitrophenylhydrazone with the corresponding derivative of cryptopinone (see above) gave a m. p. of 195—196° decomp. (all three m. ps. taken at the same time).

isodextro-*Pimarinal 2,4-dinitrophenylhydrazone. isodextro*-Pimaric acid, m. p. 155—157°, $[\alpha]_D$ + 0.6° (c, 9.59), — 2.5° (c, 7.68) in alcohol, (250 mg) was reduced with lithium aluminium hydride to *isodextro*-pimarinol and the latter oxidised with chromium trioxide as for *dextro*-pimarinol (see above). The oily *isodextro*-pimarinal was converted to the 2,4-dinitrophenylhydrazone in the usual way. Filtration in benzene solution through alumina and recrystallisation from chloroform-methanol furnished the yellow *isodextro*-pimarinal 2,4-dinitrophenylhydrazone, m. p. 180—182° decomp., $[\alpha]_D$ + 104° (c, 0.41), λ max 360 mμ, ε max 25 600. (Found: C, 67.3; H, 7.6; N, 12.2. $C_{26}H_{34}N_4O_4$ requires C, 66.95; H, 7.35; N, 12.0 %). There was a marked depression in m. p. on admixture with "cryptopinone" 2,4-dinitrophenylhydrazone (see above).

SUMMARY

"Cryptopinone" isolated from *Pinus sylvestris*. L., has been proved to be *dextro*-pimarinal by a partial synthesis of the latter from *dextro*-pimaric acid.

We thank Dr. G. C. Harris for a gift of *isodextro*-pimaric acid and Mr. C. J. W. Brooks for the preparation of the *dextro*-pimarinol.

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Ozone. Its Physiological Effects and Analytical Determination in Laboratory Air

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Since Schönbein discovered ozone in 1840, its occurrence and effects have been investigated extensively. Ozone, as is well known, occurs in the atmosphere, mainly in the upper layers where the ultraviolet radiation is more intense and the humidity lower. The latter fact is significant since ozone reacts with water vapour to form hydrogen peroxide. Ozone is formed by electrical discharges either silent or glowing, UV-radiation, and by some chemical and electrochemical processes.

Ozone has been used to purify air in storage rooms, underground spaces, cinemas *etc.* Witheridge and Yaglou [1] showed that 0.015 p. p. m. of ozone removed body odours and reduced by 50 % the amount of fresh air necessary for odour control. It seems, however, that the effect of the ozone depends more on its masking action than its oxidizing properties, and according to Elford and Ende [2], ozone does not sterilize air when used at a tolerable concentration, and gives no protection against infections. Because of its powerful oxidizing and catalyzing properties, ozone is widely used, particularly in organic chemistry.

The physiological effects of ozone have been investigated in both animal and human experiments. Hill and Flack [3] observed that a content of 1 p. p. m. of ozone in air provokes great changes in the metabolism of experimental animals, and exposure for two hours to air containing 15—20 p. p. m. of ozone causes fatal pneumonia. McDonnel [4] found that ozone administered continuously in the respiratory air for several months in concentrations even lower than 1 p. p. m. apparently shortens the lives of guinea pigs by irritating the lungs and bronchial tubes and causing pneumonia. Kunzmann [5] explains that an ozone — air mixture decomposes into oxygen at the skin surface and the oxygen enters the tissue through the sweat, diffuses into the veins and causes oxidation of organic acids, expulsion of carbon dioxide and a reduced fre-

quency of respiration. Peyre and Moricourt [6] have investigated the destructive action of ozonized oxygen on the haemolytic properties of serums. Fritz [7] attaches importance to the treatment of carbon monoxide poisoning with ozone, and points out that ozone is more poisonous than carbon monoxide. Thorp [8] claims that pure ozone is less poisonous than ozone containing nitrogen oxides. He found that ozone containing 47 % of nitrogen oxides destroys bacteria at a concentration of 3 p. p. m. of ozone, while pure ozone is ineffective below 50 p. p. m. Ewell [9] concluded on the basis of his bacteriological experiments that the addition of nitrogen oxides to ozone does not make the gas more poisonous. Thorp [10], however, maintained his opinion and found that different ozonizers produce gas mixtures of different toxicity. The alleged increase in toxicity caused by the addition of oxides of nitrogen has also been investigated by Watson [11] with negative results.

According to Dadlez [12] the presence of 4 mg ozone per cu.m. (1.5 p. p. m.) renders the atmosphere intolerable. He cites an investigation by D'Arsonval, who found that the average amount of ozone produced under normal conditions by the Hg-quartzlamp used in ultraviolet therapy does not exceed 0.5 mg/cu.m. (0.19 p. p. m.). This amount presents no danger, but when the concentration rises to 1 mg/cu.m. (0.4 p. p. m.) or more, discomfort and irritation result. These symptoms will be apparent in about 30 minutes. It is essential that rooms in which the ultraviolet installation is working should be well ventilated, especially if not spacious. Flury [13] gives the following symptoms characteristic of ozone:

0.001 mg/l (0.47 p. p. m.) causes distinct irritation.

0.002 mg/l (0.94 p. p. m.) causes, in 1 ½ hours, coughing irritation, and severe exhaustion.

0.006 mg/l (3 p. p. m.) causes sleepiness in one hour. At higher concentrations, ozone causes increased pulse frequency, sleepiness, and continued headache.

Striking data, however, have been published. According to Thorpes Dictionary [14] "the ozone content of city air is usually only (!) a few parts per million. If present in amounts larger than about one part in twenty thousand ozone is an irritant." The last mentioned concentration corresponds to 50 p. p. m. and is extremely high.

Edgar and Paneth [15] have determined the ozone content of London air and found it to be $0.4—4.5 \times 10^{-6}$ vol.-%. (C. A. *36* (1942) 988 erroneously gives these figures as 0.4—4.5 p. p. m.). For the air of Geneva, Briner and Perrottet [16, 17] have given the ozone content 7×10^{-9}, and Dauvillier [18] has determined the ozone in the air at Abisko, Swedish Lapland, with a result 1.6×10^{-7}.

According to Elkins [19] the concept of Maximum Allowable Concentration (MAC) of dusts and fumes is fundamental to industrial hygiene as practiced in the United States. The MAC values for different fumes and dusts are carefully determined by animal and human experiments, and are proposed to meet the following requirements. Illnes due to intoxication by the substance in question, and increased susceptibility to non-occupational diseases should not result from continued exposure to the MAC. There should be no marked discomfort to a major portion of exposed workers, and the capacity for working and carrying on a normal existence should not be reduced. When tolerance is acquired, the MAC should not be more than 3 times the concentration affecting unhardened persons, and should not exceed one-tenth of the concentration dangerous in a single exposure of ½ to 1 hours. A concentration 2 ½ times the MAC should fail to meet one or more of the preceeding requirements. If the MAC is based solely on animal experiment data, a safety factor of about 5 should be adopted.

For ozone, the MAC given by Elkins (*l. c.*) is 0.2 p. p. m., based on animal data. The MAC of hydrogen cyanide is 10 and that of phosgene 0.5 p. p. m., and ozone is, therefore, very poisonous. Elkins [20] points out that chronic poisoning by ozone has been reported, and the high toxicity of the substance is well established. In his opinion, ozone should never be intentionally introduced into workroom air, and when it is formed inadvertently, it should be removed by suitable ventilation. On the other hand he supposes that the concentrations of ozone produced by carbon arcs and welding arcs are usually negligible.

ANALYTICAL DETERMINATION

The characteristic strong odour of ozone is well known. The minimum concentration at which this becomes apparent is given in many handbooks as 1 : 500000, *i. e.* 2 p. p. m. This fact will be discussed later.

Of the chemical methods for the determination of ozone with sufficient sensitivity colorimetric and fluorometric methods, and methods depending on the oxidizing and catalytic properties of ozone will be mentioned. Arnold and Mentzel [21] determined ozone colorimetrically using tetramethyl-p:p′-diaminodiphenylmethane, and since then this reagent has been used extensively. Masterman [22] reports that ozone gives with this reagent colours initially blue, but changing to grassgreen, olive-green, orange, yellow and finally becoming complete bleached. This test is specific for ozone; only hypochlorites interfere. Benoist [23] determines ozone colorimetrically using fluorescein; Egorow [24] prefers the leuco-compound of fluorescein, fluorescin, which is oxided by ozone to fluorescein. This method is suitable, according to him, for concentrations of 1 p. p. m. or less. Allen [25] determines colorimetrically the iodine liberated by ozone from a KI-solution with a sensitivity of 10^{-6} g using starch as indicator. In the colorimetric method of Usher and Rao [26] $NaNO_2$-solution and the Griess-Ilosvay reagent are used. If O_3, N_2O_4

and H_2O_2 are present in the air, in the first test O_3 and H_2O_2 are removed by means of the combination $CrO_3 + MnO_2$. In a second test, H_2O_2 alone is destroyed by CrO_3, and the amount of ozone is calculated by difference. Dorta-Schaeppi and Treadwell [27] determine ozone colorimetrically in a concentration of 10^{-8} using indigodisulfonic acid in a phosphate buffered solution of pH 6.85. In the method of Konstantinowa-Schlesinger [28], the fluorescence of acridine, formed by ozone in a solution of dehydroacridine is measured.

The hypersensitive method of Briner and Perrottet [17] is based on the catalytic effect of ozone on the oxidation of aldehydes. They used benzaldehyde in carbon tetrachloride or butyraldehyde in hexane and obtained a sensitivity of 0.1—0.01 p. p. m. using samples of less than 10 litres. The sensitivity may be increased by small amounts of peracid, formed by air in the presence of light [29].

The oldest and best known method for the determination of ozone is by absorption in a solution of KI, and titration of the liberated iodine with sodium thiosulfate. In this procedure, other oxidizing agents such as nitrogen oxides, halogens, and hydrogen peroxide interfere. Teclu [30] introduced neutral instead of acid KI-solutions and Ladenburg and Quasig [31] have confirmed the correctness of this procedure. Ladenburg [32] further ascertained that a solution of $NaHSO_3$ unlike AsO_3'''-solution absorbs ozone completely, Lechner [33] recommends that the O_3-determination be made in alkaline KI-solution, because iodine should not be evaporated, but Riesenfeld [34] has shown that this method gives values which are too high due to the formation of iodate. Baskerville and Crozier [35] absorbed ozone either in acidic or neutral $CdKI_3 \cdot H_2O$-solution, and McDonnel [36] describes a rapid method in which the ozone is absorbed in a solution containing KI, $Na_2S_2O_3$ and starch, and the ozone concentration is proportional to the time required for the solution to become blue. Juliard and Silberschatz [37] used a KI-solution buffered to pH 7 by means of a boric acid-borate or mono-disodiumphosphate buffer. Ruyssen [38, 39], however, has not observed any differences in results using buffered or unbuffered solutions in the pH range 7—9.2. Between pH 5—7 and in the presence of boric acid, the results were too high by as much as 10 %. Dauvillier [40] proposes Na_3AsO_3-solution as adsorbent, but Briner and Paillard [41] have shown that a strong KI-solution is definitely superior.

Ozone may be adsorbed by silica gel at low temperatures and liberated by destillation (Briner [42] and Paneth and Edgar [43]). Separation of ozone from N_2O_4 is carried out by destillation at a temperature below — 120° C. The procedure of Ladenburg-Quasig (*l. c.*) is recommended by Paneth and Edgar for ozone determinations. Thorp [44] sensitizes the reaction between ozone and KI with a solution of $AlCl_3 \cdot 6H_2O + NH_4Cl$. Glückauf *et al.*[45] have obtained a continuous recording of the local ozone concentration with an automatic apparatus. KI-solution buffered to pH 7 is used, the liberated iodine being titrated electrometrically. As regards nitrogen peroxide, these authors made the observation that with the buffered KI-solution used the quantity of I liberated by NO_2 corresponds to less than 2 % of the amount of peroxides present. The reaction

$$NO_2 + 2\ H^+ + 2\ I^- = H_2O + NO + I_2$$

requires hydrogen ions, and the concentration of these is reduced by buffering the solution. If the concentration of NO_2 is increased (10^{-5}), the reaction occurs more rapidly.

Boelter *et al.*[46] determined ozone in higher concentrations (4 to 20 weight %) iodometrically, and found that KI-absorbents in the pH range 2.3 to 12.3 gave correct results if the solution was acidified with strong acid before titration with sodium thiosulfate. Addition of aluminum chloride to the absorption solution caused no error at concentrations up to 0.055 N. Strongly acidified potassium iodide absorbents gave high results.

Briner and Monnier[47] remove nitrogen oxides by absorption in concentrated sulfuric acid. In the presence of ozone the oxides of nitrogen are in the form of peroxide, or even nitric acid anhydride, and thus they will be almost completely absorbed by sulfuric acid. Kawamura[48] previously reported on the low solubility of ozone in concentrated sulfuric acid.

EXPERIMENTAL

In this work, the concentration of ozone in the immediate vicinity of a spectrograph and of an ultraviolet lamp has been determined. The spectrograph was used with spark excitation, the spark source being the commercial type of Heraeus, Hanau, with the inductance marked 1/10, full capacitance and a primary resistance of 40 ohm. The ultraviolet lamp was a laboratory model made by Hanovia, England, and was used without any filter. The spectrograph was in a room of about 100 cu.m. volume, the ultraviolet lamp in a room of about 50 cu.m. No ventilation was used.

Collection of the samples.

Two fritted bubblers in series were used, the first containing 50 ml of concentrated sulfuric acid to remove nitrogen oxides, the second 50 ml of KI-solution protected from light. The latter solution was either buffered to pH 7 or unbuffered. The samples, either 50 or 100 litres (measured by means of a gas meter) were collected at a rate of about 7 l/min.

Analysis.

The sample was transfered to a 250-ml Erlenmeyer flask, 1 ml 6N-H_2SO_4 and one drop of starch solution were added and the solution was titrated immediately to the colourless end-point with 0.01 N sodium thiosulfate using a micro buret. Before each sample, three blanks were run with a reproducibility of $\pm$ 0.05 ml in titration. The average of these was subtracted from the titration value of the sample.

The reaction is:

$$O_3 + 2\ KI + H_2O = 2\ KOH + O_2 + I_2$$

and hence 1 ml of 0.01 N sodium thiosulfate corresponds to 1.12 p. p. m. of ozone. The results were calculated to NTP.

Sensitivity and accuracy.

On the basis of the reproducibility of the blanks and titrations, a sensitivity of 0.1 p. p. m. and an accuracy of $\pm$ 0.05 p. p. m. are to be expected.

Reagents.

KI solution. 100 g of KI dissolved in distilled water and diluted to 500 ml.

Buffered KI solution. Prepared according to Glückauf *et al.*[45], 1.38 g of NaH_2PO_4 and 0.20 g of NaOH were added. The solutions were protected from light and used immediately.

H_2SO_4 concentrated. M & B "for analysis".

H_2SO_4 6 N.

$Na_2S_2O_3$ 0.01 N.

Starch solution. 1 gram in 100 ml hot water.

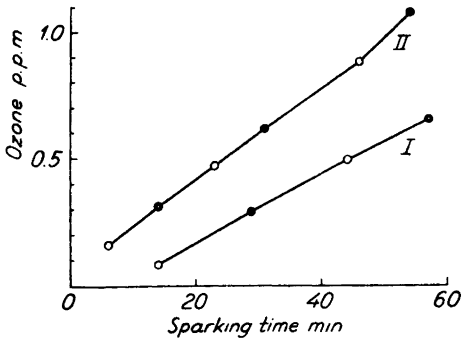

Fig. 1. The concentration of ozone in air at various distances from the spectrograph electrodes using either buffered and unbuffered KI-solutions in the analysis.

RESULTS

A. The concentration of ozone caused by the spectrograph

I. Horizontal distance from the electrodes 50 cm.

Sparking time (minutes)	Ozone p. p. m. (Air samples 100 l)
14	0.08
29 B[1]	0.29
44	0.49
57 B	1.65

[1] Buffered solution.

II. Horizontal distance from the electrodes 40 cm.

Sparking time (minutes)	Ozone p. p. m. (Air samples 50 l)
6	0.16
14 B[1]	0.31
23	0.47
31 B	0.61
46	0.87
54 B	1.07

[1] Buffered solution.

These results are shown graphically in Fig. 1. The time is measured from the start of the test to the end of each sampling. As may be seen, no differences between the buffered and unbuffered solutions are detectable.

A series of tests was carried out omitting the sulfuric acid pre-treatment, and it was observed that the increase in the titres was about 20 %, this representing the oxidizing agents absorbed by the sulfuric acid.

B. The concentration of ozone caused by the ultraviolet lamp

No significant concentration of ozone could be determined. When the lamp was lit the odour of ozone was easily noticeable, but it disappeared rapidly. This result agrees with the data given in the literature [12, 17].

DISCUSSION

The odour of ozone corresponding to a concentration of about 0.1 p. p. m. could be observed immediately after igniting the spark, and continued thereafter. A distinct irritating effect on the respiratory organs appeared in 15 minutes and increased towards the end of the test. These observations agree with those of Flury [13]. During these tests the usual ventilation system of the spectrograph, a powerful suction just above the electrodes, was not used, but during normal operation of the spectrograph the author has observed the same irritation, although after a longer working time. The author has used rather long exposure times (3—4 min.), the total working time being as long as 3—4 hours without a break. After two weeks, distinct symptoms of chronic ozone poisoning were observed — shortness of breath and continuous headache. It is evident that ventilation efficient enough to remove the hazard of contamination during the analysis is not sufficient to eliminate the possibility of ozone poisoning. Oxides of nitrogen, of course, cause irritation, too, but according to the test values, the amount of ozone is of greater significance. It seems possible that ozone may be formed by the ultraviolet radiation of the spark or an arc at a comparatively long distance from the electrodes, where it is not immediately removed by the ventilation system but is inhaled by the worker.

The hazard of ozone poisoning caused by ultraviolet lamps reported on by Dadlez [12] is worthy of note, even although the present test has not shown an unusual concentration of ozone around such a lamp. The possible effect depends on the size of the lamp and the room, on the ventilation and, of course, on individual sensitivity to ozone.

It is evident that the great toxicity of ozone is not widely appreciated, and more attention should be paid to this fact in all cases where ozone may occur in air, e. g. in electric machine halls, transformer stations, artificial sun treatment rooms and laboratories working with ozonizers, spectrographic equipment and ultraviolet lamps. The MAC-value given by Elkins [19], 0.2 p. p. m. for ozone, seems to be too high, as irritation is distinctly noticeable at this concentration. The concentration meeting all the requirements of the MAC is close to or below the minimum, at which ozone may be detected by its odour, i. e. 0.05—0.1 p. p. m.

SUMMARY

The properties, physiological activity and analytical determination of ozone are reviewed. The concentration of ozone in the vicinity of a spectrograph has been determined iodometrically and was found to rise to 1 p. p. m. in one hour. The ozone concentration near an ultraviolet lamp was found to be below the limit of determination, 0.1 p. p. m. Personal experiences of symptoms caused by ozone are presented. Finally the author stresses that the great toxicity of ozone should be better known, and considered wherever ozone may be formed.

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Quantitative Spectral Analysis of Trace Elements in Water

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In a previous paper [1] we discussed the sensitivity of the porous cup electrode technique for direct spectral analysis of solutions, first described by Feldman [2]. The application of this technique (the PCE-technique) to the detection of trace elements in water is presented in this paper.

Trace elements in waters have been widely estimated by spectrographic methods, as well as by pure chemical, polarographic and flame photometric means. Most frequently the analysis has been carried out on the dry residue obtained by evaporation of the sample. The first spectrographic investigations of trace elements in water were made by Fresenius [3] in Germany (1934) and by Braidech and Emery [4] in USA (1935). Since then, many papers have been published describing similar work, and of these those by Strock [5] on trace elements in water at Saratoga Springs, by Lopez de Azcona [6] on Spanish mineral waters, and several by Kuroda [7,8,9] on Japanese mineral spring waters are of particular interest. Schleicher and Kaiser [10] used electrolysis combined with spectral analysis for the determination of the concentration of heavy metals in mine water. Dmitriev [11] proposed the excitation by an interrupted spark of filter paper impregnated with the solution to be investigated, the paper being moved after each spark interruption. The content of Al, Fe, Mn, Mg, and Cr in natural waters has been determined using this method. The "copper spark method", first described by Gerlach and Riedl [12] has been proposed for water analysis by Nachtrieb [13]. According to him, this method does not necessarily give high precision, but is very sensitive for the detection of most metallic elements. The method employing the evapouration of samples on graphite electrodes has been widely used, but no satisfactory method for the direct spectral analysis of water samples appears to have been presented in the literature.

Most of the errors which arise in the determination of trace elements in water are due to the introduction of impurities from the flasks, chemicals, air,

etc. or to the loss of material by adsorption or volatility, and these errors will become greater as the complexity of the method increases. A systematic study of these phenomena, like that made by Heller *et al.*[14] is desirable in all investigations made by indirect means. The author has attempted to find the simplest way of proceeding from the sample to the final analysis, even when a concentration must be made to obtain sufficient sensitivity.

EXPERIMENTAL

Water samples were collected in one-half liter bottles, paraffined inside and corked with natural cork stoppers. (Samples of 25 ml are, however, sufficient for complete analysis by this method.) The paraffined bottles do not contaminate the contents, nor do they adsorb the trace elements from water kept in them for periods even longer than one month. 15 Ml quartz test-tubes, with graduation marks at one and ten milliliters, were cleaned thoroughly. 10 Ml samples of water were placed in the test-tubes, which were then heated in a clean oven at a temperature not exceeding 100° C until the volume of the samples decreased to about 0.5 ml. This was usually done by keeping the samples in the oven overnight. The internal standard solution was then added and each tube was filled to the 1 ml mark with doubly quartz distilled water. In this way samples concentrated ten times were prepared for analysis. In some cases, especially when determining the concentrations of elements, for which the sensitivity of the method is high, or which are present in large amounts, it is desirable to take spectra from the original sample. In this case the internal standard solution is added directly to the 1 ml sample, and the increase in volume, caused by the internal standard solution, must be considered in the calculation.

The apparatus, electrodes, electrical and optical arrangements were the same as described previously[1]. It was observed, however, that more reproducible results were obtained by using a pre-spark period of 60 seconds (electrode filled), refilling the electrode, and then making an exposure of 180 seconds. The analytical gap was 4 mm. Kodak Scientific Plates III-O were used, and were developed in 1 : 2 diluted Kodak D-19b developer for 5 minutes at a temperature of 18° C, rinsed in a stop bath, fixed in a Kodak F-5 fixing bath, washed and dried in air.

Because foreign elements, present in large amounts, have a considerable effect to the appearance of the lines of trace elements, it is important to have standards resembling, as closely as possible, the samples to be analysed. The main mineral components in Finnish natural waters are calcium and magnesium, the former considerably exceeding the latter, and alkalies. Because

the content and ratios of alkalies are of interest, also, only calcium and magnesium were taken as base materials. Finnish natural waters usually are rather soft, the total hardness being on the average 50 ppm calculated as CaO. Consequently the standards used with the concentrated samples contained 400 ppm of calcium oxide and 100 ppm of magnesium oxide, corresponding to 40 and 10 ppm for the original samples. The Ca- and Mg-compounds used were purified according to the procedure described by Hughes [15] and no impurities could be observed in the treated salts. The standard substances were dissolved in 3 % nitric acid (purified by distillation), and dilutions were made and the internal standard was added in the usual way, the steps being 100, 30, —, 0.03, 0.01 ppm. As a result of a preliminary qualitative investigation of water samples, standards were made of the following elements: Al, Ba, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Sr and Zn. Spectra of standards were taken on each plate together with samples.

It was observed that the greatest variations in spectra were caused by different speeds of feeding the sample into the analytical gap. Hence it is unsatisfactory to use carbon lines as reference lines, as did Bretón [16], as the internal standard should be in the solution to be analysed. Feldman (*l. c.*) proposed the use of one of the lines of the hydroxyl band as the reference line, but it would be possible to use this line for only a few elements, and an internal standard must be added in any case.

According to Feldman (*l. c.*) only the wave length of the internal standard line need be considered, the excitation potential and volatility being of minor interest. This is undoubtedly true, as, although changes in the excitation conditions produce differences in the appearance of lines of elements which have different excitation potentials, these alterations do not appear without control, if a correct spark source is used. Continuous feeding overcomes the difficulties caused by different volatilities. An internal standard element should have a reasonable number of contamination-free lines covering a wide wavelength range so that one internal standard element only is needed, and it should not be present in the samples. None of the elements discussed in the literature as internal standards seemed to fulfill these requirements. Most of the rare elements, like Au and Ag, have only a few good lines. However, it was observed that platinum is an ideal internal standard element for this purpose. It has very distinct, contamination-free and self-reversal-free lines over the whole wavelength range from 2 300 to 3 068 A, and it has not been found in Finnish waters to date.

At a concentration of *ca.* 30 ppm of Pt, the following lines were found to be useful: Pt 2440.057 for Cu 2246.995, Cd 2265.017, Co 2286.156, Ni 2287.084, Ba 2304.235, and Fe 2395.625; Pt 2646.886 for Mn 2576.104, Pb 2833.069, and

Cr 2835.633; and Pt 3064.712 for Cu 3247.540, Zn 3345.020, Sr 3380.711, and Al 3082.155. A solution of pure Pt in aqua regia was made, and a suitable amount of this solution was added to the sample and standard to give a Pt-concentration of about 30 ppm.

The densities of the lines were measured with a Hilger non-recording microphotometer, and a plate calibration curve was made in the usual way using a step sector and iron-DC-arc. Intensity ratio-concentration working curves for each element were made using the lines mentioned above. No background correction was needed when determining Cu (using the line 2246.995), Co, Ni, Cd, Ba, and Fe. Background corrections for the other metals were made by subtracting the background intensity from the (line plus background) intensity. The working curves were straight lines.

Using the procedure suggested, the elements can be determined in the following minimum concentrations in the original water: Al 0.5, Ba 0.05, Cd 0.01, Co 0.05, Cu 0.01, Cr 0.05, Fe 0.05, Mn 0.001, Ni 0.5, Pb 0.5, Sr 0.07, Zn 0.6 ppm. When these values (multiplied by ten) are compared with the sensitivities given in a previous paper [1], obtained from pure solutions of elements, it is readily apparent that the presence of foreign elements, in this case Ca and Mg, has not had any appreciable effect upon the sensitivity.

PRECISION AND ACCURACY

To determine the precision obtainable, five separate and duplicate determinations were made using the same solution of each metal. Other metals were present in the same concentration, and Ca and Mg were used as the base mixture in the amounts described above. The time from the first to the last experiment was about five months. The results are given in Table 1. It can be seen that the average percentage deviation from the mean intensity ratio (analysis line/internal standard line) varies from 1.49 % (Cd) to 4.35 % (Mn). The standard percentage deviation, calculated as shown in the table, varies from 1.96 % (Cd) to 5.66 % (Cr). Considering all factors having an influence upon the reproducibility of determinations of this type, the results are considered to be quite satisfactory.

The accuracy has not been estimated by extensive comparison of the results with those obtained by independent methods. It is questionable if chemical methods can be considered more reliable than spectrographic methods when determinations are to be made in very dilute solutions, as considerable systematic errors may arise when standard analytical methods are employed. However, the nickel content of a mine water has been determined chemically by precipitation with dimethyl glyoxime, and the result obtained 20.0 ppm

Table 1. Precision obtained making five duplicate determinations of the concentrations of trace elements in the same solution during a time of five months.

Element and concentration ppm.		Mean intensity ratio anal. line/ref. line	Average deviation from the mean intensity ratio	Standard deviation * from the mean intensity ratio
Al	10	0.909	± 2.22 %	± 3.00 %
Ba	10	1.091	4.02	5.17
Cd	10	1.470	1.49	1.96
Co	1	0.601	2.40	2.40
Cu	100	1.579	3.14	3.80
Cr	1	1.336	3.66	5.66
Fe	33	4.700	1.66	2.02
Mn	1	2.040	4.35	5.45
Ni	33	1.012	1.80	2.24
Pb	10	1.204	3.66	4.72
Sr	1	0.710	2.86	4.95
Zn	33	0.939	3.32	4.42

* Standard deviation: $s = \pm \sqrt{\frac{\Sigma d^2}{n-1}}$ if d is the percentage deviation and n the number of determinations, in this case 10.

is close to the 21.0 ppm obtained by spectrographic means, the deviation being in this case 5 %. It seems very likely that the precision of this method can be regarded as the same as the accuracy. The most common source of errors is that introduced by the use of synthetic standards, but this seems to be negligible in this procedure, as it is possible to make the standard solution very similar to the sample. Moreover, moderate changes in the concentration of basic elements do not have an appreciable influence on the working curves for different elements.

Further details and results concerning the concentrations of trace elements in Finnish ground waters and mine waters will be presented in a forthcoming communication [17].

OTHER APPLICATIONS

Undoubtedly, this procedure can be applied to liquids such as soil extracts, biological fluids etc, and in the last mentioned case especially the small amount of sample required for the complete analysis is most advantageous. Major elements as well as trace elements may be determined by this method, a step sector being used if necessary. In this case, however, because an intermittent light source is used, a special study of the working conditions should be made, as pointed out by Gillis and Eeckhout [18].

The relatively high precision obtainable by this procedure has prompted author to apply the PCE-technique to the determination of adsorption isotherms in very dilute solutions. A preliminary investigation has been made, and it has been observed that the sensitivity of the beryllium determination could be extended to 0.001 ppm without concentrating the samples. The adsorption isotherms of beryllium on glass were determined in solutions containing 0.003, 0.01 and 0.03 ppm of Be. Samples of 1 ml were taken at different time intervals, and the decrease in Be-concentration was determined spectroscopically. The advantage of the use of small samples is evident: they have no significant influence on the total volume and they can be removed quickly. Further the use of spectroscopical methods makes it possible to analyse a large number of samples in a relatively short time and to determine the concentrations of elements, the chemical analysis of which would be difficult and time-consuming. Preliminary results using this technique for adsorption studies have been quite satisfactory.

SUMMARY

The application of the PCE-technique of direct spectral analysis of solutions to the determination of trace elements in water is described. Using samples of about 25 ml each, it is possible to make a duplicate determination of different elements in the following minimum concentrations: Al 0.5, Ba 0.05, Cd 0.01, Co 0.05, Cu 0.01, Cr 0.05, Fe 0.05, Mn 0.001, Ni 0.5, Pb 0.5, Sr 0.07, Zn 0.6 ppm. The concentration of the sample is carried out in a very simple manner to avoid contamination from air and flasks, and errors due to adsorption. A precision of 1.49 %—4.35 % average deviation and 1.96 %—5.66 % standard deviation from the mean intensity ratio of lines is obtained. A preliminary report is given on the application of this method to the determination of adsorption isotherms.

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The Reaction Between Tetrathionate and Azide Ions

A Kinetic Investigation

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Dodd and Griffith [1] and Hofman-Bang [2] have found that the rate determining reaction step of the iodine-azide reaction, catalyzed by tetrathionate ions, is a bimolecular reaction between azide ions and tetrathionate ions. Further it was found [2] that potassium tetrathionate and sodium azide in aqueous solution react with the evolution of free nitrogen. Keeping in mind that the carbon disulphide catalyzed iodine-azide reaction [3] is closely related to the direct reaction between azide ions and carbon disulphide molecules [4], it would seem natural that the tetrathionate catalyzed iodine-azide reaction was also in some way related to the reaction between tetrathionate and azide ions. This seems to be the case, and the relationship will be dealt with in a following publication.

Tetrathionate and azide ions interact according to the scheme:

$$S_4O_6^{--} + 2\ N_3^{-} \rightarrow 2\ S_2O_3^{--} + 3\ N_2 \qquad (1)$$

As will appear later, this reaction is not quantitative. Even when tetrathionate is allowed to react with a very large excess of sodium azide, a precipitate of sulphur is formed after some time. The explanation of this phenomenon is presumably as follows: As soon as thiosulphate ions are formed according to (1), they start to catalyze the conversion of tetrathionate ions into trithionate and pentathionate ions:

$$2\ S_4O_6^{--} \rightarrow S_3O_6^{--} + S_5O_6^{--} \qquad (2)$$

This conversion was studied by Kurtenacker *et al.*[5] and recently by Goehring *et al.*[6]. Pentathionate ions decompose at a considerable rate:

$$S_5O_6^{--} \rightarrow S_4O_6^{--} + S \qquad (3)$$

and trithionate ions decompose slowly:

$$S_3O_6^{--} + H_2O \rightarrow S_2O_3^{--} + SO_4^{--} + 2\ H^{+} \qquad (4)$$

EXPERIMENTAL

The nitrogen evolution from a solution containing sodium azide and potassium tetrathionate was followed manometrically in the shaking apparatus used in the investigation [2,p.874] of the tetrathionate catalyzed iodine-azide reaction. The volume of the flask (including volume of the elastic connection to the manometer), in which the reaction took place, was determined in two different ways. Reaction between sodium bicarbonate, dried at 60° C, and 1 *M* hydrochloric acid gave the volume 227.9 ml. Reaction between sodium azide and an excess of 0.1 *M* ceric sulphate (1 *M* with respect to sulphuric acid) gave 228.7 ml.

Performance of an experiment: A known amount of sodium azide solution was pipetted with a Krogh syringe pipette into the reaction flask and also into the compensation flask. In the "vessel" was weighed 100 % pure potassium tetrathionate, $K_2S_4O_6$, usually 0.1000 g, which was dissolved in 2.000 ml water. The whole shaking apparatus was evacuated until the solutions began to bubble briskly, and was then allowed to stand for 30 minutes for the sake of complete temperature adjustment. The "vessel" with tetrathionate solution was caused to fall down into the azide solution by starting the shaking motor. In the following calculations the expansion of the reacting mixture from about 20° C to the experimental temperature was not compensated for. Neither was the alteration of volume due to the addition of potassium tetrathionate, nor to the mixing, taken into consideration. These corrections were of no importance compared with the experimental error.

In Table 1 are given the corresponding values of time of reaction, t, and pressure of nitrogen evolved, p, in three typical experiments. With the concentrations used (see Table), the azide ion concentration could be considered as constant during an experiment. From expt. no. 1, Table 1, is seen that the rate of reaction decreases much more than can be expected from the decrease in tetrathionate ion concentration. *E. g.* the increase in pressure is 0.29 cm Hg in the period 2—5 min., *i. e.* ca 0.1 cm Hg per min. In the period 20—30 min the increase in pressure is 0.33 cm Hg, *i. e.* ca 0.03 cm Hg per min. The last rate is ca. one third of the first, although decrease in tetrathionate concentration is only about 20 % (the concentration of azide ions is constant). It seems as though some sort of inhibitor were being produced. The immediate thought is that thiosulphate must be the inhibitor. In expt. no. 2, Table 1, 1.000 ml 0.1007 *M* sodium thiosulphate was added to the sodium azide solution in the reaction flask. The concentrations of potassium tetrathionate and

Table 1. Reaction between potassium tetrathionate and sodium azide at 25° C. In expt. no. 1 10.46 ml 4.00 M *sodium azide were used in the reaction flask, and 0.1007 g potassium tetrathionate plus 2.000 ml water in the "vessel". In expt. no. 2 10.46 ml 4.00* M *sodium azide and 1.000 ml 0.1007* M *sodium thiosulphate were used in the reaction flask, and 0.1001 g potassium tetrathionate plus 1.000 ml water in the "vessel". In expt. no. 3 10.46 ml 4.00* M *sodium azide, which at the same time was 0.0238* M *with respect to potassium iodide, were used in the reaction flask, and 0.1000 g potassium tetrathionate plus 2.000 ml water in the "vessel".*

Expt. 1		Expt. 2		Expt. 3	
t Time in min	*p* cm Hg	*t* Time in min	*p* cm Hg	*t* Time in min	*p* cm Hg
0	0	0	0	0	0
2	0.32	1	0.02	1	0.21
5	0.61	2	0.04	2	0.35
10	0.91	3	0.10	5	0.62
15	1.17	5	0.14	10	0.92
20	1.41	10	0.28	15	1.14
30	1.74	15	0.45	20	1.35
45	2.20	20	0.62	25	1.55
60	2.57	25	0.76	30	1.72
90	3.19	30	0.89	40	2.00
130	3.84	40	1.13	60	2.55
1140	6.74	50	1.40	90	3.18
1620	6.86	60	1.64	120	3.72
4030	7.00	90	2.28	150	4.11
5400	7.06	1125	6.42	180	4.47
6850	7.06	5400	6.81	435	5.99
		5820	6.82	1200	6.76
		7080	6.85		
		9840	6.86		
		14300	6.86		
82.8 % N_2		80.9 % N_2			

sodium azide were the same as in expt. no. 1. The results show, as was to be expected, that thiosulphate exercises a rather strong retarding effect on the rate of reaction.

Expt. no. 3, Table 1, shows that the addition of potassium iodide (0.02 *M*) does not alter the rate of reaction. A similar experiment, which is not tabul-

ated, was carried out with the reacting mixture 0.2 *M* with respect to potassium iodide. Neither in this case was a specific effect of the iodide ion ascertained. These two experiments with addition of potassium iodide were carried out because variation of iodide ion concentration was shown [1,2] to have a remarkable effect on the rate of the tetrathionate catalyzed iodine-azide reaction.

In Table 1 are given the final amounts of nitrogen evolved, calculated as percentages of the theoretical amounts according to (1). Quite a number of similar experiments have been carried out with variations in azide and tetrathionate concentrations. Variation of these concentrations did not effect the final amount of nitrogen evolved. All the results were between 82 and 83 %. One experiment carried out at 30° C instead of 25° C gave 83.4 %, so maybe an increase of temperature slightly increases the percentage of nitrogen. Addition of thiosulphate (see expt. no. 2, Table 1) seems to slightly decrease the total nitrogen evolution, which is not surprising, keeping in mind that thiosulphate ions catalyze degradation of polythionates.

A TENTATIVE MECHANISM OF REACTION

In the following considerations the thiosulphate catalyzed decomposition of tetrathionate will not be taken into consideration. As the over-all reaction takes place between one tetrathionate ion and two azide ions, it seems likely that the sequence of reactions consists of two bimolecular reaction steps. The striking decrease of the rate of reaction caused by thiosulphate ions can be accounted for by assuming one of the reaction steps to be reversible. The formation of free nitrogen can not be reversible, so only one possibility remains:

$$S_4O_6^{--} + N_3^- \underset{-1}{\overset{1}{\rightleftharpoons}} S_2O_3N_3^- + S_2O_3^{--} \qquad \pm \text{ I}$$

$$S_2O_3N_3^- + N_3^- \rightarrow S_2O_3^{--} + 3\ N_2 \qquad \text{II}$$

According to Christiansen [7] the reciprocal velocity is:

$$\frac{1}{s_+} = \frac{1}{a_1}\left(\frac{1}{w_1} + \frac{w_{-1}}{w_1} \cdot \frac{1}{w_2}\right) \tag{5}$$

or

$$\frac{dt}{dx} = \frac{1}{[S_4O_6^{--}]}\left(\frac{1}{k_1[N_3^-]} + \frac{k_{-1}[S_2O_3^{--}]}{k_1[N_3^-]} \cdot \frac{1}{k_2[N_3^-]}\right) \tag{6}$$

When we consider an experiment with no pre-addition of thiosulphate, and only consider very small degrees of reaction, the thiosulphate ion concentration is the only concentration that is not constant, and we have:

$$\frac{1}{k_1[S_4O_6^{--}]\,[N_3^-]} = A = \text{const. and} \frac{1}{k_1[S_4O_6^{--}]\,[N_3^-]} \cdot \frac{k_{-1}}{k_2[N_3^-]} = B = \text{const.}$$

which give:
$$\frac{dt}{dx} = A + B\,(2x) \tag{7}$$

where x is the decrease in tetrathionate concentration.
By integration of (7) we get:

$$t = Ax + Bx^2 \tag{8}$$

Let us now consider an experiment with sodium thiosulphate added in advance, but with the same initial concentrations of azide and tetrathionate ions. As before, only small degrees of reaction are considered, so that azide, tetrathionate and also thiosulphate ion concentrations are nearly constant. In this case we have:

$$\frac{dt}{dx} = A + B \cdot y \tag{9}$$

where y is the constant concentration of thiosulphate. By integration of (9) we get:

$$t = Ax + Bxy \tag{10}$$

Equation (9) was checked by carrying out a series of kinetic experiments with different concentrations of thiosulphate. In each experiment 10.46 ml 4.00 M sodium azide and 1.000 ml sodium thiosulphate (of known concentration) were used in the reaction flask, and 0.1000 $\pm$ 0.0002 g potassium tetrathionate plus 1.000 ml water in the "vessel". At first a few experiments, in which the tetrathionate, water and thiosulphate were introduced into the "vessel", were carried out. But this procedure did not give correct results, because, during the 30 minutes temperature adjustment, the thiosulphate caused some decomposition of the tetrathionate.

The experiments showed that the rate of reaction was constant as long as the degree of reaction was so small that the concentrations of tetrathionate and thiosulphate could be considered constant. The initial reciprocal rates of reaction were determined graphically. They are — together with the molar concentrations of thiosulphate — given in Table 2. The reciprocal rates,

Table 2. Reaction between potassium tetrathionate and sodium azide with simultaneous presence of sodium thiosulphate at 25° C. In each experiment 10.46 ml 4.00 **M** *sodium azide and 1.000 ml sodium thiosulphate were used in the reaction flask, and 0.1000 ± 0.0002 g potassium tetrathionate plus 1.000 ml water in the "vessel". $\frac{dt}{dx}$ min/cm Hg is the initial reciprocal rate of reaction, and* y *is the corresponding initial concentration of thiosulphate ions.*

Expt. no.	1	2	3	4	5	6	7	8	9	10
$\frac{dt}{dx}$ min/cm Hg	ca. 4	28.7	33.9	52.1	79.2	81.0	107.9	122.0	147.5	153.8
y Initial concentration of thiosulphate	0	0.00647	0.00809	0.01617	0.03234	0.03234	0.04851	0.0647	0.0809	0.0970
$\alpha = \frac{\left(\frac{dt}{dx}\right)_n - \left(\frac{dt}{dx}\right)_1}{y_n}$		3820	3700	2980	2330	2380	2140	1820	1770	1540

expressed in minutes per cm Hg nitrogen pressure, were plotted against the thiosulphate concentration (abscissa). This plot did not give a straight line in accordance with equation (9), but a curve which was downwards convex, as shown by the α-values, which are the slopes calculated from the $\frac{dt}{dx}$ and y values in expt. no. 1 and n. In other words, with increasing thiosulphate concentration the rate of reaction did not decrease quite as much as was to be expected. Nevertheless it seems likely that the sequence ± I and II represent the essentials of the mechanism. As already mentioned thiosulphate catalyzes the conversion of tetrathionate into trithionate and pentathionate. Pentathionate ions also react with azide ions to give free nitrogen — and at a considerably larger rate than do tetrathionate ions [8,p.460]. This observation explains qualitatively why the rate of nitrogen evolution, from a solution containing tetrathionate, azide and thiosulphate was higher than expected according to ± I and II.

It may be mentioned that when two experiments from Table 2 were used for the calculation of the coefficients A and B in (9), then the numerical values obtained agreed better and better with (8), the smaller the concentrations of thiosulphate were in the two first-mentioned experiments.

Table 3. Energy of activation of the reaction between tetrathionate and azide ions. In each experiment 10.46 ml 4.00 M *sodium azide were used in the reaction flask, and 0.1000 ± 0.0002 g potassium tetrathionate plus 2.000 ml water in the "vessel". The read nitrogen pressures were all corrected to 25° C. From the time of reaction-nitrogen pressure curves were found the times of reaction,* t′ *and* t″, *corresponding to 1 and 2 cm Hg nitrogen pressure respectively. The reciprocal values of* t′ *and* t″ *were used as relative rate constants.* $t'_{calc.}$ *and* $t''_{calc.}$ *were calculated by inserting the experimental temperatures in the found Arrhenius equation.*

Expt. no.	Temp. °C	t' min	$t'_{calc.}$ min	t'' min	$t''_{calc.}$ min
1	20°	21.5	21.8	73.8	73.8
2	25°	11.3	11.1	38.1	37.2
3	30°	5.75	5.73	18.6	19.1
4	33°	3.85	3.90	13.3	13.0
Energy of activation cal/mole		23590		23790	

ENERGY OF ACTIVATION

The energy of activation of the nitrogen formation was measured by running, at different temperatures, experiments with identical concentrations (the same as those used in expt. no. 1, Table 1). From the time — nitrogen pressure curves were read the times of reaction, t' and t'' in Table 3, at 1 and 2 cm Hg nitrogen pressure respectively. The reciprocal values of t' and t'' were used as relative rate constants, k. Using the method of least squares, the numerical values of H and A were calculated according to the Arrhenius equation:

$$\log k = H - \frac{A}{T}$$

where T is the absolute temperature. From this equation values of reaction times ($t_{calc.} = \frac{1}{k}$) were calculated by substituting T with the experimental temperatures. These values, $t'_{calc.}$ and $t''_{calc.}$, are, together with the experimental ones, recorded in Table 3. At the nitrogen pressure 1 cm Hg was found the energy of activation:

$$A \times 4.571 = 5161 \times 4.571 = 23590 \text{ cal/mole}$$

At the nitrogen pressure 2 cm Hg was found:

$$A \times 4.571 = 5204 \times 4.571 = 23790 \text{ cal/mole}$$

The difference between the two energies of activation is not larger than can be accounted for by the experimental error. Consequently it seems that the energy of activation does not alter through an experiment.

SUMMARY

Tetrathionate ions and azide ions react to give thiosulphate ions and free nitrogen (1). Kinetic investigations on this reaction have been carried out. The most noticeable feature is, that the rate of reaction decreases rather much with increasing concentration of thiosulphate ions. The over-all rate seems to agree qualitatively with the mechanism $\pm$ I and II. That the accordance is only qualitative can be explained by the disproportioning of tetrathionate into tri- and pentathionate (2). The energy of activation was found to be 23700 cal/mole.

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The Polarographic Determination of Ketosteroids Solubilized in Aqueous Solutions of Association Colloids

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In earlier publications [1,2] it has been shown that clear and stable aqueous solutions of water-insoluble steroid hormones can be prepared with the aid of association colloids. In view of its practical importance we have investigated whether analytical methods can be developed for the determination of the hormone contents of these solutions. The analytical procedures previously developed for the quantitative determination of the hormones cannot be employed without modification in the presence of an association colloid and in many cases it was found necessary to separate the hormone from the colloid. The present paper deals with methods for the separation and polarographic determination of testosterone propionate, desoxycorticosterone acetate and progesterone solubilized in aqueous association colloid solutions. The procedure has been used to determine the solubilities of these hormones in a number of colloid solutions of different concentrations.

Eisenbrand and Picher [3] developed a method for the polarographic determination of Δ^4-ketosteroids containing the system $O=\overset{|}{C}-\overset{|}{C}=C$, such as testosterone, desoxycorticosterone and progesterone. The hormones were reduced in an alcoholic lithium chloride solution at the dropping mercury electrode. The half wave potentials were about — 1.8 volts and the heights of the polarographic waves were under certain conditions proportional to the hormone concentration. Sartori and Bianco [4] later used this method to determine methyl testosterone and pregnenin -3-one-17-ol. The same method was applied in the present investigations.

The association colloids have a disturbing effect on the depolarization process. It is not sufficient to break up the micelles present in their aqueous solutions by adding alcohol. The hormone must be freed from the colloid substance and also from traces of sodium and potassium ions before a polaro-

graphic determination can be carried out. The most direct method to accomplish this would be to extract the hormone from the aqueous solution with a solvent that does not dissolve the association colloid. From cholate solutions it was possible to extract the hormone quantitatively directly with petroleum ether or benzene. The procedure can also be used in the case of certain nonionic association colloids. With other colloids, however, the attempts to carry out such direct extractions were unsuccessful since emulsions were formed. Experiments were therefore made to precipitate the anionic association colloids with heavy metal ions (Ag, Pb, Hg) and to extract the hormone from the precipitate but this procedure was also found to be impractical. Satisfactory results were obtained in many cases if the hormone solution was evaporated to dryness and the hormone was extracted from the solid residue with petroleum ether or benzene. The evaporation of the petroleum ether or benzene gave the pure crystalline hormone. The latter could then be dissolved in an alcoholic lithium chloride solution and determined polarographically by the method of Eisenbrand and Picher.

EXPERIMENTAL

Experiments were conducted to isolate testosterone propionate (TP), desoxycorticosterone acetate (DOCA) and progesterone (PRG) * from aqueous solutions of potassium myristate, sodium oleate, sodium lauryl sulphate, sodium myristyl sulphate, sodium cholate, polyoxyethylene sorbitan monooleate ("Tween 80", Atlas Powder Co., Wilmington, Del.) and the di-isobutyl phenyl polyethylene glycol derivative, "Triton N100", (Rohm and Haas Co., Philadelphia, Pa.). The first five colloids were purified in the same manner as in our previous study [2]; the last two were commercial products and were used as such.

Extraction. From the aqueous solutions of the fatty acid salts and the alkyl sulphates the hormones were isolated by the following procedure. A known amount of the hormone solution (containing 1–5 mg hormone) in a beaker was placed in a vacuum desiccator and the solution evaporated to dryness under reduced pressure at room temperature (about 24 hours). The hormone was extracted from the dry residue with petroleum ether (B.P. 40–60° C, May & Baker, Dagenham). It was found advantageous to allow the residue to stand covered with a layer of petroleum ether at a temperature just below the boiling point of the petroleum ether. The solution was hastened by mixing the solid colloid with a glass rod to obtain a fine suspension. A quantitative extraction was usually effected with five 20 ml portions of petroleum ether. With higher colloid concentrations and hormone contents it was necessary to employ twice this number of washings. The undissolved colloid was separated from the petroleum ether solution by filtering through an ordinary filter. The filtration was followed by a thorough washing with petroleum ether. The filtrate was transferred to a beaker and the petroleum ether was slowly evaporated. The hormone which precipitated in crystalline form was then determined polaro-

* The hormone substances were products of the American Roland Corporation and were kindly set at our disposal by Oy. Medica Ab., Helsingfors.

graphically as described below. In order to be certain that the washings were quantitative the last wash solution was analyzed separately.

This extraction procedure cannot be used when the hormone is dissolved in a sodium cholate solution because the hormone is more strongly bound in the residue obtained on evaporating such a solution than in the case of other association colloids. From the cholate the hormone can be extracted with petroleum ether only by boiling for a long time under reflux. Thus in one case the residue of a 20 per cent sodium cholate solution containing 3.32 mg progesterone per millilitre was extracted in a Soxhlet apparatus with petroleum ether. The amount of hormone extracted was only about 30 per cent after 10 hours, 86 per cent after 30 hours and about 95 per cent after 48 hours. Even poorer results were obtained in other experiments. The reason for this is apparently the known tendency of bile acids to form complex compounds with various substances.

As already mentioned, it is possible, however, to extract the hormone directly from aqueous cholate solutions. These solutions do not readily form emulsions when petroleum ether or benzene is added as the other association colloid solutions do. In the case of cholate solutions the volume of petroleum ether used in each extraction must be twice the volume of the aqueous solution. On extracting a 20 per cent cholate solution containing 3.32 mg progesterone per ml, the first extraction gave a 95 per cent yield, and after the second extraction the yield was 98.2 per cent. Three extractions with 20 ml petroleum ether were generally sufficient to effect a quantitative separation of the hormone.

The latter extraction procedure was also found to be suitable for hormones dissolved in solutions of "Tween 80". In this case the volume of the aqueous phase must be relatively small compared with the volume of petroleum ether to avoid emulsification. Six to seven extractions are required and the residue from the extracts must be redissolved in water and extracted again six or seven times to obtain the hormone uncontaminated with colloid substance.

Up to the present we have not succeeded in isolating the hormone quantitatively from aqueous solutions of "Triton N".

The polarographic measurements. The crystallized hormones obtained as described above were dissolved in a small volume of 96 per cent ethanol and the solution was transferred to a 12 ml volumetric flask. 1 ml 1 *M* aqueous lithium chloride solution was added and the flask then filled to the mark with ethanol. (The resulting solution thus contained 0.083 moles of lithium chloride per litre of 83 per cent ethanol.)

The hormone content of the alcoholic hormone solution was determined using a Leybold m-35 polarograph and a mirror galvanometer of moving coil type with a maximum sensitivity of about 3.10^{-9} amp. per mm.*

The cell used was constructed so that gas could be bubbled through the solution. The nitrogen gas used passed through an alkaline pyrogallol solution and through 96 per cent ethanol before entering the cell. From the cell the gas bubbled through an alcohol layer. Before each measurement nitrogen was passed through the solution until the galvanometer deflection was constant with a suitable constant voltage, *e. g.* 1.5 volts.

All polarograms were taken with a total voltage of 3 volts applied across the potentiometer. On the horizontal scale of the polarograms the voltage increase was 150 millivolts per cm. The galvanometer sensitivity was maintained at 1 : 20.

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* This apparatus is described, *e. g.*, in H. Hohn *Chemische Analysen mit dem Polarographen.* Berlin (1937).

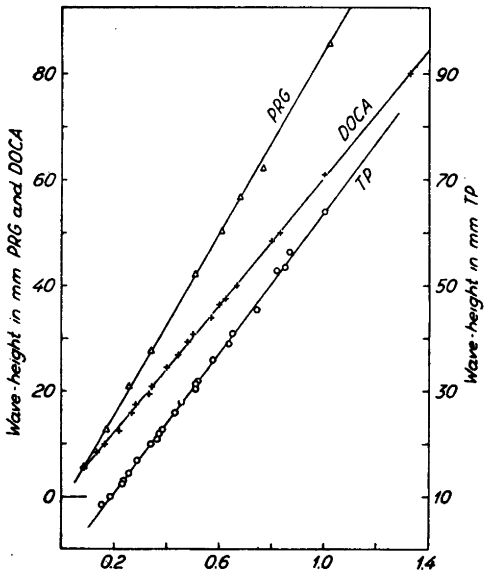

Fig. 1. Waveheight — concentration curves for progesterone, desoxycorticosterone acetate, and testosterone propionate.

The polarogram was recorded for the 1.5 — 2.25 volts range. The mercury drop rate was kept relatively high; on the average it was 0.6 seconds per drop with an applied voltage of — 1.5 volts.

RESULTS AND DISCUSSION

Our experimental results for the pure hormones dissolved in alcoholic lithium chloride solution confirmed those of Eisenbrand and Picher. The half-wave potentials of the polarographic waves are situated as given by these authors and the relation between wave height and hormone concentration was linear in all cases (Fig. 1).

The polarograms obtained with the hormones isolated from the colloid solutions had the same form as the polarograms for the pure hormones (Fig. 2). Experiments conducted with known amounts of hormone solubilized in the colloid solutions showed that the extractions were complete (Table 1). These observations prove that the hormones do not undergo any chemical change during the solubilization process.

The accuracy of the analytical method is seen from the values given in column 5 of Table 1. In the majority of cases the error is less than $\pm$ 5 per cent. A hormone concentration of 0.1 mg per ml is suitable for the recording of the polarogram. The lowest hormone concentrations in the colloid solutions were about 0.01 mg per ml. By extracting from large volumes of colloid solution, even very small hormone contents can, however, be determined. The largest

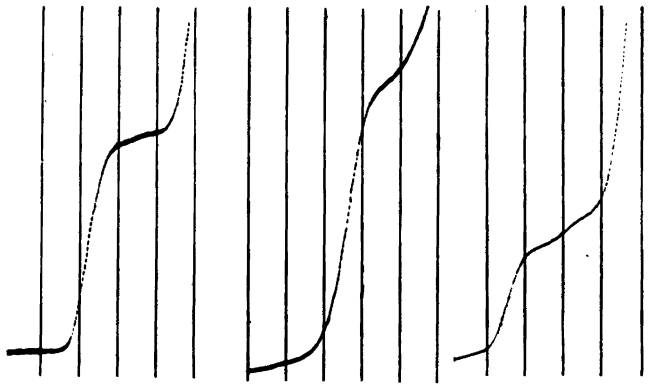

Fig. 2. Polarograms obtained with ketosteroids isolated from aqueous association colloid solutions:

a) 0.833 mg TP per ml in 12 per cent sodium oleate.
b) 1.30 mg DOCA per ml in 20 per cent potassium myristate.
c) 0.343 mg PRG per ml in 20 per cent sodium cholate.
Galvanometer sensivity 1/20.

volume of colloid solution employed was 80 ml. In the determination of the solubility of progesterone in pure water the volume of the aqueous solution was 200 ml. As the colloid concentration increases the difficulties become greater. We did not use colloid concentrations above 25 per cent.

The maximum solubilities of the three hormones were determined in aqueous solutions of the association colloids already mentioned. The colloid solutions were in most cases saturated with hormone in the manner previously described [1,2] by shaking at 40° C. The solutions in polyoxyethylene sorbitan monooleate ("Tween 80") were prepared in a different manner. In these cases the hormone was dissolved in the anhydrous colloid by warming, this solution was then diluted to the required concentration, after which the precipitated hormone was removed by filtration. The values of the solubilities of the hormones in the "Tween 80" solutions are less accurate.

The solubility values are collected in Table 2 and the variation of the solubilities with colloid concentration is shown in Figs. 3—5. In solutions of potassium myristate, sodium oleate, sodium lauryl and sodium myristyl sulphate, the solubilities of the three hormones increase above the critical concentration for micelle formation linearly with the colloid concentration. In the sodium cholate solutions the solubilities increase slowly at first and gradually more rapidly until finally at higher cholate concentrations a linear

Table 1. Polarographic determination of ketosteroids separated from aqueous association colloid solutions.

Colloid concentration in per cent	Mg hormone per ml		Average error mg/ml	Average error per cent
	Added	Found		
Testosterone propionate solubilized in sodium lauryl and sodium myristyl sulphate solutions				
2	0.00	0.00		0
2	0.20	0.22 0.21 0.22	+ 0.02	+ 10 %
10	1.00	1.08 1.00	+ 0.04	+ 4.0 %
1	1.08	1.14 1.10	+ 0.04	+ 3.7 %
2	1.10	1.20 1.20	+ 0.10	+ 9.1 %
5	3.60	3.78 3.80	+ 0.18	+ 5.0 %
10	4.78	5.16 4.80	+ 0.20	+ 4.2 %
10	5.24	5.05 5.05	− 0.19	− 3.6 %
20	5.40	5.30	− 0.10	− 1.8 %
20	10.0	9.95 9.95 10.4	+ 0.10	+ 1.0 %
20	25.90	27.0 27.4	+ 1.3	+ 5.0 %
Progesterone solubilized in sodium cholate solutions				
5	0.22	0.21	− 0.01	− 4.5 %
14	1.03	0.99	− 0.04	− 3.9 %
10	1.20	1.18	− 0.02	− 1.7 %
20	1.52	1.48	− 0.04	− 2.6 %
15	1.66	1.60 1.64	− 0.04	− 2.4 %
10	1.67	1.59	− 0.08	− 4.8 %
20	3.30	3.26 3.26	− 0.04	− 1.2 %
20	3.32	3.31 3.26	− 0.04	− 1.2 %

Table 2. The solubilities of ketosteroid hormones in aqueous solutions of different association colloids in mg hormone per ml association colloid solution. Colloid concentrations in weight per cent. 40° C.

Association colloid solution		TP	DOCA	PRG
Potassium myristate	0.5 %		0.68	
	2.0 %		3.02	
	4.0 %		5.74	
	8.0 %		10.9	
Sodium oleate	0.5 %	1.16		
	1.0 %	2.67		
	2.0 %	5.10		
	5.0 %	12.75		
Sodium lauryl sulphate	0.5 %		0.87	1.04
	1.0 %		1.78	2.07
	2.0 %		2.06	3.97
	8.0 %		7.20	16.56
	20.0 %		21.3	
Sodium myristyl sulphate	0.5 %	1.20		
	1.0 %	2.64		
	2.0 %	4.92		
	5.0 %	11.0		
	10.0 %	21.8		
	20.0 %	44.3		
Sodium cholate	0.5 %			0.012
	1.0 %			0.032
	2.0 %			0.130
	5.0 %			0.683
	10.0 %			1.90
	15.0 %			3.09
	20.0 %			4.36
Polyoxyethylene sorbitan monooleate	5.0 %	0.60	0.41	0.24
	10.0 %	1.21	0.82	0.48
	20.0 %	2.44	1.64	0.98

relation is obtained as in the case of the other colloids. A similar behaviour has also previously been noted in the power of this colloid to solubilize hydrocarbons [5,6,7]. This is due, as shown earlier [5], to the fact that the micelle forma-

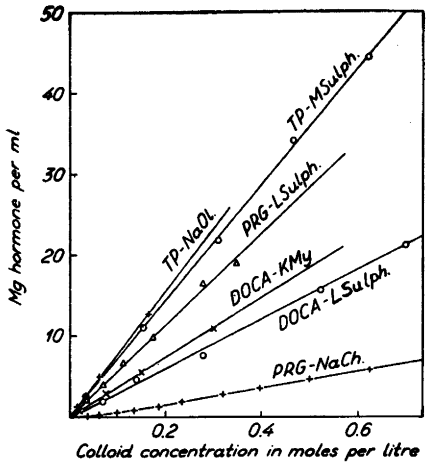

Fig. 3. The solubilities of ketosteroid hormones in various association colloid solutions. 40°C.

TP—NaOl. Testosterone propionate in sodium oleate solutions.

TP—MSulph. Testosterone propionate in sodium myristyl sulphate solutions.

PRG—LSulph. Progesterone in sodium lauryl sulphate solutions.

DOCA—KMy. Desoxycortieosterone acetate in potassium myristate solutions.

DOCA—LSulph. Desoxycorticosterone acetate in sodium lauryl sulphate solutions.

PRG—NaCh. Progesterone in sodium cholate solutions.

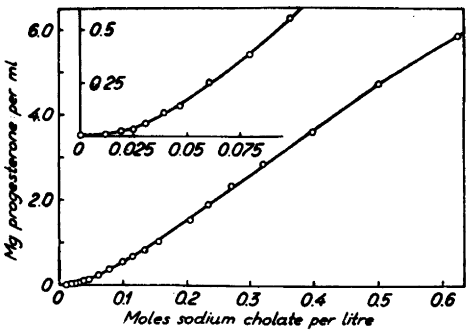

Fig. 4. The solubility of progesterone in aqueous solutions of sodium cholate. 40° C.

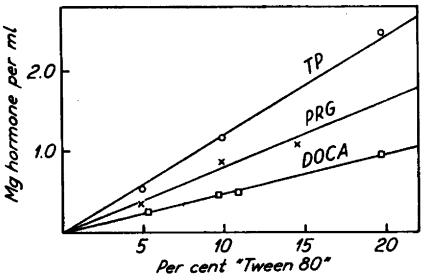

Fig. 5. The solubilities of TP, DOCA, and PRG in aqueous solutions of polyoxyethylene sorbitan monooleate ("Tween 80"). 40° C.

tion of sodium cholate occurs over a wider concentration range than in the case of the association colloids of the paraffin chain type and that the former takes place in several stages in which the micelles formed have different properties.

The saturation capacities of the micellar substances (the ratio of the amount of solubilized hormone in mg to the amount of micellar association colloid in moles) have been calculated from the linear parts of the solubility curves (Table 3). The data are still so limited that a comparison can not be made on this basis between the various hormones and between the different colloids. The differences noted are, however, fairly large. In the paraffin chain salt solutions testosterone propionate is the most soluble and desoxycorticosterone acetate the least soluble. The solubilizing power of the sodium cholate micelles

is much smaller than of the micelles of the other colloids although the hormone molecules partly have the same carbon skeleton as cholic acid.

Table 3. Maximum solubilizing powers of micellar substances for ketosteroid hormones.

Association colloid	Hormone	Mg hormone per mole micellar substance	Moles micellar substance per mole solubilized hormone
Potassium myristate	DOCA	37 200	10.0
Sodium oleate	TP	77 700	4.43
Sodium lauryl sulphate	DOCA	30 400	8.17
» » »	PRG	57 000	5.51
Sodium myristyl sulphate	TP	72 000	4.78
Sodium cholate	PRG	10 660	29.6
		Mg hormone per g Tween	Grams Tween per mole hormone
Polyoxyethylene sorbitan monooleate	TP	122	2 820
—»—	DOCA	82	4 090
—»—	PRG	49	6 410

If the micelles are assumed to contain 100 moles of the colloid substance, the number of moles of steroid hormone per micelle would be 10—20 moles in the case of paraffin chain colloids, but only about 3 moles in the cholate micelles. The degree of dispersion of the hormones solubilized in the association colloid solutions is thus relatively high.

From our results is seen that the steroid hormones in most cases are solubilized in larger amounts by the colloids than the polycyclic aromatic hydrocarbons.[7] For example, the solubility of progesterone is from about 40—50 times that of 1,2,5,6-dibenzanthracene in the solutions of most colloids. In the cholate solutions, however, the difference is smaller, the solubility of progesterone being only four times greater than that of dibenzanthracene. This is connected with the fact that whereas the solubilities of the polycyclic hydrocarbons per mole colloid are much higher in the bile acid salt solutions than in solutions of the paraffin chain salts, the opposite is the case, as we have seen, with progesterone.

These observations emphasize the special position of the bile acid salts among the association colloids. They further indicate, as do also other facts, that the solubilization differs in mechanism in the case of steroid hormones and

hydrocarbons. It seems probable that whereas the polycyclic hydrocarbons, like the lower hydrocarbons, are situated in the micelles between the hydrocarbon layers the hormones are, like alcohols and other hydropolar substances, situated between the molecules in the palisade layers of the micelles.

SUMMARY

Methods have been developed for the quantitative extraction of ketosteroids from aqueous solutions of association colloids. The isolated crystalline hormones can then be polarographically determined by the method of Eisenbrand and Picher. The methods have been used to determine the maximum solubilities of testosterone propionate, desoxycorticosterone acetate and progesterone in aqueous solutions of various association colloids.

We wish to express our indebtedness to Mr. Lars Portin for aid with the experimental work.

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Short Communications

Nitrobenzene Oxidation of the Products Formed by the Condensation of Resorcinol with Lignin Models *

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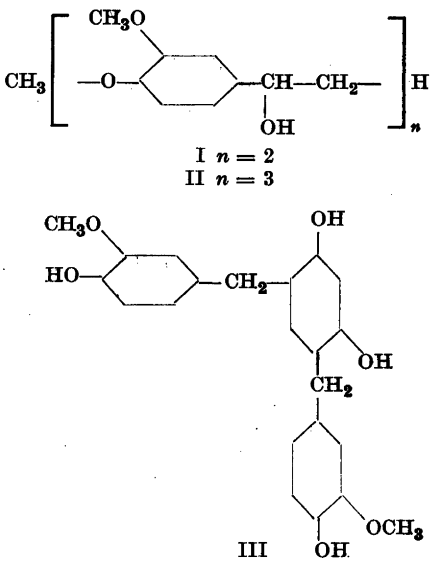
We have recently shown[1] that when lignin or lignin sulphonic acids are heated with resorcinol in acid solution, the yield of vanillin obtained on subsequent oxidation with nitrobenzene and alkali is decreased by up to 65 %. The present communication deals with the nitrobenzene oxidation of the products obtained from resorcinol and vanillyl alcohol[2] or the more complex ethers of guaiacyl glycol[3], I and II — compounds which closely resemble lignin as regards their condensation with reactive phenols, sulphonation, *etc.*

Vanillyl alcohol reacted with resorcinol in hot acid solution, yielding a crystalline product which, on the basis of its analysis and analogies with similar compounds, is considered to have the structure III. Similarly, I and II yielded amorphous products which appeared to contain one molecule of resorcinol per guaiacyl residue. The three compounds all gave vanillin on oxidation with nitrobenzene and alkali, and the yields are given in Table 1, the yield of vanillin being expressed as a percentage of the yield obtained from the corresponding compound before heating with resorcinol and acid.



I $n = 2$
II $n = 3$

Table 1. Yield of vanillin from compounds heated with resorcinol and acid.

Condensation product of	Yield of vanillin %
Vanillyl alcohol	33
I	30
II	31

When resorcinol alone was oxidized, a 50 % yield of a black, insoluble product was obtained; no unchanged resorcinol could be recovered.

It is evident that prior treatment of the compounds investigated with resorcinol has a marked effect on the subsequent yield of vanillin. However, the formation of vanillin is not entirely suppressed by the

* Part X in the series Studies on Lignin. Part IX. *Svensk Kem. Tid.* **64** (1952) 1.

initial condensation, and it is probably more than a coincidence that the decrease brought about by the reaction with resorcinol is of the same order of magnitude as was found in the case of lignin and lignin sulphonic acids subjected to similar treatment.

EXPERIMENTAL. *4,6-Bis(3-methoxy-4-hydroxybenzyl)-resorcinol (III).* A mixture of vanillyl alcohol (3 g) (conveniently synthesized by the reduction of vanillin, dissolved in dilute sodium hydroxide, with a 50 % excess of sodium borohydride), resorcinol (10 g), and 2 *N* hydrochloric acid (40 ml) was refluxed for one hour. After cooling, the solution was saturated with sodium chloride, and the precipitated oil (3 g) was dissolved in ether. The ether solution was dried over anhydrous sodium sulphate, and evaporated, and the residue was triturated with ether. A large amount of oily material was dissolved and the white powder obtained (0.5 g, 13 %) was repeatedly recrystallized from benzene, yielding transparent plates, m.p. 173–174°. Found OCH_3 16.5; required for $C_{30}H_{16}O_4(OCH_3)_2$, OCH_3 16.2.

Condensation of I and II. A mixture of I or II (0.5 g), resorcinol (2 g), 50 % aqueous ethanol (20 ml), and conc. hydrochloric acid (4 ml) was refluxed for one hour. After cooling, water (75 ml) was added and the precipitate was filtered off, washed with water and chloroform and dried.

I: Found OCH_3 17.6; required for the condensation product with two molecules of resorcinol, OCH_3 17.6.

II: Found OCH_3 15.0; required for the condensation product with three molecules of resorcinol, OCH_3 15.7.

The nitrobenzene oxidations and vanillin determinations were carried out as described previously [4].

The author expresses his thanks to Statens Tekniska Forskningsråd for financial support, and to Miss I. Malmström for skilful assistance.

1. Leopold, B. *Acta Chem. Scand.* **6** (1952) 55.
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On Quinone as Oxidising Agent in the Oppenauer-Oxidation

HENNING LUND

Department of Organic Chemistry, University of Technology, Copenhagen

In his paper on the broad applicability of the Meerwein-Ponndorf reduction of carbonyl groups by means of aluminium *iso*-propanolate Lund in 1937 [1] suggested the use of quinones for the opposite reaction: selective oxidation of >CHOH groups to >CO. The suggestion was based upon the observation that quinone was rapidly reduced to quinol by aluminium *iso*-propanolate and the quinol was immediately precipitated as aluminium quinolate. It might therefore be expected that an alcohol would react quantitatively with formation and precipitation of aluminium quinolate, as the equilibrium

$$R-CHOH-R^1 + C_6H_4O_2 \rightleftharpoons R-CO-R^1 + C_6H_4(OH)_2$$

would be displaced practically completely in favour of the right hand side, because the quinol is removed quantitatively from the solution.

In 1941 Adkins and Franklin [2], apparently without knowledge of the paper cited above, have made use of this reaction on the assumption that the high oxidation potential of quinone as compared with that of the usual aldehydes and ketones would favour the oxidation of the corresponding alcohols.

The reaction, with the use of aluminium *t*-butanolate as catalyst, was now reexamined with the purpose of finding a suitable method for the oxidation of valuable alcohols to ketones, *e.g.* in the steroid group. A few ordinary alcohols were examined first and it was found that with a reasonable excess of quinone a practically complete conversion of alcohols into ketones could be accomplished.

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The reaction, with the use of aluminium *t*-butanolate as catalyst, was now reexamined with the purpose of finding a suitable method for the oxidation of valuable alcohols to ketones, *e.g.* in the steroid group. A few ordinary alcohols were examined first and it was found that with a reasonable excess of quinone a practically complete conversion of alcohols into ketones could be accomplished.

When the reaction was carried out in benzene solution very often a violet colour appeared during the process and in certain cases the violet compound precipitated and the reduction stopped about halfway. The intensely coloured violet compound turned out to be the aluminium "salt" of quinhydrone and was formed immediately when a benzene solution of aluminium *t.*-butanolate was added to a benzene solution of quinhydrone.

It is known that cholesterol in the Oppenauer oxidation not only is oxidised to the ketone, but simultaneously the double bond between carbon atoms 5 and 6 is moved to the 4—5 position. This is the most stable configuration because the double bond comes in conjugation to the double bond in the C = O group[3]. It was hoped that under mild oxidation conditions with quinone this rearrangement might be avoided. Experiments showed that the oxidation by means of a little more than 2 mols of quinone proceeded approximately quantitatively, but the reaction product refused to crystallise. Obviously a mixture of ketonic compounds was formed and the purpose of the investigation, thus, was not reached. Distillation of the product at low pressure (~ 0.1 mm Hg) allowed the isolation of some Δ^4-cholestenone, evidently the main product of the reaction, identified through its dinitrophenylhydrazone and its semicarbazone.

1. Oxidation of benzhydrol. 18.4 g (0.1 mol) of benzhydrol, 13 g (0.12 mol) of quinone and 19.7 g (0.08 mol) of aluminium *t.*-butanolate were dissolved in 250 ml dry benzene and refluxed with stirring on a steam-bath for 1—1.5 h. A precipitate of aluminium quinolate was removed by suction. From the filtrate benzene and *t.*-butanol was removed by distillation, finally in vacuo. The residue was dissolved in ether, the solution was purified by washing with water, dried, and the ether was removed by distillation. The residue, 17.5 g of crystals with m.p. 48°, was practically pure benzophenone. 0.468 g gave 0.910 g dinitrophenylhydrazone or 98 %. Yield of benzophenone 94 %.

If the excess of quinone was reduced to 10 % the yield dropped to 88 % and the purity of the product from 98 % to 95 %.

2. Oxidation of menthol. 8 g (0.05 mol) of menthol and 8 g (0.033 mol) of aluminium *t.*-butanolate were dissolved in benzene and 6 g (0.055 mol) of quinone were added. The solution was refluxed for 0.5 h during which the violet quinhydrone-aluminium-compound precipitated. Further 6 g of quinone were added in order to finish the oxidation, and the solution was refluxed for 15 min. The precipitate was filtered off, the solution shaken with an alcaline solution of sodium sulphite (in order to remove an excess of quinone), washed with water, dried and fractionated. Between 204° and 207° 5.6 g ~ 72 % menthone were collected, identified as dinitrophenylhydrazonem m. p. 144°.

3. *Oxidation of cholesterol.* 10 g of cholesterol (0.026 mol), 10 g of aluminium *t.*-butanolate (0.040 mol) and 6 g of quinone (0.055 mol) were dissolved in 150 ml benzene and kept at 40—45° over night. The precipitate of the quinhydrone-aluminium compound was dissolved in dilute hydrochloric acid, the benzene layer was washed with water, dried and the solvent removed by distillation. The residue was dissolved in ether and methanol added. No cholestenone was precipitated. After removal of the solvents a syrup remained from which a dinitrophenylhydrazone (m. p. 236°), a semicarbazone (m. p. 234—35°), a *p*-carboxyphenylhydrazone (m. p. 275—76°) and a phenylhydrazone (m. p. 140°) were prepared. For the dinitrophenylhydrazone of Δ^4-cholestenone m. p. 238° is reported, for the semicarbazone 234° and 237°. The two other derivatives have not been recorded in the litterature.

The syrup was distilled in high-vacuum (~ 0.1 mm Hg). The distillate crystallised and was repeatedly recrystallised from ethanol. The pure substance, faint yellow crystals with m. p. 79°, m. p. of the dinitrophenylhydrazone 236°, is evidently Δ^4-cholestenone, but the syrupy consistence of the oxydation product before distillation may indicate that other cholestenones, too, are formed during the action of quinone on cholesterol. The identical m. p. of the dinitrophenylhydrazones of the substance before and after distillation proves that the rearrangement from Δ^5- to Δ^4-cholestenone takes place during the oxidation, not during the distillation.

Thanks are due to Professor Hakon Lund Department of Chemistry, The University,

Aarhus, and to Professor Stig Veibel for their interest in this work and for valuable discussions.

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On the Hexosamine Component of Seromucoid

IVAR WERNER

Institute of Medical Chemistry, Upsala, Sweden

Glucosamine has been shown by several authors to be a constituent of serum glycoproteins. Recent investigations at this institute have shown that 2-aminogalactose, chondrosamine, occurs together with sialic acid in submaxillary mucin, in gangliosides and in the acid glycoprotein of plasma [1,2]. In the two former substances the only hexosamine obtained was chondrosamine. In the latter, chondrosamine formed the minor part of the hexosamine component, the major part consisting of glucosamine.

In order to find out if chondrosamine is present in other serum glycoproteins containing sialic acid (as indicated by the reactions characteristic of that compound)[3] the present work was undertaken. The so called seromucoid, as it is usually prepared, is certainly neither a native nor a uniform serum component, but derives probably from different serum glycoproteins. As, however, the sialic acid content fis airly high in seromucoid and this substance is comparatively easy to prepare it was chosen for the investigation.

Seromucoid was prepared mainly according to Rimington [4]. The preparation contained 5.8 per cent hexosamine and 7.9 per cent hexose. The sialic acid content was calculated to about 4 per cent. One g of this material was heated at 104° for 24 h with 5 *N* hydrochloric acid. The hydrolysate was shaken with charcoal and filtered. After evaporation in vacuo with repeated additions of methanol the filtrate was finally brought to a small volume and dry methanol was added. After standing overnight in an exsiccator a crystalline deposit had formed and was collected. The crystals were shown by the X-ray powder diagram to be α-glucosamine hydrochloride. When more methanol was added glucosamine crystals continued to form for a couple of days. The mother liquor was then brought to a thick syrup and dry methanol was added again. After three weeks in the exsiccator a crystalline mass could be collected. The X-ray powder diagram of these crystals were completely identical to that of an authentic specimen of β-chondrosamine hydrochloride (Fig. 1). In total about. 5 mg of the chondrosamine salt were isolated.

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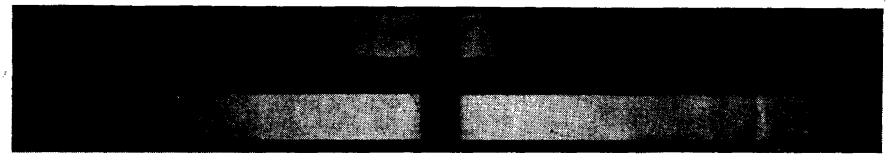

Fig. 1. I. Powder diagram of β-chondrosamine hydrochloride. II. Powder diagram of last aminosugar fraction obtained from seromucoid hydrolysate.

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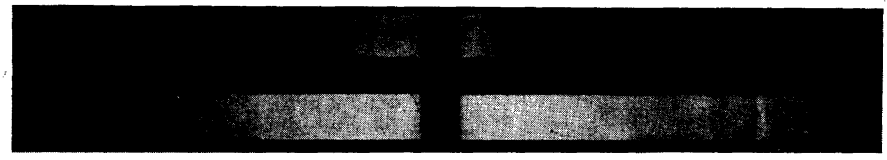


Fig. 1. I. Powder diagram of β-chondrosamine hydrochloride. II. Powder diagram of last aminosugar fraction obtained from seromucoid hydrolysate.

The Uptake of Lower Aliphatic Amines by Pea Plants

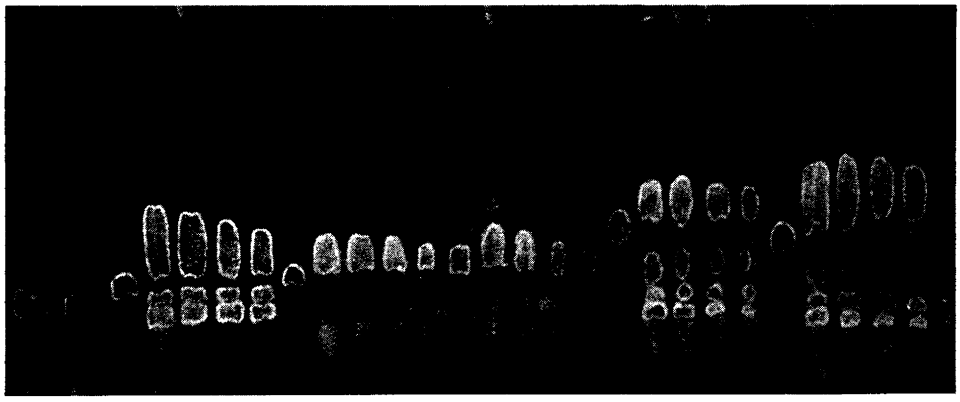
ARTTURI I. VIRTANEN and R. SCHWYZER*

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

To what extent the plants are able to take up organic compounds from the soil by their roots is still an open question. By means of the sterile culture method it has been possible to demonstrate that plants can grow on certain amino acids as their sole N-source but even then there is the possibility that the amino group splits off as ammonia already on the surface of the root. Virtanen [1] has advanced the opinion that aspartic and glutamic acids, which are good nitrogen sources for pea — surprisingly enough wheat and barley are entirely unable to utilize them — are taken up as such by the root although the deamination and transamination reactions may occur already in the root cells. The decarboxylation product of phenylalanine, phenylethylamine, which does not act as nitrogen nutrition for plants, causes even in small concentrations, according to Virtanen and Linkola [2], a curious branching on pea. *α*-Alanine, too, causes changes in the shape of pea. These observations strongly suggest that the plants really take up organic nitrogen compounds as such and also those which are not used up for N-nutrition. Steinberg [3] has later noted a transforming effect of isoleucine on the tobacco plant.

The question of the uptake of organic substances from the soil is not only of theoretical value but has a great practical bearing since some of these substances may even in small amounts affect injuriously both the animal and human nutrition.

* Visiting scientist from the Chemisches Institut der Universität Zürich, Switzerland.



40 15 µl	5γ 11.5 10 7.5 5 µl	5γ 10 7.5 5 2.5 µl	5γ 7.5 5 2.5 1.2 µl	5γ 12 10 7.5 5 µl	5γ 15 10 7.5 5µl
2	1 2	1 2	1 2	1 2	1 2
No amines	*Trimethylamine*	*Dimethylamine*	*Ethylamine*	n-*Propylamine*	i-*Propylamine*

1) Known concentration in water solution. 2) Plant extract.

Fig. 1. Uptake of amines by pea plants.

Experimental. The chromatographic methods enable us to detect in plants even small amounts of foreign organic substances, which have been added to the root support. Therefore, we have taken this method into use in this laboratory for studying accumulation of different organic substances in plants and their possible participation in the metabolism. Using the paperchromatographic detection and semiquantitative determination of aliphatic amines described by one of us [4] we have been able to show that dimethylamine, trimethylamine, ethylamine, *n*-propylamine, and *i*-propylamine are taken up by pea plants growing in sterile culture in a nutrient solution containing these amines as their HCl-salts.

A nutrient solution was prepared containing no other source of nitrogen except the amine under study in a quantity representing 25 mg of organic nitrogen per litre of nutrient solution. 6 day old pea plants were grown for three weeks in sterile culture on these nutrients. The plants growing with trimethylamine began to excrete this substance through the leaves after a few days, as could be noticed by their odour.

After three weeks the plants were dried for two days at 40° C and pulverized. Aliquots of 100 mg were thoroughly mixed with 0.50 ml of absolute alcohol containing 2 per cent glacial acetic acid. Series of volumes of 1 to 15 μl of the extracts were placed on filter paper (Munktell OB), and chromatographed in the usual manner. These dilution series were compared with known amounts of the amines run on the same chromatogram. As an average of 4 plants, the following amounts of amine were taken up: (On drying some loss of amines occurs).

	Dry weight per plant g	Amine per plant mg	Total N per plant mg
Control	0.213	0.0	5.5
Dimethylamine	0.258	2.9	7.8
Trimethylamine	0.232	1.1	6.2
Ethylamine	0.286	3.1	9.9
Propylamine (*n*-)	0.261	1.6	6.7
Propylamine (*iso*-)	0.213	1.4	7.3

A photograph of the obtained chromatograms is given in Fig. 1.

A basic compound ($R_f = 0.20$) present in the controls and in plants grown on trimethylamine and propylamines was missing in the plants grown on ethylamine and dimethylamine. The probable metabolic function of amines in the plant organism is under further investigation in this institute.

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Reduktionsversuche mit Hämoglobin an Benzhydroxamsäure und Brenztraubensäureoxim

R. SCHWYZER

Laboratorium der Stiftung für chemische Forschung, Biochemisches Institut, Helsinki, Finnland

Wegen des Auftretens organischer Derivate im lebenden Organismus wurde dem Hydroxylamin die Rolle eines Zwischenproduktes der Stickstoffassimilation zugesprochen.[1] So entstehen aus Hydroxylamin und Derivaten organischer Säuren auf enzymatischem Wege Hydroxamsäuren [2–8]. Es wäre auch denkbar, dass Oxime von Ketosäuren als Zwischenprodukte bei der Verwendung des Hydroxylamins durch den Organismus auftreten, obwohl bisher keine Enzymsysteme aufgefunden worden sind, welche einen solchen Prozess katalysieren [1].

Ausgehend von Beobachtungen von Colter und Quastel [9] über die Reduktion von Hydroxylamin durch Hämoglobin und Ascorbinsäure, versuchten wir, ob unter ähnlichen Versuchsbedingungen auch Benzhydroxamsäure und Brenztraubensäureoxim zu Benzamid resp. Alanin reduziert werden. Diese Versuche wurden spe-

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ziell in Hinsicht auf das Vorkommen von Leghämoglobin in den Knöllchen der Leguminosen ausgeführt.

Mit lysierten Erythrozyten aus Kuhblut werden in Gegenwart von Ascorbinsäure bei pH-Werten von 6,8 und 7,4 im Gegensatz zu Hydroxylamin weder Benzhydroxamsäure noch Brenztraubensäureoxim reduziert.

Experimente.

Hämoglobinlösung. Frisches, defibriniertes Kuhblut wurde zentrifugiert, die Blutzellen wurden 6-mal mit physiol. Kochsalzlösung gewaschen und zuletzt mit ihrem vierfachen Volumen an destilliertem Wasser versetzt. Die klare Lösung wurde bis zu ihrem Gebrauche in gefrorenem Zustande aufbewahrt.

Reduktion von Hydroxylamin. Um die Wirksamkeit der Hämoglobinlösung festzustellen, haben wir die Reduktion des Hydroxylamins colorimetrisch verfolgt. Ferrosalze katalysieren, wie wir festgestellt haben, die Reduktion mittels Ascorbinsäure nicht.

3 ml Hämoglobinlösung, 3 ml Ascorbinsäurelösung (frisch hergestellt, 30 mg Ascorbinsäure auf pH = 7,4 abgepuffert) sowie 0,6 ml Hydroxylaminlösung (10 mg HCl-Salz pro ml) wurden vereinigt und bei 38° C aufbewahrt. Von Zeit zu Zeit wurden Proben entnommen und der Hydroxylamingehalt colorimetrisch nach der Vorschrift von Csàky [10] bestimmt (Endverdünnung 1 000-fach). Nach drei Stunden war kein Hydroxylamin mehr nachzuweisen. In dem Kontrollansatze, welcher anstelle des Hämoglobins eine Spur Ferrosalz enthielt, war in dieser Zeit keine Abnahme des Hydroxylamingehaltes nachzuweisen.

Reduktionsversuch mit Benzhydroxamsäure. Die Versuche wurden genau gleich angesetzt, nur wurde anstelle des Hydroxylamins Benzhydroxamsäure (9 mg) verwendet. Der Hydroxamsäuregehalt der Lösungen wurde von Zeit zu Zeit colorimetrisch anhand des roten Ferri-Komplexes verfolgt (vgl. Lipmann und Tuttle [11]), nachdem in den Proben Proteine mit Trichloressigsäure entfernt worden waren. Es wurde Sorge getragen, genügend Ferrisalz zuzufügen, um alle Ascorbinsäure zu oxydieren. Auch nach 15 St. (38° C) konnte weder beim pH 7,4 noch 6,8 eine Abnahme des Hydroxamsäuregehaltes festgestellt werden.

Reduktionsversuch mit Brenztraubensäure oxim. In gleicher Weise wurde versucht, 10 mg Brenztraubensäureoxim (in Lösung aus äquivalenten Mengen Na-Pyruvat und $NH_2OH \cdot HCl$ hergestellt) bei pH 6,8 und 7,4 zu reduzieren. Nach 20 St. bei 38° C wurde dem Ansatze soviel abs. Alkohol zugesetzt, bis der Alkohol 75 %ig war. Nach Aufkochen, Filtrieren und Verjagen des Lösungsmittels im Vakuum wurde der Rückstand in 1 ml Wasser aufgenommen und verschiedene Mengen davon der Papierchromatographie unterworfen (eindimensional, Phenol als Entwicklungsflüssigkeit). Weder in dieser Lösung, noch in einer Kontrolle, welche gleich, aber ohne Brenztraubensäureoxim, hergestellt worden war, liess sich Alanin nachweisen. Dagegen gelang der Nachweis leicht in einem Kontrollansatze, dem zu Beginn Alanin zugesetzt worden war.

Herrn Prof. A. I. Virtanen möchte ich für die Anregung zu dieser Arbeit und für die gastfreundliche Aufnahme an seinem Institute, sowie der »Stiftung zur Förderung des akademischen Nachwuchses» des Kantons Zürich, Schweiz, für ein Reisestipendium bestens danken.

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Influence of Compound Formation on Activity Factor Curves for Binary Mixtures

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Dolezalek [1] has shown that for liquid mixtures, deviations from Raoult's law can in some cases be explained by assuming association of one of the components or compound formation. He assumed the molecular species present to obey Raoult's law; the problem was to correctly identify the various species present and their amounts. Denoting the components by A and B he developed equations in two cases: the formation of the symmetric compound AB, and the association of A to A_2. Later on Hildebrand and Eastman [2] in their study of thallium amalgams developed expressions assuming the formation of the compound AB_2.

The present author has made an extension of Dolezalek's theory to the general case A_uB_v, this work being part of a critical comparison of a number of such simplified assumptions to the observed deviations from ideality in binary mixtures such as ion exchangers.

We make the following assumptions: we have two components A and B in a two phase equilibrium. The ideal laws are valid, but in one phase we have compound formation or association. In the following only the case of compound formation will be dealt with

A and B interact to form the compound A_uB_v according to the reaction:

$$\underset{x_1}{uA} + \underset{x_2}{vB} \rightleftharpoons \underset{x_3}{A_uB_v} \qquad (1)$$

$$k = \frac{[A_uB_v]}{[A]^u \cdot [B]^v} = \frac{x_3}{x_1^u \cdot x_2^v} \qquad (2)$$

where x_1, x_2 and x_3 are the true mole fractions of A, B and A_uB_v. The apparent mole fractions X_1 and X_2 of A and B are connected with the true mole fractions by:

$$X_1 = (x_1 + ux_3)/(x_1 + x_2 + (u+v)x_3)$$
$$X_2 = (x_2 + vx_3)/(x_1 + x_2 + (u+v)x_3) \qquad (3)$$

Since the ideal laws are valid we have the following expressions for the apparent activities a_1, a_2 and activity factors g_1, g_2, if we take the pure components as standard states:

$$a_1 = x_1 \qquad (4) \qquad g_1 = x_1/X_1 \qquad (5)$$
$$a_2 = x_2 \qquad\qquad g_2 = x_2/X_2$$

By experimental methods it is possible to find the $g_1(X_1)$ and $g_2(X_2)$ curves. As shown below it can be seen from these curves, whether they may be due to compound formation or not. From (2), (3) and (5) we find the following limiting values for g_1 and g_2, remembering that $x_1 = 1$, $x_2 = 0$ at $X_2 = 0$ and that $x_1 = 0$, $x_2 = 1$ at $X_2 = 1$:

$X_2 = 0$: $g_1 = 1$; $g_2 = 1$ for $v > 1$ and
$g_2 = 1/(1+k)$ for $v = 1$ (6)
$X_2 = 1$: $g_2 = 1$; $g_1 = 1$ for $u > 1$ and
$g_1 = 1/(1+k)$ for $u = 1$

By setting $g_1 = g_2$ one finds:

$$X_2 = v/(u+v) \qquad (7)$$

From (6) and (7) it is seen that the point of intersection of g_1 and g_2 gives the composition of the compound and the limiting values of g_1 and g_2 will give the value of k if one of u or $v = 1$. If, however, the g curves do not have the limiting values predicted from (6), they cannot be explained by compound formation only.

In Figs. 1 and 2 the a and g values are given as functions of X_2 assuming the compound A_3B to be formed. The func-

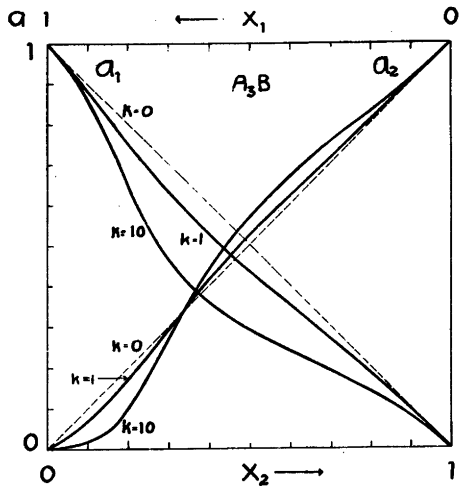

Fig. 1. The activities a_1 and a_2 are plotted against X_2, assuming the compound A_3B to be formed. The activities have been calculated for $k = 1$ and 10. The dotted lines give a_1 and a_2 when Raoult's law is valid.

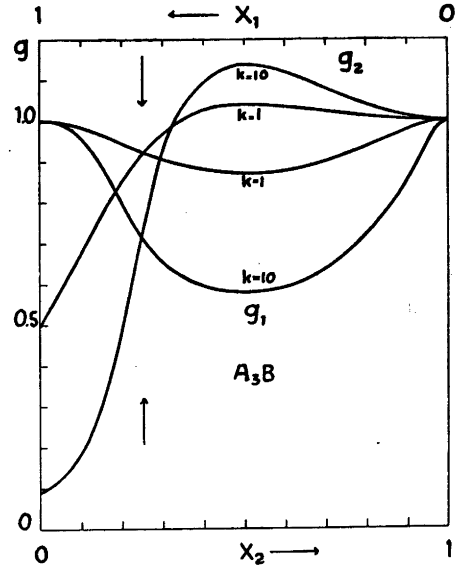

Fig. 2. The activity factors g_1 and g_2 are plotted against X_2, assuming the compound A_3B to be formed. The activity factors have been calculated for $k = 1$ and 10. The arrows indicate the points of intersection at $X_2 = 0.25$.

tions have been calculated for $k = 1$ and 10. It may be noted that depending upon the symmetry of equations (2)—(5), the a and g functions, assuming AB_3 to be formed, are mirror images of those given in Figs. 1 and 2.

Application to ion exchange equilibria:

The equilibrium quotient λ_{21} for the ion exchange reaction:

$$z_1 A_2^{z_2+} + z_2 A_1 R_{z_1} \rightleftharpoons z_2 A_1^{z_1+} + z_1 A_2 R_{z_2} \quad (8)$$

is defined by:

$$\lambda_{21} = \frac{\left\{A_1^{z_1+}\right\}^{z_2} \quad X_2^{z_1}}{\left\{A_2^{z_2+}\right\}^{z_1} \quad X_1^{z_2}} = K_{21} \frac{g_1^{z_2}}{g_2^{z_1}} \quad (9)$$

where K_{21} is the thermodynamic equilibrium constant of (8).

In Fig. 3 log λ_{21} calculated from (9) is plotted against X_2 for $k = 10$, $K_{21} = 10$ and $z_1 = z_2 = 1$ assuming that one of

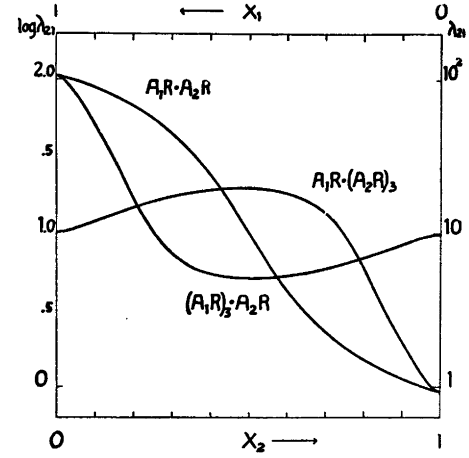

Fig. 3. Log λ_{21} is plotted against X_2, assuming one of the following compounds $A_1R \cdot A_2R$, $A_1R \cdot (A_2R)_3$ and $(A_1R)_3 \cdot A_2R$ to be formed. The equilibrium quotient has been calculated for $k = 10$, $K_{21} = 10$ and $z_1 = z_2 = 1$.

the following compounds is formed: 1) $A_1R \cdot A_2R$, 2) $A_1R \cdot (A_2R)_3$ and 3) $(A_1R)_3 \cdot A_2R$. From Fig. 3 is seen that the symmetric compound gives a decreasing log $\lambda_{21}(X_2)$ curve, the asymmetric compounds AB_3 and A_3B give curves with maximum or minimum. For arbitrary values of z_1 and z_2 the log $\lambda_{21}(X_2)$ curves have essentially the same form as those reproduced in Fig. 3.

By comparing Figs. 1—3 with the corresponding Figs. in the preliminary communications by Högfeldt *et. al.*[3-4] or in the full paper by Högfeldt [5] it is seen that they have the same general form. Especially the g curves calculated from Marinsky's measurements have a form almost identical with those caused by compound formation. The point of intersection gives $X_2 = 0.17$. From (6) and (7) $X_2 = 0.17$ gives $v = 1$, $u = 5$. The compound $(HR)_5 \cdot BaR_2$ has also been proposed by Marinsky and Coryell [6], who found by trial that it fitted the experimental data. The first to use this approach was probably Kielland [7], who attributed the deviations from Duhem-Margules equation he found in ion exchange systems to compound formation.

Although certainly over-simplified, the assumption of compound formation is, so far as I know, the only approach hitherto published that gives an explanation of the existence of extreme values of the equilibrium quotients. Any theory dealing with ion exchange equilibria must allow for such extremes, and the striking qualitative agreement of this simple assumption with experimental data may perhaps focus the attention on some kind of interaction between the components in the exchanger.

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Dissolving velocity of Metals in Deutero-Electrolytes

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The present paper is a preliminary report on experiments dealing with the dissolution of metals in acids and bases, the hydrogen content of which had been partly or completely replaced by deuterium.

Dissolving velocity experiments. The dissolving velocity of metals in non-oxidizing acids is usually determined by measuring the rate of hydrogen evolution. This method was also employed in the present study. Some results are given here for zinc, aluminium and iron.

1. Zinc. 2 mm wire, prepared from a very pure zinc was used. The reagents were 1 *N* hydrochloric acid and 2 *N* sulphuric acid with varying content of deuterium. Curves representing the mean values from the experiments with sulphuric acid at 25° C are shown in the diagram Fig. 1. Similar curves were obtained in the experiments with hydrochlorid acid.

Further it was attemped to etablish a sort of "model local element", the cathodic area of which could be maintained constant during the dissolving process. The anode of the element, 2 mm zinc wire, was fixed axially in a nickel cylinder, and the ends of the wire connected to the cylinder.

When this element was immersed in acid practically all the hydrogen liberation took place at the inside area of the nickel cylinder.

The curves obtained were of a different character than those shown in Fig. 1. There

the following compounds is formed: 1) $A_1R \cdot A_2R$, 2) $A_1R \cdot (A_2R)_3$ and 3) $(A_1R)_3 \cdot A_2R$. From Fig. 3 is seen that the symmetric compound gives a decreasing log $\lambda_{21}(X_2)$ curve, the asymmetric compounds AB_3 and A_3B give curves with maximum or minimum. For arbitrary values of z_1 and z_2 the log $\lambda_{21}(X_2)$ curves have essentially the same form as those reproduced in Fig. 3.

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When this element was immersed in acid practically all the hydrogen liberation took place at the inside area of the nickel cylinder.

The curves obtained were of a different character than those shown in Fig. 1. There

was no period of induction. The average dissolving velocities at different D-concentrations are given in Table 1.

Table 1. Dissolving velocity ϱ in ml hydrogen/min.sq.cm.

% D	ϱ
0	2.59
30	2.48
50	2.11
75	1.96
90	1.83
99	1.63

2. *Aluminium.* Johnston and Davies [1] have reported some preliminary observations on reaction rates of aluminium with 4.5 *N* sulphuric acid at 50° C. The present investigations on aluminium began with repetition of the experiment reported in [1]. High purity aluminium wire was used. The

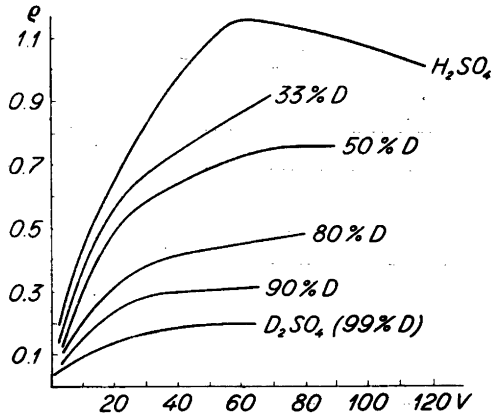

Fig. 1.
ϱ denotes ml hydrogen developed per min. and sq. cm. metal surface, thus representing the dissolving velocity.
V denotes ml hydrogen liberated since the beginning of an experiment, thus representing the quantity of zinc dissolved.

Table. 2. Dissolving velocity ϱ of aluminium, in ml hydrogen per hour. T denotes time in hours elapsed since the beginning of an experiment.

4.5 *N* sulphuric acid (50° C)		4.5 *N* hydrochloric acid (25° C)		2 *N* potassium hydroxide (25° C)	
T.	ϱ	T.	ϱ	T.	ϱ
	H_2SO_4		*HCl*		*KOH*
2	1.25	0.25	4.7	0.04	12.6
25	0.30	1.7	2.3	0.45	8.4
40	0.29	35	0.15	1.1	8.4
		35	0.15		
	30 % D.		*50 % D.*		*30 % D.*
2.8	0.73	0.3	13.5	0.05	10.2
25	0.24	1.6	3.0	0.57	7.2
45	0.24	35	0.16	0.9	7.2
	50 % D.	56	0.17		*50 % D.*
3	0.80		*99 % D.*	0.04	7
37	0.21	0.35	22.7	0.55	6.2
46	0.23	1.4	4.8	0.88	6.1
	70 % D.	34	0.4		*70 % D.*
2.7	0.9	56	0.33	0.04	8.8
28	0.2			0.52	5.6
52	0.2			0.74	5.5
	99 % D.				*98 % D.*
2	0.7			0.04	7.8
25	0.15			0.94	4.6
52	0.145			1.8	4.5

Table 3.

η *denotes overvoltage in millivolts.*
C. D. » *current density (amp/sq.cm.)*

	% D.	C.D.	η
Copper in 1 *N* hydrochloric acid	0	10^{-3}	525
	75		546
	99.5		587
Nickel in 1 *N* hydrochloric acid	0	»	178
	30		186
	50		209
	75		235
	99.5		260
Lead in 1 *N* hydrochloric acid	0	»	1 044
	50		1 088
	80		1 127
	99.5		1 175
Iron in *N* potassium hydroxide	0	»	170
	50		188
	80		232
	98		269
Mercury in 0.2 *N* sulphuric acid	0	10^{-4}	828
	50		831
	80		894
	99.5		903

observations showed satisfactory agreement with the results reported in [1]. Some mean values are given in Table 2.

Experiments with aluminium in 4.5 *N* hydrochloric acid at 25° C gave the surprising result that the reaction rate was much higher with DCl (99 % D) than with HCl. Some mean values are given in Table 2. Similar results were obtained with 2 *N* hydrochloric acid.

Further a number of observations were made on the dissolving velocity of aluminium in 2 *N* potassium hydroxide. Some mean values are given in Table 2.

3. Iron. Experiments with commercial mild steel and high purity iron in 2 *N* hydrochloric acid also showed a marked reducing effect of the deuterium content on the dissolving rate.

Measurements of overvoltage. In the present work are included measurements on mercury, copper, nickel, lead and iron at 25° C.

Some mean values from the overvoltage-C. D. curves are tabulated in Table 3.

1. Johnston, H. L., and Davies, C. D. *J. Am. Chem. Soc.* **64** (1942) 2613.

Received November 8, 1951.

Molecular Configuration of 1,2-Dichloro-4,5-Dibromocyclohexane ($\varepsilon\,\varepsilon$—$\varkappa\,\varkappa \rightleftharpoons \varkappa\,\varkappa$—$\varepsilon\,\varepsilon$) in the Vapour State

O. BASTIANSEN and O. HASSEL

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Some months ago the 1,2-dichloro-4,5-dibromocyclohexane corresponding to the 1,2,4,5,-tetrachlorocyclohexane of m.p. 176° C and the tetrabromocyclohexane of m.p. 187° C was prepared in our laboratory [1]. We wanted to decide which of the two possible configurations is the energetically preferred one, that in which the chlorine atoms occupy $\varkappa$ positions and the bromine atoms ε positions or the reversed configuration (Fig. 1).

The substance (m.p. 171°) turned out to crystallize isomorphous with the two substances mentioned above, the only element of symmetry required by the space group being a two-fold axis of symmetry. An X-Ray investigation, the result of which is now being published in this journal [2], showed the chlorine atoms to be in ε positions and the bromine atoms in $\varkappa$ positions (Fig. 1 a).

Although this result seemed entirely acceptable from a theoretical point of view, the objection could be raised that the

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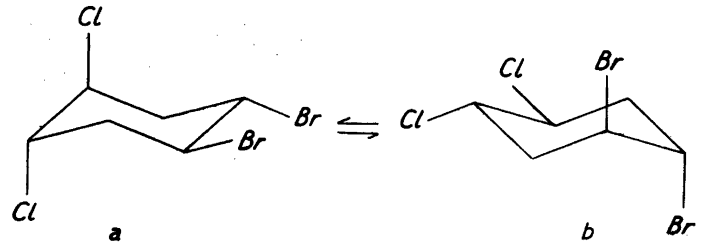

Fig. 1.

configuration b (Fig. 1) might be present to a considerable extent in the vapour. We have therefore investigated the substance in the gaseous state using the electron diffraction technique based upon the rotating sector.

The experimentally obtained distance distribution curve ($\frac{\sigma(r)}{r}$ curve) is reproduced in Fig. 2, and below this curve line diagrams giving the internuclear distances (and their relative weights) for three

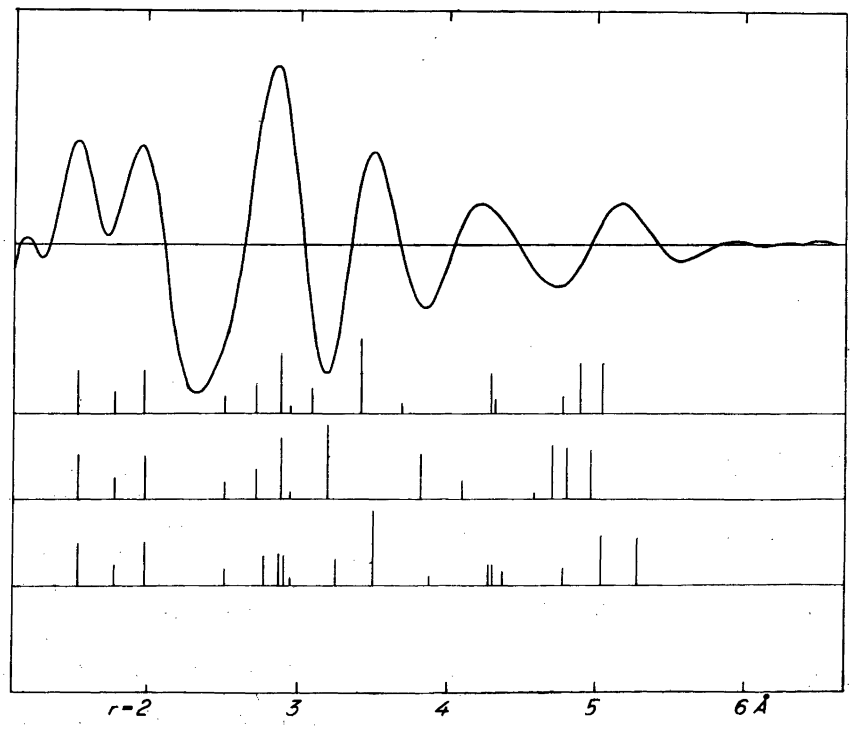

Fig. 2.

possible models: a) a model having the chlorine atoms in ε position, the bromine atoms in ϰ positions (Fig. 1 a), and with all valency angles strictly tetrahedral, b) a corresponding idealized model of the second possible configuration (the chlorine atoms in ϰ positions, the bromine atoms in ε positions, Fig. 1 b), c) a *distorted* model having the chlorine atoms in ε position and the bromine atoms in ϰ position. It is easily recognized that the second line diagram cannot account for the maxima of the experimental curve and that the first is in fair agreement with the experimental result. It seems obvious, however, that some minor distortions of the valency angles will be necessary to bring the line diagram in full agreement with the experimental curve. First of all the C-Cl bond will have to be bent away from the chief axis of the carbon ring. We found that an angle of 8° between this bond and the directions of the chief axis leads to the best agreement with experiment. It is interesting to note that a distortion of the same kind and very nearly the same magnitude was observed in the corresponding tetrachloro-compound both in the crystal and in the vapour [3]. It is no doubt due to repulsive forces acting between the chlorine atoms and the nearest (ε bonded) hydrogen atoms. In addition to this deviation from strictly tetrahedral valency angle a smaller distortion (3°) of the C-C-Br angle bringing the neighbouring bromine atoms a little further apart than in the idealized structure was introduced.

1. Hassel, O., and Lunde, K. To be published shortly.

2. Hassel, O., and Wang Lund, E. *Acta Chem. Scand.* In publication.

3. Hassel, O., and Wang Lund, E. *Acta Cryst.* **2** (1949) 309.

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Hydrogen Peroxide Decomposition Catalyzed by Ferric Salt in Solutions acidified with Perchloric Acid

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In a previous work [1] the author has shown that the course of hydrogen peroxide decomposition catalyzed by ferric salt in solutions containing nitric acid deviates from the unimolecular scheme. Bray and Peterson [2] states in a paper published recently that this deviation should be ascribed to the presence of nitrate ions, as they have been able to reproduce the deviation in experiments made in solutions acidified with nitric acid, but not in solutions acidified with perchloric acid.

Abel[3] is likewise of the opinion that the deviation must be due to the influence of nitrate ions, although this author has not any experimental results on which to base this contention.

As the said deviation forms the actual experimental basis on which the author of the present work has established the mechanism proposed for the hydrogen peroxide decomposition catalyzed by ferric salt [1,4,5] the author considers it necessary once more to revert to this subject quite briefly by presenting the results of a single experiment with two repetitions. The experiment was made with hydrogen peroxide, ferric salt and acid in the same concentrations as those used for the purpose of the experiments of table 1 of the paper quoted above [1], but this time the ferric nitrate and the nitric acid were replaced with ferric perchlorate and perchloric acid. The ferric perchlorate used was prepared by Robertson * during his

* C. A. Robertson, who has published several works dealing with this problem, *e. g. J. Am. Chem. Soc.* **45** (1923) 2493.

possible models: a) a model having the chlorine atoms in ε position, the bromine atoms in ϰ positions (Fig. 1 a), and with all valency angles strictly tetrahedral, b) a corresponding idealized model of the second possible configuration (the chlorine atoms in ϰ positions, the bromine atoms in ε positions, Fig. 1 b), c) a *distorted* model having the chlorine atoms in ε position and the bromine atoms in ϰ position. It is easily recognized that the second line diagram cannot account for the maxima of the experimental curve and that the first is in fair agreement with the experimental result. It seems obvious, however, that some minor distortions of the valency angles will be necessary to bring the line diagram in full agreement with the experimental curve. First of all the C-Cl bond will have to be bent away from the chief axis of the carbon ring. We found that an angle of 8° between this bond and the directions of the chief axis leads to the best agreement with experiment. It is interesting to note that a distortion of the same kind and very nearly the same magnitude was observed in the corresponding tetrachloro-compound both in the crystal and in the vapour [3]. It is no doubt due to repulsive forces acting between the chlorine atoms and the nearest (ε bonded) hydrogen atoms. In addition to this deviation from strictly tetrahedral valency angle a smaller distortion (3°) of the C-C-Br angle bringing the neighbouring bromine atoms a little further apart than in the idealized structure was introduced.

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stay in 1933 at the Institute of Physical Chemistry of the University of Copenhagen, the hydrogen peroxide used was Merck's Perhydrol, which was redestilled in an apparatus of glass to avoid all traces of inhibitors, and the perchloric acid used was of Merck's quality "analytical reagent".

The experimental results have been calculated by means of the formula applied previously [1].

The constants found were (average of 3 experiments):

$$A = (0.93 \pm 0.03) \cdot 10^{-3}$$

and

$$B = (1.66 \pm 0.02) \cdot 10^{-2}$$

An experiment made by Arne Nielsen, the Institute of Physical Chemistry of the University of Copenhagen, gave a similar result.

The corresponding experiment with a solution containing nitric acid provided the following results: $A = 1.50 \cdot 10^{-3}$ and $B = 1.78 \cdot 10^{-2}$.

In proof of the applicability of the formula the two functions $\log \frac{a}{x}$ and $f(x) = \log \frac{a}{x} + A \left(\frac{1}{x} - \frac{1}{a}\right)$ have been plottet against t in Fig. 1 curve I and curve II respectively.

The figure shows quite clearly:

1. that the reaction cannot be considered to be a unimolecular reaction, if so, curve I should have been a straight line.

2. that the reaction, even when it has proceeded almost to completion, conforms with close approximation to the velocity expression used for calculation of the experiments [1]. Curve II is a straight line.

The difference between the magnitudes of the constants A and B as calculated from the experiment on decomposition in a solution containing nitric acid and as calculated from the experiment on decomposition in a solution containing perchloric acid should no doubt be ascribed to a difference in the salt effect.

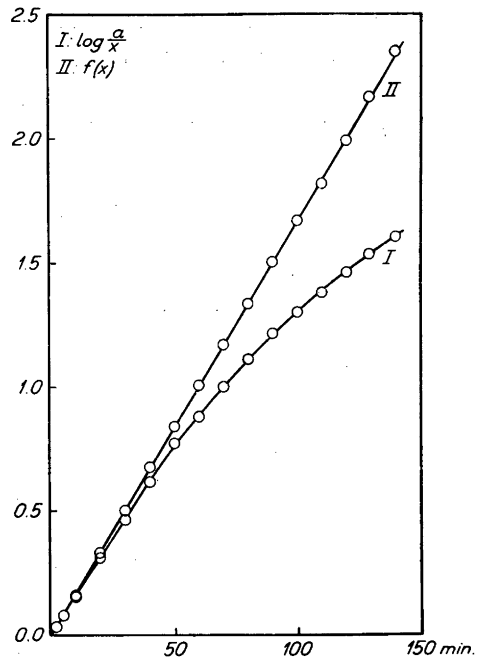

Fig. 1. The decomposition of hydrogen peroxide at 25° C in solution 0.004 M as to ferric perchlorate and 0.01 M as to perchloric acid. The initial concentration of hydrogen peroxide was 0.05 M. Curve I is a plot of $\log \frac{a}{x}$ *against time t. Curve II represents the function* $f(x) = B \cdot t = \log \frac{a}{x} + A \cdot \left(\frac{1}{x} - \frac{1}{a}\right)$ *plotted against time t;* $A = 0.95 \cdot 10^{-3}$; $B = 1.67 \cdot 10^{-2}$.

I wish to express my gratitude to Professor J. A. Christiansen for his kind interest in my work. Further I wish to thank Mr. Arne Nielsen for carrying out one of the experiments.

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m- and *p*-Nitrobenzazide

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m-Nitrobenzazide is recommended [1–5] as an excellent reagent for the characterization and estimation of aliphatic and aromatic hydroxyl compounds, as it forms well crystallizing *m*-nitrophenylcarbamic esters [3–6] in which the nitro group may be titrated with titanous chloride. Recently [7,8] it has been employed as a reagent also for amines, with which it reacts to form substituted *m*-nitrophenylureas.

The preparation previously given [3,5], involves the treatment of methyl *m*-nitrobenzoate with hydrazine hydrate and the subsequent treatment of *m*-nitrobenzohydrazide with nitrous acid. As hydrazine hydrate is expensive and frequently unavailable, it was considered desirable to develop a method of preparation from cheaper starting materials. The method of treating the acid chloride with sodium azide, as mentioned by Naegeli and Tyabji [9] has proved to be successful in this case. Sodium azide is commercially available and comparatively cheap and the method described below is somewhat simpler than that previously described and gives as good a yield.

m-Nitrobenzoyl chloride, b. p. 153—154° at 12 mm (m. p. 33°) is obtained from m-nitrobenzoic acid [10] and excess of thionyl chloride in about 90 % yield.

m-Nitrobenzazide. As small amounts of the toxic hydrazoic acid may be liberated, the reaction should be carried out in a hood. A solution of 78 g (1.2 moles) of sodium azide in 500 ml of water, kept at room temperature (20—25°) by means of a water-bath, is efficiently stirred, and a solution of 185.5 g (1 mole) of m-nitrobenzoyl chloride in 300 ml of acetone is added from a dropping funnel during one hour. The m-nitrobenzazide separates at once as a white precipitate. The reaction mixture is stirred for ½ hour, 500 ml of water added and stirring continued for ½ hour. The azide is filtered, washed with water and dried in the air. The yield is almost quantitative. The product melts at 68° as reported in the literature [5] and is, in general, sufficiently pure as a reagent. It may, however, be recrystallized with about 15 % loss from a mixture of equal parts of benzene and ligroin (b. p. 100—140°), the maximum temperature used for the dissolution being below 50°. *m*-Nitrobenzazide is then obtained as almost colorless crystals, m. p. 68° (the melted substance decomposes with liberation of nitrogen).

Similarly *p-nitrobenzazide* was prepared. In this case the crude product, obtained in 90 % yield, was slightly discolored. It was, therefore, dissolved in a small amount of benzene, decolorizing charcoal added and the solution filtered. An equal volume of ligroin was added to the resultant reddish yellow solution. On seeding, p-nitrobenzazide crystallized in yellow crystals, m. p. 71—72° (reported 69°), yield 70 %.

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A Method for Determination of the Amino Acid Sequence of Proteins

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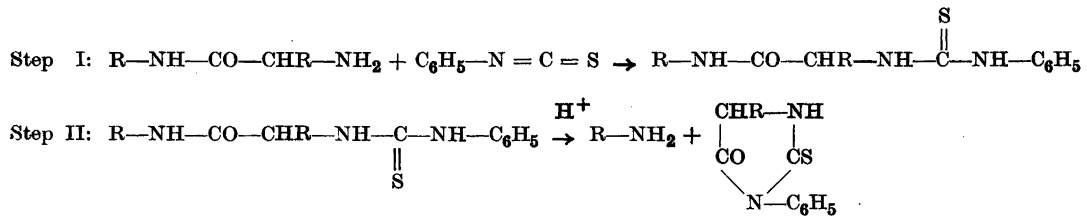
Edman [1] has proposed an ingeneous method for stepwise degradation of peptides by means of phenyl isothiocyanate. It has now been attempted to adapt this method for use with proteins.

The reaction occurs in two steps, as indicated:



Step I occurs under similar conditions to those of other protein amino end group methods [2,3], *i.e.* in aqueous pyridine at pH 9 and 40°. Step II may be carried out in aqueous hydrochloric acid (0.6—1.2 *N*), a modification which was suggested by M. Ottesen for the analysis of peptides. In constrast to other methods, hydrolysis of the protein is not required for identification of the terminal amino acid. Actually, the number of end groups seemed not to be increased upon subjecting the protein to the reaction for a second and third time, which indicates that measurable hydrolysis of peptide bonds had been avoided. There appears therefore to be no necessary limit to the complete elucidation of the peptide sequence of a protein.

The proteins used for this preliminary study of the method were insulin ovomucoid, conalbumin, and β-lactoglobulin. Insulin has yielded 3—3.5 end groups per subunit of MW 12 000. Recovery experiments in which tripeptides were analysed by the same technique suggest that losses of 15—20 % must at present be counted on; thus the value for the end groups of insulin appears to approach that found by the DNP method (4 per 12 000) [2]. Similarly in β-lactoglobulin we have found about 2.5 (uncorrected) while Porter found 3 terminal amino groups (corrected value) by the DNP method [4]. With the two other proteins, approximately one end group was found, in agreement with DNP-results [5].

Qualitatively, phenylalanine and glycine, but, unexpectedly, also small amounts of alanine were found in insulin, alanine in ovomucoid, and leucine and some glycine in β-lactoglobulin.

Identification of the second and third amino acids from the amino end is as yet incomplete, but the results appear to be compatible with those of Sanger in the case of insulin [6].

The technique is briefly as follows: Step I is performed exactly according to Edman at a constant pH of 9.0 or 9.8 for 0.5—3 hours; also the extraction with benzene is the same. The product is then suspended or dissolved in little water and continuously extracted with ether until no more U.V. absorbing material is eluted. Addition of guanidine halide (1.4 g/ml of the aqueous solution) is of advantage during this extraction. It seems to be necessary for the subsequent quantitative extraction of the thiohydantoin, which

* Rockefeller Foundation Fellow.

is formed upon the addition of 6 *N* HCl to final concentrations of 0.6—1.2 *N* (Step II). The thiohydantoins which appear in the ether after acidification of the reaction mixture, show a characteristic maximum at 267—268 mμ, with molar extinction coefficient close to 16 000. Determination of the number of terminal amino acids is based on this property. Identification is done chromatographically after regenerating the amino acid by baryta hydrolysis according to Edman [1, 2].

The authors wish to express their profound gratitude to Professor K. Linderstrøm-Lang and Dr. M. Ottesen for stimulation, help and suggestions given in the course of this work.

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On the Use of Rayleigh-Calvet-Philpot Interference Fringes for the Measurement of Diffusion Coefficients According to the Moment Method

HARRY SVENSSON

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The astigmatic modification of the Rayleigh interferometer, characterized by giving interference fringes which take the form of the refractive index function in the cell, was described by Philpot and Cook [1] in 1948. The author has therefore previously called these fringes Rayleigh-Philpot-Cook fringes. However, the optical arrangement described by Calvet [2–4] earlier contains the essential features of this interferometer. This fact has hitherto escaped the author's attention, largely because it was concealed by Calvet's addition of some nonessential features and of a slit and rotating drum arrangement. Hereby he produced interferograms with the cell coordinate and time on two perpendicular axes, which have quite another appearence than Philpot-Cook's interferograms with the cell coordinate and the refractive index on these axes. Although Calvet's priority is undisputable, Philpot and Cook have no doubt carried out their work independently. It is therefore suggested that the fringes in question be called as in the heading of this article. These fringes are a special kind of *integral interference fringes*, which are produced by bringing to interference one light beam passing through the cell and an other passing through a comparison cell with a constant refractive index, whether the basic interferometer is according to Rayleigh or of some other kind. Hence the observation methods of Labhart and Staub [5] and Antweiler [6] also belong to the integral fringe methods. On the other hand, the Gouy method (Kegeles and Gosting [7]; Longsworth [8]; Coulson, Cox, Ogston and Philpot [9]) and the gradient-recording method described recently by the author [10] might be called *differential interference methods*, and the fringes *differential interference fringes*, since the two interfering light pencils are both passing through the cell.

In a previous article [11], the author described the calculation of diffusion coefficients from integral fringe interferograms according to two methods, the conventional height-area method (this name, of course, refers to the gradient curve), and a method based upon a direct comparison between the position of the fringes and the course of the integral of the error function. In this article, the calculation of the diffusion coefficient according to the moment method will be outlined.

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This diffusion coefficient is defined by the equation:

$$D_m = \frac{\int_{-\infty}^{+\infty} x^2 y \, dx}{2t \int_{-\infty}^{+\infty} y \, dx} \qquad (1)$$

where x is the cell coordinate (with $x = 0$ in the centre of the boundary), y the refractive index gradient, dn/dx, and t the time. The above formula is used in the scale method and in other methods which give records of the refractive index gradient. When an integral interference method is used, equation (1) should be given in the form:

$$D_m = \frac{\int_{n_1}^{n_2} x_2 \, dn}{2t(n_2 - n_1)} \qquad (2)$$

where n_1 and n_2 are the refractive indices of top and bottom solutions in the diffusion cell, respectively. Equation (2) is directly applicable to integral fringe interferograms, since $(n_1 - n_2)$ is the total number of fringes and the integral can be computed directly from the comparator readings of the fringes. The refractive index appears in the same power in numerator and denominator, hence its unit is arbitrary. We will use the number of fringes as the most convenient refractive index unit.

In order to show how this calculation is carried out in detail, we will use the same interferogram as that presented in Table 4 of the previous communication. Table 1 below contains in its first column the number of the fringes for which readings have been taken, and the second column gives the thickness of the intervals in the fringe number. In the third column, we find the plate coordinates Gx (G is the optical magnification factor; numerical value: 2.5307), which have been obtained from the comparator readings in the former table by subtraction of 30.179, the reading corresponding to the centre of the boundary ($z = 0$). The fourth column, finally, contains the products of the figures in the second and the squares of those in the third column. The sum of the values in the second column must equal the total number of fringes, and the sum of those in the last column is the numerical value of the integral in equation (2). The individual values in the last column need not be

Table 1.

Fringe number	Thickness of fringe number interval Δ	Distance from centre of boundary Gx	$\Delta \cdot G^2x^2$
0.5	0.75	−13.179	130.2645
1	0.75	−13.224	112.0695
2	1	−10.600	112.3600
3	1	− 9.564	91.4700
4	1.5	− 8.775	115.5015
6	2	− 7.566	114.4880
8	2	− 6.615	87.5160
10	2	− 5.829	67.9540
12	2.5	− 5.151	66.3325
15	3	− 4.241	53.9580
18	3	− 3.428	35.2530
21	3	− 2.690	21.7080
24	3	− 1.989	11.8680
27	3	− 1.326	5.2740
30	4	− 0.666	1.7760
35	5	0.398	0.7900
40	4	1.479	8.7480
43	3	2.146	13.8150
46	2	2.857	16.3240
47	2	3.102	19.2440
50	3	3.875	45.0480
53	2.5	4.725	55.8150
55	2	5.361	57.4800
57	2	6.076	73.8360
59	2	6.897	95.1380
61	2	7.901	124.8520
63	2	9.268	171.7920
65	1.5	11.603	201.9450
66	0.83	14.706	179.5008
Sum	66.33		2092.1208

noted separately; the sum can be computed directly on the calculating machine.

The sum is in this case 20.9212 cm^2, and after division by the square of the magnification factor, one obtains the figure 3.2667 cm^2. This has to be divided by $2t(n_2-n_1) = 2 \times 5220 \times 66.33 = 692\,485$ sec. Consequently the diffusion coefficient is $4.717 \cdot 10^{-6}$ cm^2/sec. After recalculation from the temperature during the experiment, 21.1°, to 25.0°, and to infinite dilution as described by Gosting and Morris [12], one obtains $D_m = 5.254 \cdot 10^{-6}$ cm^2/sec. This figure differs by less than 0.1 per cent from the value obtained in the same experiment by the height-area method, and by 0.45 per cent from the value given by Gosting and Morris.

This investigation is part of a research program for the development of improved methods of optical analysis of stationary and flowing liquids, which program is generously supported by the Swedish Technical Research Council. Laboratory facilities and additional financial aid has been given by *LKB-Produkter Fabriksaktiebolag*, Stockholm.

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On the Presence of a Tuberculostatic Factor in Organ Extracts from Cow *

Preliminary report

K. B. BJÖRNESJÖ

Department of Medical Chemistry, University of Uppsala, Uppsala, Sweden

The presence of a thermostabile substance in human urine, which dialyses through cellophane, and has a bacteriostatic and bactericidal effect toward tubercle bacilli has been reported earlier [1]. Furthermore, it could be shown that this substance was not identical with urea, hippuric acid, creatinine, urinary phenols, or salts. Experiments with urine from patients with tuberculosis of the kidney seemed to indicate that this type of urine had a weaker tuberculostatic effect than normal urine.

Because of this observation and because it had proved difficult to purify the tuberculostatic substance from urine, attempts were made to isolate a similar tuberculostatic substance from other sources. Bovine urine appeared to have an inhibitive effect toward tubercle bacteria of about the same order as human urine, and for this reason extracts from several organs from the cow were tested.

It could be shown that water extracts of spleen, lung, liver, kidney and muscle did have a tuberculostatic effect. The active substance from these extracts is similar to the urinary factor in that it is thermostabile and is adsorbed on activated charcoal. Furthermore, it can also be eluted from the charcoal with acetic acid.

* After the completion of this work I became aware a note by R. J. Dubos on the occurence of a tuberculostatic agent in animal tissues [*Am. Rev. Tuberc.* **63** (1951) 119]. The active factor had been extracted by acid alcohol from several organs of cattle, rabbits and guinea pigs.

noted separately; the sum can be computed directly on the calculating machine.

The sum is in this case 20.9212 cm^2, and after division by the square of the magnification factor, one obtains the figure 3.2667 cm^2. This has to be divided by $2t(n_2-n_1) = 2 \times 5220 \times 66.33 = 692\,485$ sec. Consequently the diffusion coefficient is $4.717 \cdot 10^{-6}$ cm^2/sec. After recalculation from the temperature during the experiment, 21.1°, to 25.0°, and to infinite dilution as described by Gosting and Morris [12], one obtains $D_m = 5.254 \cdot 10^{-6}$ cm^2/sec. This figure differs by less than 0.1 per cent from the value obtained in the same experiment by the height-area method, and by 0.45 per cent from the value given by Gosting and Morris.

This investigation is part of a research program for the development of improved methods of optical analysis of stationary and flowing liquids, which program is generously supported by the Swedish Technical Research Council. Laboratory facilities and additional financial aid has been given by *LKB-Produkter Fabriksaktiebolag*, Stockholm.

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A Disregarded Complication in the Synthesis of β-Ketoesters by the Base Catalysed Acidolysis of Diethyl Acylmalonates

ARNE BRÄNDSTRÖM

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The author has recently described a method for preparing β-ketoesters using a base catalysed acidolysis of diethyl acylmalonates according to the following equation [1]:

$$RCOCH(CO_2C_2H_5)_2 + RCO_2H \rightarrow RCOCH_2\,CO_2C_2H_5 + RCO_2C_2H_5 + CO_2$$

This reaction was found to give mixtures of β-ketoesters if the acyl group of the acid was not the same as that of the acylmalonate used [2].

This fact and some other observations have called my attention to the possibility that the β-ketoesters obtained are not quite pure even if both the acyl groups are the same. The main impurities resulting from the acyl group mobility [2] would be the corresponding β-diketone and diethyl malonate. The reaction leading to these products probably is:

$$RCOCH(CO_2C_2H_5)_2 + RCOCH_2CO_2C_2H_5 \rightarrow (RCO)_2CHCO_2C_2H_5 + CH_2(CO_2C_2H_5)_2$$
$$(RCO)_2CHCO_2C_2H_5 + RCO_2H \rightarrow RCOCH_2COR + RCO_2C_2H_5 + CO_2$$

In my first preparation of ethyl propionylacetate the possibility of an impurity in the form of diethyl malonate was fully realised and a test according to Breslow, Baumgarten, and Hauser [3] was made for this compound. This test was negative. These authors stated, that the complete solubility of the product in 5 % sodium hydroxide will show the absence of any appreciable quantities of diethyl malonate. However, such a statement is false as diethyl malonate itself is readily soluble in 5 % sodium hydroxide. Later experiments have shown, indeed, that the product was contaminated by appreciable quantities of diethyl malonate and dipropionylmethane.

It can be demonstrated that the ethyl diacylacetate is a probable intermediate product since ethyl diacetoacetate can be isolated in appreciable quantities from the reaction of diethyl acylmalonate and ethyl acetoacetate.

The second step in the reaction, the acidolysis of the ethyl diacylacetate with an organic acid is a well-known reaction for the preparation of β-diketones [4-6].

In order to see the extent to which these side reactions have proceeded in the preparations of the β-ketoesters reported in the previous publication, the products have been tested for the diketone and the diethyl malonate.

The synthesis of ethyl propionylacetate was repeated on a 2 mole scale. When the product was carefully fractionated through a very efficient Pyrex Widmer column, a forerun of 47 g b.p. 40—68°/7.5 mm was obtained. This fraction was precipitated with a solution of copper acetate, yielding the copper derivative of dipropionylmethane m.p. 212° corresponding to 31 g of dipropionylmethane b. p. 59°/9 mm, or a 12 % yield based on the diethyl propionylmalonate.

The forefraction was followed by a fraction boiling at 68—69°/7.5 mm and a fraction at 72—73°/7.5 mm together with a very small middle fraction b.p. 69—72°/7.5 mm. A determination of the refractive indices of the two main fractions showed that the low boiling product had a somewhat higher

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initial value $n_D^{20} = 1.4351$ than the high-boiling one, but this value rapidly decreased to that of the latter, *viz.* $n_D^{20} = 1.4315$. No attempts were made to obtain the highest value of the refractive index for the low-boiling product, but it is obvious that the two products are the enol- and keto-forms of ethyl propionylacetate.

The residue in the distillation flask consisted of diethyl malonate b.p. 79—81°/7.5 mm amounting to about 10 % of the initial product, together with a small quantity of high-boiling products.

From this experiment it is obvious that the reaction does not give a pure product, but that the mixture can be separated to give ethyl propionylacetate in a rather good yield (over 50 %). This seems at present to be the best method of preparing this compound.

The product obtained from the reaction of *iso*butyric acid with diethyl *iso*butyrylmalonate was carefully fractionated. In this way a very small forerun b.p. 50—72°/8 mm was obtained, followed by an absolutely constant boiling fraction 74.4°/7.5 mm leaving a very small residue. From the forerun di*iso*butyrylmethane was isolated in the form of its copper derivative in a yield of about 1 %. M.P. (after recrystallisation) 124°. This indicates that the reaction gives a rather pure product in this case. The method is, no doubt, the best one of preparing ethyl *iso*butyrylacetate.

In the preparation of ethyl *n*-butyrylacetate by the reaction of *n*-butyric acid with diethyl *n*-butyrylmalonate a very troublesome mixture is obtained as the boiling-points of all the three main products are in the region 198—203°. In this case a separation by distillation is quite impossible. In order to test the product for impurities 10 g of the distilled product were refluxed with 100 ml of 20 % sulphuric acid for 3 hours and the product then steam distilled. This treatment decomposes all components except the dibutyrylmethane, which was precipitated in the form of the copper derivative m.p. 157° in a yield of 11 %. As the product must contain a corresponding quantity of diethyl malonate it will be only about 78 % pure. It may be pointed out that as the product is entirely free from homologues it may be very useful in the preparation of certain derivatives where the contamination of diethyl malonate and dibutyrylmethane is harmless.

In order to prove the existence of ethyl diacylacetate as an probable intermediate in the formation of the diketone, a mixture of equimolar amounts of diethyl acetylmalonate and ethyl acetoacetate were refluxed with a little magnesium oxide and copper acetate as a catalyst. After three hours heating the mixture was fractionated under reduced pressure. From a 0.2 mole run the following fractions were taken: (I) 30—60°/7.5 mm 3 g, (II) 60—75°/7.5 mm 6 g, (III) 75—83°/7.5 mm 22 g, (IV) 83—100°/7.5 mm 10 g, together with a considerable quantity of high-boiling products. From fraction (I) a little (1.5 g) acetylacetone was isolated as the copper derivative. Fraction (II) consists no doubt of unchanged ethyl acetoacetate. Fraction (III) gives on redistillation a fraction b.p. 79—81°/7.5 mm which is diethyl malonate. S-Benzyl-*iso*thiuronium salt m.p. 147°. From fraction (IV) ethyl diacetoacetate was isolated in the form of the copper derivative, m.p. 152°, (5.7 g). The formation of acetylacetone is presumably a result of secondary reactions consequent from the high temperature employed.

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Die Bildungsweise der Chlorhydrine. X. Ein quantitativer Vergleich zwischen Addition von HOCl an Doppelbindungen und von HCl an Äthylenoxyde

LENNART SMITH und STURE SKYLE

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Vor einiger Zeit haben Smith und Skyle [1] in dieser Zeitschrift einige Angaben veröffentlicht über die Mengenverhältnisse der isomeren Chlorhydrine, die bei Addition von unterchloriger Säure an Äthylenderivate und von Chlorwasserstoff an Oxydoverbindungen gebildet werden. Das Zahlenmaterial war, insbesondere in Bezug auf die erstgenannte Reaktion, indessen ziemlich knapp, teilweise auch nicht so genau und ist seitdem von Skyle durch neue Untersuchungen erweitert worden. Diese bestätigen völlig die in der vorigen Mitteilung gezogenen Schlüsse (Seite 43) und erlauben auch eine Erweiterung derselben. Wir haben es deshalb für angemessen erachtet, eine neue, vollständigere Vergleichstabelle vorzulegen. Die gefundene Gesetzmässigkeit bei den erwähnten Reaktionen ist unseres Wissens vollkommen neu.

In der Tabelle bezieht sich die Bezeichnung α auf die Stellung des *zutretenden Choratoms*. Die Angabe, dass der Anteil der α-Verbindung am Isomerengemisch 1,0 beträgt, soll nur andeuten, dass die β-Verbindung in den betreffenden Gemischen nicht oder kaum nachgewiesen werden konnte.

A Addition von HOCl	B Addition von HCl	Anteil der α-Halogenverbindung am Isomerengemisch (abgerundet) A	 B
I. $ClCH_2 \cdot CH : CH_2$	$ClCH_2 \cdot CH(OH) \cdot CH_2Cl$ / $ClCH_2 \cdot CHCl \cdot CH_2OH$; $ClCH_2 \cdot CH-CH_2$ (O)	0,35	1,0
II. $HOCH_2 \cdot CH : CH_2$	$HOCH_2 \cdot CH(OH) \cdot CH_2Cl$ / $HOCH_2 \cdot CHCl \cdot CH_2OH$; $HOCH_2 \cdot CH-CH_2$ (O)	0,75	0,9
III. $C_2H_5 \cdot CH : CH_2$	$C_2H_5 \cdot CH(OH) \cdot CH_2Cl$ / $C_2H_5 \cdot CHCl \cdot CH_2OH$; $C_2H_5 \cdot CH-CH_2$ (O)	0,85 *	0,8 **
IV. $CH_3 \cdot CH : CH_2$	$CH_3 \cdot CH(OH) \cdot CH_2Cl$ / $CH_3 \cdot CHCl \cdot CH_2OH$; $CH_3 \cdot CH-CH_2$ (O)	0,95 *	0,75
V. $(CH_3)_2 \cdot C : CH_2$	$(CH_3)_2 \cdot C(OH) \cdot CH_2Cl$ / $(CH_3)_2 \cdot CCl \cdot CH_2OH$; $(CH_3)_2 \cdot C-CH_2$ (O)	1,0 *	0,45
Bei den Bromhydrinen ergaben die Reaktionen I		0,2	1,0
» » » » » » II		0,65	0,9

* Neue Bestimmung von S. Skyle.
** Privatmitteilung von Göran Forsberg.

Die regelmässige Veränderung der relativen Ausbeuten an α- und β-Verbindung ist hier noch ausgeprägter als in der Tabelle der vorigen Mitteilung und findet, wie schon dort hervorgehoben wurde, grösstenteils ihre Erklärung in den verschiedenen elektrischen Ladungen der Chloratome in HOCl und HCl. Die Reaktionen V bieten hier als Extremfall ein Gegenstück zu den Reaktionen I. Besonders für den Reaktionstypus V können aber wahrscheinlich auch sterische Einflüsse eine nicht zu unterschätzende Rolle spielen.

1. Smith, L., und Skyle, S. *Acta Chem. Scand.* **4** (1950) 39.

Eingegangen am 23. November 1951.

Influence of Nitrate Concentration upon Chlorate Toxicity in Microorganisms

G. FÅHRAEUS

Institute of Microbiology, Royal Agricultural College, Uppsala, Sweden

Following the discovery that sodium chlorate is effective as a weed eradicant under certain conditions, soil microbiologists became interested in the action of this substance on microorganisms. One of the most important contributions in this field is that of Stapp and Bucksteeg [1], who found that fungi and bacteria are as a rule very resistant to chlorate. A few exceptions have been demonstrated by earlier investigators, for instance the nitrifying bacteria, which are sensitive to chlorate.

It has also been shown that nitrate reduces the toxic effect of chlorate on higher plants, and Hurd-Karrer [2] suggested that chlorate specifically interferes with the reduction of nitrate in plants. A theory for the mechanism of chlorate action was developed and experimentally supported by Åberg [3].

In the experiments of Stapp and Bucksteeg cited above no special attention was given to the nitrogen source, since the importance of this was not clearly established at that time. On the basis of the hypothesis of chlorate action one should not, however, expect chlorate to be toxic to ordinary microorganisms, unless the nitrogen assimilated is offered in the form of nitrate. Further, the effect of chlorate should be more pronounced at low nitrate concentrations, because higher concentrations would prevent the nitrate-reducing enzyme, by competition, from combining with the chlorate.

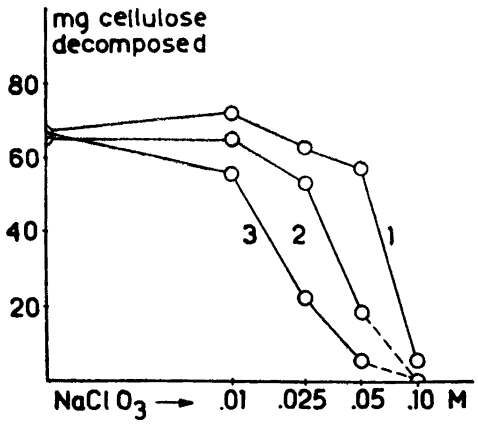

Fig. 1. Decomposition of cellulose by Cytophaga in the presence of varying amounts of sodium nitrate and sodium chlorate. 7 days. Each point represents the mean of 4 replicate samples. 1) 0.02 M $NaNO_3$, 2) 0.01 M $NaNO_3$, 3) 0.005 M $NaNO_3$.

However, in the recent work of Goksöyr [4] *Aspergillus oryzae* was shown to be strongly inhibited by chlorate in the presence of KNO_3, irrespective of the nitrate concentration. The molar ratio nitrate/chlorate was, in one case, as large as 100:1. In the presence of ammonium salt no inhibition occurred.

The present author studied cellulose-decomposing bacteria (also included in the work of Stapp and Bucksteeg [1]). Quantitative experiments were carried out with a *Cytophaga* strain, "W" [5, p. 226]. The decom-

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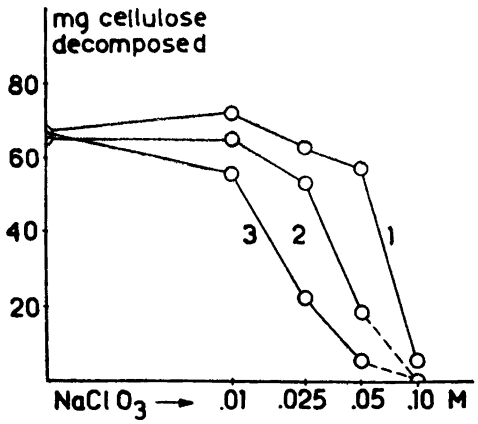

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position of cotton cellulose was followed according to methods described earlier [6, p. 17–19]. Samples from flasks were withdrawn after 7, 14, and 21 days for determination of residual cellulose. If ammonium phosphate was used as a nitrogen source, the addition of up to 1 % (0.1 *M*) Na ClO_3 had very little effect on the cellulose decomposition. The results obtained with a nitrate medium were different. The values from this experiment are given in Fig. 1. The diagram has been drawn in the same fashion as Goksöyr's [4] Fig. 1 to permit direct comparison.

It is evident that chlorate affects the decomposition of cellulose in *Cytophaga* cultures containing nitrate and that the inhibition by chlorate is dependent on the amount of nitrate present. At a ratio chlorate/nitrate of 1:1 or even 2:1, there is no appreciable inhibition of the cellulose decomposition, but at higher ratios there is a significant effect. This is at variance with the results obtained by Goksöyr [4] with *Aspergillus*.

Admittedly, *Aspergillus* and *Cytophaga* are widely different organisms, and although the evidence brought forward favours the hypothesis that chlorate is able to combine with the nitrate-reducing enzyme, the affinity of this to chlorate may perhaps vary from one organism to another. Further studies on different microorganisms are therefore needed.

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The Crystal Structure of the Methanethiosulphonates of Divalent Sulphur, Selenium and Tellurium

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The synthesis and the unit cells and space group of these compounds were described by one of us recently [1]. The crystals are isomorphous, with a four-molecule unit cell based on the space group $C_{2h}^5 - P2_1/n$. The dimensions are:

	a, Å	*b*, Å	*c*, Å	β
$S(S_2O_2CH_3)_2$	11.33	5.21	16.14	91°
$Se(S_2O_2CH_3)_2$	11.38	5.23	16.23	91°
$Te(S_2O_2CH_3)_2$	11.43	5.29	16.32	91°

Weissenberg photographs were taken with CuK radiation on multiple films, and the intensities estimated visually.

A Patterson synthesis based on the *h0l* data for the tellurium compound indicated four possible tellurium positions. A Fourier map for one of the positions, using signs of the reflections calculated from the tellurium contributions alone, gave a clear resolution of the sulphur atoms. Inclusion of the calculated structure factors from these atoms changed the sign of 13 % of the reflections, and a second two-dimensional Fourier analysis was made, with the resulting tellurium and sulphur parameters:

	S_I	S_{II}	Te	S_{IV}	S_V
x	0.133	0.123	0.234	0.422	0.436
z	0.117	−0.017	−0.068	−0.054	−0.163

A Fourier analysis based on the *h0l* data for the sulphur compound was subsequently carried out, using signs of the reflections obtained from the tellurium compound revised by subtracting two thirds of the calculated tellurium structure factors. The five sulphur atoms were clearly resolved, and after three successive

position of cotton cellulose was followed according to methods described earlier [6, p. 17–19]. Samples from flasks were withdrawn after 7, 14, and 21 days for determination of residual cellulose. If ammonium phosphate was used as a nitrogen source, the addition of up to 1 % (0.1 *M*) Na ClO_3 had very little effect on the cellulose decomposition. The results obtained with a nitrate medium were different. The values from this experiment are given in Fig. 1. The diagram has been drawn in the same fashion as Goksöyr's [4] Fig. 1 to permit direct comparison.

It is evident that chlorate affects the decomposition of cellulose in *Cytophaga* cultures containing nitrate and that the inhibition by chlorate is dependent on the amount of nitrate present. At a ratio chlorate/nitrate of 1:1 or even 2:1, there is no appreciable inhibition of the cellulose decomposition, but at higher ratios there is a significant effect. This is at variance with the results obtained by Goksöyr [4] with *Aspergillus*.

Admittedly, *Aspergillus* and *Cytophaga* are widely different organisms, and although the evidence brought forward favours the hypothesis that chlorate is able to combine with the nitrate-reducing enzyme, the affinity of this to chlorate may perhaps vary from one organism to another. Further studies on different microorganisms are therefore needed.

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The Crystal Structure of the Methanethiosulphonates of Divalent Sulphur, Selenium and Tellurium

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The synthesis and the unit cells and space group of these compounds were described by one of us recently [1]. The crystals are isomorphous, with a four-molecule unit cell based on the space group $C_{2h}^5 - P2_1/n$. The dimensions are:

	a, Å	*b*, Å	*c*, Å	β
$S(S_2O_2CH_3)_2$	11.33	5.21	16.14	91°
$Se(S_2O_2CH_3)_2$	11.38	5.23	16.23	91°
$Te(S_2O_2CH_3)_2$	11.43	5.29	16.32	91°

Weissenberg photographs were taken with CuK radiation on multiple films, and the intensities estimated visually.

A Patterson synthesis based on the *h0l* data for the tellurium compound indicated four possible tellurium positions. A Fourier map for one of the positions, using signs of the reflections calculated from the tellurium contributions alone, gave a clear resolution of the sulphur atoms. Inclusion of the calculated structure factors from these atoms changed the sign of 13 % of the reflections, and a second two-dimensional Fourier analysis was made, with the resulting tellurium and sulphur parameters:

	S_I	S_{II}	Te	S_{IV}	S_V
x	0.133	0.123	0.234	0.422	0.436
z	0.117	−0.017	−0.068	−0.054	−0.163

A Fourier analysis based on the *h0l* data for the sulphur compound was subsequently carried out, using signs of the reflections obtained from the tellurium compound revised by subtracting two thirds of the calculated tellurium structure factors. The five sulphur atoms were clearly resolved, and after three successive

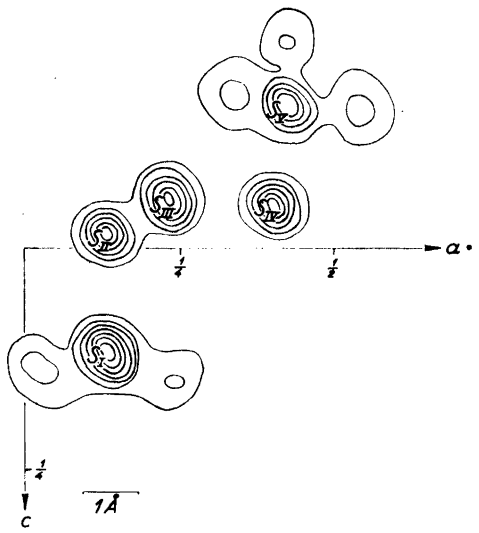

Fig. 1. Projection of $S(S_2O_2CH_3)_2$ along the b axis.

refinements the electron density map shown in Fig. 1 was obtained. The reliability factor $\Sigma||F|_{obs} - |F|_{calc}|/\Sigma|F|_{obs}$ is at this stage 0.22, and the structure is being further refined. The present sulphur parameters are:

	S_I	S_{II}	S_{III}	S_{IV}	S_V
x	0.129	0.133	0.234	0.403	0.428
z	0.119	−0.015	−0.053	−0.047	−0.159

Fig. 1 shows that the compound possesses an unbranched chain structure, the distances $S_I - S_{III}$, $S_{II} - S_{IV}$ and $S_{III} - S_V$ in the projection being 2.91 Å, 3.11 Å and 2.93 Å, respectively, *i.e.*, too large for bonds to exist between those atoms.

This is the first structure determination reported for a pentathionic compound. Details of the complete structure analyses of the sulphur, selenium and tellurium methanethiosulphonates will be published in due course.

The work is carried out by the aid of grants from the *Norges Almenvitenskapelige Forskningsråd.*

1. Foss, O. *Acta Chem. Scand.* **5** (1951) 115.

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The Fluorometric Measurement of 4-Pyridoxic Acid in Normal Urine

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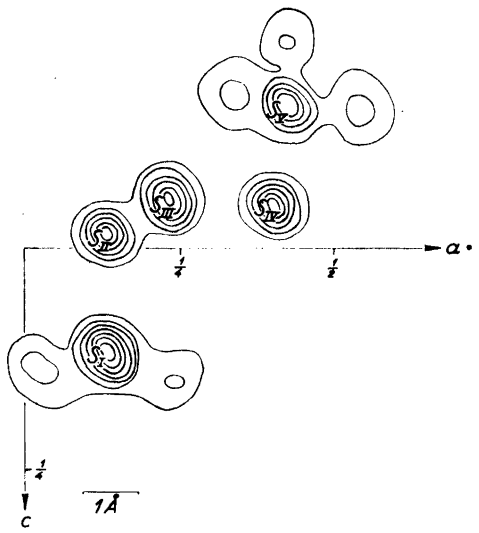
Huff and Perlzweig [1] have isolated and identified the excretion product 4-pyridoxic acid (2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine), which is supposed to be the main excretion product of vitamin B_6. In the same work they described a fluorometric method for quantitative determination of 4-pyridoxic acid in urine. The fluorometric determination is based on the fact that the lactone form of 4-pyridoxic acid gives a fluorescent intensity 25 times that of the free acid, which in itself is highly fluorescent. In human urine the product occurs in the free acid form. By heating the urine in acid solution this compound is converted into its lactone form. The blank is an untreated urine sample. With this method it has been found that the average 24 hour urinary excretion in man is from 3 to 4 mg [2–4].

In a critical study of the Huff and Perlzweig method Sarrett [5] has shown that the blank is not reliable in measurements of normal urine. Sarrett reports of unsuccesful attempts to separate the 4-pyridoxid acid from interfering substances by means of adsorption or precipitation.

Using the same method we have found that other substances than 4-pyridoxic acid present in the urine increase in fluorescence by treatment with acid. We have therefore used a blank prepared in a different way. Our technique is based on the reconversion of the lactone into the free acid by heating the urine in alkaline solution. The first step is to heat the urine with acid. By this treatment the free acid is converted into its lactone form and the fluorescence of this sample containing the



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refinements the electron density map shown in Fig. 1 was obtained. The reliability factor $\Sigma||F|_{obs} - |F|_{calc}|/\Sigma|F|_{obs}$ is at this stage 0.22, and the structure is being further refined. The present sulphur parameters are:

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In a critical study of the Huff and Perlzweig method Sarrett [5] has shown that the blank is not reliable in measurements of normal urine. Sarrett reports of unsuccesful attempts to separate the 4-pyridoxid acid from interfering substances by means of adsorption or precipitation.

Using the same method we have found that other substances than 4-pyridoxic acid present in the urine increase in fluorescence by treatment with acid. We have therefore used a blank prepared in a different way. Our technique is based on the reconversion of the lactone into the free acid by heating the urine in alkaline solution. The first step is to heat the urine with acid. By this treatment the free acid is converted into its lactone form and the fluorescence of this sample containing the

Table 1. Fluorescence measurement of 4-pyridoxic acid in normal urine and in urine after ingestion of 25 mg pyridoxine hydrochloride.

		Fluorometer-reading
4-Pyridoxic acid standard solution	μg 0.3	31
4-Pyridoxic acid standard solution	μg 0.6	57
Blank for the standard		5
Standard treated with NaOH	μg 0.6	5
Normal urine (final dilution 1—200)		
Sample A		72
Blank B		43
Blank C		74
Blank B treated with NaOH without preliminary treatment with acid		47
Urine after ingestion of pyridoxine (final dilution 1—1 000)		
Sample A		51
Blank B		10
Blank C		12

lactone is measured. Thereafter the sample is heated with alkali. By this procedure the lactone ring opens again. The fluorescence of this sample is taken as the blank. When this new blank is used in the determination of 4-pyridoxic acid in normal urine the values obtained are considerably smaller. This seems to indicate that the previous findings of rather high amounts of 4-pyridoxic acid in normal urine are incorrect.

Technique: Sample A and blank B are prepared according to an unpublished modification of the technique described by Huff and Perlzweig (from Duke University, Dept. of Biochemistry, 1946, personal communication). Blank C is the new blank. Fluorescence measurements were done with a Farrand microphotofluorometer with filters: primary Corning 5 860, secondary Corning 4 308 + 3 389.

Sample A: Normal urine is diluted 10 to 20 times and 1.00 ml of the diluted urine is measured into a test tube graduated at 10 ml. 1.00 ml of 2 *N* HCl is added and test tube placed in a boiling bath for 20 minutes. After cooling 1.00 ml of 2 *N* NaOH is added and the sample is diluted to the 10 ml mark with a saturated borax solution.

Blank B: Consists of the same reagents, but heating is omitted.

Blank C: After heating with acid and neutralization as described with sample A 1.00 ml of 2 *N* NaOH is added and the tube again immersed in a boiling bath for 15 minutes. After cooling and addition of 1.00 ml of 2 *N* HCl saturated borax is added to 10 ml mark.

Table 1 shows the results with normal urine and with urine after ingestion of 25 mg pyridoxine hydrochloride. It is seen that the increase in fluorescence after acid treatment of normal urine *does not* disappear by alkali treatment. The increased fluorescence does disappear in the standard solution of 4-pyridoxic acid and in the urine obtained after ingestion of pyridoxine. (Alkali treatment in itself produces only a slight increase in fluorescence intensity.)

The conclusion is that the increase in fluorescence of normal human urine after treatment with acid must be caused by substances other than 4-pyridoxic acid as this increase in fluorescence is unremovable by alkali treatment. According to this free 4-pyridoxic acid is not excreted on a normal diet and occurs only after test doses of vitamin B_6. In order to determine the least possible quantity of pyridoxine which after ingestion will produce a mea-

Table 2. Analysis of 4 hour urine samples after ingestion of pyridoxine hydrochloride.

Amount of pyridoxine ingested	Volume of 4 hour urine sample	4-Pyridoxic acid excreted	
		Huff and Perlzweig method	New method
1 mg	93 ml	0.69 mg	0 mg
2 mg	270 ml	0.84 mg	0 mg
3 mg	325 ml	0.79 mg	0.09 mg
4 mg	260 ml	2.14 mg	0.73 mg
5 mg	380 ml	1.28 mg	0.59 mg

surable excretion of 4-pyridoxic acid, 4 hour urine samples after intake of 1, 2, 3, 4 and 5 mg of pyridoxine were analysed with the new technique. The results are shown in Table 2 and compared with results obtained with the Huff and Perlzweig method.

The author wants to express his thanks to Professor R. Ege for laboratory facilities and to Dr. E. Hoff-Jørgensen for encouragement and criticism. Thanks are due to Professor W. A. Perlzweig, Duke University, for a sample of 4-pyridoxic acid, to Dr. H. M. Kalckar, Copenhagen University, for the fluorescence apparatus kindly placed at the authors disposal and to the technical research fund for financial support.

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On the Hexosamine Component of Ovomucin

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In the course of a study on the carbohydrate groups of some protein constituents of hen's eggs and especially of the eggwhite proteins it was found that ovomucin gave the colour reactions characteristic for sialic acid. The other substances investigated gave none or only weak reactions. Later it was shown that ovomucin as well as several other substances contain considerable amounts of this acid [1]. As recently reported sialic acid occurs together with chondrosamine in submaxillary mucin, in gangliosides, in an acid glycoprotein from plasma and in seromucoid [2-4]. The hexosamine of ovomucin has therefore been examined too. So far glucosamine only has been isolated from the total eggwhite [5,6] and yolk [6,7] and from some of their constituents, as ovomucoid [6,8] and ovalbumin [6,9,10].

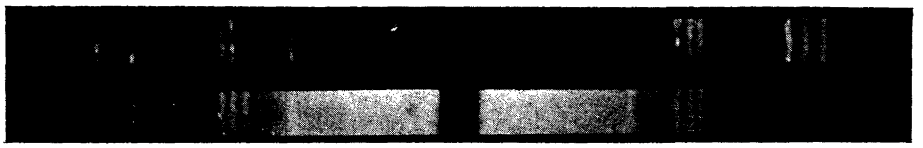

Fig 1. A. Powder diagram of α-glucosamine hydrochloride. B. Powder diagram of first hexosamine fraction from an acid hydrolysate of ovomycin.

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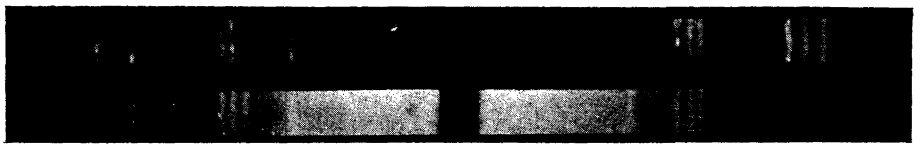

Fig 1. A. Powder diagram of α-glucosamine hydrochloride. B. Powder diagram of first hexosamine fraction from an acid hydrolysate of ovomycin.

Fig. 2. A. Powder diagram of β-chondrosamine hydrochloride. B. Powder diagram of last hexosamine fraction from the hydrolysate of ovomucin.

Ovomucin was prepared according to Sörensen [11]. It contained 11.8 per cent hexosamine and 7—8 per cent sialic acid [1]. The isolation and the identification of the hexosamine were performed mainly in the same way as described in an earlier paper [2].

One g of ovomucin was heated with 5 *N* hydrochloric acid under reflux on a boiling water bath for 18 hrs. After filtration and evaporation the material was boiled again with 1 *N* hydrochloric acid for 14 hrs. It was then treated with charcoal, filtered and brought to dryness. The residue was several times taken up in methanol and the extracts evaporated. Finally, the residue was dissolved in a small amount of dry methanol and the solution placed in an desiccator at room temperature. The crystalline precipitates obtained were identified by their x-ray diffraction patterns.

The x-ray powder diagrams of the first fractions were identical with that of α-glucosamine hydrochloride (Fig. 1), those of the last fractions with that of β-chondrosamine hydrochloride (Fig. 2). The glucosamine hydrochloride formed the main part of the crystalline material.

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