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Liquid Structure of Aqueous Solutions of Sodium Chloride: Solubility, Vapor Pressure, and Conductivity

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Solubility of NaCl in H_2O and vapor pressure and conductivity of such solutions are related to the structure of the solution. It is concluded from those properties that the NaCl dissolved is completely hydrolyzed into NaOH and HCl. The two molecules are arranged in pairs such that the Cl atom in the HCl molecule is separated by one H_2O molecule from the Na atom and by one H_2O molecule from the O atom in the NaOH molecule. Solubility is determined by the possibility of formation of $NaCl \cdot 2\ H_2O$ which is precipitated.

1. INTRODUCTION

In a preceding paper[1] on the solubilities of silver and lead halides in water, the solubility was calculated from the formula (1) valid for ideal solutions and by applying the statistical theory of ideal mixtures to the hydrolysis of the salt.

The fundamental formula is

$$X = e^{-\frac{\Delta E_s}{R}\left(\frac{1}{T}-\frac{1}{T_f}\right)} \tag{1}$$

when X is the mole fraction of the salt dissolved, T_f its melting point, $-\Delta E_s$ the heat of solution. When the dissolved salt is hydrolyzed we have to add a factor W related to the configurational entropy of hydrolysis, and (1) takes the form

$$X = W\ e^{-\frac{\Delta E_s}{R}\left(\frac{1}{T}-\frac{1}{T_f}\right)} \tag{2}$$

The solubilities of those salts were taken from conductivity measurements. As has been pointed out elsewhere [2,3] only dissociated solute molecules contribute to the conductivity. Hence, conductivity measurements do not give the total amount of salt dissolved but only the fraction of the salt which is hydro-

lyzed, *e.g.* in the case of AgCl, only the Ag present in the form of AgOH but not that in the form of AgCl.

It follows from these considerations that the slope of the plot of log solubility versus $\frac{1}{T}$ gives a heat of dissolution which is equal to ΔE_s divided by the number of molecules formed by the hydrolysis. Hence, the state of hydrolysis may be determined from that plot and the calorimetric value of ΔE_s. It was found that the states of hydrolysis are independent of the temperature. Thus, $PbCl_2$ and $PbBr_2$ give $PbOHX + HX$ but no $Pb(OH)_2 + 2\,HX$, whereas PbI_2 gives $Pb(OH)_2 + 2\,HX$ but no $PbOHX + HX$.

When the two atoms M and X occupy adjacent lattice sites, they form a molecule of MX which does not contribute to the conductivity. When they occupy non-adjacent sites, they are present in the form of MOH and HX, respectively. The number of MX molecules present is determined by (1) or (2) while the numbers of MOH and HX molecules are determined by the statistics of the liquid structure, *viz.* by the numbers of ways in which bonds may be broken and reformed and in which the M and X atoms may be distributed on the lattice sites.

In this communication we will apply similar arguments to aqueous solutions of sodium chloride. It turns out, however, that the structure of the solution is not random but that there is a preferred distribution of Na and Cl atoms relative to each other. In order to discuss this structure we also have to consider other properties of the solution, *viz.* conductivity and vapor pressure. The fundamentals of those properties have been treated elsewhere (conductivity [2], vapor pressure [4]) and reference will therefore be made to those papers in order to avoid repetition.

Unfortunately, the data available are not sufficient for a complete treatment of the subject. The data missing include the temperature-dependence of the conductivity. Further, the geometry of the structure is not known. According to X-ray determinations by Finbak [5] the structure of NaOH is similar to that of HCl from which result we may conclude that the coordination numbers are the same in both structures.

2. SOLUBILITY

The solubilities of NaCl in H_2O and in H_2O-HCl mixtures depend very little on the temperature as is shown by the data given by various investigators [6]. Hence, we have approximately $\Delta E_s = 0$ in both cases. The solubility is then determined almost entirely by the configurational entropy. Since the observed solubility includes Na both as NaOH and as NaCl and since $\Delta E_s = 0$, we can not conclude upon the degree of hydrolysis from the same arguments as in the case of the Ag and Pb halides.

Table 1 reproduces the data of Fricke and Brümmer [6,7] on the solubility of NaCl in aqueous HCl solutions at 18°.

Table 1.

HCl mol/100 g solution	NaCl	HCl	NaCl mole fractions	H_2O	$\frac{[H_2O] + [NaCl]}{[HCl] + [NaCl]}$
0.0	4.513	0.0	9.950	90.050	10.055
0.0438	4.474	0.0960	9.838	90.065	10.057
0.0870	4.433	0.1913	9.720	90.059	10.068
0.1756	4.346	0.3853	9.5365	90.078	10.040
0.3610	4.151	0.7879	9.0593	90.153	10.075
0.9263	3.605	1.9935	7.7583	90.248	10.049
1.6677	2.889	3.5247	6.1060	90.369	10.017
2.6740	2.055	5.5529	4.2675	90.180	(9.618)
3.5670	1.408	7.3209	2.8898	89.789	(9.070)
				Average	10.053 ± 0.005

In the last column we have computed the ratio $\frac{[H_2O] + [NaCl]}{[HCl] + [NaCl]}$, the brackets denoting the mole fraction of the substance put into the solution. This ratio is a constant, 10.053 ± 0.005, say 10, below 3.5 mol. % of HCl.

On the assumption that there are no NaCl molecules present in the solution, all NaCl dissolved being hydrolyzed into NaOH and HCl, we have the following actual concentrations in the solution:

$$\left.\begin{array}{ll} H_2O: & [H_2O] - [NaCl] \\ HCl: & [HCl] + [NaCl] \\ NaOH: & [NaCl] \end{array}\right\} \quad (3)$$

corresponding to a reaction which we may write symbolically

$$NaCl + H_2O \rightarrow NaOH + HCl, \quad (4)$$

indicating that the Na and Cl atoms are separated from each other by H_2O molecules in the structure of the solution.

From vapor pressure [4] and conductivity [2] data we have concluded that when there are more than 10 H_2O molecules per HCl molecule on the average, each HCl molecule is surrounded by 10 neighbors, all of which are H_2O molecules and none of which is a HCl molecule. The NaOH molecule, like the AgOH molecule [1], may be assumed to occupy two lattice sites. We will also assume that a NaOH molecule may replace two H_2O molecules among the neighbors of the HCl molecule [1].

The number of sites occupied by H_2O and NaOH molecules is then according to (3)

$$[H_2O] - [NaCl] + 2\ [NaCl] = [H_2O] + [NaCl]$$

and the number of sites occupied by HCl molecules is

$$[HCl] + [NaCl].$$

Hence, when

$$\frac{[H_2O] + [NaCl]}{[HCl] + [NaCl]} \geqq 10 \quad (5)$$

each HCl molecule is surrounded by 10 H_2O or NaOH molecules and there is no H_2O or NaOH molecule belonging simultaneously to the two groups of 10 neighbors of two different HCl molecules.

The data in Table 1 show that (5) is the condition for solubility. Putting the ratio (5) equal to 10 we obtain for the solubility of NaCl in aqueous solutions of HCl

$$[NaCl] = \frac{1}{9} \{[H_2O] - 10\ [HCl]\} \qquad (6)$$

The form of (6) suggests the simple interpretation that the HCl molecules added to H_2O associate with themselves 10 H_2O molecules each and that the NaCl dissolves according to (4) in whatever water is left. This changes nothing in the previous argument, if the HCl molecules directly added are undistinguishable from those created by (4).

According to (6), no NaCl at all should dissolve in aqueous solutions of less than 10 H_2O per HCl. However, there is some slight solubility even at very high concentrations as shown by the data of Seidel and Fischer [6,8] at 0°. Those data are reproduced in Table 2.

Table 2.

HCl percent by weight	NaCl	HCl	NaCl mole fractions	H_2O	$\frac{[H_2O] + [NaCl]}{[HCl] + [NaCl]}$
0	26.2	0	9.86	90.14	10.14
22.6	1.15	12.72	0.40	86.88	6.65
26.7	0.41	15.30	0.15	84.55	5.48
39.0	0.047	24.01	0.018	75.97	3.16
43.9	0.034	27.89	0.014	72.10	2.58
44.5	0.032	28.38	0.012	71.61	2.52

Data solubilities of NaCl in H_2O-NaOH mixtures given by von Antropoff [6,9] show a very slight dependence upon temperature, *i.e.* approximately $\Delta E_s = 0$.

Table 3 gives the solubility at 25° according to Åkerlöf and Short [6,10].

Table 3.

NaOH mol/1 000 g H_2O	NaCl	H_2O	$\frac{[H_2O] - 5\ [NaOH] + [NaCl]}{[NaCl]}$	$\frac{[H_2O] - 9\ [NaCl]}{[NaOH]}$
0.920	5.613	55.506	10.07	5.42
1.858	5.048		10.21	5.43
2.714	4.672		9.97	4.96
3.070	44.86		9.95	4.93
3.812	4.048		10.00	5.01
4.798	3.610		9.73	4.80
5.721	3.206		9.39	4.66
6.688	2.635		9.37	4.75
7.141	2.442		8.86	4.70
8.031	2.173		8.06	4.48
8.876	1.956		6.69	4.27
9.824	1.558		5.10	4.22
11.437	1.280		−0.28	3.85
11.582	1.253		−0.9	3.82

In Table 3 we have also given the ratios

$$\frac{[H_2O] - 5\ [NaOH] + [NaCl]}{[NaCl]} \tag{7}$$

and

$$\frac{[H_2O] - 9\ [NaCl]}{[NaOH]} \tag{8}$$

The ratio (7) should be 10, just as in the case of H_2O-HCl solutions, the ratio (8) should be 5, *viz.* the number of H_2O molecules bound to one NaOH molecule. At low concentrations, below 4 *N* NaOH, those values are actually obtained. At higher concentrations, they are lower, just as was found in H_2O-HCl mixtures. This phenomenon will be discussed presently.

We find, thus, that the same condition of solubility is valid whether we add HCl or NaOH to the solution.

For the hydrolysis of AgX we have found the relation [1]

$$c_{AgOH} \cdot c_{HX} = 10\ c_{AgX} \tag{9}$$

the factor 10 being derived from the probability that an Ag atom and a X atom occupy adjacent lattice sites. This is the equilibrium equation of hydrolysis, the factor 10 being the equilibrium constant. However, we may also look upon (9) as an expression for the solubility product, if

$$c_{AgX} = \frac{55.55}{1\,215}\ e^{\frac{\Delta E_s}{R}\left(\frac{1}{T} - \frac{1}{T_f}\right)} \tag{10}$$

is inserted for c_{AgX} in (9). We would therefore expect the solubility of AgX, *i.e.* c_{AgOH}, to decrease when HCl is added to the solution. Experimental data show, on the contrary, that the solubility of AgX increases steadily with the concentration of HCl. This deviation from the rule of the constant solubility product is accompanied by a marked reduction of ΔE_s with increasing HCl concentration as shown by the data of Dede and Walther [6,11].

In the case of NaCl solutions we find that the solubility of NaCl decreases when HCl or NaOH is added to the solution. However, the rule of the constant solubility product is still not applicable, although $\Delta E_s = 0$ is unaffected by those additions, because the hydrolysis does not follow the same statistics, owing to a preferred distribution of NaOH and HCl molecules relative to each other (section 4).

In order that the solubility product be constant, two conditions have to be fulfilled:

1. A random distribution of all molecules on the lattice sites.
2. A constant value of ΔE_s, independent of the concentrations of the various species in the solution.

Both conditions exclude the formation of soluble complex salts, since this corresponds to a preferred distribution and because the formation of the complex is connected with a heat of formation.

Similarly, deviations from any law of solubility which does not take into account changes in distribution and in ΔE_s, may be due to those causes. Thus, according to von Antropoff [6,9], the solubility of NaCl in H_2O—NaOH mixtures increases by 0.5 % between 20° and 30° at 5 % NaOH and by 25 % at 50 % NaOH by weight. The increase of the solubility of AgCN with the concentration of added HCN (Randall and Halford [6,12]) is attributed to formation of the acid $HAg(CN)_2$ with the complex ion $Ag(CN)^-_2$. The same applies to AgCl.

Corresponding complexes of NaCl with HCl and NaOH do not seem to be known. It is therefore difficult to explain why more NaCl than given by the rule just discussed is dissolved at high concentrations of NaOH or HCl. However, the essence of the structure of NaCl in H_2O and its relation to solubility is covered by the agreement at low concentrations of NaOH and HCl.

3. VAPOR PRESSURE

The partial vapor pressure of water above aqueous solutions of NaCl has been measured by a number of investigators [13]. The data show that the relative partial vapor pressure p, *i. e.* the actual partial vapor pressure above the solution divided by that above pure water of the same temperature, is independent of the temperature. Hence, the differential heat of vaporization is $\Delta E_{H_2O} = 0$.

For aqueous solutions of HCl we have found [4] that $\Delta E_{H_2O} = 0$ for $m > 10$. This condition is fulfilled for all NaCl solutions, if we in the number of H_2O molecules m include twice the number of NaOH molecules as discussed in section 2, because this total number of H_2O and NaOH neighbors of the HCl molecule is always >10. For NaOH solutions [4] $\Delta E_{H_2O} = 0$ for $m > 5$.

Fig. 1 shows $p = f\left(\frac{1}{m+1}\right)$ for NaCl solution at 25° on the data of Olnyk and Gordon [14] and Robinson [15]. At low concentrations we find

$$p = 1 - \frac{2}{m+1} = \frac{m-1}{m+1}, \quad m > 24 \tag{11}$$

Fig. 2 shows the difference

$$\Delta p = \frac{m-1}{m+1} - p \tag{12}$$

For $m > 24$, $\Delta p = 0$.

For HCl solutions we have found [4]

$$p = 1 - \frac{3}{m+1} = \frac{m-2}{m+1}, \quad m > 26 \tag{13}$$

When mixing one NaCl with m H_2O we obtain in the solution as discussed in section 2, one NaOH, one HCl, and $(m-1)$ H_2O. Thus, the concentration of H_2O is $\frac{m-1}{m+1}$, and (10) is therefore identical with Raoult's law.

An analysis of Δp for NaCl solutions is difficult owing to the small range of $\Delta p \neq 0$. We will observe, however, that the sudden increase of Δp at $m = 24$ for NaCl solutions corresponds to that at $m = 26$ for HCl solutions.

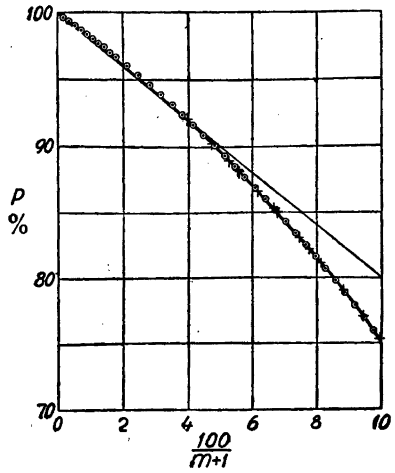

Fig. 1. $p = f\left(\frac{1}{m+1}\right)$ *for NaCl solutions.* + *Olnyk & Gordon,* ⊙ *Robinson.*

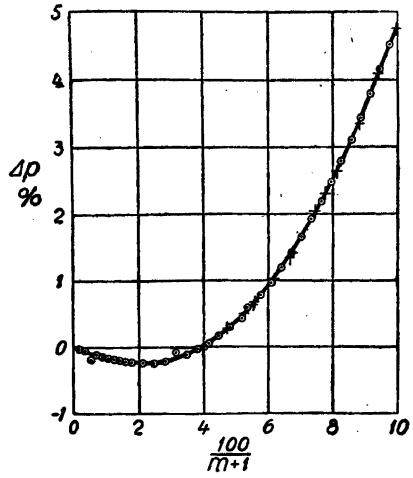

Fig. 2. $\Delta p = f\left(\frac{1}{m+1}\right)$ *for NaCl solutions on the data of Olnyk & Gordon (+) and Robinson (⊙).*

The interpretation given for the fact that $\Delta E_{H_2O} = 0$ for $m > 10$ in the case of HCl solutions is that 10 H_2O molecules are bound to each HCl molecule and that, thus, when $m > 10$ there are H_2O molecules which are bound to other H_2O molecules only [4]. If $m = 28$ is substituted for $m = 10$ the same applies to H_2SO_4 solutions [4]. Applying this interpretation to NaCl solutions we must conclude that there are H_2O molecules not bound to a HCl molecule as long as the sum of the number of H_2O molecules and twice that of NaOH molecules is greater than 10. Hence, two of the 10 sites arround the HCl molecule are preferentially occupied by NaOH molecules, *i. e.*, since there is one NaOH molecule per HCl molecule, two of the 10 sites are always occupied by an NaOH molecule independently of the number of H_2O molecules per HCl molecule.

4. CONDUCTIVITY

The data employed are listed in Table 4.

Table 4.

Solute	Experimental data by	Temp.	Fig.	x	ΔS_0
NaCl	Kohlrausch & Grotrian [13,16]	18°	3	7.64	1.52
KOH	Kohlrausch [13,17]	15°	4	7.53	1.49
NaOH	Kohlrausch [13,17]	18°	5	11.59	2.30
	Bousfield & Lowry [13,18]	18°	5	11.59	2.30

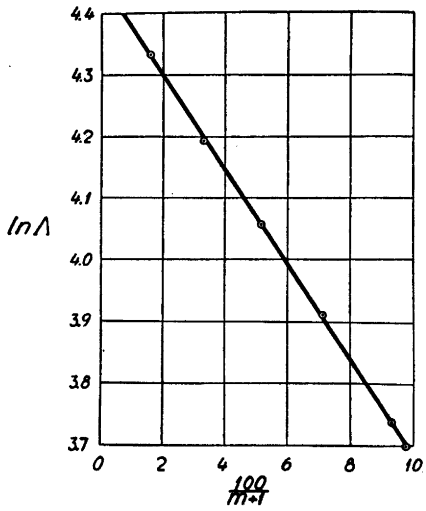

Fig. 3. $\ln \Lambda = f\left(\frac{1}{m+1}\right)$ *for NaCl solutions at 18°.*

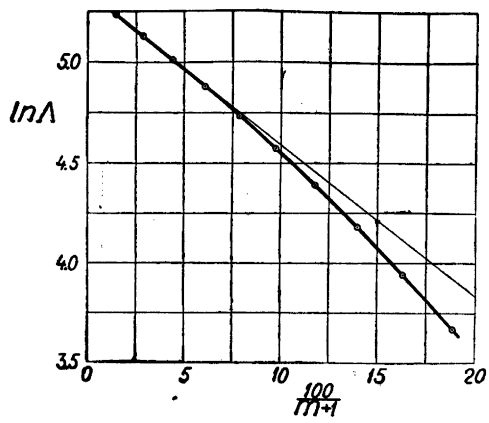

Fig. 4. $\ln \Lambda = f\left(\frac{1}{m+1}\right)$ *for KOH solutions at 15°.*

Figs. 3—5 show the plots of $\ln \Lambda = f\left(\frac{1}{m+1}\right)$ when Λ is the equivalent conductance and m is the number of water molecules per molecule of solute. At low concentrations those plots are of the form [2]

$$\ln \Lambda = A - \frac{x}{m+1}. \tag{14}$$

The values of x obtained from the curves are given in Table 3. From x we have computed ΔS_0 in the formula for the entropy of activation [2]

$$\Delta S = \frac{10}{m+1} \Delta S_0. \tag{15}$$

The data given by Darken and Meier [19] can not be represented by (14), because they fall much quicker at low concentrations, giving a non-linear curve for $\ln \Lambda = f\left(\frac{1}{m+1}\right)$ at low concentrations. The same effect was found in some data on acid solutions [2].

The coordination numbers may be assumed to be the same in the aqueous solutions of NaOH, KOH, and HCl [5].

For NaCl the formula (14) is valid in the entire range of solubility. For NaOH and KOH the validity is less extended. For HCl it is valid in the entire range of complete dissociation, $m > 5$. If we may call ΔS_0 for NaCl and KOH normal, ΔS_0 for NaOH is abnormal. Without further data, particularly on the temperature-dependence of Λ, it is difficult to account for this anomaly. However, we may conclude that the presence of HCl restores normality to NaOH.

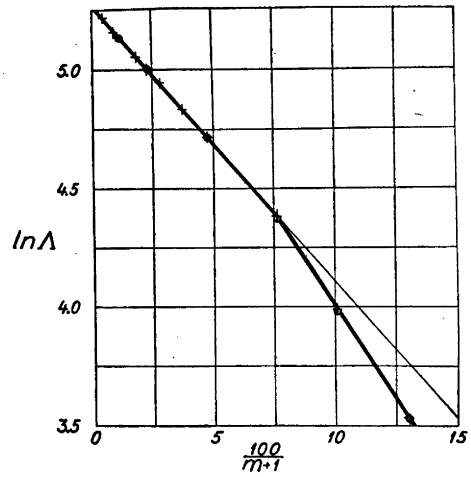

Fig. 5. ln $\Lambda = f\left(\frac{1}{m+1}\right)$ for NaOH solutions at 18°. ⊙ Kohlrausch, + Bousfield & Lowry.

The HCl molecule is surrounded by 5 nearest neighbors. For HCl we have derived [2]

$$\Delta S_0 = R \ln \left(\frac{4}{5} \cdot \frac{1}{5}\right)^{1/3} = -1.90 \text{ cal./mole}^0. \quad (16)$$

The KOH molecule conducts with its OH radical only just as an acid molecule conducts with its proton only [2]. The O atom has 4 nearest neighbors one of which is the K atom. Hence,

$$\Delta S_0 = R \ln \left(\frac{2}{3} \cdot \frac{1}{3}\right)^{1/3} = -1.49 \text{ cal./mole}^0 \quad (17)$$

in agreement with the empirical values for KOH and NaCl.

From these considerations we may conclude that the OH group is bound in the same manner in the two cases to which (17) applies. Hence, we must conclude that the NaOH molecule is not bound to a HCl molecule with its OH group adjacent to the Cl atom in the range where (17) is valid, *i. e.* below and at saturation.

In the structure of HCl in H_2O there are two coordination numbers, 5 and 10. The HCl molecule can form hydrogen bonds with 5 H_2O molecules directly and bind another 5 H_2O molecules indirectly. The number 5 has been found in the following properties: conductivity [2], vapor pressure [4], dissociation [20], all indicating pairs of HCl molecules when $m < 5$ and no such pairs when $m \geqq 5$. The number 10 has been found in the heats of vaporization of H_2O and HCl [4] and in the solubilities of halides [1] (and section 2). We must conclude that the HCl molecule is surrounded by two shells of H_2O molecules, each consisting of 5 H_2O molecules. For H_2O molecules within the outer shell, the binding energy is greater than that of H_2O molecules outside the outer shell, ΔE_{H_2O} being zero for $m > 10$ and increasing rapidly with decreasing m in the range $m < 10$ [4]. Since $\Delta E_{H_2O} = 0$ for NaCl solutions too, we must conclude

that there are H_2O molecules outside the outer shell in the entire range of solubility. This implies that, at least when $m < 10$, the NaOH molecules preferentially populate the outer shell around the HCl molecule. Assuming that this preference remains at all concentrations, we are led to assume that the NaOH molecule occupies two sites in the outer shell, *i. e.* the Na atom and the O atom are both located in the outer shell, and occupies thus a fixed position and a fixed orientation relative to the HCl molecule.

This discussion on the structure of NaCl solutions may explain why the presence of HCl molecules affects the value of ΔS_0 of the NaOH molecule, although it does not explain why it is rendered normal. Nor, of course, does it explain the anomaly of ΔS_0 in absence of HCl molecules.

5. CONCLUSIONS

The solubility data show that pairs of Na and Cl atoms, *i. e.* NaCl molecules, are not present in the solution, the NaCl dissolved being completely hydrolyzed into NaOH and HCl. This result is supported by vapor pressure data. The heat of vaporization of H_2O shows a preferential arrangement of the NaOH and HCl molecules, probably due to attraction between dipoles. From the activation entropy of conductivity it follows further, that not only the Na but also the O atom in the NaOH molecule is separated from the Cl atom by a H_2O molecule. The structure contains, thus, pairs of one NaOH molecule and one HCl molecule in a fixed position and a fixed orientation relative to one another, the two molecules in the pair being separated from each other by two H_2O molecules, one between Cl and Na, the other between Cl and O.

When two such pairs are separated by water they can not combine. But if the water molecules are not many enough, such pairs may combine to form a complex which we may write in the form $2(HCl \cdot 2\ H_2O \cdot NaOH)$. This complex may form when the ratio (5) is equal to 10. When it is less than 10, one H_2O molecule may be missing in the complex which may then precipitate as two molecules of the di-hydrate, $NaCl \cdot 2\ H_2O$. Thus, the condition of solubility (4) implies that the precipitate is $NaCl \cdot 2\ H_2O$ which is also actually found at low temperatures. Above 0.1° the precipitate contains $NaCl \cdot 2\ H_2O$ + NaCl, above 20° it contains NaCl only [21]. The two-phase precipitate $NaCl \cdot 2\ H_2O$ + NaCl seems to indicate that the process

$$NaCl \cdot 2\ H_2O \rightarrow NaCl + 2\ H_2O$$

is sluggish in the temperature interval 0.1 to 20°, and that equilibrium was not attained in the experiments quoted.

At equilibrium the free energy of solution is

$$\Delta F_s = \Delta E_s - T\ \Delta S_s = 0.$$

In this case ΔE_s and ΔS_s are both zero, and the entropy, *i. e.* the configuration, and the energy are both the same in the solution and in the precipitate.

Both structures consist, thus, at low temperatures, of aggregates of 2 Na atoms, 2 Cl atoms, and 4 H_2O molecules held together by hydrogen bonds. At higher temperatures, the precipitate gives off its crystal water.

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Die *cis-trans*-Konfigurationen und die Ultrarot-Absorptionsspektren der Ester einiger Cyclopentan-β-oxycarbonsäuren

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Die Ester der *cis*-β-Oxycarbonsäuren der Cyclopentanreihe besitzen die Fähigkeit, mit Hilfe der Wasserstoffbindung intramolekular einen Chelatring zu bilden. Den Estern der *trans*-β-Oxycarbonsäuren der erwähnten Reihe ist dies hingegen nicht möglich (starrer Cyclopentanring). Die Moleküle der Ester beider Säuren, sowohl die der *cis*- als die der *trans*-Säure, können sich gleichfalls mit Hilfe von Wasserstoffbindungen durch Vermittlung ihres Alkoholhydroxyls mit anderen Molekülen derselben Verbindung zu assoziieren.

Diese Assoziation ist eine Gleichgewichtsreaktion, die der allgemeinen Gleichung (1) folgt, in welcher A das sich assoziierende Molekül, B das Assoziat und K_1 die Gleichgewichtskonstante bedeuten. Aus der Gleichung (2) kann, wie bekannt, der Schluss gezogen werden, dass, wenn man einem solchen System, in welchem das Gleichgewicht (1) herrscht, ein indifferentes Lösungsmittel zufügt, die relative Menge der assoziierten Moleküle und damit zugleich auch die der durch dieselbe Wasserstoffbrücke gebundenen O—H-Gruppen bei sinkender Konzentration der Lösung rasch abnimmt.

$$A + A \underset{}{\overset{K_1}{\rightleftharpoons}} B \qquad (1) \qquad\qquad K_1 = \frac{(B)}{(A)^2} \qquad (2)$$

Die Einstellung des Gleichgewichts der Chelatbildung befolgt als intramolekulare Reaktion der Reaktionsgleichung (3), in welcher A ein offenes Einzelmolekül, C ein Chelatmolekül und K_2 die Gleichgewichtskonstante bedeuten. Gemäss Gleichung (4) werden die relativen Mengen der Einzel- und der Chelatmoleküle durch Zusatz eines indifferenten Lösungsmittels nicht verändert. Der Chelatisierungsgrad ist m.a.W. von der Totalkonzentration unabhängig.

$$A \underset{}{\overset{K_2}{\rightleftharpoons}} C \qquad (3) \qquad\qquad K_2 = \frac{(C)}{(A)} \qquad (4)$$

Neben der Chelatbildung kann es stets auch zur Assoziation kommen, wobei im Gleichgewichtszustand sowohl Gleichung (3) als (1) nebeneinander gelten. Der Zusatz eines indifferenten Lösungsmittels beeinflusst dann nicht das relative Mengenverhältnis der Einzel- und der Chelatmoleküle; dagegen nimmt die relative Menge der assoziierten Moleküle bei sinkender Konzentration der Lösung rasch ab.

An und für sich ist das System der Assoziation weit verwickelter, als es die oben wiedergegebene allgemeine Reaktionsgleichung (1) angibt. Die Assoziierung kann so geschehen, dass die Wasserstoffbindung zwischen dem Wasserstoffatom und entweder a) dem Carbonylsauerstoff, b) dem Hydroxylsauerstoff oder c) dem Äthersauerstoff der Estergruppe eines anderen Moleküls zustandekommt. In dem so entstandenen Assoziat bleibt eine Hydroxyl-OH-Bindung frei, welche ihrerseits wieder a) innerhalb des Assoziats selbst, b) zwischen diesem und einem Einzelmolekül oder c) zwischen den Assoziaten unter sich in sämtlichen oben aufgezählten Kombinationen zur Bildung einer Wasserstoffbindung befähigt ist. So kann dann der Vorgang weitergehen.

Aus Obigem kann gefolgert werden, dass, wenn die relativen Mengen des freien und gebundenen Hydroxyls bei Estern der β-Oxycarbonsäure in Lösungen eines indifferenten Lösungsmittels von der Konzentration unabhängig sind, wir es mit einem reinen Chelatbildungsgleichgewicht zu tun haben und sofern es sich um einen Ester der β-Oxycarbonsäure der Cyclopentanreihe handelt, muss dieser die *cis*-Form haben.

Da es möglich ist, mit Hilfe des Ultrarot-Absorptionsspektrums die relativen Mengen der durch eine Wasserstoffbrücke gebundenen Moleküle einerseits und der eine freie Hydroxylgruppe enthaltenden Moleküle anderseits zu beurteilen, kann man durch Untersuchung der Assoziations- und Chelatbildungsverhältnisse der β-Oxycarbonsäureester der Cyclopentanreihe nach dieser Methode bestimmte Schlüsse betreffs der *cis-trans*-Konfiguration ziehen.

In der vorliegenden Arbeit wird die Ultrarot-Absorption der O—H-Bindung folgender Verbindungen, bei denen die *cis-trans*-Konfiguration der Hydroxyl- und Carboxylgruppen chemisch festgestellt worden ist, untersucht:

5-Oxycamphersäure-dimethylester der Borneolreihe.
5-Oxycamphersäure-dimethylester der Isoborneolreihe.
5-Oxyisofenchosäure-dimethylester der α-Fencholreihe.
5-Oxyisofenchosäure-dimethylester der β-Fencholreihe.
cis-Borneol-3-carbonsäure-methylester.
trans-Borneol-3-carbonsäure methylester.

Vergleichshalber sind auch die OH-Absorptionen der entsprechenden Säuren sowie diejenigen von *n*-Propanol und α-Fenchol in die zusammenfassende Tabelle mit aufgenommen.

Mit Hilfe der Statistik über die Ultrarot-Absorptionsspektren solcher Verbindungen, deren Konfiguration auf anderen Wegen (chemisch, Röntgenspektrum, Elektrondiffraktion) festgestellt worden ist, können aus den Ultrarot-Absorptionsspektren anderer typverwandter Verbindungen Schlüsse betreffs der Konfiguration gezogen werden.

DIE KONFIGURATIONEN DER UNTERSUCHTEN VERBINDUNGEN

Borneol-3-carbonsäuren [1]. Böeseken, Slooff und Lutgerhorst [2] haben aus der niedriger schmelzenden Borneol-3-carbonsäure (Schmp. 102—103°) ein Chloralid und eine Acetonverbindung dargestellt, die sich nicht aus der höher schmelzenden Säure (Schmp. 175°) herstellen lassen. Das elektrische Leitvermögen der wässrigen Lösung der niedriger schmelzenden Säure ist grösser als das der höher schmelzenden. Zusatz von Borsäure verursacht bei ersterer eine

bedeutende Steigerung, bei letzterer hingegen eine gelinde Abnahme des Leitvermögens. Die Steigerung des Leitvermögens wird auf die Entstehung einer cyklischen, komplexen Borsäureverbindung zurückgeführt, die anscheinend nur bei der *cis*-Form möglich ist. Aus diesen Tatsachen haben Böeseken, Slooff und Lutgerhorst den Schluss gezogen, dass das niedriger schmelzende Isomere die *cis*-, das höher schmelzende die *trans*-Form ist. (Die *endo-exo*-Konfigurationsverhältnisse sind vorläufig ungeklärt.) Bredt [1] hielt schon vordem die niedriger schmelzende Säure für die *cis*-Form, ohne indessen eingehendere Gründe für seine Auffassung anzuführen.

5-Oxycamphersäuren [3]. Beide Säuren lassen sich in die Anhydride überführen, und diese gehen wiederum durch Hydrolyse in die betreffenden Ausgangssäuren zurück, was besagt, dass sich bei beiden die Carboxylgruppen im Verhältnis zueinander in der *cis*-Stellung befinden. Aus der 5-Oxycamphersäure der Borneolreihe kann man die β-Lactonsäure und ein Chloralid, aus deren α-Methylester das α-Methylester-β-lacton und aus dem β-Methylester das β-Methylester-γ-lacton darstellen, die sich aus der 5-Oxycamphersäure der Isoborneolreihe und den entsprechenden Estern dagegen nicht gewinnen lassen. Diese Tatsachen erweisen, wie auch schon früher dargelegt wurde [3d], dass sich bei den Verbindungen der Borneolreihe die Hydroxylgruppe in der *cis*-Stellung und bei denen der Isoborneolreihe in der *trans*-Stellung zu den Carboxylen befindet.

5-Oxyisofenchosäuren [3b, 4]. Nach demselben Verfahren wie bei den 5-Oxycamphersäuren ist erwiesen worden [3b, 4], dass sich bei der 5-Oxyisofenchosäure der α-Fencholreihe das Hydroxyl und die Carboxyle gegenseitig durchgehend in der *cis*-Stellung befinden. Bei der 5-Oxyisofenchosäure der β-Fencholreihe liegen die Carboxyle im Verhältnis zueinander in der *cis*-Stellung, das Hydroxyl aber zu diesen in der *trans*-Stellung. Aus der 5-Oxyisofenchosäure der α-Fencholreihe ist es gelungen, die β-Lactonsäure und ein Chloralid und aus deren α-Methylester das α-Methylester-β-lacton darzustellen, die sich aus den entsprechenden Verbindungen der β-Fencholreihe nicht herstellen lassen.

In Anschluss an diese Konfigurationsbeweise habe ich nun das elektrische Leitvermögen von Lösungen der 5-Oxycampher- und 5-Oxyisofenchosäuren in Wasser sowie die Einwirkung eines Zusatzes von Borsäure untersucht. Die gewonnenen Ergebnisse dieser Versuche stehen mit dem Obigen im Einklang.

VERSUCHE

Die Darstellung der 5-Oxycampher- und 5-Oxyisofenchosäuren sowie deren Dimethylester ist bereits früher in anderem Zusammenhang beschrieben worden [3d, 4b].

Rac. 5-Oxycamphersäure der Borneolreihe, Schmp. 194° (Zers.). Dimethylester, Schmp. 48—50°.

Rac. 5-Oxycamphersäure der Isoborneolreihe, Schmp. 207—208° (Zers.) *Dimethylester*, flüssig, Sdp.$_6$ 158—160°.

5-Oxyisofenchosäure der D-*a-Fencholreihe* * schmolz bei 147°, kristallisierte erneut (Umwandlungspunkt) und schmolz dann wieder unter gleichzeitiger Zersetzung bei 164°. *Dimethylester*, Schmp. 59—61°.

* In der vorliegenden Arbeit bedeuten die Vorzeichen D- bzw. L- wie bei Wallach [6], dass die betreffenden Verbindungen vom rechts- bzw. linksdrehenden Fenchon herstammen.

Rac. 5-Oxyisofenchosäure der α-Fencholreihe, Schmp. 196—197°. *Dimethylester*, Schmp. 47,5—49°.

5-Oxyisofenchosäure der L-*β-Fencholreihe*, Schmp. 188—189,5° (Zers.). *Dimethylester:* Aus dem Dikaliumsalz mit Dimethylsulfat wie früher beim 5-Oxyisofenchosäure-dimethylester der α-Fencholreihe beschrieben [4]. Er ist flüssig und wurde nicht destilliert.

Borneol-3-carbonsäuren. Aus den aus der rac. Camphocarbonsäure durch Reduktion nach Bredt [1] erhaltenen *cis-* und *trans*-Borneol-3-carbonsäuren hatte Mag. phil. E. Avela die betreffenden Methylester dargestellt, die er mir freundlicherweise zur Verfügung stellte *.

Messung des elektrischen Leitvermögens

Die Leitvermögensmessungen wurden in etwa 10 ml fassenden, mit platinierten Platinelektroden ausgerüsteten Gefässen unter Benutzung der Wechselstrommessbrücke "Philoscop" von Philips (Frequenz 1 000) ausgeführt. Die Widerstandskapazität der Gefässe wurde mit 0,01 *M* Kaliumchloridlösung bestimmt. Die Messungen erfolgten bei 25,00° C; die Temperatur des Thermostaten liess sich mit einer Genauigkeit von 0,01° regulieren. Das Thermometer wurde mit einem Standardthermometer verglichen.

Von jeder Oxycarbonsäure wurde durch Lösen einer gewogenen Menge der Säure in Wasser in einer Messflasche eine Stammlösung bereitet. Aus analysenreiner Borsäure wurde eine 1/2 *M* wässrige Lösung hergestellt. Von jeder Stammlösung wurden durch Pipettieren und Verdünnen mit Wasser oder mit der 1/2 *M* Borsäurelösung zwei Verdünnungsreihen hergestellt, eine wässrige und eine in bezug auf die Borsäure 1/4-molare. Das Leitvermögen der verschiedenen Lösungen ein und derselben Oxysäure wurde in einer Folge in demselben Gefäss gemessen, das nach jeder Messung mehrmals mit reinem Wasser und analysenreinem Aceton ausgespült und danach getrocknet wurde. Die Ergebnisse sind in den Tabellen 1—4 zusammengefasst.

Die Ultrarot-Absorptionsspektren

Die Ultrarot-Absorptionsspektren wurden mit einem automatischen Spektrometer (Perkin-Elmer Modell 12 C) aufgenommen. Prisma und Fenster bestanden aus Natriumchlorid. Der Apparat war in einem Zimmer aufgestellt, dessen Temperatur bei 20° ± 1° gehalten wurde und in welchem die relative Feuchtigkeit der Luft zwischen 20 und 45 % variierte. Die Schlitzweite wurde konstant (etwa 0,02 mm) gehalten. Die Schichtdicke der Natriumchloridzelle betrug etwa 0,1 mm und die der Quarzzelle 10,03 mm. Die erhaltenen Ultrarot-Absorptionsspektren sind im Wellenlängenbereich von 2,5—3,5 μ in Abb. 1—8 wiedergegeben. In diesen findet man die 2,7 μ-Absorption der Atmosphäre. In jeder Abbildung ist ausser den Spektren der verschieden konzentrierten Lösungen oben auch das Spektrum des Lösungsmittels wiedergegeben. Die Wellenlängen der scharfen Absorptionsmaxima in diesem Bereich waren mit einer Genauigkeit von $< 14\ cm^{-1}$ messbar. Über die Kalibrierung des Apparats und die Reproduzierbarkeit der Absorptionskurven ist bereits früher berichtet worden [4b]. In dem hier untersuchten Spektrumbereich ist leider das Trennungsvermögen des NaCl-Prismas am schlechtesten. Weil jedoch zwischen den Spektren der entsprechenden *cis-* und *trans*-Verbindungen durchgehend qualitative Unterschiede vorkommen, mag aus diesem Umstand kein Nachteil erwachsen sein (man vergleiche auch die Bandenbreite der 2,7 μ-Absorptionen der Atmosphäre mit den O—H-Bindungsabsorptionen der untersuchten Stoffe). In den Begleittabellen 5—10 der Abbildungen 1—8 sind angegeben die aus den Kurven ermittelten Werte für P und P_0 sowie der aus der Gleichung (5) berechnete Wert für k. In der Gleichung (5) bedeuten P_0 die vom reinen Lösungsmittel, P die von einer Lösung durchgelassene Strahlung, deren Oxysäurekonzentration gleich c Mol $\cdot$ l^{-1} ist; $k = a \cdot b$, wobei a den molaren Extinktionskoeffizienten, und b die Schichtdicke der Probe in der Zelle bezeichnen.

$$\log_{10} \frac{P}{P_0} = -k \cdot c \qquad (5)$$

* Die Eigenschaften der erwähnten Ester werden demnächst von Avela in einer anderen Arbeit beschrieben.

Da bei den 1—1/32 molaren Lösungen jedesmal dieselbe Zelle benutzt wurde, deren Dicke nicht genau bekannt war, wurde k anstelle des molaren Extinktionskoeffizienten berechnet. Dieselbe Berechnungsweise wurde auch beim Gebrauch einer 1 cm-Zelle benutzt. In der Tabelle 11 sind ausserdem die Wellenlängen der Maximalabsorptionen der Absorptionsbanden der freien und der durch eine Wasserstoffbrücke gebundenen O—H- und O—H ← O-Bindungen für die untersuchten Verbindungen wiedergegeben. Von den Faktoren, die die Messung der in den Tabellen angegebenen P- und P_0-Werte und die Messungsgenauigkeit derselben beeinflussen, möge folgendes angeführt werden: Die in der Stabilität des Röhrenverstärkers während der Messungsdauer sowie in der Linearität derselben festgestellten Fehler verblieben unter ± 0,5 % der gesamten Skala des »Recorders». Wegen der Dämpfung der Apparatur findet der Stift des Recorders nicht Zeit, die Absorptionsbanden bis zu deren richtigen Tiefe auf das Papier zu übertragen, sondern man erhält je nach der Stärke der betreffenden Bande mehr oder weniger zu hohe P-Werte. Beim P/P_0-Wert von etwa 0,5 belief sich der hierdurch hervorgerufene Fehler auf + 1 % der ganzen Skala. Den grössten durch die Apparatur bedingten Fehler verursacht die Intensitätsschwankung der Lichtquelle, die wiederum ihrerseits wahrscheinlich zur Hauptsache durch die Temperaturschwankungen des zur Kühlung benutzten Leitungswassers verursacht wurde. (Bei den Kontrollversuchen konnte in einem der unbefriedigendsten Fälle bei fester Einstellung auf eine bestimmte Wellenlänge während drei Stunden eine offensichtlich auf Temperaturerniedrigung des Kühlwassers zurückzuführende gleichmässige Abnahme von P_0 um etwa 5 % festgestellt werden. — Die Aufnahme der Spektren erforderte durchschnittlich eine Stunde je Lösungsreihe.) Die Lösungen wurden so bereitet, dass man von dem zu untersuchenden Stoff durch Wägen in einer 5 ml-Messflasche und Auffüllen mit dem Lösungsmittel bis zur Marke bei 20,0° eine Stammlösung bereitete. Aus dieser und demselben Lösungsmittel wurde dann mit Hilfe derselben 2 ml-Pipette die abgestufte Verdünnungsreihe hergestellt. Die Konzentrationen der Lösungen sind daraufhin berechnet worden und wurden nicht durch Bestimmungen nachgeprüft.

ERGEBNISSE

Leitvermögen. Aus den Tabellen 1—4 ist ersichtlich, dass von den 5-Oxycamphersäuren das elektrische Leitvermögen der *cis*-Form bedeutend grösser als das der *trans*-Form ist. Bei den 5-Oxyisofenchosäuren ist dieser Unterschied geringer.

Tabelle 1. Elektrisches Leitvermögen der racemischen 5-Oxycamphersäure der Borneolreihe in Wasser. Stammlösung 1/16 M. Widerstandskapazität des Gefässes 0,466; Wasser $\varkappa^{25}$ 2,59 · 10⁻⁶ Ohm⁻¹; 1/4 M Borsäure-Wasserlösung $\varkappa^{25}$ 12,9 · 10⁻⁶ Ohm⁻¹.

Oxysäurekonz. der Lösung, Mol · l⁻¹	Lösungsmittel			
	Wasser		1/4 *M* Borsäure	
	Widerstand der Lösung, Ohm	Spezifisches Leitvermögen der Lösung, $\varkappa \cdot 10^4$ Ohm⁻¹	Widerstand der Lösung, Ohm	Spezifisches Leitvermögen der Lösung, $\varkappa \cdot 10^4$ Ohm⁻¹
1/32	680	6,85	184	25,32
1/64	980	4,75	351	13,27
1/128	1 420	3,28	635	7,34
1/256	2 010	2,32	1 150	4,05
1/512	2 910	1,60	1 890	2,46
1/1 024	4 350	1,07	3 050	1,53

Tabelle 2. Elektrisches Leitvermögen der racemischen 5-Oxycamphersäure der Isoborneolreihe in Wasser. Stammlösung 1/8 M. Widerstandskapazität des Gefässes 0,466; Wasser $\varkappa^{25}$ 2,59 · 10⁻⁶ Ohm⁻¹; 1/4 M Borsäure-Wasserlösung $\varkappa^{25}$ 12,9 · 10⁻⁶ Ohm⁻¹.

Oxysäurekonz. der Lösung, Mol · l⁻¹	Lösungsmittel			
	Wasser		1/4 *M* Borsäure	
	Widerstand der Lösung, Ohm	Spezifisches Leitvermögen der Lösung, $\varkappa \cdot 10^4$ Ohm⁻¹	Widerstand der Lösung, Ohm	Spezifisches Leitvermögen der Lösung, $\varkappa \cdot 10^4$ Ohm⁻¹
1/16	880	5,29	900	5,18
1/32	1 260	3,70	1 290	3,61
1/64	1 740	2,68	1 760	2,65
1/128	—	—	—	—
1/256	3 510	1,33	3 510	1,33

Tabelle 3. Elektrisches Leitvermögen der 5-Oxyisofenchosäure der D-α-Fencholreihe in Wasser a) "Schmp." 147°, b) Schmp. 164°. Stammlösungen (a und b) 1/8 M. Widerstandskapazität des Gefässes 0,466; Wasser a^{25} 3,53 · 10⁻⁶ Ohm⁻¹, 1/4 M Borsäure-Wasserlösung $\varkappa^{25}$ 14,4 · 10⁻⁶ Ohm⁻¹.

	Oxysäurekonz. der Lösung, Mol · l⁻¹	Lösungsmittel			
		Wasser		1/4 *M* Borsäure	
		Widerstand der Lösung, Ohm	Spezifisches Leitvermögen der Lösung, $\varkappa \cdot 10^4$ Ohm⁻¹	Widerstand der Lösung, Ohm	Spezifisches Leitvermögen der Lösung, $\varkappa \cdot 10^4$ Ohm⁻¹
a)					
	1/16	448	10,40	402	11,59
	1/32	620	7,52	580	8,04
	1/64	880	5,30	845	5,44
	1/128	1 280	3,63	1 270	3,67
	1/256	1 830	2,55	1 830	2,55
	1/512	2 600	1,77	2 590	1,78
	1/1 024	3 860	1,19	3 800	1,21
b)	1/16	449	10,38	404	11,53
	1/32	630	7,38	585	7,97
	1/64	890	5,24	860	5,42
	1/128	1 300	3,59	1 280	3,64
	1/256	1 840	2,53	1 830	2,55
	1/512	2 700	1,73	2 660	1,75
	1/1 024	3 870	1,19	3 810	1,21

Zusatz von Borsäure bedingt bei der 5-Oxycamphersäure der Borneolreihe eine sehr beträchtliche Steigerung des Leitvermögens (1/64 *M*, Δ 842 · 10⁻⁶ Ohm⁻¹), die von derselben Grössenordnung ist, wie die von Böeseken, Slooff

Tabelle 4. Elektrisches Leitvermögen der 5-Oxyisofenchosäure der L-*β-Fencholreihe in Wasser. Stammlösung 1/16 M. Widerstandskapazität des Gefässes 0,340; Wasser* $\varkappa^{25}$ $4{,}60 \cdot 10^{-6}$ Ohm^{-1}; *1/4 M Borsäure-Wasserlösung* $\varkappa^{25}$ $10{,}8 \cdot 10^{-6}$ Ohm^{-1}.

Oxysäurekonz. der Lösung, $Mol \cdot l^{-1}$	Lösungsmittel			
	Wasser		1/4 *M* Borsäure	
	Widerstand der Lösung, Ohm	Spezifisches Leitvermögen der Lösung, $\varkappa \cdot 10^4$ Ohm^{-1}	Widerstand der Lösung, Ohm	Spezifisches Leitvermögen der Lösung $\varkappa \cdot 10^4$ Ohm^{-1}
1/16	420	8,10	—	—
1/32	580	5,86	605	5,62
1/64	830	4,10	870	3,91
1/128	1 250	2,72	1 290	2,64
1/256	1 800	1,89	1 830	1,86
1/512	2 600	1,31	2 670	1,27
1/1 024	3 900	0,87	4 080	0,83
0.000089	21 300	0,156	—	—

und Lutgerhorst [2] bei der *cis*-Borneol-3-carbonsäure gefundene (1/64 *M*, Λ $530 \cdot 10^{-6}$ Ohm^{-1}) *. Bei der 5-Oxycamphersäure der Isoborneolreihe führt der Borsäurezusatz zu einer geringen Abnahme des Leitvermögens.

Das Leitvermögen der 5-Oxyisofenchosäure der *α*-Fencholreihe ist in 1/4 *M* Borsäurelösung deutlich, obwohl nur wenig grösser als in Wasser, dagegen ist es bei der entsprechenden Säure in der *α*-Fencholreihe in der Borsäurelösung etwas geringer als in Wasser. Die bei der *cis*-Form wahrnehmbare Steigerung des Leitvermögens (1/16 *M*, Λ $106 \cdot 10^{-6}$ Ohm^{-1}) ist jetzt von der gleichen Grössenordnung wie diejenige, die von Böeseken, Slooff, Hoeffelman und Hirsch [5] für die *cis*-2-Oxycyclopentancarbonsäure gefunden wurde (1/8 *M*, Λ $96 \cdot 10^{-6}$ Ohm^{-1}). Aus dieser letztgenannten Verbindung haben die erwähnten Autoren eine Acetonverbindung und ein Chloralid dargestellt, und es darf demnach die *cis*-Konfiguration der Verbindung als erwiesen gelten. — Die bedeutenden Unterschiede in der Gleichgewichtskonstante der komplexen Borsäureverbindung, wiedergegeben durch das Leitvermögen in der Borsäurelösung, können sterisch bedingt sein. Als mit der Gleichgewichtseinstellung konkurrierende Reaktion dürfte Chelatbildung auftreten. Sterische Umstände, u.a. die abschirmende Wirkung der Methylgruppen bei der *cis*-Borneol-3-carbonsäure und der 5-Oxycamphersäure der Borneolreihe, dürften im Vergleich mit der 5-Oxyisofenchosäure der *α*-Fencholreihe und der *cis*-2-Oxycyclopentancarbonsäure auf die Gleichgewichtskonstante der Chelatbildung herabsetzend einwirken. Der *cis*-Borneol-3-carbonsäure-methylester und der 5-Oxycamphersäure-dimethylester der Borneolreihe besitzen, nach dem Ultrarot-Absorptionsspektrum zu schliessen, in Tetrachlormethanlösung mehr freie (nicht durch eine Wasserstoffbrücke gebundene) O—H-Bindungen als der 5-Oxyisofenchosäure-dimethylester der *α*-Fencholreihe unter gleichen Bedingungen.

* Diese Autoren benutzten in ihren Arbeiten ½ *M* Borsäurelösungen.

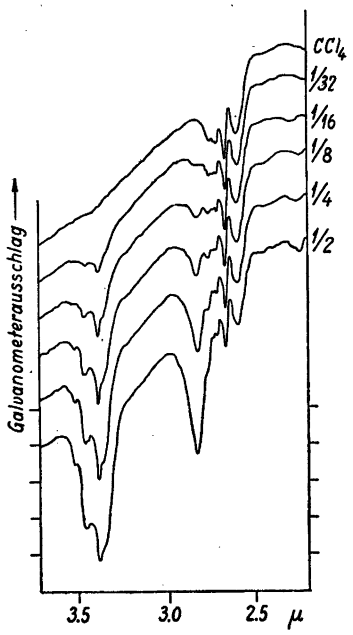

Abb. 1. Rac. 5-Oxycamphersäure-dimethylester der Borneolreihe. Ultrarot-Absorptionsspektren von 1/2—1/32-molaren CCl_4-Lösungen (Tab. 5).

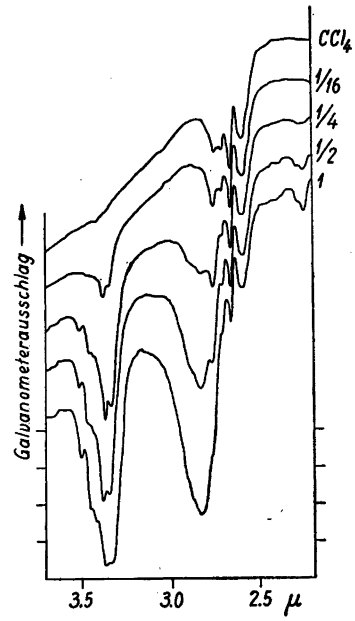

Abb. 2. Rac. 5-Oxycamphersäure-dimethylester der Isoborneolreihe. Ultrarot-Absorptionsspektren von 1—1/16-molaren CCl_4-Lösungen (Tab. 6).

Die in Wasser und in Borsäurelösung ausgeführten Leitvermögenmessungen stützen die oben geäusserte Auffassung über *cis*- und *trans*-Konfigurationen der in Rede stehenden Verbindungen.

Die Ultrarot-Absorptionsspektren. Aus den Abbildungen 5, 7, 1 und 3 und den anschliessenden Tabellen 9, 5 und 7 kann festgestellt werden, dass in den Ultrarot-Absorptionsspektren der (1—1/32 *M*) Tetrachlormethanlösungen von *cis*-Borneol-3-carbonsäure-methylester (bei dieser Verbindung auch in 1/100—1/3 200 *M* Lösungen), 5-Oxycamphersäure-dimethylester der Borneolreihe und 5-Oxyisofenchosäure der α-Fencholreihe bei 3 476 cm^{-1}, beziehungsweise bei 3 525 cm^{-1} und 3 468 cm^{-1} eine verhältnismässig scharfe Absorptionsbande der durch eine Wasserstoffbrücke gebundenen O—H-Bindung vorkommt. Bei allen diesen Verbindungen hält sich der bei der Maximalabsorption der betreffenden Bande berechnete *k*-Wert im geprüften Konzentrationsbereich innerhalb der Fehlergrenzen konstant. Dies bedeutet, dass die relative Menge der durch eine Wasserstoffbrücke gebundenen O—H-Bindungen konstant bleibt, was als Beweis für eintretende Chelatbildung und zugleich für die *cis*-Konfiguration der Verbindungen gedeutet wurde. Bei sämtlichen obengenannten Verbindungen findet man bei 3 616 cm^{-1}, beziehungsweise bei 3 602 cm^{-1} und 3 610 cm^{-1} eine sehr schwache Absorptionsbande der freien O—H-Bindung; diese ist beim 5-Oxyisofenchosäure-dimethylester der α-Fencholreihe schwä-

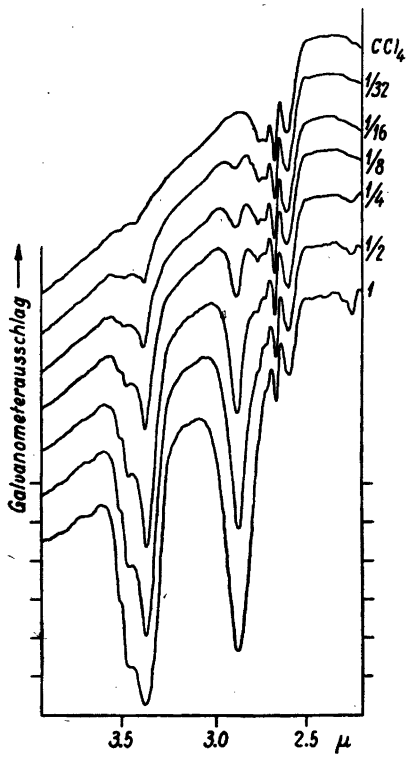

Abb. 3. 5-Oxyisofenchosäure-dimethylester der D-α-*Fencholreihe. Ultrarot-Absorptionsspektren von 1—1/32-molaren CCl_4-Lösungen (Tab. 7).*

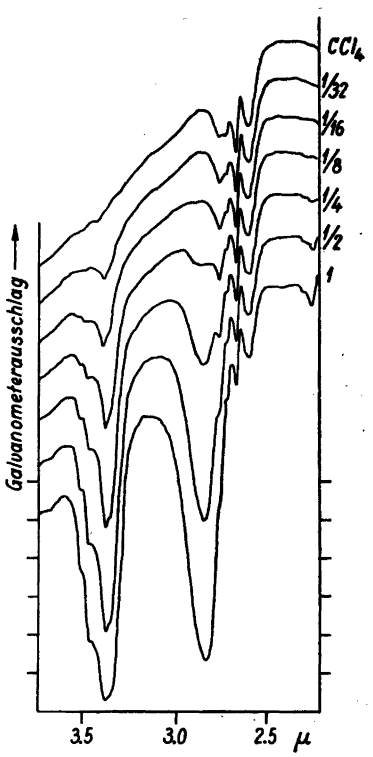

Abb. 4. 5-Oxyisofenchosäure-dimethylester der L-β-*Fencholreihe. Ultrarot-Absorptionsspektren von 1—1/32-molaren CCl_4-Lösungen (Tab. 8).*

cher ausgebildet als bei den beiden anderen. Die relativ geringe Absorption und die gegenseitige Überdeckung derselben mit der Absorptionsbande der gebundenen O—H-Bindung haben es unmöglich gemacht, die Konstanz von k bei der Maximalabsorption dieser Bande festzustellen.

Tabelle 5. Racemischer 5-Oxycamphersäure-dimethylester der Borneolreihe. Transmission in Tetrachlormethanlösungen bei der Maximalabsorption (3 525 cm^{-1}) der Absorptionsbande der gebundenen O—H-Bindung, berechnet auf Grund der Kurven in Abb. 1.

Konzentration Mol · l^{-1}	$\log_{10} \frac{P}{P_0}$	k 1 · Mol^{-1}
1/2	0,671 −1	0,66
1/4	0,840 −1	0,64
1/8	0,919 −1	0,65
1/16	0,960 −1	0,64
1/32	0,980 −1	0,64
	Mittel	0,65

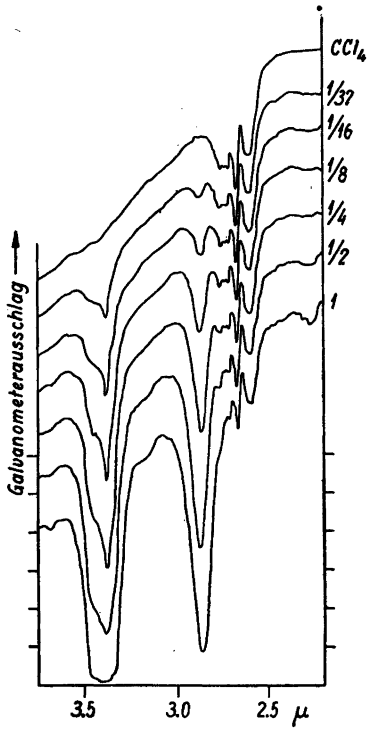

Abb. 5. c i s-Borneol-3-carbonsäure-methylester. Ultrarot-Absorptionsspektren von 1—1/32-molaren CCl_4-Lösungen (Tab. 9).

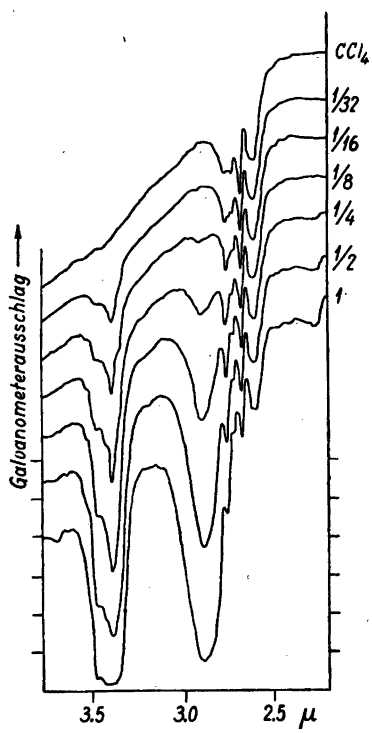

Abb. 6. t r a n s-Borneol-3-carbonsäure-methylester. Ultrarot-Absorptionsspektren von 1—1/32-molaren CCl_4-Lösungen (Tab. 10).

In den Ultrarot-Absorptionsspektren der 1—1/32 *M* Tetrachlormethanlösungen von *trans*-Borneol-3-carbonsäure-Methylester, 5-Oxycamphersäure-dimethylester der Isoborneolreihe und 5-Oxyisofenchosäure-dimethylester der β-Fencholreihe (bei der ersteren Verbindung auch in 1/100—1/3 200 *M* Lösungen) begegnet man der Absorptionsbande der durch eine Was-

Tabelle 6. Racemischer 5-Oxycamphersäure-dimethylester der Isoborneolreihe. Transmission in Tetrachlormethanlösungen bei der Maximalabsorption der Absorptionsbanden der gebundenen (3 563 cm^{-1}) und der freien O—H-Bindung (3 608 cm^{-1}), berechnet auf Grund der Kurven in Abb. 2.

Konzentration Mol · l^{-1}	O—H gebunden		O—H frei	
	$\log_{10}\frac{P}{P_0}$	k 1 · Mol^{-1}	$\log_{10}\frac{P}{P_0}$	k 1 · Mol^{-1}
1	0,330 −1	0,67	0,681 −1	0,32
1/2	0,699 −1	0,60	0,810 −1	0,38
1/4	0,871 −1	0,52	0,890 −1	0,44
1/16	0,984 −1	0,26	0,970 −1	0,48

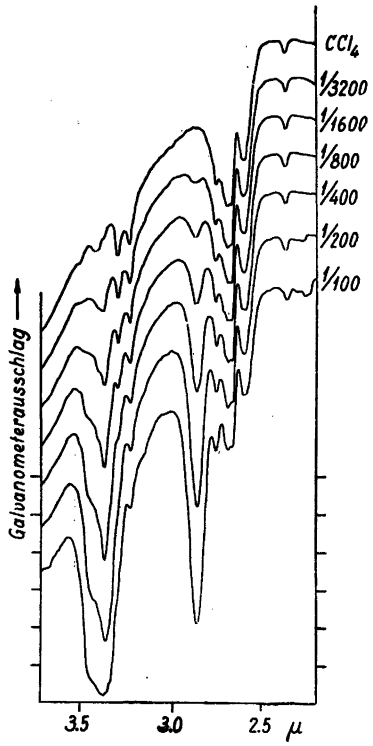

Abb. 7. c i s-Borneol-3-carbonsäure-methylester. Ultrarot-Absorptionsspektren von 1/100 —1/3 200-molaren CCl_4-Lösungen (Tab. 9).

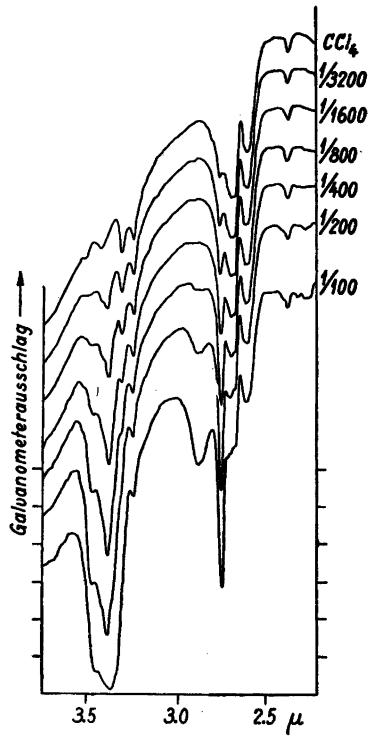

Abb. 8. t r a n s-Borneol-3-carbonsäure-methylester. Ultrarot-Absorptionsspektren von 1/100—1/3 200-molaren CCl_4-Lösungen (Tab. 10).

Tabelle 7. 5-Oxyisofenchosäure-dimethylester der D-α-*Fencholreihe. Transmission in Tetrachlormethanlösungen bei der Maximalabsorption der Absorptionsbande der gebundenen O—H-Bindung (3 468 cm^{-1}), berechnet auf Grund der Kurven in Abb. 3.*

Konzentration, Mol · l^{-1}	$\log_{10} \frac{P}{P_0}$	k 1 · Mol^{-1}
1	0,233 −1	0,77
1/2	0,595 −1	0,81
1/4	0,786 −1	0,86
1/8	0,903 −1	0,78
1/16	0,950 −1	0,77
1/32	0,976 −1	0,77
	Mittel	0,80

Tabelle 8. 5-Oxyisofenchosäure-dimethylester der L-β-Fencholreihe. Transmission in Tetrachlormethanlösungen bei der Maximalabsorption der Absorptionsbanden der gebundenen (3 506 cm^{-1}) und der freien O—H-Bindung (3 636 cm^{-1}), berechnet auf Grund der Kurven in Abb. 4.

Konzentration Mol · l^{-1}	O—H gebunden		O—H frei	
	$\log_{10}\frac{P}{P_0}$	k l · Mol^{-1}	$\log_{10}\frac{P}{P_0}$	k l · Mol^{-1}
1	0,144 −1	0,86	0,776 −1	0,22
1/2	0,610 −1	0,78	0,873 −1	0,26
1/4	0,861 −1	0,65	0,935 −1	0,26
1/8	0,950 −1	0,40	0,965 −1	0,27
1/16	0,984 −1	0,26	0,978 −1	0,36
1/32	0,996 −1	0,14	0,988 −1	0,39

serstoffbrücke gebundenen O—H-Bindung bei 3 453 cm^{-1}, beziehungsweise bei 3 513 cm^{-1} und 3 506 cm^{-1} (Abb. 6, 8, 2 und 4 nebst anschliessenden Tabellen 10, 6 und 8). Sie ist hier etwas breiter als bei den obenbeschriebenen *cis*-Verbindungen. Bei zunehmender Verdünnung der Lösung verbleibt der bei der Maximalabsorption berechnete Wert für k nicht konstant, sondern sinkt beträchtlich. Dementsprechend erfährt die Absorptionsbande der freien O—H-Bindung (3 623 cm^{-1}, 3 608 cm^{-1} und 3 636 cm^{-1}) bei sinkender Konzentration der Lösung eine relative Zunahme. Dieselben Ursachen wie vorhin bei den entsprechenden *cis*-Verbindungen, erschweren die Bestimmung von k bei der

Tabelle 9. C i s-Borneol-3-carbonsäure-methylester. Transmission in Tetrachlormethanlösungen bei der Maximalabsorption der Absorptionsbande der gebundenen O—H-Bindung (3 476 cm^{-1}), berechnet auf Grund der Kurven in Abb. 5 und 7.

Konzentration Mol · l^{-1}	$\log_{10}\frac{P}{P_0}$	k l · Mol^{-1}
0,1 mm Natriumchloridzelle		
1	0,035 −1	0,96
1/2	0,486 −1	1,03
1/4	0,743 −1	1,03
1/8	0,877 −1	0,98
1/16	0,938 −1	0,99
1/32	0,970 −1	0,96
	Mittel	0,99
10,03 mm Quarzzelle		
1/100	0,340 −1	66,0
1/200	0,657 −1	68,1
1/400	0,830 −1	68,0
1/800	0,912 −1	70,4
1/1 600	0,955 −1	72,0
1/3 200	0,979 −1	68,8
	Mittel	69,0

Tabelle 10. Trans-Borneol-3-carbonsäure-methylester. Transmission in Tetrachlormethanlösungen bei der Maximalabsorption der Absorptionsbanden der gebundenen (3 453 cm^{-1}) und der freien (3 623 cm^{-1}) O—H-Bindung, berechnet auf Grund der Kurven in Abb. 6 und 8.

Konzentration Mol · l^{-1}	O—H gebunden		O—H frei	
	$\log_{10}\frac{P}{P_0}$	k l · Mol^{-1}	$\log_{10}\frac{P}{P_0}$	k l · Mol^{-1}
0,1 mm Natriumchloridzelle				
1	0,956 −2	1,04	0,794 −1	0,21
1/2	0,499 −1	1,02	0,863 −1	0,27
1/4	0,780 −1	0,88	0,920 −1	0,32
1/8	0,910 −1	0,72	0,941 −1	0,47
1/16	0,965 −1	0,56	0,972 −1	0,45
1/32	0,989 −1	0,35	0,985 −1	0,48
10,03 mm Quartzzelle				
1/100	0,817 −1	18,3	0,507 −1	49,3
1/200	0,939 −1	12,3	0,725 −1	55,0
1/400	0,975 −1	10,1	0,859 −1	56,4
1/800	0,988 −1	9,5	0,925 −1	60,0
1/1 600	0,9953−1	7,5	0,975 −1	70,4
1/3 200	0,9977−1	7,5		81,5

*Tabelle 11. Übersicht über die Absorptionsmaxima der Alkohol-*O—H- *und* O ← H—O- *Bindungen bei den untersuchten Isomerenpaaren in verschiedenen Lösungsmitteln, in cm^{-1}.*

Verbindung	Lösungsmittel	O—H frei	O—H gebunden
Rac. 5-Oxyisofenchosäure-dimethylester	CCl_4	3 610	3 468
5-Oxyisofenchosäure-dimethylester der D-α-Fencholreihe	»	3 610	3 468
Rac. 5-Oxyisofenchosäure-dimethylester	Paraffinölpaste	3 610	3 454
5-Oxyisofenchosäure-dimethylester der D-α-Fencholreihe	»	3 610	3 435
5-Oxyisofenchosäure-dimethylester der L-β-Fencholreihe	CCl_4	3 636	3 506
5-Oxyisofenchosäure-dimethylester der D-α-Fencholreihe	Dioxan	— *	3 441
5-Oxyisofenchosäure der L-α-Fencholreihe	»	— *	3 435
5-Oxyisofenchosäure der L-β-Fencholreihe	»	— *	3 453
Rac. 5-Oxycamphersäure-dimethylester der Borneolreihe	CCl_4	3 602	3 525
Rac. 5-Oxycamphersäure-dimethylester der Isoborneolreihe	»	3 608	3 513
cis-Borneol-3-carbonsäure-methylester	»	3 616	3 476
trans-Borneol-3-carbonsäure-methylester	»	3 623	3 453
D-α-Fenchol	»	3 623	3 484
n-Propanol	»	3 621	3 311
n-Propanol	Dioxan	— *	3 497

* Ermittlung unmöglich wegen der starken Absorption des Lösungsmittels.

Maximalabsorption dieser Bande. Lässt man die gegenseitige Überdeckung der Banden unberücksichtigt, so wächst der für k berechnete Wert bei zunehmender Verdünnung. (Bei den *cis*- und *trans*-Formen einer Verbindung sind die bei der Maximalabsorption der durch eine Wasserstoffbrücke gebundenen O—H-Bindung berechneten k Werte in 1 M Lösungen praktisch gleich gross). Die parallel mit der Konzentration stattfindende Abnahme von k bedeutet, dass die relative Konzentration der durch eine O—H-Bindung gebundenen Hydroxylgruppen bei abnehmender absoluter Konzentration der Oxysäureester sinkt, was, wie eingangs dargetan wurde, eine Folge der Einstellung des Assoziationsgleichgewichts sein kann.

Die oben angeführten, einerseits für die *cis*- und andererseits für die *trans*-Formen eindeutigen Resultate erweisen, dass es bei den *cis*-Formen der β-Oxycarbonsäureester der Cyclopentanreihe zu einer starken Chelatbildung kommt, sowie dass sich deren Nachweis mit Hilfe der Ultrarot-Absorption der O—H-Bindung zur Klärung der *cis-trans*-Konfiguration der erwähnten verbindungen verwenden lässt. Weil sich die *endo-exo*-Isomerieverhältnisse der meisten bicyklischen Terpenalkohole von der *cis-trans*-Isomerie einiger α-Oxycarbonsäuren der Cyclopentanreihe ableiten lassen [3,4], kommt diesem Ergebnis eine allgemeinere Bedeutung in der Terpenchemie zu.

ZUSAMMENFASSUNG

1. Ein Verfahren zur Klärung der *cis-trans*-Isomerieverhältnisse der β-Oxycarbonsäureester der Cyclopentanreihe mit Hilfe der OH-Absorptionsbanden der Ultrarot-Absorptionsspektren wird beschrieben.

2. Die Einwirkung zugesetzter Borsäure auf das elektrische Leitvermögen der wässrigen Lösungen von 5-Oxycamphersäuren der Borneol- und Isoborneolreihe sowie 5-Oxyisofenchosäuren der α- und β-Fencholreihe wurde untersucht. Die Ergebnisse bestätigen die früher auf rein chemischem Wege gewonnene Auffassung von der sterischen Konfiguration der erwähnten Verbindungen.

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Studies Related to Naturally Occurring Acetylene Compounds. XIII. The Occurrence of *trans*-Methyl-*n*-Dec-2-en-4 : 6 : 8-triyonate in the Genus *Tripleurospermum* Schultz Bipontinus

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The matricaria ester (I), named so after the discovery of this substance in

$$CH_3—CH=CH—C\equiv C—C\equiv C—CH=CH—COOCH_3 \quad (2\text{-}cis,\ 8\text{-}cis) \qquad (I)$$

scentless mayweed [1] (*Matricaria inodora* L) is the acetylenic compound most frequently found in the essential oils of plants belonging to the family of the *Compositae*. It is, however, not in the tribus *Anthemideae* to which *Matricaria* belongs (subtribus *Chrysantheminae*) that (I) has been found to be of general occurrence, but in some genera within the tribus *Astereae*, especially *Amellus* [2], *Erigeron* [3,4] and *Felicia* [2].

We have, of course, long sought for the matricaria ester in other members of the genus *Matricaria*, but for many years without success. As mentioned in the ninth communication of this series [2] the oil of chamomile (from *M. chamomilla* L.) is devoid of matricaria ester and other acetylenic compounds. *M. discoidea* DC, which in the last century has invaded large areas of Norway, gives large amounts of essential oil. The dominating chromophores seem to be derivatives of 7-hydroxy-coumarines. Professor Dr. G. Ledyard Stebbins jr. most kindly provided a seed sample of *M. occidentalis* Greene which is endemic to some districts of California. *M. occidentalis* which botanically is closely related to *M. discoidea* DC gave an essential oil, the UV-spectrum of which agreed closely with that of *M. discoidea* in the main chromophores.

Coumarin itself was isolated from *M. glabra* (Lag) Ball from the Mediterranean. In South-Africa there occurs a number of *Matricaria* species, most of them in modern botanical literature transferred to the genus *Pentzia*. Through the most kind assistance of Director R. H. Compton, National Botanic Gardens, Kirstenbosch and chem.eng. F. Hougen, we obtained seed samples of *M. globifera* Fenzl. and *M. multiflora* (Thunb.) Fenzl ex Harv. & Sond. Of these

only *M. multiflora* seemed to contain acetylenic compounds. But the acetylenic compound which is present in the root of *M. multiflora* is different both from (I) and from any other of the acetylenic compounds so far isolated in a pure state. Finally, when in 1952 we cultivated *M. oreades* Boiss. we again obtained a *Matricaria* which contained large amounts of (I) both in the flowers and in the leaves. At that time we became aware, through a note in the newspapers, of a dissertation by the swedish botanist Gunnar Harling [5] concerning the embryology of different subtribes of the *Compositae*. It turned out that Dr. Harling had treated very extensively the two tribes *Asteraea* and *Anthemideae* which had been investigated chemically in our institute. It was very interesting to experience that Dr. Harling for embryological reasons had separated *M. inodora* and *M. oreades* from the other *Matricaria* and transferred both to the genus *Tripleurospermum*, erected by C. W. Schultz Bipontinus in 1844 on the basis of the structure of the seed wall and the achenes; a separation partly recommended by other specialists on the *Compositae* in the last century [6,7]. To this Genus *Tripleurospermum* Harling further transferred for embryological reasons a plant mostly named *Chrysanthemum caucasicum* Pers.

If this transfer was justified, it was to be expected that *Chrysanthemum caucasicum* should behave chemically as *M. inodora* and *M. oreades*. Through the obligingness of the botanical garden of the University of Copenhagen we obtained a small sample of fresh *Chr. caucasicum* plants. The leaves, which constituted most of this late-autumn sample (150 g), furnished apreciable amounts of (I), which according to the U.V.-spectrum is the only acetylene present in this crude essential oil. Only traces of roots (7.4 g) were present in the small sample; the crude essential oil showed a very broad absorption in U.V. from 3 800 ÅU with very little fine structure. This — not very promising spectrum — agreed closely with that of the oil from the root of *M. oreades*. Since both these samples were very small, we turned to the root of *M. inodora* L. The root of scentless mayweed had never been investigated separately in our laboratory. All investigations on scentless mayweed had been carried out before the investigation of European mugwort (*Artemisia vulgaris* L.) had disclosed that the root chemically may differ completely from the rest of the plant.

The root from scentless mayweed afforded a crude oil with the same broad and uncharacteristic spectrum as obtained from *M. oreades* and *Chrysanthemum caucasicum*. It turned out, however, that the component responsible for a small, sharp peak at 2 556 ÅU was very insoluble in petroleum ether. After one recrystallization the m.p. was constant at 105.5° C. The spectrum of this crystalline substance is reproduced in Fig. 1. In this spectrum the maxima 3 440, 3 210, 3 012, 2 832 and 2 556 ÅU coincided with the maxima given by Christensen and Sörensen for the *trans*-isomer of methyl-*n*-dec-2-ene-4:6:8-triynoate (II) m.p. 95.5°

$$CH_3—C\equiv C—C\equiv C—C\equiv C—CH=CH—COOCH_3 \qquad \text{(II)}$$

the only one of the four possible dehydromatricaria esters so far prepared synthetically. The spectrum of synthetic (II) differed in the lack of a maximum at 2 445 ÅU and in the occurrence of a small additional maximum on the long-wave side at 3 630 ÅU. As the coincidence in the 5 main maxima and in the

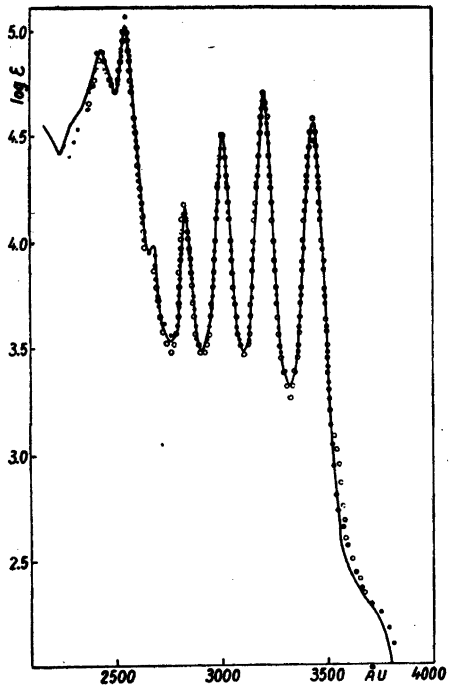

Fig. 1. Ultraviolet absorption in hexane of methyl-n-dec-2-ene-4 : 6 : 8-triynoate.
——— *synthetic*
○ ○ ○ ○ *from root of Matricaria inodora L.*
● ● ● ● *from the root of Matricaria oreades Boiss.*

general shape of the curve was quite surprising, the original spectrograms taken on synthetic (II) were inspected in the plate archive. It turned out that the maximum at 2 445 ÅU was present on the spectrograms of both synthetic preparations. The position of this band falls in the border region between the Fe-W spectra used for wave-lengths >2 400 ÅU and the Ni spectra used for the region 2 150—2 450 and through this position in the border region this maximum had been completely overlooked. The 3 630 ÅU maximum was definitely present in both preparations and so only the possibility remained that this extra maximum belonged to an unknown impurity which at the same time depressed the m.p. from 105.5° to 95.5°. Primarily, it did not seem very likely that an impurity should be present in exactly the same amount in two parallel syntheses where the isolation of (II) was achieved through chromatography. The resynthesis of (II), carried out by one of us (T. B.) under slightly altered coupling conditions, gave appreciably higher yields and this time (II) was easily purified to m.p. 105° and the U.V.-spectrum of this pure substance was completely identical. (*cf.* Fig. 1) with that of the substance from the root of scentless mayweed and the identity of the two preparations was further confirmed by a mixed m.p. determination.

The isolation of pure II, m.p. 105.2—105.5° from the root of *M. oreades* was successful too. The amount of oil from *Chrysanthemum caucasicum* was, however, too small to allow the isolation of any pure compound from the complex mixture present. A large scale cultivation of *Chr. caucasicum* in the sum-

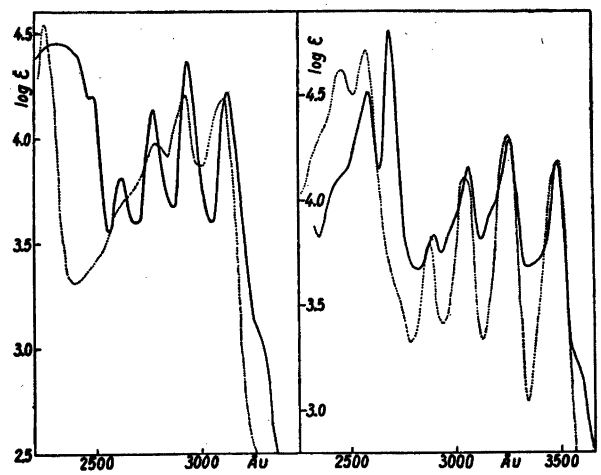

Fig. 2. Ultraviolet absorption in hexane of

left: ——— $-CH=CH-C\equiv C-C\equiv C-CH=CH-$ *measured on* 2-trans : 8-trans-*matricarianol*

····· $-C\equiv C-C\equiv C-CH=CH-COOCH_3$ » » cis-*lachnophyllum ester*

right: —— $-CH=CH-C\equiv C-C\equiv C-C\equiv C-CH=CH-$ » » *"Centaur X" from Artemisia vulgaris*

····· $-C\equiv C-C\equiv C-C\equiv C-CH=CH-COOCH_3$ » » *dehydromatricaria ester from Artemisia vulgaris*

mer of 1953 is hoped to provide the starting material necessary for the confirmation of the presence of the *trans*-isomer of (II) as a general component of the oil from roots of members of the genus *Tripleurospermum*.

The occurrence of *trans*-methyl-*n*-dec-2-ene-4 : 6 : 8-triynoate (II) in nature and the corrected spectrum of this dehydromatricaria ester give valuable hints to the constitution of the dehydromatricaria ester of m.p. 112.5 found in the root of *Artemisia vulgaris* [8]. As is seen from Fig. 1 the two known dehydromatricaria esters differ about 40 ÅU in the positions of the maxima in the long-wave band group, whereas the position of the short-wave band group is identical. As mentioned earlier [9] only 3 possibilities exist for the structure of dehydromatricaria ester from *A. vulgaris, viz.* the *cis*-form of II and the *cis*- and *trans*-isomers of (III)

$$CH_3—CH=CH—C\equiv C—C\equiv C—C\equiv C—COOCH_3 \qquad \text{(III)}$$

Our attempts to synthesise 8-*trans*-III so far have been unsuccessful. From evidence accumulated in later years concerning the spectra of polyacetylenes we are, however, inclined to conclude that the shortwave band group in the U.V.-spectrum of (III) should be displaced some 130 ÅU towards longer wavelengths as compared with the *cis*- and *trans*-isomers of (II).

Two examples supporting this view are collected in Fig. 2.

The lachnophyllum ester [3] (IV) and symmetrical diene-diyne compounds (V) [10,11] very nearly agree in the position of the long-wave band group.

$$CH_3—CH_2—CH_2—C\equiv C—C\equiv C—CH=CH—COOCH_3 \qquad (IV)$$

$$R—CH=CH—C\equiv C—C\equiv C—CH=CH—R' \qquad (V)$$

The first maximum is found at about 3 100 ÅU, but the positions of the short-wave band group differ not less than 200 ÅU.

In the same way the *trans*-dehydromatricaria ester (II) and the "Centaur-X"-class hydrocarbons [8,12] (VI) agree in the long-wave group, but differ 130 ÅU in the strong, short-wave band group.

$$R—CH=CH—C\equiv C—C\equiv C—C\equiv C—CH=CH—R' \qquad (VI)$$

It has turned out that the carbomethoxy group is spectroscopically nearly equivalent to one double bond in en-ynes as in polyenes [13,14,15]. Obviously then (III) should agree in the position of both bond groups with the symmetrical "Centaur-X"-class (VI). So these spectral rules — which in recent months have been strengthened through the outstanding constitutional work of B. Lythgoe [16] on the diyne-triene toxins of *Cicuta* and *Oenanthe* — make very probable that the constitution of the dehydromatricaria ester from *Artemisia vulgaris* is the 2-*cis*-isomer of (II), that is the *cis*-isomer of the second dehydromatricaria ester found in nature, which so far is known only from the oils from roots of three members of the genus *Tripleurospermum*.

The U.V.-spectrum of (II) has one of the most outstanding fine-structures reported for solution spectra. As the crude oils from the root of the three investigated members of the genus *Tripleurospermum*, as mentioned above, show very broad and undistinctive U.V.-spectra it will be clear that (II) is only a minor component of these oils. The oils from the roots of *M. inodora* and *M. oreades* have been separated into different fractions by chromatography on alumina as described in the experimental part. Two of the components have been obtained in a crystalline state, *viz.* from the 10—25 % benzene in petroleum ether eluates a substance of m.p. 47.5—48.5° with a rather unsharp spectrum (*cf.* curve 3A), and the dehydromatricaria ester (II) which is concentrated in the 50 % benzene petroleum ether eluates. With *M. oreades* root there has been obtained in the 75—90 % benzene-petroleum ether eluates another new compound, m.p. 44—45°, again with a rather undistinctive U.V.-spectrum (curve 3B) with the first maximum at 3 650 ÅU. These two compounds seem to be the main components and it will be obvious that a mixture of these two will give a very broad and very undistinctive spectrum.

Both these substances are very unstable; we hope to obtain sufficient quantities of oil in the summer season of 1953 to get some insight into their constitutions. These preliminary investigations of the oil of the roots of *Matricaria inodora* and its relatives — which in agreement with the proposal of Harling undoubtedly should be transferred to the genus *Tripleurospermum* — demonstrate on the one hand that the composition of the root differs remarkably from that of the overground part of the plants and on the other hand that the roots are good sources for new members of the naturally occurring acetylenic compounds.

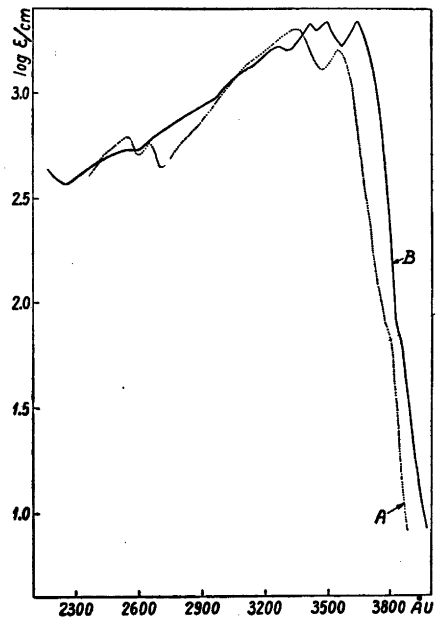

Fig. 3. Ultraviolet absorption in hexane of
A: Substance m. p. 47.5—48.5 from Matricaria inodora L.
B: » » 44—45 » » oreades Boiss.

EXPERIMENTAL

1. *Matricaria inodora* L. Root. Portions of fresh root were collected in the vicinity of Trondheim at the end of October. 2.5 kg were distilled with steam giving 1.1 g of oil, semi-solid in the cold. U.V.-spectrum of the crude oil: very broad absorption from 3 800 with only a small peak at 2 555 ÅU. When the oil was dissolved in petroleum ether slightly yellow crystalls separated, which after recrystallization from ether-petroleum ether melted constantly at 105.5°—106° (corr.). Mixed m.p. with synthetic methyl-*n*-dec-2-en-4 : 6 : 8-triynoate 105.5°.

Another portion of 480 g of roots was extracted with acetone in the cold. The aceton extract was diluted with much water and extracted with ether. The ether solution was washed free from acetone, dried and evaporated in a vacuum at room temperature. Residue, 72.4 mg, yellow oil, which solidified in the cold. Methyl-*n*-dec-2-en-4 : 6 : 8-triynoate was isolated as above, m.p. 105.5°, so there should be no doubt that this dehydromatricaria ester is no artefact.

All mother liquors were combined and the solvents removed. There followed chromatography of the petroleum ether solution on slightly de-activated alumina followed by elution with mixtures of benzene-petroleum ether. In the eluates with 25—40 % benzene-petroleum ether a substance was concentrated with the spectrum given in curve 3A. A very similar chromophore has been observed in the root of *Rudbeckia hirta*, but as this chromophore is eluted with the petroleum ether washings and so far has remained liquid they are scarcely identical. This substance can be distilled at 50—60°/0.0001 mm, but decomposes if the temperature exceeds 70°. The distillate solidified; after recrystallization from petroleum ether, the m.p. was 47.5—48.5°. The substance is rather unstable and the amounts obtained pure were too small for further experiments.

2. *Matricaria oreades* Boiss. Cultivated at Trondheim 1951/52 from a seed sample from *Botanischer Garten München-Nymphenburg.*

a) Flowers (115 g) were distilled with steam; the essential oil, 68.5 mg, crystallized at room temperature. The crystalls were recrystallized once from petroleum ether, m.p. 34—34.5°, undepressed on admixture with authentic matricaria ester. The U.V.-spectra of these crystalls and of the mother-liquor were measured. The spectrum of the former was completely identical with that of matricaria ester, that of the latter was conformed with the matricaria ester spectrum. Thus no other chromophore seems to be present in this oil.

b) Leaves (1 600 g) were distilled with steam. This essential oil (1.3 g) solidified in the cold; the U.V.-spectrum of the crude oil was not entirely identical with that of matricaria ester, but crystallization from petroleum ether at once afforded pure matricaria ester.

c) Roots (460 g) were also distilled with steam. The essential oil (1.9 g) was semisolid at room temperature. The U.V.-spectrum of the crude oil: strong absorption from 3 800 ÅU downwards with undistinct maxima at 2 550 and 2 430 ÅU. The oil was dissolved in petroleum ether and then at once there deposited slightly yellow crystalls, which were recrystallized from ethanol-water, m.p. 105—105.2°. Spectrum curve 1.

ε_{max}	35 970	45 290	32 810	15 170	8 630	106 200	71 800
λ_{max}	3 435	3 204	3 008	2 835	2 680	2 550	2 447
$\nu_{max}\ 10^{-13}$	873.4	936.3	997.3	1 058.2	1 119.4	1 176.5	1 226.0
$\Delta\nu$ —»—	62.9	61.0	60.9	61.2		49.5	

The mother liquors were chromatographed on alumina of the same quality as above. The chromophore observed with *M. inodora* was concentrated in the 10 % benzene-petroleum ether eluates. Traces of methyl-*n*-dec-2-ene-4 : 6 : 8-triyn-1-oate followed in the eluates with about 1 : 1 benzene-petroleum ether. In the 70 % benzene-petroleum ether eluates there was concentrated a new substance with the spectrum given in Fig. 3B. This fraction distilled at 90°/0.0001 mm; the distillate was solid and was recrystallized from petroleum ether, m.p. 44—45°.

3. *Chrysanthemum caucasicum* Pers. The Botanical Garden at the University of Copenhagen most amiably sent us a small sample of the living plant October 1952. Only traces of flowers were present at this late time. The leaves and the stem were separated from the root and distilled with steam, the essential oil (6.3 ‰) partly solidified in the cooler. One single crystallization from petroleum ether furnished pure matricaria ester, m.p. 34.6—34.8°, undepressed on admixture with authentic matricaria ester. U.V.-spectra were measured both of the pure crystalls and of the mother liquors. That of the former was identical with the prototype spectrum, that of the latter revealed that the matricaria ester was the only chromophore present in this essential oil.

4. *trans-Methyl-n-dec-2-ene-4 : 6 : 8-triynoate.* Methyl-pent-2-en-4-ynoate (3.75 g) and pentadiyne (2.2 g) in a solution of copper(I)chloride (49.5 g) ammonium chloride (76.4 g) and iron(III)chloride (4.0 g) in water (190 ml) were coupled oxidatively by passing air through the solution for 6.5 hours. Isolation of the methyl-*n*-dec-2-ene-4 : 6 : 8-triynoate as in [9] followed by crystallization from petroleum ether, m.p. 105—106°, yielded approximately 0.5 g of crude ester.

SUMMARY

trans-Methyl-*n*-dec-2-ene-4 : 6 : 8-triynoate (II) was resynthesised in better yield which enabled the removal of an impurity present in the preparation described in the first synthesis [9]. This dehydromatricaria ester was further isolated from the essential oil of the root of *Matricaria inodora* L. and *M. oreades* Boiss. and proved by isolation from acetone extracts in the cold to be no artefact. The corrected spectrum of *trans*-methyl-*n*-dec-2-ene-4 : 6 : 8-triyonate makes very probable that the dehydromatricaria ester m.p. 112.5° found in the root of *Artemisia vulgaris* L. is the 2-*cis*-isomer of (II).

The matricaria ester (I) has been isolated from *M. oreades* and *Chrysanthemum caucasicum* Pers. which, according to recent embryological investigations, together with *M. inodora*, belongs to a special genus *Tripleurospermum* Schultz Bipontinus. Six other species of the genus *Matricaria* belonging to other sections, of this genus have been investigated. They contained neither (I) nor (II) and most of them seemed quite devoid of acetylenic compounds altogether. The power to synthesise acetylenic compounds thus separates *Tripleurospermum* distinctly from *Matricaria*.

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Studies Related to Naturally Occurring Acetylene Compounds. XIV. The Occurrence of 2-*trans* : 8-*trans* Deca-2 : 8-diene-4 : 6-diyn-1-ol = *trans* : *trans*-Matricarianol in Nature

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The 2-*cis*:8-*cis* matricaria ester (I) is so far the acetylenic compound, which seems to be of most general occurrence in essential oils from plants be-

$$CH_3—CH=CH—C\equiv C—C\equiv C—CH=CH—COOCH_3 \qquad (I)$$

longing to the family of the *Compositae*. Table 1 summarises the results of our investigations so far concerning the occurrence of (I) in nature.
In this table ordinary types denote that (I) has been isolated in a pure state, small types that (I) because of the small amounts present has been made probable only through U.V.-spectrography. In the tribus VII = *Anthemideae*, where this ester was first found, its occurrence seems restricted to the small sub-genus *Tripleurospermum*, separated from *Matricaria* some 100 years ago on pure botanical indications [1]. Table 1 demonstrates, however, that a lot of genera in the tribus III = *Astereae* contain (I), mostly in large quantities. Besides (I), its α,β-dihydro derivative also occurs in this tribus [2]. During our investigation of some further members of this tribus, deviating results were obtained. One of us (D.H.) [3] in 1951 demonstrated that the root of *Aster tripoleum* L. contained a chromophore, resembling very much that of the two isomers (2-*trans* : 8-*trans* and 2-*trans* : 8-*cis*) of deca-2 : 8-diene-4 : 6-diyn-1-ol (II), synthesized by Bruun *et al.*[4] as intermediates in their syntheses of some stereoisomers of (I), and for this reason were named "matricarianols".

$$CH_3—CH=CH—C\equiv C—C\equiv C—CH=CH—CH_2OH \qquad (II)$$

Aster tripoleum L. is the only common wild *Aster* of Norway. This rather tall plant contains only small amounts of essential oil in all parts of the plant. The oils from the flowers and the leaves are — to judge from the U.V.-spectra of the crude oils — quite devoid of acetylenic compounds. The root, which is surprisingly small, contains very little oil but definitely with an acetylenic

Table 1.

The Occurrence of 2-cis:8-cis Matricaria Ester in the 13 tribes of the Compositae Family.

I	II	III
Vernonieae (not investigated)	*Eupatorieae*	*Astereae* ***Amellus strigosus*** (flowers and leaves) ***Aster mongolicus*** (root) » ***lautureanus*** (root) *Aster Novae Angliae* (root) *Brachycome iberidifolia* (flowers) ***Erigeron*** 19 species (all parts) *Erigeron* 10 species (all parts) *Felicia amelloides* (all parts) » *fragilis* » » » *rotundifolia* » » *Grindelia arenicola* (root) *Solidago virga aurea* (root) *Townsendia Parryi* (all parts)
IV	V	VI
Inuleae *Ammobium alatum* (leaves)	*Heliantheae* —	*Helenieae* *Gaillardia aristata* (leaves)
VII	VIII	IX
Anthemideae *Matricaria caucasicum* » *inodora* » *oreades*	*Senecioeae* —	*Calenduleae* *Dimorphotheca aurantiacus* (flower)
X	XI	XII	XIII
Arctotideae —	*Cynareae* *Saussurea alpina* (all parts)	*Mutisieae* (not investigated)	*Cichorieae* —

chromophore but in a low concentration and so one of the main difficulties in this work has been to procure enough starting material. We are greatly indebted to Mrs. Helene Sörensen and Mrs. J. Stene Sörensen for their kind assistance on this point.

Because of the small amounts of acetylenic compounds present in *Aster tripoleum* L. this work extended over several years. In the meantime two species of *Grindelia viz. arenicola* f. *trichophora* Steyermark and *stricta* D.C., of which Dr. Philip A. Munz, Rancho Santa Ana Botanic Gardens, had most kindly sent us seed samples, had been cultivated at Norges Tekniske Högskole, Trondheim.

Grindelia arenicola did not flower the first year at N.T.H. Most of the plants were distilled without flowers, because we did not trust the plants to stand the winter climate of Trondheim. Both the leaves and the root of *Grindelia arenicola* were rich in oil (1.8 ‰), and the crude oil showed an U.V.-spectrum in general agreement with the matricarianols (see Fig. 1), contents

$\sim$ 50—70 % calculated as free alcohol. After high vacuum distillation the extinction coefficients had changed very little. Analysis agreed with a substance $C_{12}H_{12}O_2$, corresponding to the acetate of a matricarianol. The ester structure was confirmed through strong infra red maxima at 1745 and 1230 cm^{-1}, characteristic of an ester of acetic acid. Catalytic hydrogenation furnished *n*-decyl-acetate identical in m.p., mixed m.p. and infra-red spectrum with synthetic *n*-decyl-acetate.

Thus only the configuration at the double bonds remained to be established. Saponification under cautious conditions afforded immediately a solid alcohol, the m.p. of which could not be raised above 93° by recrystallisation. After further purification by chromatography, this crude matricarianol was esterified with 3:5-dinitro-benzoyl chloride in pyridine and the ester purified by recrystallisation from acetone-petroleum ether. The 3:5-dinitrobenzoate then melted at 126—128° undepressed by an authentic sample of synthetic 3:5-dinitrobenzoate of 2-*trans* : 8-*trans*-matricarianol. Dr. H. Sörum had the kindness to take Debye Scherrer diagrams of these two dinitrobenzoates. The diagrams were completely identical.

The reason for the low m.p. of the crude matricarianol might be the presence of some *cis*-isomer. Thus in the infrared spectrum of the native acetate there occurred the characteristic 720 cm^{-1} band of the *cis*-double bond. Pure 2-*trans* : 8-*trans*-matricarianol was devoid of selective absorption in this region.

As described in the experimental part the isolation of 2-*trans* : 8-*trans*-matricarianol then was successful also from *Grindelia stricta* (leaves and root) and from the root of *Aster tripoleum*, where this chromophore was first discovered. In these cases also the primary compound was an ester. The amounts and purity of the oils did not allow the preparation of the original esters in a pure state, and therefore nothing is known about the acid moiety.

It then became tempting to see whether the matricarianol was a general component of the essential oil of the genus *Aster*. Since no further wild representatives are available in Norway, we cultivated some of the common garden *Asters* from seed samples, most kindly put at our disposal by the Botanical Garden of The University of Copenhagen. The details of this investigation are given in the experimental part. The results may briefly be summarised as follows: Two species: *A. Herveyi*, A. Gray and *A. ptarmicoides* (Nees) T. & G., were lacking acetylenes in all parts of the plants. *A. ptarmicoides* seemed to contain coumarins in the root. Three species: *A. novae angliae* L., *A. lautureanus* Fr. and *A. mongolicus* Fr. were lacking acetylenic compounds in the overground part of the plants, but contained appreciable amounts of the 2-*cis* : 8-*cis* matricaria ester (I) in the root. One species: *A. novi belgii* L. also did not contain acetylenes in the overground part of the plant. The root contained a chromophore with acetylenic spacing between the maxima, which were found at 3 075, 2 900 and (2 710) ÅU, positions close to, but significantly different from the symmetrical diene-diyne grouping of the matricarianols and intermediate between the *cis* and *trans* lachnophyllum esters. Since only traces of oils were available, larger cultivations of this *Aster* are laid out in 1953.

These six *Asters* resemble *Aster tripoleum* in so far as none of them has shown any indication of acetylenic compounds in the flowers or green parts,

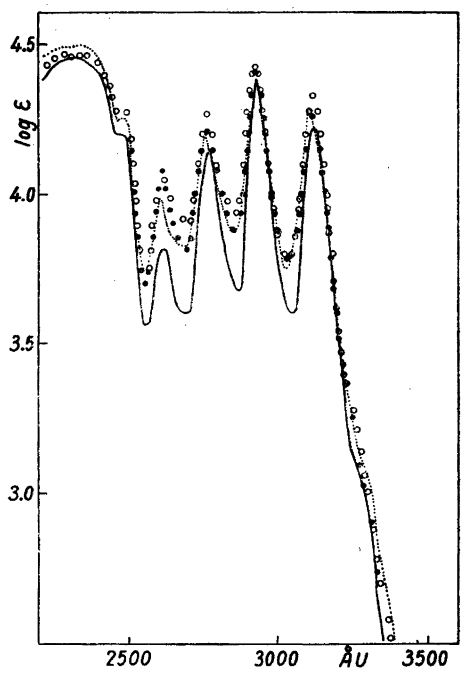

Fig. 1. Ultraviolet absorption in hexane of 2-trans : 8-trans-matricarianol
($CH_3-CH=CH-C\equiv C-C\equiv C-CH=CH-CH_2OH$)

——— *synthetic*
····· *from root of Aster tripoleum L.*
⊙ ⊙ ⊙ ⊙ *from leaves of Grindelia arenicola f. trichophora Steyermark.*
● ● ● ● *from root of Grindelia stricta D.C.*

none of these six, however, has given indications of the presence of matricarianols; four of them contain other acetylenic compounds in the root. It has to be borne in mind that the botanists have put *A. tripoleum* in its own section, *Tripoleum* Nees, A. *ptarmicoides* belongs to the section *Orthomeris* T. & G. *A. novae-angliae* and *A. novi-belgii* belongs to the section *Euaster* Gray. *A. lautureanus* and *A. mongolicus* are by modern botanists mostly transferred into the genus *Asteromoea* Blume [5].

Aster is a very large genus with at least some twohundred good species, and is subdivided by O. Hoffmann into not less than 15 sections. It is obviously premature to draw any conclusion from their contents of acetylenic compounds, but already it is evident that *Aster* is not at all so constant with regard to the occurrence and number of acetylenic compounds as the botanically closely related genus *Erigeron*, where the occurrence of matricaria and lachnophyllum ester in all parts of the plants is a real chemical characteristic of the genus. If any conclusions should be drawn with regard to *Aster*, the seven species investigated have at least that in common that all of them are lacking acetylenic compounds in the overground parts of the plants, whereas such may occur in the roots.

The chemical similarities between *A. tripoleum* and the two investigated species of the genus *Grindelia* is interesting. The botanists long ago placed *Grindelia* in the tribus *Astereae* sub-tribus *Solidagininae*, whereas Harling [5] in his recent embryological work points out relations to the tribus *Heliantheae*. In the sub-tribus *Solidagininae* only some three species of the very large genus

Solidago has been investigated as to their contents of acetylenic compounds. As with *Aster* the results are rather conflicting:

Solidago canadensis L. seems to lack acetylenic compounds in all parts of the plant.

Solidago sempervirens L. seems to contain the α,β-dihydro-matricaria ester in the root.

Solidago virga aurea L. lacks acetylenic compounds in the overground part. The roots, however, are the richest source so far known of 2-*cis* : 8-*cis* matricaria ester (8 g of pure (I) from 1 kg of fresh roots).

Only further extensive chemical investigations of the tribus *Astereae* may clear up the rather obscure connections. Since very few representatives of this tribus are available in Europe and seed samples are difficult to obtain, progress will be slow.

EXPERIMENTAL

1. *Grindelia arenicola* f. *trichophora* Steyermark. Cultivated at Trondheim 1952. The young plants did not develop flowers.

a) *Leaves and stems*, 800 g, gave 1.8 ‰ of essential oil. The U.V. spectrum of the crude oil was in good agreement with that of matricarianol. The oil distilled at 70° at 0.001 mm practically without forerun and residue, and, as might then be expected, with very little change in the U.V.-spectrum

$C_{13}H_{12}O_2$	Found	C 76.28, 75.98	H 6.87, 6.96
	Calc.	» 76.60	» 6.38

This formula corresponds to the acetate of matricarianol; in the infrared spectrum there were strong peaks at 1 745 and 1 230 cm^{-1}, characteristic of acetates.

92.4 mg of this ester was hydrogenated with a $Pd/BaSO_4$-catalyst in alcoholic solution and consumed 80.3 ml H_2, calculated for $C_{13}H_{12}O_2$, 6/= 79.7 ml. The perhydrocompound was distilled and purified through freezing out at −20°. The crystals then melted at −16°, synthetic *n*-decyl-acetate at −14°, mixed m.p. −15°. Of both preparations infrared spectrograms were made with the same cell and on the same recording sheet. From 5−15 μ the two curves coalesced through all 11 maxima.

The acetate from *Grindelia* leaves was saponified under nitrogen with potassium hydroxide. Acetone was added to obtain a homogenous solution. The unsaponifiable part which was isolated in the usual way, crystallised immediately, m.p. after recrystallisation from petroleum ether 80° rose after several recrystallisations to 88−93°, further recrystallisations did not improve the m.p. These 93° crystals were chromatographed from petroleum ether solution on Floridin XXX; elution with benzene-petroleum ether mixtures. The main part of the matricarianol went in the 70 % benzene-petroleum ether eluates; m.p. after recrystallisation from petroleum ether 97°−99°.

3:5-dinitro-benzoate of 2-*trans* : 8-*trans*-matricarianol from *Grindelia arenicola*. 40 mg of the matricarianol fraction above was esterified with 155 mg 3:5-dinitrobenzoyl chloride in anhydrous pyridin at room temperature; and the dinitrobenzoate isolated in the usual way after 24 hours and purified by crystallisation from acetone-petroleum ether. After one recrystallisation from the same solvent mixture the crystals melted at 126.5°−128.2°, given by Bruun *et al.* 126−128°, no depression of m.p. in a 1 : 1 mixture. Debye-Scherrer diagrams (Dr. H. Sörum) were identical.

b) The *roots* of *Grindelia arenicola* (185 g) were distilled with steam; essential oil 1.9 ‰. The U.V.-spectrum of the crude oil revealed that this oil besides matricarianol seemed to contain (I) since there occurred a pronounced peak at 3 300 ÅU. The crude essential oil was therefore distilled slowly at 0.001 mm. The matricaria ester became enriched in the fraction b.p. <52°, whereas the matricarianol esters concentrated above 70°; both fractions were still liquid mixtures.

The fraction b.p. <52° was saponified as above, the acid fraction isolated, and in the crude state immediately converted to the methyl ester with diazomethane. The methyl ester was distilled at 50° and 10^{-3} mm. The distillate remained liquid at room temperature, but solidified below zero and then showed m.p. 0°. The spectral curve was identical with

that of 8-*cis* : 2-*trans* matricaria ester m.p. + 2°, which resembles very much the 2-*cis* : 8-*cis* isomer, but differs in a sharper peak at 3 355 and a much narrower inflexion at 3 600 ÅU. *Grindelia arenicola* thus does not seem to contain the more widely distributed 2-*cis* : 8-*cis* ester, but only the low melting 2-*trans* : 8-*cis* isomer.

The fraction b.p. >70° was saponified as above and the unsaponifiable part purified by chromatography on slightly deactivated alumina. The matricarianol was eluted with ether and showed the correct spectrum of 2-*trans* : 8-*trans*-matricarianol, cf. Fig. 1.

c) *Flowers* of *Grindelia arenicola* (125 g) collected in the summer 1953, were distilled with steam, essential oil 2.4 ‰. The U.V.-spectrum of the crude oil was conformable with that of matricarianol, calculated contents as matricarianol acetate 7.7 %. Because of the low concentration no further investigations were carried out; the flowering heads of *G. arenicola* have very herbaceous phyllaries and the origin of the small amount of acetylenic compounds may be these green parts.

2. *Grindelia stricta* D.C., cultivated at Trondheim 1952. Late in the autumn the young plants developed buds, and they were then transferred to a greenhouse, where some of them went into flower. The rest flowered in the spring 1953.

a) The *flowers* gave about 6 ‰ essential oil with only indistinct absorption in U. V. By chromatography on alumina and elution with benzene-petroleum ether mixture followed by pure ether, the oil was divided into six fractions. The two main ones were the petroleum ether washings (47 %) and the ether eluate (32 %). None of them seemed to contain acetylenic compounds. The first fraction showed only a step out below 2 500 ÅU, the second, which had a strong borneol-like odour exhibited a diene maximum at 2 340 ÅU. The material in 50 % benzene eluate amounted to 10 % and showed a very unsharp matricarianol curve in U. V., from which a maximum contents of 2 % of matricarianol could be estimated. Since *Grindelia stricta* has very great and herbaceous phyllaries these traces of matricarianol might originate from the green part of the flowers, which themselves seem to be devoid of acetylenic compounds.

b) The *leaves and stems* (2 953 g) afforded 1.1 ‰ essential oil. The crude oil has a fair matricarianol spectrum in U.V., but in low concentrations. The oil was chromatographed from petroleum ether solution on slightly deactivated alumina and separated into six fractions as above. Only the material in the 50 % benzene eluate, which amounted to 4 %, contained appreciable amounts of matricarianol, about 60 % calculated as the free alcohol. These contents were not changed by distillation, whereby the main part distilled at 60°/0.001 mm. The distillate was saponified as above and the unsaponifiable part showed an U.V.-spectrum identical with that of matricarianol.

c) The roots (740 g) furnished 3.6 ‰ essential oil with a typical matricarianol spectrum in U.V., but again the concentration was low. Distillation at 0.001 brought out a fraction b.p. ~70° amounting to 6.5 % of the oil, which showed a good matricarianol spectrum in concentration of about 65 %, calculated as free alcohol. This fraction was saponified as above. The unsaponifiable part, however, which showed the correct spectrum of pure matricarianol, remained liquid. In the infrared spectrum there occurred a strong maximum at 716 cm^{-1}, indicating the presence of appreciable amounts of *cis*-bonds. From petroleum ether solution in the cold this matricarianol concentrate deposited crystals, m.p. 87—89°, raised after one recrystallisation to 96—98°, and by further crystallisation finally to 100.5—103.0°. Mixed m.p. with synthetic 2-*trans* : 8-*trans*-matricarianol of m.p. 104° was 102°.

The contents of *cis*-isomers in the *Grindelia stricta* oil, seemed to be appreciably higher than in *G. arenicola*. Trials to isolate one of these liquid *cis*-isomers as a 3:5-dinitrobenzoate were unsuccessful.

3. *Aster tripoleum* L. This plant was collected at different sea-shores in the surroundings of Trondheim. When the available quantities turned out to be insufficient, further quantities of the root were collected on Kadettangen in Sandvika near Oslo, where this *Aster* was abundant.

a) The *flowers* yielded 0.3 ‰ essential oil, which, to judge from the U.V.-absorption, was devoid of acetylenic compounds.

b) The *leaves and stem* gave 0.3 ‰ essential oil, which also seemed to lack acetylenic chromophores.

c) The *roots* afforded only 0.2 ‰ of essential oil, but this oil showed definite absorption maxima in U.V. in accordance with the symmetrical diene-diyne systems. The contents calculated as free matricarianol was about 10 % in the different batches. A portion

of *A. tripoleum* root was extracted with acetone in the cold, and the lipoids isolated in the usual way. The yield of crude lipoids was appreciable, (2.5 ‰), but the yield of matricarianol was the same as when the roots were steam distilled. Some of the crude essential oils were distilled at 0.001 mm; a chromophore with the 3 355 peak of the matricaria esters became enriched in the most volatile fractions (< 55°), but the main fraction, boiling about 70°, was still contaminated with this chromophore and also with another substance with absorption maxima close to those of lachnophyllol (that is at 2 825, 2 665 and 2 520 ÅU). The fractions boiling at 70° were chromatographed on alumina and eluted with petroleum ether-benzene mixtures. The matricarianol chromophore went mainly in the 50 % eluates. The best chromatographic fractions from several batches were combined and rechromatographed on alumina and eluted with large quantities of 20, 40 and 60 % benzene-petroleum ether mixtures. Each elution mixture was separated into three parts which were investigated separately in U.V. About 20 % of the combined fractions was recovered in the first part of the 40 % eluate. This fraction showed an almost perfect U.V.-absorption curve for matricarianol with only a weak maximum at 3 355 ÅU.

This fraction was saponified in the usual way; the unsaponifiable part (25 mg) crystallised spontaneously; after recrystallisation from chloroform-petroleum ether the crystals melted at 98.5—102°; the mixed m.p. with authentic 2-*trans* : 8-*trans*-matricarianol of m.p. 103—104 was also 98.5—102°. The U.V.-spectrum of this purest fraction was in complete agreement with that of 2-*trans* : 8-*trans*-matricarianol.

4. *Aster Herveyi A. Gray.* Seed sample *Hortus Botanicus Hauniensis.* Cultivated Trondheim 1952.

	g	Ess. oil ‰	U.V.-absorption
Flowers	16.5	1.8	step out below 3 600 ÅU, no selective absorption
Leaves + stems	177	0.5	» » » 3 600 » »
Roots	297	0.5	» » » 3 500 » »

5. *Aster lautureanus Franchet.* Seed samples *Hortus Botanicus Hauniensis*, and *Hortus Bergianus*, cultivated Trondheim 1952—53.

	g	Ess. oil ‰	
Flowers	55	2.1	step out below 3 100 ÅU
Leaves	693	0.22	» » » 3 400, broad max. at 2 580 ÅU
Roots	470	3.7	close to the standard curve for matricaria ester (I), contents 72 %. The crude oil solidified in the cold and pure (I) was obtained after one crystallisation from petroleum ether, m. p. 32.8—33°, undepressed by authentic (I).

6. *Aster mongolicus Franchet.* Seed samples *Hortus Botanicus Hauniensis* and *Hortus Bergianus.*

	g	Ess. oil ‰	broad max.
Flowers	320	1.5	step out below 2 800 ÅU, at 2 600 ÅU
Leaves	615	0.1	» » » 3 400 » » 2 600 »
Roots	815	2.3	close to the standard curve for matricaria ester (I), contents 72 % as with *A. lautureanus*. Pure (I) was also isolated by one single crystallisation, m.p. 33°.

7. *Aster novae angleae* L. Seed sample *Hortus Botanicus Hauniensis.* Cultivated Trondheim 1952. The plant material was in good agreement with the botanical descriptions, but about one half of it developed curled leaves. Since no infections could be detected, the curled and normal plants were investigated separately, but when no chemical differences was found, the data have been summarised. The plants developed buds only late in the autumn.

	g	Ess. oil ‰	U.V.-absorption
Buds + leaves + stems	815	0.5	step out below 3 600 ÅU, no selective abs.
Root	540	1.1	close to the standard curve for matricaria ester although with definite indications of presence of *cis*-lachnophyllum ester, contents

41 %. The crude oil solidified in the cold and furnished crystals, m.p. 19—20° after one crystallisation from petroleum ether.

8. *Aster novi-belgii* L. Cultivated by Mr. R. Persson, Trondheim, season 1952.

	g	Ess. oil ‰	U.V.-absorption
Flowers	33	0.6	step out below 3 200 ÅU, no selective abs.
Leaves + stem	190	0.2	» » » 3 200 » » » »
Roots	55	0.1	maxima at 3 075, 2 900, (2 710) ÅU

These maxima are some 25 ÅU shorter than those of the symmetrical diene-diyne grouping as present in the matricarianols. They are intermediate between those of *cis*- and *trans*-lachnophyllum ester.

9. *Aster ptarmicoides* (Nees.) T. & G. Seed sample *Hortus Botanicus Hauniensis*, cultivated Trondheim 1952.

	g	Ess. oil ‰	U.V.-absorption
Flowers	32	2.2	step out below 3 200 ÅU, no selective abs.
Leaves + stem	1 067	0.6	» » » 3 200 » » » »
Roots	270	0.5	maxima at 3 160, 2 670 and 2 565 ÅU

The shape of the curve might indicate the presence of coumarins.

10. *Solidago virga aurea* L. The plants were collected in the hills around Trondheim, July 1951.

	g	Ess. oil ‰	U.V.-absorption
Flowers	410	1	step out below 3 500 ÅU, no selective abs.
Leaves + stems	2 360	1	» » » 3 500 » » »
Roots	674	10	the spectrum of the crude oil showed perfect

agreement with that of (I). The root oil solidified in the cooler; one single crystallisation from petroleum ether gave pure matricaria ester, m.p. 32.8—33.3°; mixed m.p. with an authentic sample 32.8—33.3°. U.V.-spectrogram identical with the standard curve for (I).

SUMMARY

2-*trans* : 8-*trans*-Matricarianol (II) has been isolated from the essential oil of *Aster tripoleum* L., *Grindelia arenicola* f. *trichophora* Steyermark and *G. stricta* D.C. In all three cases (II) occurs as an ester, but only in the case of *G. arenicola* the native ester, which turned out to be the acetate, could be isolated. In the crude esters *cis*-isomers of matricarianol might be present. Some 6 further species of *Aster* investigated did not contain matricarianols, but three of them had 2-*cis* : 8-*cis*-matricaria ester in the root. The overground part of members of the genus *Aster* seems to lack acetylenic chromophores.

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The Crystal Structure of Selenium Dibenzenesulphinate

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The crystal structure of sulphur dibenzenesulphinate $S(SO_2C_6H_5)_2$ (bisphenylsulphonyl sulphide) was determined by Mathieson and Robertson in 1949[1]. Foss has since then prepared the analogous selenium compound [2], $Se(SO_2C_6H_5)_2$, and has also shown that the two compounds are isomorphous [3]. We thought it worth while to determine the exact atomic positions also in the selenium compound, as relatively little is known about the stereochemistry of substances of this type. Thus no direct determinations of the covalent bond distance Se—S and the angle S—Se—S have been reported in the literature. The need for more structural information on selenium compounds has recently been stressed [4].

THE STRUCTURE DETERMINATION

The unit cell and space group was determined by Foss [3], and we have confirmed his results. The crystals are monoclinic, elongated along b, with $\{10\bar{1}\}$ predominant, and with the following cell dimensions: $a = 16.14$ Å, $b = 5.60$ Å, $c = 15.84$ Å, and $\beta = 112°$ (all $\pm$ 0.5 %). Cu$K\alpha$ radiation ($\lambda = 1.542$ Å) was used throughout the investigation.

The systematic absences in the X-ray reflexions are consistent with the space groups $A2/a$ and Aa. The structure determination of the isomorphous compound (cell dimensions $a = 15.88$ Å, $b = 5.52$ Å, $c = 15.88$ Å, and $\beta = 112.9°$) shows that the former ($A2/a$) is the correct one. The density was found to be 1.79_5 g cm^{-3} (flotation method), corresponding to four (calc. 3.97) molecules in the unit cell. The selenium atoms occupy consequently fourfold positions, and the molecule must have a twofold axis of symmetry. The origin and the axes are chosen as in Mathieson and Robertson's paper.

For the structure determination Weissenberg diagrams were taken about the b and c axes, using crystals of cross-sections about 0.1 × 0.1 mm. To correlate the strong and weak reflexions, three diagrams were taken of each zone, and intensities were obtained over a range of about 6 500 to 3. The intensities were estimated visually and corrected for the Lorentz polarization factor. They were converted to an approximately absolute scale later by comparison of observed and calculated structure factors.

The signs of most of the structure factors were derived from the data given on the isomorphous compound [1], making allowance for the effect of replacing

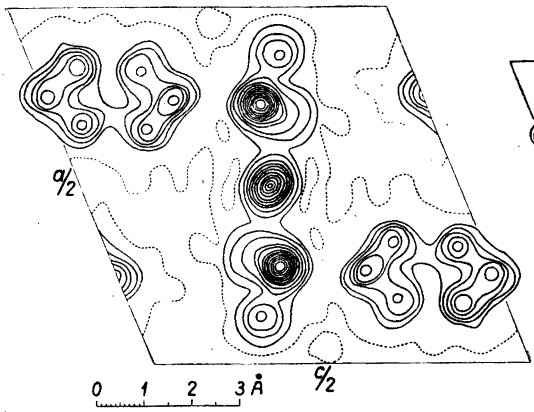

Fig. 1. Fourier projection in direction of b *axis. Contours at intervals of 5 eÅ⁻² for Se, 2 eÅ⁻² for S and O, and 1 eÅ⁻² for C. The first contour line corresponds to 3 eÅ⁻². The one-electron line is dotted.*

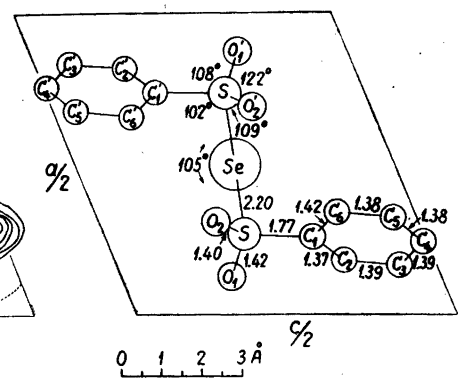

Fig. 2. Bond lengths and bond angles in $Se(SO_2C_6H_5)_2$.

the central sulphur atom by selenium. The electron density maps corresponding to projections along the *b* and *c* axes could now be calculated, and from these more accurate atomic positions were derived. After a number of refinements, the final electron density maps given in Figs. 1 and 3 were obtained. In the Fourier projection along the *b* axis (Fig. 1) all the atoms are well resolved with the exception of O_2, and fairly reliable values of the *x* and *z* coordinates of the atoms can be derived directly from the map. The interpretation of Fig. 1 is obvious from a comparison with Fig. 2, which shows the corresponding projection of the molecule. In the *c* projection (Fig. 3) bad overlapping occurs, and only very approximate *y* coordinates can therefore be obtained, except for the selenium and sulphur atoms. The former is well resolved, and the *y* coordinate of the latter could be determined by applying a graphical

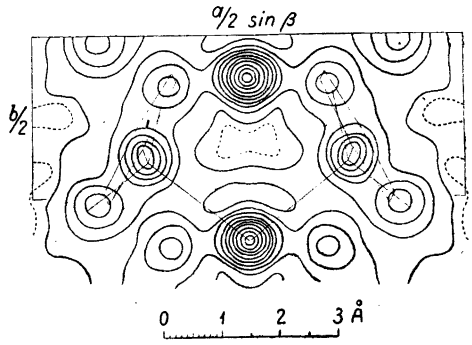

Fig. 3. Fourier projection in direction of c *axis. Contours at intervals of 5 eÅ⁻², starting at 3 eÅ⁻². The one-electron line is dotted.*

correction for the overlapping effect from the atoms C_1 and C_4. In Table 1 the atomic coordinates are given.

Table 1. Atomic coordinates as fractions of the corresponding cell edge.

Atom	x	y	z
Se	0.250	0.871	0.250
S	0.134	0.110	0.219
O_1	0.062	0.959	0.167
O_2	0.153	0.323	0.183
C_1	0.130	0.158	0.327
C_2	0.090	0.980	0.358
C_3	0.083	0.008	0.443
C_4	0.120	0.209	0.495
C_5	0.162	0.388	0.466
C_6	0.168	0.360	0.382

The agreement between observed and calculated structure factors is satisfactory, the reliability factor $R = \Sigma \| F_{obs.} | - | F_{calc.} \| / | \Sigma F_{obs.} |$ being 0.17 for both projections. For the non-observed reflexions half the lowest observable value of F_{obs} was used in the calculation of R. The contribution from the hydrogen atoms was neglected. The atomic scattering curves of the *International Tables* were employed, with a temperature factor of $B = 3.7$ Å^2. A list of observed and calculated structure factors can be supplied by the authors on request.

DESCRIPTION AND DISCUSSION OF THE STRUCTURE

From the coordinates in Table 1 the bond lengths and bond angles given in Table 2 were calculated. They are also shown in Fig. 2. For comparison, the values reported for the sulphur compound are included in the table.

Table 2. Bond lengths (in Å units) and bond angles.

Bond	$Se(SO_2C_6H_5)_2$	$S(SO_2C_6H_5)_2$	
Se—S	2.20	2.07	(S—S)
S—O_1	1.42	1.41	
S—O_2	1.40	1.41	
S—C_1	1.77	1.76	
C—C	1.37—1.42	1.39	(average)

Bond angles.

$Se(SO_2C_6H_5)_2$		$S(SO_2C_6H_5)_2$	
S—Se—S′	105.1°	$S_2—S_1—S_2'$	106.5°
Se—S—O_1	102°	$S_1—S_2—O_1$	104.2°
Se—S—O_2	109°	$S_1—S_2—O_2$	111.9°
Se—S—C_1	102°	$S_1—S_2—C_1$	101.7°
O_1—S—O_2	122°	$O_1—S_2—O_2$	117.2°
O_1—S—C_1	108°	$O_1—S_2—C_1$	107.5°
O_2—S—C_1	111°	$O_2—S_2—C_1$	115.9°

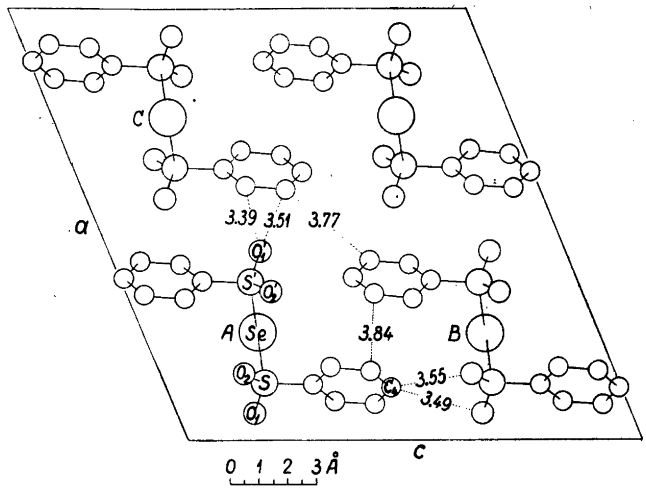

Fig. 4. The b *projection of the structure, showing the packing of the molecules and some of the intermolecular distances.*

The non-bonded intramolecular distance S—S′ is 3.49 Å, whereas the corresponding distance in the sulphur compound is 3.32 Å.

It is believed that the error in the Se—S distance is less than 0.03 Å and in the S—Se—S′ angle less than about 2°. The errors in the other distances and angles may be considerably greater due to the overlapping of atoms and the distorting effect of the heavy atoms, but it is seen that the values in general agree well with those reported for the sulphur compound.

The Se—S distance is found to be 2.20 Å, corresponding closely to the sum of the covalent radii for selenium and sulphur (1.17 Å and 1.04 Å respectively) derived from crystal structure investigations of the elements. The angle S—Se—S′ is slightly smaller than the corresponding angle S—S—S in the isomorphous compound, the values being 105° and 106.5°. As selenium and sulphur are supposed to form p-bonds, the ideal bond angle would be 90°. It is therefore to be expected that the angle is somewhat smaller in the selenium compound, as the repulsive forces between the non-bonded sulphur atoms are probably weaker in this case.

Table 3. Intermolecular distances (in Å units).

	$Se(SO_2C_6H_5)_2$	$S(SO_2C_6H_5)_2$
$C_4(A)-O_1(B)$	3.49	3.43
$C_4(A)-O_2(B)$	3.55	3.43
$C_3'(B)-C_3(C)$	3.77	3.7
$O_1'(A)-C_2(C)$	3.39	3.31
$O_1'(A)-C_3(C)$	3.51	3.58

The shortest intermolecular distances are given in Table 3 and Fig. 4. They are in general greater than those reported for the sulphur compound, where certain intermolecular attractions are indicated [1], and correspond roughly to normal van der Waals separations. The packing of the molecules is shown in Fig. 4.

SUMMARY

The crystal structure of selenium dibenzenesulphinate is determined with the aid of the known structure of the isomorphous sulphur compound. The Se—S bond distance is found to be 2.20 ± 0.03 Å and the S—Se—S′ angle to be 105° ± 2°.

We wish to thank Dr. O. Foss for suggesting the investigation and supplying the crystals, and Prof. H. Haraldsen for working facilities.

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Über die Umsetzung von Formaldehyd mit Ketocarbonsäuren

I. Brenztraubensäure

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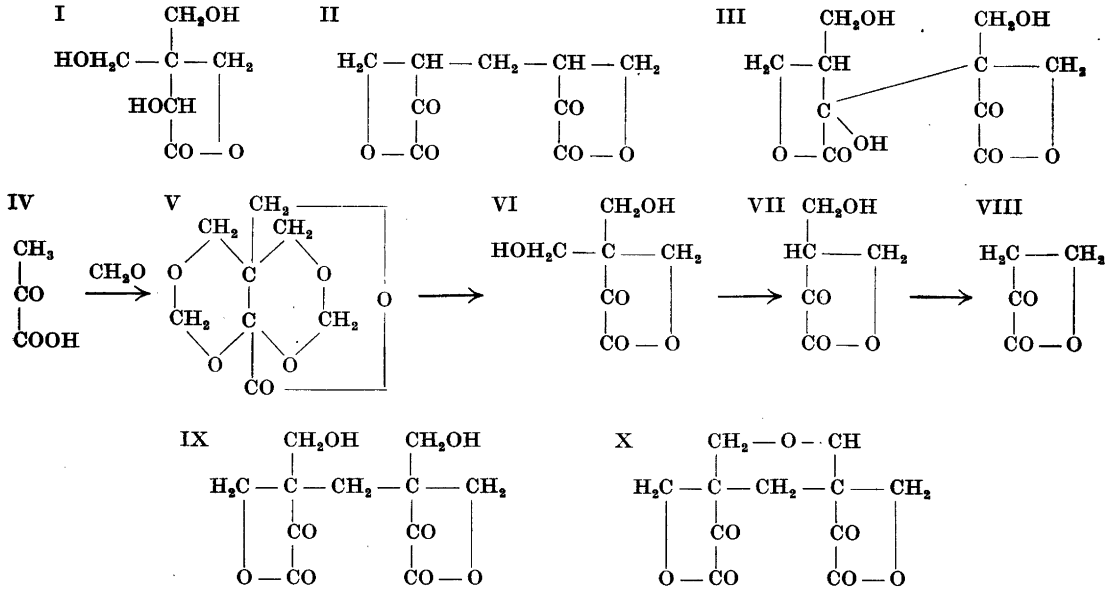
Es wurde kürzlich an einfachen Carbonylverbindungen gezeigt, dass ein stufenweiser Methylolaufbau durch Formaldehyd auch in saurem Medium möglich ist und dass dieser gegenüber dem durch Tollens bekannt gewordenen alkalischen Aufbau keine grundsätzlichen Unterschiede aufweist [1]. Ein typischer Unterschied ergibt sich erst im Anschluss an den vollständigen Methylolaufbau, indem nach unseren bisherigen Erfahrungen durch überschüssigen Formaldehyd bei Aldehyden die Carbonylgruppe auch in saurem Medium zur Carbinolgruppe reduziert wird, bei Ketonen dagegen nicht. Natürlich wird man bei der sauren Kondensation für die normalen alkoholischen Aufbauprodukte mit Sekundärreaktionen (z.B. Ringschlüssen, Methylenätherbildungen, intermolekularen Kondensationen) rechnen müssen, die bei der alkalischen Umsetzung nicht in Erscheinung treten oder hier einen anderen Verlauf nehmen. Diese Verhältnisse zeichnen sich bereits andeutungsweise beim Vergleich früherer Untersuchungsergebnisse über die Kondensation der Brenztraubensäure mit Formaldehyd ab. Hosaeus [2], der nach Tollens in alkalischem Milieu mit überschüssigem Formaldehyd arbeitete, erhielt das „*Trimethylolmilchsäure-lacton*" (I), Kaltwasser [3] dagegen beim Erwärmen der Komponenten im Molverhältnis 1 : 1.5 mit konzentrierter Schwefelsäure eine Verbindung, der nach Asahina und Terada [4] die Formel eines „*Methylen-bis-α-keto-butyrolactons*" (II) zukommt. Die Richtigkeit dieser Formel wurde unabhängig durch Feofilaktow [5] bestätigt; dieser isolierte ausserdem eine neutrale Verbindung $C_{10}H_{12}O_8$, für die er die Formel des *Dilactons* (III) vorschlägt.

Die drastischen Reaktionsbedingungen Kaltwasser's und das von ihm gewählte Umsetzungsverhältnis sind jedoch keineswegs geeignet, die gemeinsamen und trennenden Züge der alkalischen und sauren Kondensation herauszustellen. Nach unseren Beobachtungen an einfachen Oxoverbindungen schien es uns durchaus nicht plausibel, weshalb man bei der sauren Kondensation nicht die normalen einfachen Aufbauprodukte vom Trimethyloltypus sollte abfangen können und statt ihrer nur Verbindungen erhält, die durch intermolekulare Sekundärreaktion zwischen diesen einfachen Individuen entstanden sein müssen. Weiter interessierten wir uns im Zusammenhange mit ande-



ren synthetischen Versuchen für das Schicksal der in der Brenztraubensäure vorhandenen Ketogruppe.

Das Ergebnis unserer Untersuchung lässt sich wie folgt zusammenfassen: *Brenztraubensäure* (IV) reagiert mit überschüssigem Formaldehyd in Eisessig-Schwefelsäure unter Bildung des *Bismethylenäthers des α,α-Dioxy-β,β-dimethylolbutyrolactons* (V). Der überschüssige Formaldehyd, der die Ketogruppe also nicht reduziert, wird anstatt dessen zur Bildung der Methylenätherbrükken zwischen den freien Methylolgruppen und der hydratisierten Carbonylgruppe verbraucht. Der Bismethylenäther wird beim Erhitzen mit Mineralsäuren zum *α-Oxo-β,β-dimethylol-butyrolacton* (VI) gespalten. Es gelang, dieses als 2,4-Dinitro-phenylhydrazon abzufangen. Das α-Oxo-β,β-dimethylol-butyrolacton wird jedoch durch die Mineralsäure weiter abgebaut, indem die Methylolgruppen sukzessive in Gestalt von Formaldehyd wieder abgetrennt werden, wobei nacheinander das *α-Oxo-β-methylol-butyrolacton* (VII) und das *α-Oxo-butyrolacton* (VIII) entstehen. Diese Art der Formaldehydabspaltung ist, worauf wir bereits hingewiesen haben [1], für die β-Keton-alkohol-Gruppierung charakteristisch und überraschte uns daher nicht. Die zuletzt erwähnten Lactone (VII und VIII) haben wir nicht als solche isoliert, sondern nur deren intermolekulares Kondensationsprodukt, das *β,β'-Methylen-bis-[α-oxo-butyrolacton]* (II) Kaltwasser's. — Beim Säureabbau des Bismethylenäthers sollte man im Reaktionsgemisch auch das intermolekulare Kondensationsprodukt aus den Lactonen VI und VII, nämlich das *β,β'-Methylen-bis-[α-oxo-β-methylol-butyrolacton]* (IX) bzw. dessen *Anhydrid* (X) erwarten. Die Isolierung dieser Verbindungen aus dem Spaltungsgemisch gelang freilich nicht. Das Anhydrid (X) wurde jedoch ohne weiteres bei der Umsetzung von Kaltwas-

ser's Dilacton (II) mit Formalin in Salzsäure erhalten. — Um die Darstellung des von Feofilaktow beschriebenen Dilactons (III) haben wir uns bei der Nacharbeitung seines Experimentes vergeblich bemüht, wohl aber haben wir dabei den Bismethylenäther (V) erhalten. — Über die Umsetzung anderer Keto-carbonsäuren mit Formaldehyd soll in Kürze berichtet werden.

EXPERIMENTELLER TEIL

Umsetzung von Brenztraubensäure mit Formaldehyd

Eine Lösung von 72 g Paraformaldehyd und 12 ml konz. Schwefelsäure in 173 g Eisessig wurde bei 90° mit 45 g Brenztraubensäure versetzt. Beim weiteren Erhitzen des Gemisches setzte bei 100° Selbstreaktion ein, wobei die Temperatur auf 113° stieg und hier längere Zeit konstant blieb. Nach dem Abklingen der exothermen Reaktion wurde 2 Stunden unter Rückflusskühlung gekocht. Nach dem Erkalten schieden sich Krystalle aus, die abgesaugt und mit Wasser gewaschen wurden (40 g). Umkrystallisiert aus Alkohol. Schmp. 163—165°. Die Verbindung reagiert in der Kälte nicht mit salzsaurem 2,4-Dinitro-phenylhydrazin. *Bismethylenäther des α,α-Dioxy-β,β-dimethylol-butyrolactons* (V)

$C_8H_{16}O_6$ (202,2) Ber. C 47,52 H 4,99
Gef. » 47,52 » 5,02

Beim Kochen des Bismethylenäthers (V) mit 2,4-Dinitro-phenylhydrazin und Salzsäure erhielt man eine gelbbraune Substanz, die zwecks Abtrennung des begleitenden Formaldehyd-2,4-dinitrophenylhydrazons bis zur Schmelzpunktkonstanz aus verd. Essigsäure umkrystallisiert wurde. Schmp. 225—230°. *2,4-Dinitro-phenylhydrazon des α-Oxo-β,β-dimethylol-butyrolactons* (VI)

$C_{13}H_{12}O_8N_4$ (340,3) Ber. C 42,36 H 3,55 N 16,47
Gef. » 42,54 » 3,65 » 16,65

Abbau des Bismethylenäthers mit methylalkoholischer Schwefelsäure

10 g des Bismethylenäthers (V) wurden nach Price und Krishnamurti [6] mit methylalkoholischer Schwefelsäure destilliert, bis im Destillat mit salzsaurem 2,4-Dinitro-phenylhydrazin kein Formaldehyd mehr nachweisbar war. Der Destillationsrückstand wurde erschöpfend ausgeäthert. Nach dem Trocknen und Eindampfen der Ätherlösung hinterblieb ein gelbes Öl, das fast vollständig krystallisierte. Durch Absaugen erhielt man 3,6 g Rohprodukt, das aus Methanol umkrystallisiert wurde. Schmp. und Mischschmp. mit einem nach Kaltwasser hergestellten Präparat unverändert 236—238° (Zers.). *β,β'-Methylen-bis-[α-oxo-butyrolacton]* (II)

$C_9H_8O_6$ (212,2) Ber. C 50,95 H 3,80
Gef. » 50,93 » 3,79

Umsetzung von Kaltwasser's Dilacton mit Formalin-Salzsäure

Eine Mischung von 1,13 g des Dilactons (II), 15 ml Formalin und 15 ml konz. Salzsäure wurde 90 Minuten auf dem Wasserbade erhitzt, wobei das Dilacton allmählich in Lösung ging. Aus der gelben Reaktionsflüssigkeit schied sich beim 24-stündigen Stehen im Kühlschrank eine geringe Menge Krystalle aus, die nach dem Umkrystallisieren aus Aceton-Alkohol den Schmp. 266—268° hatten. *Anhydrid des β,β'-Methylen-bis-[α-oxo-β-methylol-butyrolacton]* (X)

$C_{11}H_{10}O_7$ (254.2) Ber. C 51.97 H 3.97
Gef. » 51.80 » 4.04

ZUSAMMENFASSUNG

Bei der Umsetzung von Brenztraubensäure mit Formaldehyd verläuft der Methylolaufbau in saurer und in alkalischer Lösung völlig gleichartig. Zum Unterschiede wird aber danach in saurer Lösung die Oxogruppe durch überschüssigen Formaldehyd nicht zur Carbinolgruppe reduziert. Die durch Aufbau entstandenen α-Oxo-methylol-derivate bzw. deren Methylenäther sind empfindlich gegen heisse Mineralsäuren, durch die die Methylenätherbrücken und die Methylolgruppen sukzessive wieder als Formaldehyd abgespalten werden. Die hierbei entstandenen Spaltprodukte sind in saurem Medium zu intermolekularen Kondensationen befähigt. Diese Befunde erklären die bisher isoliert dastehenden Einzelergebnisse unter einem gemeinsamen Gesichtspunkt.

Der Eine von uns dankt dem *Fridtjof Nansen-Fond* für finanzielle Unterstützung.

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Formation of Methyl*cyclo*pentenolone by Digestion of Spruce Wood or Galactose with Sodium Hydroxide Solutions at 100° C

TERJE ENKVIST
Experiments by BO ALFREDSSON*, MAUNU MERIKALLIO, PAAVO PÄÄKKÖNEN and OIVA JÄRVELÄ

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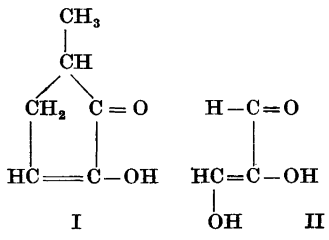
During studies on the degradation products of lignin [1], spruce wood was digested with 1.7 % sodium hydroxide solution for 6 hours at 100° C under pressure. From the liquor obtained, a crystalline substance could be isolated, which is soluble in ether and in sodium hydroxide but not in sodium bicarbonate solution, and which can be sublimated in vacuum. Its ultraviolet absorption spectrum (Fig. 1) is fairly sharp and shows a strong bathochromic shift on addition of alkali, indicating the presence of an ionizable group. The substance gives a red enol reaction with ferric chloride solution. It has now been identified as 1-methyl-*cyclo*penten-(3)-ol-(3)-one-(2), (formula 1) [2–5] by elementary analysis and mixed melting point determinations for the substance itself, and for its *bis*-2,4-dinitrophenylhydrazone $C_6H_8[:N \cdot NH \cdot C_6H_3(NO_2)_2]_2$. The best yield of crude crystals obtained is 0.06 % of the wood, but of recrystallized, practically pure substance only about 0.016 % has been recovered.



I II

Compound I has previously been found in soluble wood tar [6–9], and can also be prepared by synthesis [5, 10–12], but it seems never before to have been obtained at so low a temperature as 100° C. Moreover, its isolation after alkaline digestion was rather unexpected, since the compound has been reported not to be very stable in alkaline solution [4]. Therefore it seemed to be of interest to trace the wood constituent from which it originated.

In Fig. 3 a summary is given of a series of preliminary experiments where spruce wood was treated in different ways and methylcyclopentenolone sought for in the ether extracts of the reaction solutions, ferric chloride and 2,4-

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dinitrophenylhydrazine ("DNPh") being used as testing reagents. No methyl*cyclo*pentenolone could be detected after simple boiling of the wood with water. Hence the substance seems not to be present as such in the wood. Treatment of spruce wood with dilute sodium hydroxide at room temperature resulted in a slight positive reaction, and heating of the wood with dilute sodium hydroxide at 100° C led to strongly positive tests. Hence the substance seems to be formed only on treatment of the wood with sodium hydroxide solution.

Since methyl*cyclo*pentenolone contains no methoxy-groups and no benzene nucleus, it can hardly be a degradation product of lignin, but must be formed from some substance of carbohydrate nature. A series of experiments with various carbohydrate materials were carried out. Some of the results obtained are given in Table 1, which shows the absorption maxima of ether-soluble, vacuum-sublimated enolic fractions obtained after pressure heating of various materials with sodium hydroxide solutions at 100° C. For comparison, absorption maxima are also given for several known substances. It can be seen that the wave lengths for the absorption maxima of methyl*cyclo*pentenolone from spruce wood or from soluble tar are, within the limits of error, the same as for the enol fraction obtained through alkaline digestion at 100° C of a solution of spruce wood polyoses produced by heating the wood with water at 120° C (*cf.*[13]). In contrast, after analogous treatment the corresponding uronic acids gave only a very poor yield of the sublimated enol fraction, and the sublimate had absorption maxima at wave-lengths quite different from those of methyl*cyclo*pentenolone. Also the absorption maxima of a series of lignin preparations, represented in Table 1 by thiolignin, and of several lignin model substances, for instance vanillyl alcohol and acetoguaiacone, lie far from the position of the maxima of the spectrum of methyl*cyclo*pentenolone.

The extinction values ($E\,{}^{1\%}_{1\,cm}$) reported in Table 1 give essentially the same impression as the wave lengths. It must be observed that the extinction

Table 1. Absorption maxima for ether-soluble, vacuum-sublimated enolic fractions obtained after pressure heating of various starting materials with dilute NaOH solutions at 100° C, compared with the absorption maxima of several known substances.

Enolic fractions from alkaline pressure heating of:	Not alkaline		Alkaline	
	mμ	$E\,{}^{1\%}_{1\,cm}$	mμ	$E\,{}^{1\%}_{1\,cm}$
Spruce wood, washed with acetone	258	729	297	553
Galactose	259	679	298	547
Glucose	260	541	(297)	764
Fructose [a]	260 (305)	164	295	180
Neutral substance from spruce wood hemicellulose	261	368	299	464
Uronic acid from same	276 (305)	477	348	1 057
Spectra of known substances:				
Methyl*cyclo*pentenolone from soluble wood tar	258	709	295	—
Vanillyl alcohol [23]	230, 281	190 [b]	294	245
Acetoguaiacone [23]	230, 276, 303	513 [c]	248, 348	1 450 [d]
Thiolignin (80°, H_2S [24])	(230) 280	161	287	158

[a]) In this case the whole ether extract was sublimated instead of the enolic fraction. — [b]) At 281 mμ. — [c]) At 303 mμ. — [d]) At 348 mμ.

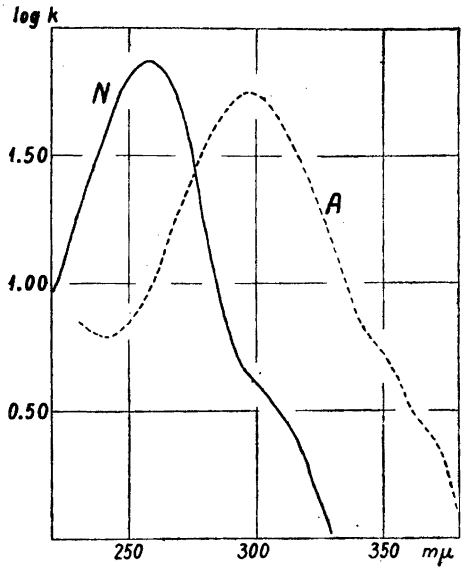

Fig. 1. Absorption spectrum of methylcyclopentenolone obtained by alkaline digestion of spruce wood at 100° C. Substance: expt. 2, "cr, recr.". Concentration: 10 mg/l. Curve N in 96 % ethanol, A in 0.5 N ethanolic KOH. Curve N at 258 mμ: $E^{1\,\%}_{1\,cm} = 729$.

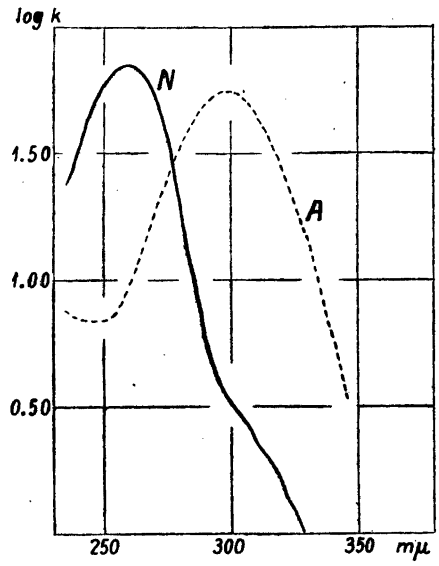

Fig. 2. Absorption spectrum of methylcyclopentenolone obtained by alkaline digestion of galactose at 100° C. Substance: expt. C, sublimate 71—92° C (8 mm). Concentration: 11.8 mg/l. Curve N in 96 % ethanol, A in 0.5 N ethanolic KOH. Curve N at 260 mμ: $E^{1\,\%}_{1\,cm} = 679$.

value is dependent on the purity of the substance, and that it decreases if the substance is so unstable that its concentration drops markedly before the measurement can be made. This seems to be the case with methyl*cyclo*pentenolone, especially in alkaline solution [4].

On pressure heating with sodium hydroxide solutions at 100° C, several hexoses gave low yields of ether-soluble enolic substances showing absorption spectra which in the case of galactose and glucose were rather like that of methyl*cyclo*pentenolone (Table 1). The closest resemblance was seen with the spectrum of the enolic fraction formed from galactose (*cf.* Figs. 1 and 2). In fact, on sublimation in vacuum, this enolic fraction gave crystals which could be identified with the methyl*cyclo*pentenolone of formula I from soluble wood tar by determination of the mixed melting points of the enols themselves as well as of their dinitrophenylhydrazones. The yield of pure crystals of methyl*cyclo*pentenolone from galactose was very small, but a calculation from the absorption spectrum of a vacuum sublimate (expt. B, see experimental part) corresponds to a yield of 1.8 % of a substance with a spectrum resembling that of methyl*cyclo*pentenolone.

The question arises of what type of reaction can be involved in the formation of methyl*cyclo*pentenolone from hexoses. v. Euler and Martius [14] have found that hexoses such as glucose, galactose and fructose, as well as dihydroxyacetone and pentoses, give reductones, especially triose-reductone(formula

II), on heating with sodium hydroxide solution at 92° C, *i. e.* under almost the same reaction conditions as are used in the formation of methyl*cyclo*pentenolone as described above. In fact, the water solutions obtained on preparation of methyl*cyclo*pentenolone by alkaline digestion of spruce wood or galactose were found to give the reactions of reductone with Tillmans' reagent [15] and with iodine in acid solution. Moreover, a solution obtained by boiling the raw spruce wood with water gave no positive test for reductone (expt. a—e).

As two molecules of triose-reductone contain the same number of carbon and oxygen atoms, but four hydrogen atoms less than one molecule of hexose, it seems probable that the reductone is formed as a dehydrogenation product of hexose by a disproportionation reaction. In the case of dihydroxyacetone, v. Euler [16] has presumed that the corresponding hydrogenation product would be glycerol, according to scheme III. However, it seems that the presence of glycerol has not been experimentally proved by him. In the present investigation therefore, tests were made for glycerol in the reaction solution resulting from treatment of galactose with sodium hydroxide at 100° C, but the result was negative. Therefore it seems that the most probable scheme for the formation of methyl*cyclo*pentenolone at present would be the disproportionation reaction IV:

$$\underset{\text{Dihydroxy-acetone}}{2\ C_3H_6O_3} \rightarrow \underset{\text{Reduc-tone}}{C_3H_4O_3} + \underset{\text{Glycerol}}{C_3H_8O_3} \qquad \text{(III)}$$

$$\underset{\text{Hexose}}{2\ C_6H_{12}O_6} = 2\ \underset{\text{Reductone}}{C_3H_4O_3} + \underset{\text{Methyl-}\textit{cyclo}\text{pentenolone}}{C_6H_8O_2} + 4\ H_2O \qquad \text{(IV)}$$

Spruce wood is reported to contain about 1.9 % of galactan [13]. Hence it could be thought possible that the methyl*cyclo*pentenolone formed on alkaline digestion of spruce wood originates from the galactose produced on hydrolysis of galactan, but it is not impossible that other hexosans also give methyl-*cyclo*pentenolone.

EXPERIMENTAL

Methyl*cyclo*pentenolone from spruce wood

Four experiments were made employing essentially the same procedure: Expt. 1: Spruce wood, ground in a Nelco mill to splits about 1 × 5 mm, was extracted with acetone until the amount of acetone solubles had decreased to < 0.1 %. 630 g of this wood, calculated on a dry basis, was digested for 6 hours at 100° ± 1° C with 1.7 % NaOH solution in a rotating 10 litre digester of stainless steel, in which the air had been replaced by N_2. Ratio NaOH (g): wood (dry basis, g) = 10 : 100, of liquor (including moisture, ml): wood (dry basis, g) = 6 : 1. After filtration: yield of undissolved wood (dry basis) 83.0 %, containing 30.9 % of Klason lignin [17], 4.96 % OCH_3 and 2.09 % of ash. The filtrate and the wash waters were evaporated in vacuum to about 1 700 ml, acidified with HCl to about pH 3, and filtered. The precipitate (lignin) amounted to 2.3 % of the wood (dry and ash-free basis). The filtrate was thoroughly extracted with ether in an extraction apparatus for liquids. A small amount of ethanol was added in order to obtain good separation of the layers. The ether extract was divided by shaking successively with 21 % $NaHSO_3$, 8 % $NaHCO_3$ and 5 % NaOH [18] in an aldehydic (0.127 %), an acidic (0.335 %), a phenolic-enolic (1.229 g, 0.195 %) and a neutral fraction (0.029 % of the original wood, dry basis).

418.9 mg of the *aldehydic fraction* was sublimated in an apparatus with a cup for collection of oily sublimate beneath the finger, at 9 mm Hg and 160° C, measured in the oil bath. Sublimate: 263.1 mg, with 3.95 % OCH_3. Residue: 122.4 mg, with 6.22 % OCH_3. The absorption spectrum of the sublimate was very like that of vanillin.

141.0 mg of the *acidic fraction* was sublimated in a corresponding way at 12 mm Hg, and gave 63.9 mg of a partly crystallizing sublimate containing 4.1 % OCH_3 and showing an absorption spectrum with a maximum in neutral solution at 260 mμ and $E^{1\,\%}_{1\,cm} = 156$.

488.3 mg of the *enolic fraction* was correspondingly sublimated at about 6 mm Hg. Yields in percent of the wood: 80—160° C in oil bath, crystals on the finger 14.6 mg = 0.0058 %, oil in the cup 51.6 mg = 0.0206 %, with 2.8 % OCH_3, 160—245° C viscous oil 0.0309 %, residue 0.1059 %. The crude crystals showed m.p. about 80° C, and after recrystallization from light petroleum-ethyl ether 100° C.

Expt. 2 gave better yields of crystals: 80—97° C, 24 mm Hg ("cr") 0.051 % of the wood, at 97—150° C 0.012 %, in all 0.063 % of the wood. Color reaction with ferric chloride in neutral solution: red-violet. The substance reduces Fehling's solution and ammoniacal silver nitrate solution. 275.8 mg of "cr" on recrystallization from light petroleum-ethyl ether gave 45.3 mg of a substance "cr recr": M.P. 97—98° C, absorption spectrum in Fig. 1. Analysis (A. Schoeller, Kronach): OCH_3 negative; C 63.97; H 7.12. Calc. for $C_6H_8O_2$: C 64.25; H 7.20.

Expt. 3: The corresponding recrystallized crystals showed m.p. 104.5° C. A mixture with methyl*cyclo*pentenolone (m.p. 103.5—104.5°) prepared from soluble wood tar had m.p. 104.5° C (Rothes app.).

Dinitrophenylhydrazone: 61.0 mg of crude crystals from expt. 3 was dissolved in water. 150 ml of a 0.5 % solution of 2,4-dinitrophenylhydrazine ("DNPh") in 2 *N* HCl was added. After short heating on the water bath and standing overnight at room temperature the brick red precipitate obtained was washed with HCl (yield 130 mg), purified by recrystallization from warm benzene after passing the benzene solution through a 3 cm layer of aluminium oxide (Brockmann) and finally dried for 2 hours at 100° C and 20 mm Hg. Analysis (K. Salo, Helsingfors): N 23.25 (Dumas); C 46.12; H 3.52. Calc. for $C_{18}H_{16}N_8O_8$: N 23.73; C 45.74; H 3.42. m.p. 238° C; m. p. of a mixture with the corresponding derivative (m. p. 235° C) of methyl*cyclo*pentenolone prepared from soluble wood tar 238° C.

Qualitative tests for methyl*cyclo*pentenolone on extracts of wood obtained by various treatments: 40 g of original spruce wood sawdust was digested for 5 hours with 350 ml of distilled water in a loosely stoppered flask on the water bath. Filtration gave the filtrate f1. Thereafter 400 ml of 4 % NaOH solution was added to the sawdust, and the mixture allowed to stand for 3 hours at about 20° C with occasional mixing. Filtration gave the filtrate f2. 100 ml of this was heated in a sealed tube of stainless steel for 4 hours at 100° C (solution s1). Another 100 ml of f2 was refluxed for 4 hours in a glass vessel in a current of N_2 (solution s2).

A sample of spruce wood chips was digested with NaOH solution in a sealed tube of stainless steel for 3 hours at 100° C. Ratio of liquor (ml): wood (dry basis, g) = 4 : 1, of NaOH (g): wood (dry basis, g) = 10 : 100. Filtration gave the filtrate f3.

From each of the five solutions obtained, a volume corresponding to 2 g of wood was acidified with hydrochloric acid and extracted with ether. The ether solutions were all evaporated to the same small volume. 5 drops of each solution was put on strips of filter paper, the ether allowed to evaporate and the spots obtained tested with a 0.5 % solution of DNPh in 2 *N* HCl. An orange color was regarded as a positive test. The rest of the ether solutions were evaporated at room temperature, the residues dissolved in water, neutralized with dilute ammonia and tested with dilute $FeCl_3$solution. A red or violet color reaction was taken as a positive test.

The tests with DNPh and with $FeCl_3$ gave the same results, positive or negative, for each extract tested. The findings are given in Fig. 3.

Alkaline digestions of wood polyoses

A sample of 1 000 g, calculated on a dry basis, of spruce wood sawdust was digested with 8 l of distilled water for 8 hours at 120° C in a rotating digester of stainless steel. The sawdust was filtered off and washed with water. The aqueous solution obtained had a

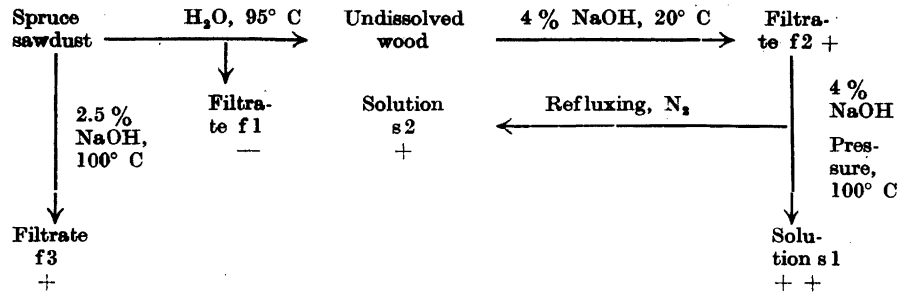

Fig. 3. Tests on methylcyclopentenolone with DNPh and $FeCl_3$ on ether extracts obtained by various treatments from spruce wood.

volume of 9.08 l. 8.58 l of this was evaporated *in vacuo* in an evaporator in accordance with Bartholomew's method [19] to a volume of 690 ml. This concentrated solution was allowed to pass an anion exchanger containing the strongly basic exchange resin "Levatit II". The pH of the solution after this treatment was 6.9. On evaporation to dryness of a sample of the solution, it was found to give a brown, transparent solid (neutral part of the hemicellulose) corresponding to 11.9 % of the original wood. 108.8 g of NaOH dissolved in water was added to the bulk of the solution, which contained the same amount by weight of organic solids. This solution was evaporated in vacuum at 40—60° C to a volume of 350 ml and heated for 15 hours at 100° C in a tube of stainless steel. After cooling, the solution was acidified with dilute H_2SO_4, extracted with ether, and the enolic fraction (347.1 mg) of the ether extract was isolated as in the case of NaOH digestion of wood (Expt. 1). 317 mg of the enolic fraction was sublimated. The fraction obtained at 68—100° C and 8—10 mm weighed 6.1 mg and contained some crystals. Its absorption maximum in ultraviolet light is given in Table 1.

The uronic acid fraction was eluted from the anion exchanger with 40 ml of 4 % and 80 ml of 2 % NaOH solution and finally with 380 ml of water. The eluate was evaporated to 325 ml and heated for 15 hours at 100° C in a tube of stainless steel. An ethersoluble enolic fraction was isolated in an amount of 50 mg, which, on sublimation at 8—10 mm Hg gave only 0.15 mg of sublimate at 67—100° C. For absorption maximum of sublimate see Table 1.

Table 2. Alkaline digestions of various monosaccharides.

Expt.	Monosaccharides	Alkali	Heating vessel	Heating °C	Heating Time	Yield of ether solubles [c], as % of sugar used
D	Galactose, 1 g	4 %NaOH, 30 ml	Glass flask	100	8 h	3.6
E	» 2 g	8 %NaOH, 62.5 »	Steel [a] tube	100	3 h	3.5
F	» 2 g	—»—	—»—	170	3 h	2.9
G	» 2 g	71 %KOH, 28 g	Ni crucible [b]	100	3 min	10.5
H	» 2 g	—»—	—»—	170	3 »	3.9
I	Glucose, 100 g	5 %NaOH, 600 ml	Steel [a] tubes	100	46 h	6.6 [d]
K	Fructose, 5 g	4 » » 150 »	—»—	100	8 h	6.0 [e]
L	Mannose, 1 g	4 » » 150 »	Glass flask	100	8 h	2.3
M	Arabinose, 1 g	4 » » 30 »	—»—	100	8 h	2.0
N	Xylose, 1 g	4 » » 30 »	—»—	100	8 h	2.9

[a]) Stainless. — [b]) As in exp. B. — [c]) After acidification. — [d]) Enolic ether fraction isolated and sublimed in vacuum. Yield of sublimate at about 100° (9 mm) 0.013 % of the glucose used. Spectrum: Table 1. — e) Ether extract sublimated in vacuum. Yield of sublimate at 80—100° (10 mm) 0.026 % of the fructose used. Absorption spectrum: Table 1.

Alkaline digestions of galactose solutions

Expt. A: 150 g of galactose were heated in a sealed tube of stainless steel with 4 500 ml of 4 % NaOH solution for 8 hours at 100° C. After cooling, the reaction solution was evaporated *in vacuo* to a volume of about 1 000 ml and acidified with HCl. The ether-soluble enolic fraction was isolated in the manner described. The ether was evaporated *in vacuo* at room temperature, H_2 being led through the capillary tube. Sublimation at 10—12 mm: until 70° C, 48.6 mg of yellow oil; 70—95° C, 70.2 mg, white crystals and yellow oil. The crystals were recrystallized 5 times from ethyl ether-light petroleum, each time allowing them to stand on porous clay *in vacuo*; m.p. thereafter 100—102° C; m. p. of a mixture with methylcyclopentenolone of m. p. 99—101° C, isolated from soluble wood tar, was found to be 99—101° C.

Negative test for glycerol: 2 l of the acidified aqueous solution, which had been extracted with ether, was treated with 25 g of charcoal, filtered and evaporated *in vacuo*. Some NaCl crystallized and was filtered off. Blank tests showed that galactose alone could give the color reactions of glycerol (conversion to acrolein, test with sodium nitroprussiate and piperidine [21]; oxidation with bromine water to dihydroxyacetone, color reactions with resorcinol and codeine, respectively, according to Dènigés [20]). Therefore the carbohydrates were removed according to the method of Fresenius [22]: 25 g of $CaCO_3$ were added to the concentrated solution, from which the water was evaporated. The dry residue was ground to a fine powder and digested twice with 50 ml of a mixture of ethanol (2 vol.) and ethyl ether (1 vol.). The solvent was evaporated, but no residue was left. Glycerol was thus found to be absent.

Expt. B: 80 g of KOH was dissolved in 32 ml of water in a nickel crucible. 8 g of galactose, which had been washed with ethyl ether, was introduced into the crucible in a current of N_2 at 78° C. The heat of reaction raised the temperature to 97° C and the crucible was further kept for 3 min. at 100° C. After dissolving in 160 ml of water, the solution was acidified with 2 *N* HCl and extracted with ethyl ether. Weight of the ether extract: 1.216 g, which on sublimation at 60—95° C at 6—8 mm gave 494 mg of a dark orange-brown oil, which showed the absorption spectrum of Fig. 4 and gave a precipitate with DNPh in hydrochloric acid solution. Calculation of the content of methyl*cyclo*pentenolone: It is assumed that the absorption at 259 mμ in neutral solution is due to this compound exclusively, that the value of $E^{1\,\%}_{1\,cm}$ at that wave-length is proportional to the content of methyl*cyclo*pentenolone and that the $E^{1\,\%}_{1\,cm}$ for the pure compound is 729 (Fig. 1). Thus the observed $E^{1\,\%}_{1\,cm} = 209$ corresponds to 142 mg of methyl*cyclo*pentenolone, or 1.8 % of the original galactose.

Expt. C: 20.04 g of galactose, which had been washed with ethyl ether, was heated for 8 hours at 100° C with 600 ml of 4 % NaOH solution in a sealed tube of stainless steel.

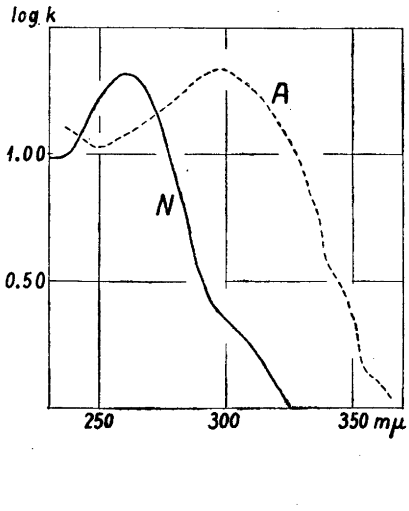

Fig. 4. Absorption spectrum of sublimate of total ether extract obtained after alkaline digestion of galactose (expt. B). Concentration: 10 mg/l. Curve N: in 96 % ethanol, A in 0.5 N ethanolic KOH. Curve N at 259 mμ: $E^{1\,\%}_{1\,cm} = 209$.

After cooling, the solution was acidified and the enolic ether-soluble fraction isolated as before. It weighed 53.8 mg and, on sublimation at 8 mm between 71 and 92° C, gave a sublimate which partly crystallized. Absorption spectrum in Fig. 2. The sublimate was dissolved in ethanol, and on addition of DNPh in 2 *N* HCl gave a 2,4-dinitrophenylhydrazone, which was crystallized from benzene; m. p. 235—236° C; m. p. of a mixture with dinitrophenylhydrazone of methyl*cyclo*pentenolone prepared from soluble wood tar (m. p. 229—230° C) was 229—231° C.

Alkaline digestions of various monosaccharides

Several monosaccharides were digested with alkali in excess, in some cases with concentrated KOH solution in a current of N_2 in an open nickel crucible, in others with dilute NaOH in sealed tubes of stainless steel. After digestion, the solutions were acidified and extracted with ether. Table 2 shows the results. The maxima of the absorption spectra of vacuum sublimates of some of the ether extracts are given in Table 1. Before the vacuum sublimation, the ether extracts showed very flat absorption curves. Even then, the curve from experiment D with galactose was the sharpest.

Titrations of reductone

Expt. a: 1.01 g of galactose, 10 g of KOH and 4 ml of H_2O were digested in a nickel crucible in a current of N_2 for 3 min. at 100° C. The content of the crucible was then dissolved in water and diluted to 350 ml. 20 ml samples of the solution were titrated with dichlorophenolindophenol (Tillmans' reagent "TR") after addition of phosphoric acid to pH 9, and with 0.1 *N* I_2 solution after acidification with about 2 *N* acetic acid.

Expt. b: The experiment was repeated in the same way with another 1.0 g of galactose, but with the difference that the titration with TR was made at pH 5.

Expt. c was made in a corresponding way, but heating the crucible only 2.5 min. at 92° C (cf. [14]).

Expt. d: 5 g of raw air-dry spruce sawdust (moisture content 7.5 %) was boiled 5 min. with 100 ml of distilled water. The mixture was filtered and after addition of Na_2HPO_4 to pH 9 the filtrate was titrated with TR and I_2.

Expt. e: 5 g of raw sawdust of the kind used in expt. d was refluxed for 30 min. with 100 ml of 4 % NaOH solution. The mixture was filtered, and samples of the filtrate treated and titrated in a way corresponding to that of the experiments a and b.

The results are given as mg reductone/g of original galactose or sawdust: expt. a) TR 39 mg at pH 9, I_2 85 mg. — b) TR 67.7 mg at pH 5, I_2 55.3 mg. — c) TR 16.5 mg at pH 9, I_2 33.3 mg. — d) TR 0 at pH 9, I_2 0. — e) TR 1.7 mg at pH 9, I_2 3.1 mg.

SUMMARY

Methyl*cyclo*pentenolone (Formula I) could be isolated in low yields after digestion of spruce wood or of pure galactose with sodium hydroxide solution at 100° C. It could not be found in the raw wood, but seems to be formed from galactan and perhaps also from other neutral wood polyoses on alkaline digestion. The mechanism of the formation of methyl*cyclo*pentenolone is discussed.

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Dielectric Increments and Dispersions in Aqueous Solutions of a Dextran Ester Sulphate

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The sodium salts of a number of acid polyelectrolytes containing carboxylic or phosphoric acid groups have been shown to give high dielectric increments in aqueous solutions, and also to give dielectric dispersions at radio frequencies [1–5]. The dielectric properties of these polyelectrolytes also show anomalous concentration effects as compared with globular proteins and simple dipolar ions. Recently it has been found that chondroitin sulphate much resembles the other acid polyelectrolytes in respect of dielectric increment and dispersion [6]. It was to obtain more information concerning the influence of sulphate groups in the molecule on the dielectric properties that this investigation of the sodium salt of a dextran sulphuric acid [7] was carried out.

Table 1. Dielectric increments and critical wavelengths of dextran sulphate.

Concn. %	ε_l	ε_h	Increment $(\varepsilon_l - \varepsilon_w)$	I_g	λ_c (meters)
0.10	87.0	82.3	6.7	6.7	25
0.10	86.8	—	6.5	6.5	—
0.08	86.2	—	5.9	7.4	—
0.06	85.2	—	4.9	8.2	—
0.05	84.7	81.7	4.4	8.8	39
0.04	84.0	—	3.7	9.3	—
0.03	83.3	—	3.0	10	—
0.025	83.3	80.8	3.0	12	51
0.02	82.7	—	2.4	12	—

Symbols: ε_l = DC at frequencies low in relation to those of the dispersion region.
ε_h = DC at frequencies high in relation to those of the dispersion region.
ε_w = DC of solvent (80.3 for water at 20° C).
λ_c = critical wavelength, obtained graphically.

EXPERIMENTAL

The dextran sulphate * used (abbreviated to DS hereafter) contained 17.3 % sulphur. The molecular weight of the specimen was about 40 000 (calculated from the sulphur content and the molecular weight of the dextran starting material — about 19 000 — determined viscometrically). The sulphur content corresponds to about two ester sulphate groups per glucose unit in the macromolecule.

The dielectric measurements were carried out by the ellipsoid method as modified by G. Jungner and coworkers [8, 2, 3] for measurements at various radio frequencies between 30 Mc/s and 30 kc/s. All measurements were made at 20.0° C. Fresh aqueous solutions below 0.1 % (w/v) were investigated. pH about 6.0.

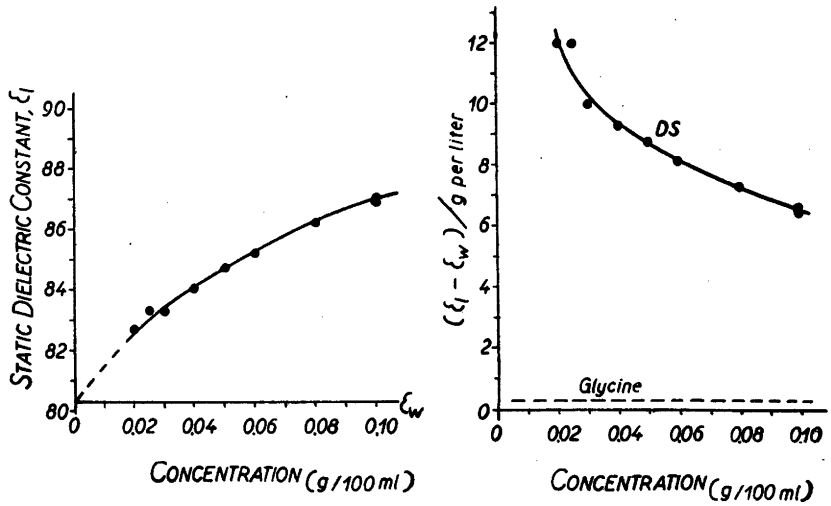

Fig. 1A (left), static dielectric constant and, Fig. 1B (right), increment per gram per liter as a function of concentration for DS in aqueous solutions.

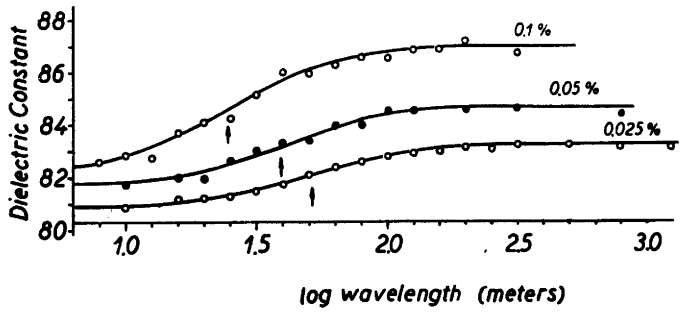

Fig. 2. Dielectric dispersion curves of dextran sulphate in aqueous solution at various concentrations.

The arrows indicate the critical wavelengths. The fulldrawn curves are the theoretical curves according to the Debye theory fitting the experimental points.

* kindly supplied by Dr B. Ingelman, Pharmacia Ltd., Upsala.

RESULTS

A dielectric increment was obtained for DS in dilute aqueous solution. The increment per gram per liter (I_g) was of the order of 10 dielectric constant (DC) units at the lowest concentrations studied, corresponding to a molar increment of about $4 \cdot 10^5$.

As is shown in Figs. 1A and B and Table 1, the increments are not linear functions of concentration. I_g increases as concentration decreases (Fig. 1B).

Dielectric dispersions were obtained in the frequency range used. The dispersions approached theoretical dispersions according to Debye (for a solute with a single relaxation time). The critical wavelengths (Fig. 2 and Table 1) varied considerably with concentration, being longer at lower concentrations. The relaxation times calculated according to the Debye theory and assuming that the DS molecule orientates in the electric field around its long axis, were $1.3 \cdot 10^{-8}$ sec. at 0.1 % and $2.6 \cdot 10^{-8}$ sec. at 0.025 % concentration.

DISCUSSION

A specimen of the neutral polyglucose dextran has been shown previously to give *no* dielectric increment in aqueous solution at 0.5 % concentration [5]. It is therefore natural to assume that the high dielectric increment obtained with DS in this study is closely connected with the presence of charged groups, $-SO_3^-$, in this molecule.

DS is an elongated molecule with side chains, which gives it a large surface compared with, *e. g.*, globular proteins in the same molecular weight class. DS will then have a high "surface charge" compared with such proteins, in which the charges — positive and negative — will be internally compensated to a large extent. This might be the explanation of the much higher dielectric increments obtained for DS and similar polyions (I_g about 10 for DS and less than 2 for globular proteins).

The cause of the dielectric increment in the case of proteins and simple dipolar ions is assumed to be an orientation polarization due to orientation of dipoles in the electric field in accordance with the Debye theory. Such an orientation effect implies a more or less asymmetric distribution of the charges in the molecule. It is, however, difficult to assume such an electric asymmetry for the DS molecule, and thus it is doubtful whether the Debye theory will apply to this case. Fuoss [9] has suggested that a modified Wagner theory [10, 11] will explain the polarization phenomena in the case of polyelectrolytes, and thus for DS.

Qualitatively there is no difference between the dielectric properties in aqueous solution of DS and other polyelectrolytes studied. All give, *e. g.*, nonlinear increment *vs.* concentration curves and displacement of the dispersion curves to longer wavelengths with lower concentrations. Quantitatively there are considerable differences, however. Thus the dielectric increments show large variations between different substances (and in some cases different molecular weights of one substance) even if comparisons are made at corresponding concentrations on an equivalents per unit volume basis, in pure aqueous solutions.

The course of the I_g *vs.* concentration curve (Fig. 1B) has previously been interpreted in similar cases [1–5] as being due to a prevalence of free polar macromolecules or polar aggregates in the solution at very low concentrations and of aggregates less polar at higher concentrations, *i. e.* association-dissociation phenomena on a macromolecular scale. It might, however, also be interpreted as being due to an increasing "dissociation" of counter ions (Na^+) from the macromolecule with increasing dilution, *i. e.* a lessening of the screening effect of the counter ions. Thus the net charge of the polyion increases with increasing dilution, giving rise to higher I_g values. This is well compatible with the dependence of viscosity and d.c. conductivity of polyelectrolyte solutions on concentration [12–15] and does not seem to be influenced by the theory chosen for the dielectric polarization and relaxation phenomena.

Finally it may be mentioned that two other polysaccharide ester sulphates with relatively low molecular weights — a cellulose sulphate and the above-mentioned chondroitin sulphate — have been shown [6] to have similar dielectric properties in aqueous solution to DS.

SUMMARY

The dielectric properties of DS in aqueous solution are similar to those of other acid polyelectrolytes studied, containing —COOH, $= PO_4H$ or $—SO_3H$ groups. As might be expected the dielectric increments seem to be mainly dependent upon the actual charge of the molecules.

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The Optically Active Hexahydrohomophthalic Acids and their Anhydrides

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The two stereoisomeric hexahydrohomophthalic acids, *trans* m.p. 161° and *cis* m.p. 147°, which were prepared by catalytic hydrogenation of homophthalic acid as described by Windaus, Hückel and Reverey [1], have now been resolved into optically active components by fractional crystallization of the brucine salts. The components of the *trans*-acid had m.p. 116° and a specific rotation of ± 40.0°, while those of the *cis*-acid had m.p. 82° and a rotation of ± 12.5°. By treatment with acetic anhydride the active acids were easily transformed into the corresponding anhydrides without any racemization taking place. The sign of the rotation changed, however, during this process, a dextrorotatory acid giving a levorotatory anhydride and vice versa. The active anhydrides of the *trans*-acid had m.p. 111° and a specific rotation of ± 71.5°, and those of the *cis*-acid m.p. 88° and a rotation of about ± 30°.

Stereochemically the anhydrides of the hexahydrohomophthalic acids can be compared with the isomeric decalins. It should, however, be kept in mind that the six-membered anhydride ring contains two carbonyl groups and one oxygen atom instead of methylene groups as in a cyclohexane ring, and consequently the valency angles at these points will deviate somewhat from the tetrahedral angle. This will obviously result in a certain flattening of the heterocyclic ring, but as a whole it should be justifiable to assume that the molecules of the anhydrides and those of the decalins are similar in shape. In the following some simplified models will demonstrate the possible steric forms of the hexahydrohomophthalic acids and their anhydrides. Only one of the active components is considered in each case. In the models of the acids a small circle indicates a carboxyl group and a greater circle the group $CH_2 \cdot COOH$.

Hassel [2] has shown that the cyclohexane itself and its derivatives generally exist in the centrosymmetrical, so-called "staircase", model. Using his notations ε and $\varkappa$ for the two types of bonds at the ring carbon atoms the substituents in the case of *trans*-hexahydrohomophthalic acid will be either $\varepsilon\varepsilon$ or $\varkappa\varkappa$ as shown in the models T_1 and T_2 in Fig. 1. The one model may be converted into the other in this way that the three ring carbon atoms in the upper plane

move downwards and the three in the lower plane move upwards. The two models are not enanthiomorphic and no racemization would therefore follow an interconversion.

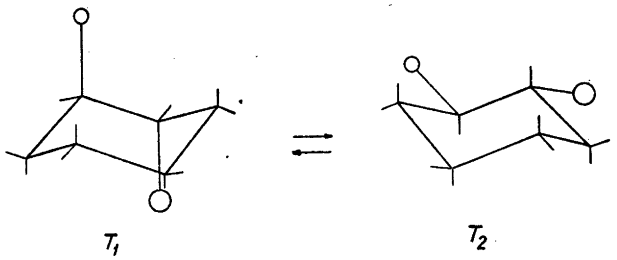

Fig. 1. Models of trans-hexahydrohomophthalic acid

No investigation has as yet been carried out in order to show if any of the two models is predominating. Neither can theoretical considerations give any certain indication of the one model being more stable than the other. Actually the distance between the two substituents in T_1 (about 3.9 Å) is 1.3 times as great as that in T_2 and for that reason T_1 might be more stable than T_2. (The figure given refers to the distance between the carbon atoms bound directly to the ring.) On the other hand the substituents in T_1 will be relatively near (2.5 Å) to two ε-hydrogen atoms, while the substituents in T_2 will have only one of the ring carbon atoms at the same short distance. The van der Waals forces arising at such a distance will certainly cause some deformation of the molecule, but it would not be safe to predict their influence on the interconversion of the two models. The only conclusion we can draw at the present time is that *trans*-hexahydrohomophthalic acid probably consists of an equilibrium between the models T_1 and T_2.

Turning now to the anhydride of *trans*-hexahydrohomophthalic acid it is evident that it stereochemically must be derived from model T_2 as shown in Fig. 2. Consequently, if any part of the acid is present as model T_1 it must pass over to T_2 before it can form the anhydride. As in the case of *trans*-decalin the model in Fig. 2 is locked and no conversion can take place.

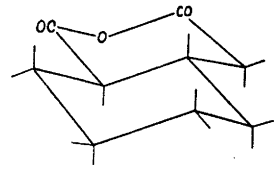

Fig. 2. Model of trans-hexahydrohomophthalic anhydride

In *cis*-hexahydrohomophthalic acid one substituent will be ε and the other $\varkappa$ and accordingly there will be the two interconvertible models C_1 and C_2 as shown in Fig. 3. The models are again not enanthiomorphic and no racemization would accompany a conversion. The distance between the substituents is

the same in both models as are also the other main intramolecular distances. The acid is therefore probably a mixture of approximately equal parts of the models C_1 and C_2.

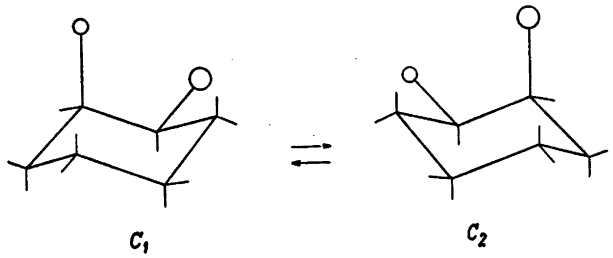

Fig. 3. Models of cis-hexahydrohomophthalic acid

The model of the anhydride of the *cis*-acid may be derived either from C_1 or C_2 giving thus the interconvertible models A_1 and A_2 respectively (Fig. 4). In A_2 one of the hydrogen atoms of the methylene group in the heterocyclic ring (marked with a cross) will come very near to the two ε-hydrogen atoms in the cyclohexane ring, also marked with a cross. This will not be the case in A_1 in which both hydrogen atoms of the methylene group in question are pointing away from the cyclohexane ring. Model A_1 is therefore most probably the dominating form of the *cis*-anhydride molecule.

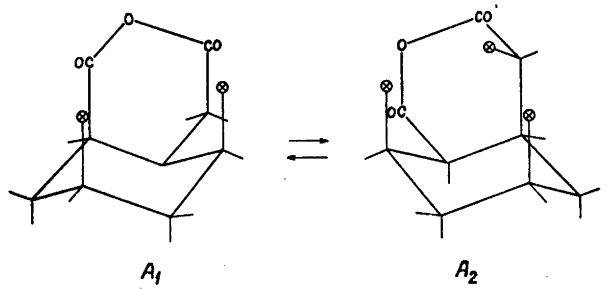

Fig. 4. Models of cis-hexahydrohomophthalic anhydride

EXPERIMENTAL PART

Preparation of the hexahydrohomophthalic acids

By the hydrogenation of homophthalic acid according to Windaus, Hückel and Reverey [1] a mixture of *cis*- and *trans*-hexahydrohomophthalic acids was obtained. After decoloration by boiling an aqueous solution with charcoal the acid-mixture melted from 120° to 140°. The separation of the two acids by fractional crystallization from water is a tedious process as there is only a small difference in the solubility of the isomers. For the isolation of the *cis*-acid it was found advantageous to proceed in the following way. The finely powdered acid mixture was heated 4—5 times with warm carbon tetrachloride stirring vigorously and filtering the still warm solution after each treatment. In this way most of the *trans*-acid dissolved and a *cis*-acid with m.p. 145° remained undissolved. For

further purification the acid was recrystallized twice from water when the m.p. rose to 147°. A renewed crystallization did not change the m.p. and on evaporation of the mother liquor an acid with the same m.p. resulted

$C_9H_{14}O_4$ (186.2) Calc. C 58.05 H 7.58
Found » 58.17 » 8.00

The isolation of the *trans*-acid was carried out as described by the authors mentioned above, *viz.* after most of the *cis*-acid in the mixture had been converted into the *trans*-acid by heating with concentrated hydrochloric acid. After recrystallization several times from water the *trans*-acid melted at 161° and did not change its m.p. on a renewed crystallization. Windaus, Hückel and Reverey found m.p. 146° and 157° for the *cis*- and the *trans*-acid respectively.

Resolution of *cis*-hexahydrohomophthalic acid

To a solution of the *cis*-acid (9 g) in warm water (200 ml) 45 g of brucine (2 mole alkaloid to 1 mole acid) were added in portions. It was necessary to keep the solution warm and to stir continuously in order to dissolve the alkaloid. One should, however, be careful not to heat too long as the solution then would darken and the subsequent crystallization of the salt be impeded. Generally the crystallization of the brucine salt took place over night especially on keeping the solution at a cool place. The salt was recrystallized 3—4 times from water, the purified salt dissolved in hydrochloric acid and the *cis*-acid extracted with ether. From the dried ethereal solution a residue was obtained which partly crystallized and obviously consisted of a mixture of active and racemic acid. Great difficulties were encountered in obtaining the active acid in the crystalline state. After decolorizing the crude acid with charcoal crystallizations from water gave fractions of practically inactive acid, the active acid being enriched in the mother liquor. From the latter the active acid was obtained as a thick syrup the crystallization of which in the end was achieved in the following way. The syrup was placed at the bottom of a low glass dish and covered with a layer of water. Placing the dish at zero degree for several days big clear crystals of the pure dextrorotatory *cis*-acid separated which after washing and drying had m.p. 82°.

$C_9H_{14}O_4$ (186.2) Calc. C 58.05 H 7.58
Found » 57.76 » 7.53

The levorotatory *cis*-acid was isolated from the easily soluble brucine salt in the same way as described for the dextrorotatory acid, even greater difficulties being met with, however, in obtaining the pure (−) -acid which also had m.p. 82°.

The specific rotations of the optical components at various concentrations in dioxan are given in Table 1. The concentration c is g acid in 100 ml solution; all measurements taken in a 1 dm tube. The specific gravity at various concentrations was determined on solutions of the racemic acid and could be expressed sufficient accurately by means of the linear equation.

$$d_4^{20} = 1.035 + c \cdot 0.00168.$$

Table 1. Specific rotations of the cis-acids in dioxan.

(+)-*cis*-acid				(−)-*cis*-acid			
c	d_4^{20}	a_D^{20}	$[a]_D^{20}$	c	d_4^{20}	a_D^{20}	$[a]_D^{20}$
4.80	1.043	+0.60°	+12.0°	3.42	1.041	−0.39°	−11.0
9.49	1.051	1.25	12.5	5.48	1.044	0.67	11.6
12.33	1.056	1.62	12.5	10.29	1.052	1.31	12.1
14.35	1.059	1.95	12.8	14.57	1.059	1.93	12.5
19.89	1.068	2.65	12.5	23.41	1.074	3.28	13.0
26.70	1.080	3.84	13.3				

The melting point curve obtained for mixtures of the inactive *cis*-acid with its levorotatory component (see Fig. 5) shows that the inactive acid is a true racemic form.

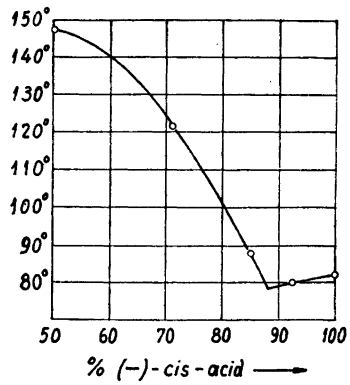

Fig. 5. Mixtures of inactive and leverotatory cis-acids

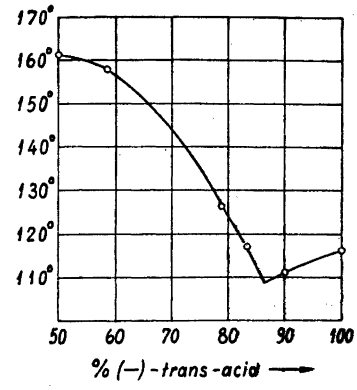

Fig. 6. Mixtures of inactive and levorotatory trans-acids.

Resolution of *trans*-hexahydrohomophthalic acid

The resolution of the *trans*-acid met with no difficulties especially because the active components crystallized very easily. The neutral brucine salt was used also in this case. After recrystallizing the first fraction of the brucine salt 3—4 times from water a crude dextrorotatory *trans*-acid was isolated in the usual way. Although the active acid was more soluble than the inactive it was easily obtained in a pure state by repeated crystallizations from water. It formed big clear crystals with m.p. 116°. The leverotatory *trans*-acid, isolated in a similar way from the easily soluble brucine salt, formed the same kind of clear crystals with m.p. 116°. The rotation of the two components will be found in Table 2.

Table 2. Specific rotations of the trans-acids in dioxan.

(+)-*trans*-acid				(−)-*trans*-acid			
c	d_4^{20}	α_D^{20}	$[\alpha]_D^{20}$	c	d_4^{20}	α_D^{20}	$[\alpha]_D^{20}$
5.11	1.044	+2.14°	+40.2°	4.95	1.044	−2.07°	−40.1°
9.42	1.051	3.98	40.2	9.50	1.051	4.02	40.1
14.86	1.060	6.22	40.0	18.42	1.066	7.84	39.9
31.85	1.092	13.86	39.9	30.14	1.086	13.14	40.2

The melting point curve for mixtures of active and inactive *trans*-acids again shows that the inactive acid is a true racemic form; see Fig. 6.

Anhydrides of the active *cis*-hexahydrohomophthalic acids

On boiling a solution of an active component of the *cis*-acid in acetic anhydride the acid was transformed into the corresponding acid anhydride. For isolation of the anhydride the solution was simply evaporated at room temperature when the anhydride

crystallized. Both the active anhydrides had m.p. 88°. The anhydride prepared from the levorotatory acid was dextrorotatory.

$C_9H_{12}O_3$ (168.2) Calc. C 64.27 H 7.19
Found » 63.96, 64.12 » 7.16, 7.22

The optical rotation of the two *cis*-anhydrides will be found in Table 3.

Table 3. Specific rotations of the cis-anhydrides in dioxan.

(+)-*cis*-anhydride				(−)-*cis*-anhydride			
c	d_4^{20}	α_D^{20}	$[\alpha]_D^{20}$	c	d_4^{20}	α_D^{20}	$[\alpha]_D^{20}$
10.50	1.051	+3.35°	+30.3°	9.40	1.051	−2.98°	−30.2°
15.10	1.060	4.90	30.6	18.60	1.066	6.06	30.5

The active *cis*-anhydrides could be distilled in a vacuum without any racemization taking place, the specific rotation being the same after as before the distillation. When (+)-*cis*-anhydride was boiled with water it was transformed into the same levorotatory acid from which it had been prepared, the specific rotation of the recovered acid being −12.5° (c = 11.4 in dioxan) and the m.p. 82°.

Anhydrides of the active *trans*-hexahydrohomophthalic acids

Both the active components of the *trans*-acid were transformed into the corresponding anhydrides in the same way as described for the *cis*-acids. Also in this case the sign of the rotation changed on formation of an anhydride. The *trans*-anhydrides were crystalline with m.p. 111°. Their optical rotations are given in Table 4.

Table 4. Specific rotations of the trans-anhydrides in dioxan.

(+)-*trans*-anhydride				(−)-*trans*-anhydride			
c	d_4^{20}	α_D^{20}	$[\alpha]_D^{20}$	c	d_4^{20}	α_D^{20}	$[\alpha]_D^{20}$
4.56	1.043	+3.40°	+71.5°	10.93	1.053	−8.30°	−72.1°
10.54	1.053	7.90	71.5				

As in the case of the *cis*-anhydrides distillation in a vacuum did not cause any racemization. On boiling (−)-*trans*-anhydride with water an acid was obtained which had the specific rotation +40.2° (c = 11.3 in dioxan) and the m.p. 116°.

SUMMARY

The *cis*- and *trans*-forms of hexahydrohomophthalic acid were resolved into optically active components by means of brucine. The active acids could easily be transformed into the corresponding active anhydrides by treatment

with acetic anhydride. By this process the sign of the rotation changed as well in the case of the *cis*-acid as in that of the *trans*-acid but no racemization took place. Both the inactive hexahydrohomophthalic acids were found to be true racemic forms.

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Triterpenoids in Lichens

I. The Occurrence of Friedelin and *epi*Friedelinol *

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Whilst acidic substances found in lichens have been extensively investigated, little is known about neutral constituents of lichens. In particular, triterpenoids seem to be scarce, the only ones known so far being zeorin (for a summary see [1], compare also [2]), ursolic acid [3] and leucotylin [2]. The classifying of zeorin as a triterpenoid substance is due to Asahina and Akagi [2], who deduced this from the fact that on dehydrogenation with selenium naphtalene hydrocarbons characteristic as dehydrogenation products of triterpenoids were isolated. Its relationship to the known triterpenoid compounds is obscure, and the basic hydrocarbon, zeorinane, is not identical with any of the known triterpenoid hydrocarbons [4].

In an attempt to establish such a relationship it was decided to investigate lichens other than those known to contain zeorin, to see whether there might occur triterpenoids which on the one hand could be related to zeorin and on the other to a triterpenoid of known constitution. As a first object *Cetraria nivalis* (L.) Ach. was chosen, because it could be collected relatively easily in large quantities. During purification of the collected material some other, easily recognisable lichens were taken care of: *C. cucullata* (Bell.) Ach., *Cladonia alpestris* (L.) Rabh., *Alectoria ochroleuca* (Ehrh.) Nyl. and *Stereocaulon paschale* (L.) Fr. The remaining waste lichens, consisting mainly of various *Cladonias*, were also extracted, to take advantage of the fact that some of the isolated substances could thus be obtained in larger quantities. Furthermore were investigated *Cetraria islandica* (L.) Ach., since this lichen can be easily obtained commercially as a drug, and *C. Delisei* (Bory) Th. Fr. [= *hiascens* (Fr.)] and *C. crispa* (Ach.) Nyl., both readily collected in a pure state.

As it happened, the extraction was carried out without the use of cork. After sparingly soluble substances had been removed by filtration and acidic substances had been completely extracted by alkali, the neutral fraction was subjected to a chromatographic purification. *C. nivalis* was first investigated,

* Parts of this and the following paper were presented to the *8. Nordiske Kjemikermöte*, Oslo, June 14th—17th, 1953.

With petroleum-benzene (1 : 1) and with benzene there was eluted a colourless substance, m.p. 262—263° (vac.), $[\alpha]_D$—21°, which analysed as $C_{30}H_{50}O$, gave a 2 : 4-dinitrophenylhydrazone, m.p. 300° (dec.), light abs. max. at 3 700 Å, and showed an absorption band at 1 708 cm^{-1} in chloroform solution in the infra-red. Indications, therefore, were that the isolated substance might be a triterpenoid ketone with the carbonyl function in a six-membered ring. Wolff-Kishner reduction of the ketone afforded the basic hydrocarbon, m.p. 246—247°, $[\alpha]_D$ + 21°. Inspection of the literature revealed that friedelin had m.p. 255—261°, $[\alpha]_D$ —29° [5], m.p. 264—265° (vac., corr.), $[\alpha]_D$ —28° [6], 2 : 4-dinitrophenylhydrazone, m.p. 297—299° [7], whilst the basic hydrocarbon, friedelane, prepared by various methods, is reported to melt at 245—249° [5], m.p. 243—244° (corr.), $[\alpha]_D$ + 42° [6], m.p. 244°, $[\alpha]_D$ + 42° [8]. For comparison friedelin, together with cerin, was extracted from cork [5,6]. The obtained specimen showed m.p. 262—263° (vac.), $[\alpha]_D$ —21°, 2 : 4-dinitrophenylhydrazone, m.p. 297° (dec.), light abs. max. at 3 710 Å, and there was no depression of m.p. on admixture of the respective substances. Such was also the case with friedelane, prepared by the Wolff-Kishner method, m.p. 245—246°, $[\alpha]_D$ +18° *.

In a similar way friedelin was obtained from the other lichens mentioned above. The identity was secured by comparison of the ketone and its 2 : 4-dinitrophenylhydrazone with authentic specimens. The yield of friedelin was quite small, 250 mg having been obtained from nearly 4 kg of *Cetraria nivalis*, and relatively less from the other pure lichens. Quite astonishing, in view of this, is the amount isolated from the waste lichens, 5 g from 16 kg. This fact strongly indicates that some lichen(s) must contain friedelin in large amounts, but there are no hints as to which this might be.

By elution with benzene/ether (1 : 1) and with ether a mixture of substances was obtained from *C. nivalis*. Nearly all of it was very soluble in chloroform, but when treated with this solvent on the filter a small amount of a colourless crystalline substance was left. It was crystallised from chloroform, m.p. 280—281° (vac.), $[\alpha]_D$ +23°. This substance also occurred, in somewhat larger quantities, in the waste lichen extract, m.p. 281—282° (vac.), $[\alpha]_D$ +15°. It analysed as $C_{30}H_{52}O$ and in the usual way gave an acetate, m.p. 275—276° (vac.), $[\alpha]_D$ +12° and a benzoate, m.p. 232—233° (vac.), $[\alpha]_D$ +26°. Chromic acid oxidation furnished friedelin; this substance, therefore, must be a friedelinol. In literature the preparation of a friedelinol, m.p. 301—304°, by sodium-alcohol reduction of friedelin has been described [9], and it is reported that a lower melting friedelinol, m.p. 278—282° has been prepared, compare [10], foot-note 3. This low melting friedelinol must contain the hydroxyl group in the polar position, since the other isomer has been prepared by a method, which gives an alcohol with the hydroxyl in the thermodynamically more stable equatorial position, compare [11]. This isolated friedelinol presumably is identical with the low-melting friedelinol of the literature. The view that it contains the hydroxyl function in the polar position was confirmed by dehydration with phosphorus oxychloride in pyridine, which afforded a hydrocarbon mixture. Assuming that the hydroxyl group of this alcohol is situated at a

* It will be noticed that there is a large discrepancy between reported and found values for $[\alpha]_D$. This will be dealt with in the following paper.

position corresponding to the position 2 of the usual triterpenoids, this friedelinol should be designated *epi*friedelinol, in analogy with the commonly accepted nomenclature.

EXPERIMENTAL

M.p.'s are uncorrected. All rotations were measured in chloroform solution in a 1 dm tube at room temperature (20—25°) unless specified to the contrary. The values recorded have been approximated to the nearest degree. Petroleum refers to the fraction of b.r. 40—70°. Ether solutions were dried, when necessary, with anhydrous sodium sulphate, before removal of the solvent. 2 : 4-Dinitrophenylhydrazones were purified by filtration through alumina and crystallisation from a suitable solvent (mixture). Their light absorption was measured in chloroform solution. Infra-red absorption spectra were recorded in chloroform solution on a Perkin-Elmer double beam instrument. The term "in the usual way" refers to pouring into water, extraction of organic material with ether and washing of the ether solution successively with alkali and dilute sulphuric acid solutions, when necessary. Saponifications were carried out by refluxing for 1—2 hours with several equivalents of potassium hydroxide in methanol. Substances were acetylated and benzoylated with acetic anhydride and benzoyl chloride, respectively, in pyridine solution at room temperature.

The lichens used in these investigations were collected in the Oppdal region, some 75 miles south of Trondheim in early September. The samples were purified, dried at 50° and ground coarsely before extraction with ether in an all-glass, ground-joint Soxhlet extractor. The waste lichens were extracted in a large metal Soxhlet extractor. The general procedure by working up the extracts was as follows: Substances which had precipitated during extraction were removed by filtration, and then the ether solution was concentrated to a small volume, cooled and again filtered. Finally the filtered ether extract was extracted with potassium hydroxide solution to remove all acidic substances. The ether solution was evaporated to dryness and the residue redissolved in petroleum and chromatographed on alumina.

Cetraria nivalis *(L.) Ach.* 3 950 g of dried lichen furnished 17.5 g of neutral material. Careful chromatography afforded several fractions eluted with benzene, and with benzene/ether (99 : 1) and (49 : 1). All of them gave the same colourless substance from acetone with m.p. ranging from 259—260° to 262—263° (vac.). They were combined and crystallised to constant m.p. 262—263° (vac.) and constant rotation, $[\alpha]_D$ −21° (*c*, 2.34). (Found C 84.0, 84.4; H 11.8, 11.95. $C_{30}H_{50}O$ (426.70) requires C 84.45; H 11.8). The total yield of this substance in the slightly impure form was 250 mg. It formed a 2 : 4-dinitrophenylhydrazone, m.p. 300° (dec.), abs.max. at 3 700 Å, ε 28 000. There was no depression of m.p. on admixture of the ketone and its 2 : 4-dinitrophenylhydrazone with specimens of friedelin and its 2 : 4-dinitrophenylhydrazone obtained from cork (see below).

The ketone (60 mg) was heated for 18 hours at 180° with hydrazine (2 ml) and sodium (0.25 g) in ethanol (6 ml). The product, isolated in the usual way, was filtered through alumina in petroleum, and the eluted substance crystallised from chloroform, m.p. 246—247°, $[\alpha]_D$ +21° (*c*, 0.98, 2 dm tube); +22° (*c*, 0.36, 2 dm tube). The hydrocarbon gave no depression on admixture with authentic friedelane (see below).

epi*Friedelinol.* The material eluted with benzene/ether (1 : 1) and with ether (0.8 g) was dissolved in petroleum, when crystals separated. Treatment on the filter with cold chloroform removed most of the material. The substance sparingly soluble in chloroform was crystallised and recrystallised from chloroform, m.p. 280—281° (vac.), $[\alpha]_D$ +23° (*c*, 0.52). There was no depression on admixture with a substance of the same m.p. obtained from the waste lichens (see below). Oxidation with chromic acid in acetic acid at room temperature afforded friedelin, m.p. 260—262°, converted to the 2:4-dinitrophenylhydrazone, m.p. 298—299° (dec.), no depression on admixture with authentic specimens.

The waste lichens. 16.2 kg of dried material furnished 179 g of neutral fraction, which, when subjected to a treatment similar to that reported for *Cetraria nivalis* (see above), afforded altogether 5 g of a slightly impure friedelin. It was crystallised (very wastefully) from chloroform to give a sample of m.p. 263—264° (vac.), $[\alpha]_D$ −21° (*c*, 1.99, 2 dm tube), no depression on admixture with friedelin obtained from *C. nivalis* (see above).

epi*Friedelinol.* This compound precipitated in admixture with other substances from a benzene solution of material which was sparingly soluble in ether, and from a benzene

solution of the unsaponifiable matter resulting after saponification of the neutral fraction. It was crystallised from chloroform to constant m.p. 281–282° (vac.) and constant rotation, $[\alpha]_D$ +15° (*c*, 1.00, 2 dm tube), (67 mg). (Found C 83.9; H 12.25. $C_{30}H_{52}O$ (428.72) requires C 84.0; H 12.2). It gave an *acetate*, m.p. 275–276° (vac.), from chloroform/methanol, depressed to 255–260° on admixture with *epi*friedelinol, $[\alpha]_D$ +12° (*c*, 0.81). (Found *C 80.95; H 11.5. $C_{32}H_{54}O_2$ (470.75) requires C 81.5; H 11.55) and a *benzoate*, m.p. 232–233° (vac.), from chloroform/methanol, $[\alpha]_D$ +25° (*c*, 1.29), +27° (*c*, 1.35). (Found *C 83.3; H 11.25. $C_{37}H_{58}O_2$ (532.82) requires C 83.4; H 10.6). Oxidation of *epi*friedelinol with chromic acid in acetic acid at room temperature furnished friedelin, m.p. 263–264° (vac.), no depression on admixture with an authentic specimen, $[\alpha]_D$ −15° (*c*, 0.98), converted to the 2:4-dinitrophenylhydrazone, m.p. 300–301° (dec.), no depression on admixture with authentic material.

*epi*Friedelinol was dehydrated with phosphorus oxychloride (1 ml) in pyridine (12 ml) with standing overnight at room temperature. The dehydration product was isolated with petroleum and filtered through alumina. After crystallisation from chloroform the eluted material melted at 250–258°.

Cetraria islandica *(L.) Ach.* 10 kg of dried lichen, purchased through the local branch of Tollef Bredal, and said to originate from Poland, afforded 19 g of neutral matter which was chromatographed on a column 34 × 4.8 cm of alumina. Benzene/ether (99 : 1) and (49 : 1) eluted 0.3 g and 0.1 g, respectively, which on treatment with petroleum furnished altogether 30 mg of friedelin of m.p. 259–260°, $[\alpha]_D$ −21° (*c*, 2.21), 2:4-dinitrophenylhydrazone, m.p. 296–297° (dec.), no depression on admixture with authentic specimens.

Cetraria cucullata *(Bell.) Ach.* 945 g of dried material afforded 4.5 g of neutral matter. After chromatography on alumina friedelin (23 mg) was obtained, m.p. 250–255° (vac.), from chloroform/methanol, $[\alpha]_D$ −18° (*c*, 2.11), converted to the 2:4-dinitrophenylhydrazone, m.p. 297–298° (dec.), no depression on admixture with authentic specimens.

Cetraria crispa *(Ach.) Nyl.* 896 g of dried lichen furnished 4.9 g of neutral matter. Friedelin (22 mg) was obtained as above, m.p. 250–255° (vac.) from acetone, $[\alpha]_D$ −17° (*c*, 2.00), converted to the 2:4-dinitrophenylhydrazone, m.p. 295–296° (dec.), from chloroform-ethyl acetate, no depression on admixture with authentic specimens.

Cetraria Delisei *(Bory) Th. Fr.* [= hiascens *(Fr.)*]. 763 g of dried lichen gave 4.1 g of neutral material. Chromatography as above afforded friedelin (20 mg), m.p. 253–257° (vac.) from chloroform/acetone, $[\alpha]_D$ −22° (*c*, 1.93), converted to the 2:4-dinitrophenylhydrazone, m.p. 297–298° (dec.), from chloroform/ethyl acetate, no depression on admixture with authentic specimens.

Cladonia alpestris *(L.) Rabh.* From 648 g of dried lichen 3.0 g of neutral material was obtained. When chromatographed, a very small amount of a very impure friedelin was obtained, m.r. 240–255°, admixture with authentic friedelin, m.r. 258–262°, all three taken at the same time and in evacuated tubes.

All neutral material was combined and saponified. The unsaponifiable matter (0.6 g) was isolated with ether and chromatographed on alumina. Benzene eluted 0.1 g, which, from chloroform/acetone, furnished a substance of m.p. 262–263° (vac.) undepressed on admixture with friedelin, $[\alpha]_D$ −13° (*c*, 0.64), converted to the 2:4-dinitrophenylhydrazone, m.p. 299–300° (dec.), undepressed on admixture with friedelin 2:4-dinitrophenylhydrazone.

Alectoria ochroleuca *(Ehrh.) Nyl.* 612 g of dried lichen afforded 3.5 g of neutral material, which was chromatographed on alumina to give 0.5 g eluted with benzene. When this material was dissolved in petroleum and left standing, there gradually separated 17 mg of a mixture of substances, m.r. 225–250°. The mixture was heated twice with fresh amounts of petroleum and each time filtered whilst hot. The small amount of substance finally left on the filter melted at 262–263° (vac.), and did not depress a sample of authentic friedelin.

All material was combined and saponified, and the unsaponifiable matter (0.6 g) was isolated with ether and chromatographed on alumina. Benzene eluted material, which, from acetone furnished friedelin, m.p. 261–263° (vac.), undepressed on admixture with the authentic compound, $[\alpha]_D$ −21° (*c*, 0.96).

Stereocaulon paschale *(L.) Fr.* 1.4 g of neutral matter was obtained from 360 g of dried lichen. When this was chromatographed, benzene eluted 0.1 g, which was treated with petroleum as above. Undissolved material was crystallised from chloroform/acetone, m.p. 261–263° (vac.), undepressed on admixture with friedelin, $[\alpha]_D$ −18° (*c*, 1.11),

converted to the 2:4-dinitrophenylhydrazone, m.p. 298—299° (dec.), no depression on admixture with friedelin 2:4-dinitrophenylhydrazone.

Isolation of friedelin and cerin from cork. Cork dust (1 kg) was extracted with hot ethyl acetate (compare [5,6]). A small sample of the deposited material was chromatographed on alumina. The crystals eluted with benzene were crystallised from chloroform/acetone, and finally from benzene to give friedelin, m.p. 262—263° (vac.), $[\alpha]_D$ −21° (*c*, 2.40), 2:4-dinitrophenylhydrazone, m.p. 297° (dec.), light abs. max. at 3 710 Å, ε 28 000.

The main part of the material which separated from the ethyl acetate solution was crystallised from chloroform until a substance of constant m.p. 260—262° (vac.), and constant rotation, $[\alpha]_D$ −49° (*c*, 0.68); −50° (*c*, 0.50, 4 dm tube) was obtained (cerin). A mixture of cerin and friedelin melted at 264—275° (vac.), all three taken at the same time.

Friedelane. Friedelin (0.2 g) was heated for 18 hours at 180° with sodium (0.25 g) and hydrazine (2 ml) in ethanol (6 ml). The reaction product, isolated in the usual way, was filtered through alumina in petroleum and the eluted substance crystallised from chloroform, m.p. 245—246°, $[\alpha]_D$ +18° (*c*, 0.57).

SUMMARY

Friedelin has been isolated from the following lichens: *Cetraria nivalis* (L.) Ach., *C. islandica* (L.) Ach., *C. cucullata* (Bell.) Ach., *C. crispa* (Ach.) Nyl., *C. Delisei* (Bory) Th. Fr. [= *hiascens* (Fr.)], *Cladonia alpestris* (L.), Rabh., *Alectoria ochroleuca* (Ehrh.) Nyl. and *Stereocaulon paschale* (L.) Fr.

The occurrence of *epi*friedelinol in *Cetraria nivalis* (L.) Ach. has been demonstrated.

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The Isomeric Friedelanes

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As mentioned in the preceding paper there was observed a discrepancy between the rotation measured by us for friedelane, $[\alpha]_D$ +21°, and the one recorded in literature, $[\alpha]_D$ +42° [1], compare [2]. Furthermore, it had been impossible to prepare solutions for rotation measurements of concentrations above 1 % at 20° (compare [1]). Such attempts invariably led to precipitation of crystals, either at once on cooling to room temperature, or (in cases of the concentration only slightly exceeding 1 %) in the tube. The discrepancy seemed so serious that it was deemed worth while to investigate it more closely, and the more so since the structure of friedelane is unknown.

It has been shown that friedelin should be classified as a pentacyclic triterpenoid ketone, with the formula $C_{30}H_{50}O$ [3-5] and this view has been accepted by Ruzicka *et al.*[1]. Friedelin may be represented by the partially resolved formula —CH—CH—CO—CH_2—CH_2—$C_{25}H_{44}$ [1]. The ketonic function is present in a ring, which must be at least six-membered, since friedelin dicarboxylic acid, represented by —CH—CH—COOH HOOC—CH_2—$C_{25}H_{44}$ on pyrolysis furnished a ketone, represented by —CH—CH—CO—CH_2—$C_{25}H_{44}$ [1], compare also [6] and I.R. evidence of a ketone in a six-membered ring reported in the preceding paper. The partial formula of friedelin may be extended further to —CH—CH—CO—CH_2—CH_2—$C_{24}H_{44}$, since friedelonic (friedonic) acid is a keto-acid [1,7]. Friedelin, therefore, has an enolisable tertiary centre adjacent to the carbonyl group. For this reason there might exist two isomers of friedelin, which could give rise to two isomeric friedelanes. The other isomer of friedelin, however, can be present only in minute amounts, since it has been claimed by Drake and Jacobsen [3] that friedelin is easily obtained in a state of high purity when purified through its enol benzoate, and this statement, that friedelin is regenerated from its enol benzoate, has not been disputed by Ruzicka *et al.* [1]. Nevertheless, such isomer might be predominant under the reduction conditions recorded in literature. It, therefore, was undertaken to repeat as exactly as possible all the reported procedures,

except the reduction of cerin to friedelane by the Clemmensen method. In all cases the main product showed m.p. and rotation values close to those reported in the preceding paper. In the cases of reduction of friedelin and cerin according to Huang-Minlon [2], the mother liquors were successively concentrated and the rotation and m.p. as well as mixed m.p. with a reference specimen were determined (for details see the experimental part). No deviations from the values of the physical constants of the reference specimen could be observed, until 10—20 mg of substance were left. This material consisted almost exclusively of a very much more soluble component, which crystallised in fine needles from chloroform/acetone or from chloroform/methanol.

It should be emphasised that when cerin was reduced, the precautions recommended by Huang-Minlon [2] for alkali-sensitive substances were strictly maintained, whilst they were ignored when friedelin was reduced, in order to obtain as large amount as possible of a second isomer. Apparently there was no difference between the relative amounts of the second isomer in the two cases.

The possibility existed that this new hydrocarbon originated from an impurity in friedelin. To establish whether this were so, friedelin was purified *via* its semicarbazone, which was prepared in analogy with the oxime [4]. Since it melted very unsharply at about 340° (vac.), the m.p. could not be used as a criterion of purity. It, therefore, was crystallised to constant rotation, $[\alpha]_D$ +1.7°. The determination was carried out in 8 % solution and 2 dm tube, *i. e.* with a reading accuracy of ±0.02° $[\alpha]_D$ should be accurate within *ca.* ± 0.1°.

One part of this semicarbazone was reduced according to Huang-Minlon [2], but the yield of hydrocarbon was quite low, 0.1 g from 0.8 g of semicarbazone. When crystallised as above, there were finally left 3—4 mg, which were crystallised from chloroform/methanol to give less than 2 mg of material of m.r. 155—215°. Concentration of the chloroform/methanol mother liquor furnished more material, of m.r. 105—125°.

The other part of the semicarbazone was treated with aqueous oxalic acid [8]. The regenerated friedelin showed m.p. 262—263° (vac.), $[\alpha]_D$—22° after crystallisation from chloroform/methanol, and was reduced according to Huang-Minlon [2]. After crystallisation etc. of the reduction product as above there finally resulted about 20 mg of material from which friedelane was removed as far as possible by treatment with petroleum. Finally there resulted a substance, supposedly the isomer friedelane II, of m.p. 207—208°, $[\alpha]_D$ +2°.

EXPERIMENTAL

M.p.'s are uncorrected. All rotations were measured in chloroform solution in a 1 dm tube at room temperature (20—25°), unless specified to the contrary; the values recorded have been approximated to the nearest degree. Petroleum refers to the fraction of b. r. 40/70°. In all cases of mixed m. p. determination the m. p. of the two compared substances and their admixture were taken all three at the same time.

The isolation of friedelin and cerin as well as the preparation of the reference specimen of friedelane are described in the preceding paper.

Friedelane

a) A specimen of friedelane provided by Dr. J. F. McGhie, through the good offices of Professor D. H. R. Barton and Mr. P. de Mayo, showed m. p. 242—244°, $[\alpha]_D$ +10° (*c*, 0.60). It was crystallised from chloroform, m. p. 247—248°, $[\alpha]_D$ +18° (*c*, 0.50). It gave no depression on admixture with the reference specimen.

b) Friedelane according to Ruzicka *et al.*[1]

The amounts of friedelin and reagents given by these authors were heated to 215° during 2 hours and kept at this temperature for 6 hours. The obtained hydrocarbon, isolated with ether and filtered through alumina in petroleum, was crystallised once from chloroform, m.p. 245—246°, $[\alpha]_D$ +20° (*c*, 0.69), undepressed on admixture with the reference specimen.

c) Friedelane according to Drake and Jacobsen[3]

Friedelin (100 mg) was refluxed in acetic acid (50 ml) with zinc amalgam (prepared from zinc (4 g) and mercuric chloride (4 g) in 50 % ethanol (50 ml)) for three hours. During this time hydrochloric acid (20 ml, *d*, 1.19) was added in portions of 2.5 ml, the last portion 1/2 hour before the reaction was interrupted. The reaction product was isolated and purified as above, and crystallised from chloroform, m.p. 245—246°, $[\alpha]_D$ +19° (*c*, 0.68), undepressed on admixture with the reference specimen.

d) Friedelane according to Huang-Minlon[2]

1. Cerin (0.6 g) was heated for 1/2 hour with diethylene glycol (10 ml) and hydrazine hydrate (1 ml). Then potassium hydroxide (1 g) in conc. aqueous solution was added, and the heating continued for further 20 min., when water was distilled off until the temperature had reached 200° in the vapours at the top of the flask. The reaction mixture was refluxed for 2 hours. The reaction product was isolated and purified in the usual way and crystallised once from chloroform, m.p. 245—246°, $[\alpha]_D$ +22° (*c*, 0.69), undepressed on admixture with the reference specimen.

The mother liquor from this crystallisation was concentrated to about 1/2 of its original volume. The substance which crystallised on cooling, showed m.p. 240—242°, undepressed on admixture with the reference specimen, $[\alpha]_D$ +23° (*c*, 0.70).

The mother liquor was concentrated once more, to a very small volume. The obtained crystals were no longer completely colourless, m.p. 241—244°, $[\alpha]_D$ +26° (*c*, 0.47), undepressed on admixture with the reference specimen.

Finally the mother liquor was evaporated to dryness, and the residue dissolved in a small amount of petroleum. The precipitated, minute amount of crystals was somewhat miscoloured, m.p. 242—244°, no colour with tetranitromethane. The residue melted at 170—190°, $[\alpha]_D$ +17° (*c*, 0.53).

This petroleum mother liquor was concentrated to a small volume, when a substance crystallised on cooling as fine, somewhat felted needles, m.p. 192—195°; a mixture with friedelane melted at 195—235°. The substance was recrystallised from chloroform/methanol, m.p. 193—194°, $[\alpha]_D$ +2° (*c*, 0.65); no colour with tetranitromethane.

2. Friedelin (1 g) was heated for 1/2 hour with diethylene glycol (30 ml), potassium hydroxide (3 g) and hydrazine hydrate (1 ml). Water was distilled off until about 200° in the vapours, and then the heating was continued for 2 hours. The reaction product was isolated and purified as above and crystallised from chloroform, m.p. 245—246°, undepressed on admixture with the reference specimen, $[\alpha]_D$ +22° (*c*, 0.83).

The mother liquor was concentrated. The crystals which separated, melted at 246—247°, undepressed on admixture with the reference specimen, $[\alpha]_D$ +22° (*c*, 0.59).

Again the mother liquor was concentrated. The precipitated substance showed m.p. 242—244°, undepressed on admixture with the reference specimen, $[\alpha]_D$ +23° (*c*, 0.53).

This mother liquor was evaporated to dryness, and the residue dissolved in a little petroleum. On standing a small amount of somewhat miscoloured crystals was obtained, m.p. 241—243°, undepressed on admixture with the reference specimen. The filtrate was concentrated further and another small crop of crystals was obtained, m.p. 240—242°, also undepressed on admixture with the reference specimen.

Concentration of the mother liquor to a very small volume furnished fine, colourless needles, which were crystallised from chloroform/methanol, m.p. 195—196°, $[\alpha]_D$ +14° (*c*, 1.05), recrystallised from chloroform/acetone, m.p. 195—202°, $[\alpha]_D$ +14° (*c*, 0.87).

At this stage of the investigations the rotation of the reference specimen was checked: $[\alpha]_D$ +20° (*c*, 0.64).

Friedelin semicarbazone. Friedelin (3.6 g) and semicarbazide hydrochloride (2.3 g) were heated with benzene (72 ml) and ethanol (18 ml). Sodium acetate (2.1 g) in ethanol (18 ml) was added and the mixture was refluxed for 1 1/2 hours [4]. The reaction mixture was poured into water and the reaction product isolated with benzene. The benzene solution was evaporated until incipient crystallisation. The precipitated substance was filtered after standing for a considerable time, m.p. 342—345° (vac., dec.), $[\alpha]_D$ +2.7° (*c*, 8.09, 2 dm tube), crystallised from chloroform/methanol, m.p. 341—345° (vac., dec.), $[\alpha]_D$ +1.7° (*c*, 8.09, 2 dm tube), recrystallised, $[\alpha]_D$ +1.6° (*c*, 8.05, 2 dm tube); yield 0.9 g. (Found C 75.6, 75.4; H 10.65, 10.7. $(C_{31}H_{53}N_3O)_3 \cdot CH_3OH$ (999.56) requires C 75.7, H 11.09.) The mother liquor from the recrystallisation was evaporated to dryness and the residue, together with some crystallised material, was used for the reduction of the semicarbazone (see below). The remaining mother liquors were combined and evaporated and crystallised to give further 0.3 g of the semicarbazone with $[\alpha]_D$ +1.8° (*c*, 8.49, 2 dm tube).

Friedelane from the semicarbazone. Friedelin semicarbazone (0.8 g, compare above) was heated with diethylene glycol (10 ml) and potassium hydroxide (1 g) until the temperature was 200° in the vapour at the top of the flask. The mixture was refluxed for 2 hours and the reaction product isolated and filtered through alumina as above. 0.1 g was eluted and crystallised from chloroform, m.p. 245—246°, no depression with the reference specimen, $[\alpha]_D$ +17° (*c*, 0.65). Two subsequent concentrations of the mother liquors furnished material of m.p. 244—245° and 240—241°, respectively, no depression with the reference specimen.

The final mother liquor was evaporated to dryness and the residue (3—4 mg) crystallised from chloroform/methanol to give less than 2 mg of m.r. 155—215°. Concentration of the mother liquor afforded material with m.r. 105—125°.

Regenerated friedelin. Friedelin semicarbazone (0.7 g) was heated overnight in a boiling water-bath with oxalic acid (1.5 g) and water (1.1 ml) [8]. The reaction product was isolated with ether and crystallised from chloroform/methanol, m.p. 262—263° (vac.), $[\alpha]_D$ −22° (*c*, 1.16, 2 dm tube).

Friedelane and friedelane II from regenerated friedelin

The regenerated friedelin (0.6 g) was treated with diethylene glycol (50 ml), potassium hydroxide (5 g) and hydrazine hydrate (1 ml) and the reaction product isolated and filtered through alumina (0.5 g) and crystallised as above. The first crystallisate melted at 245—246°, $[\alpha]_D$ +27° (*c*, 0.66), the second at 244—245° $[\alpha]_D$ +28° (*c*, 0.58) and the third at 244—245°, $[\alpha]_D$ +26° (*c*, 0.93). The material in the mother liquor from the last crystallisation was repeatedly crystallised from chloroform/methanol. The product melted constantly at 197—204°, but the rotation steadily decreased. The material, therefore, was repeatedly treated with small amounts of petroleum to remove as far as possible any friedelane present. The success of this procedure depended on the fact that friedelane was much less soluble in petroleum than friedelane II. Finally the material was crystallised three times from chloroform/methanol m.p. 207—208°, $[\alpha]_D$ +2° (*c*, 1.47); +3°, (*c*, 1.09). (Found * C 87.05; H 12.75. $C_{30}H_{52}$ (412.72) requires C 87.3; H 12.7).

SUMMARY

The isomeric friedelanes, friedelane, m.p. 244—245°, $[\alpha]_D$ +22° and, in a state of high purity, friedelane II, m.p. 207—208°, $[\alpha]_D$ +2°, have been prepared.

The author likes to thank Professor N. A. Sörensen for the interest he has taken in this work, and Dr. J. F. McGhie and Professor D. H. R. Barton and Mr. P. de Mayo for samples of friedelin and friedelane.

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The Structure of $Pb(NH_4)_2(SO_4)_2$ and Related Compounds

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Double salts of the type $PbMe_2(SO_4)_2$, where Me represents K or NH_4, have been described in the literature [1], and it is now found that those with Me = Rb or Tl may also be prepared. Although the structure of the potassium compound has been determined by X-ray methods by Bellanca [2], the structure of the other double salts, where the monovalent ion has a somewhat larger ionic radius (about 1.49 Å as against 1.32 Å), need not necessarily be the same. Hence it was thought worthwhile to investigate these double salts by X-ray methods and compare the results with Bellanca's. However, the Rb- and Tl-salts have been investigated by the powder method only because it so far has not been possible to obtain crystals large enough for single crystal work in these cases.

PREPARATION

All the above mentioned double salts were prepared in the microcrystalline state by stirring a precipitate of lead sulphate with a sufficiently concentrated solution of the sulphate of the monovalent ion, filtering, and washing with dilute alcohol. In the case of $Pb(NH_4)_2(SO_4)_2$ rather large single crystals were prepared in the following way. Freshly precipitated lead sulphate was boiled with a strong solution of ammonium acetate and ammonium sulphate; upon filtering the hot solution, to get rid of excess of lead sulphate, and leaving the filtrate to cool slowly, nice crystals with dimensions up to about 1/2 mm precipitated. They were filtered off, washed with dilute alcohol, and dried at room temperature. The usual qualitative tests for NH_4^+, SO_4^{--} and Pb^{++} were all positive, and on heating to about 450° C there was a loss in weight of 30.1 % (the literature [1] gives 30.3 %).

OPTICAL AND OTHER TESTS

Under the polarizing microscope the crystals of $Pb(NH_4)_2(SO_4)_2$ appeared to be hexagonal prisms, uniaxial negative and strongly birefringent. The refractive indices for sodium light were determined by observing the behaviour of 'Becke's line' for the crystals embedded in mixtures of α-monobromo-

naphthalene with paraffin oil or methylene iodide, the refractive indices being measured with a Pulfrich refractometer:

$$n_\omega = 1.721 \quad n_\varepsilon = 1.642$$

The density was determined with a small-scale pycnometer using absolute alcohol as reference substance:

$$d = 3.69 \pm 0.03 \text{ g/cm}^3$$

No piezoelectric effect could be detected by the electrostatic method with a very sensitive Lindemann electrometer [3].

X-RAY INVESTIGATION

Rotation and Weissenberg photographs of single crystals of the ammonium compound were taken in a 57.3 mm camera using unfiltered Cu-radiation. From the diagrams the space group and approximate values of the axes were derived. More exact values were obtained from powder photographs of the same substance in a 19 cm Bradley camera using CoK_α-radiation ($\lambda(K_\alpha)$ = 1.7889 Å). Assuming next that the K-, Rb- and Tl-compounds also are hexagonal with axes not very different from those of the NH_4-salt, powder photographs of these in the same camera could be indexed and hence the space group and crystal axes determined. In all cases the crystals turned out to be hexagonal; as the Weissenberg and rotation diagrams indicated a diad axis or a plane of symmetry, and reflections *(hkil)* were present only for $h-k+l=3n$ where n is a whole number, the space group can be either $R32$, $R3m$ or $R\bar{3}m$, the last being most probable since the single crystals of the K- or NH_4-compound show no piezoelectric effect [2]. The axes of the hexagonal unit cells are given in Table 1. The uncertainty is estimated to be ± 0.01 Å on the a-axes and ± 0.05 Å on the c-axes.

From the density and volume of the unit cell the number of molecules per unit cell is calculated to be 3.00 for the NH_4-compound and it seems reasonable to assume that it is 3 for the Rb- and Tl-salts also.

Taking Weissenberg diagrams about the a-axis and applying a multiple film technique the relative intensities of reflections with indices $(h0\bar{h}l)$ could be roughly estimated by visual comparison with a calibrated scale. However, for small sin ϑ-values the absorption was so strong that in certain cases only a narrow edge of the reflection was recorded on the films. As it seemed rather hopeless to correct for the absorption, the maximum intensity, and not the

Table 1. Unit cell dimensions.

Compound	a-axis	c-axis	Volume of unit cell
$PbK_2(SO_4)_2$	5.49 Å	20.83 Å	544 Å^3
$Pb(NH_4)_2(SO_4)_2$	5.58 Å	21.84 Å	589 Å^3
$PbRb_2(SO_4)_2$	5.60 Å	21.58 Å	585 Å^3
$PbTl_2(SO_4)_2$	5.59 Å	22.12 Å	598 Å^3

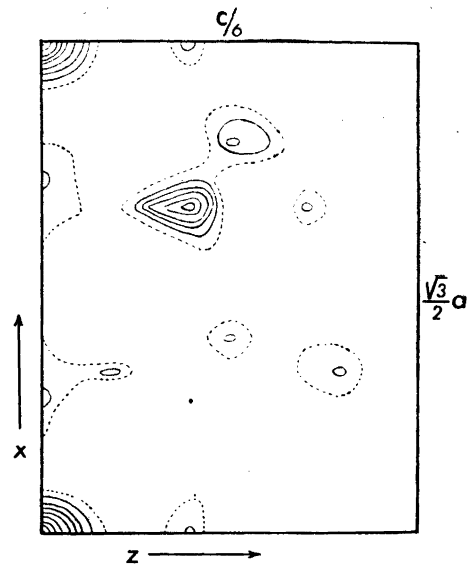

Fig. 1. Electron projection on the $(1\bar{2}10)$-plane. Contour lines are drawn at intervals of roughly 2.5 e/Å² except for the Pb-atoms at the origin where the intervals are roughly 10 e/Å². The broken line is at ca. 2 e/Å².

integrated intensity, has been used for Fourier calculations, the absorption being neglected. These intensities were corrected for the Lorentz and polarization effects. From the $F(h0\bar{h}l)$-values finally obtained an electron projection on the $(1\bar{2}10)$-plane was calculated. The space group $R\bar{3}m$ requires that the Pb-atoms are located in centres of symmetry [4], either (0 0 0), $(\frac{1}{3}\,\frac{2}{3}\,\frac{1}{3})$, $(\frac{2}{3}\,\frac{1}{3}\,\frac{2}{3})$ or in $(0\ 0\ \frac{1}{2})$, $(\frac{1}{3}\,\frac{2}{3}\,\frac{5}{6})$ and $(\frac{2}{3}\,\frac{1}{3}\,\frac{1}{6})$. Assuming the first set to be appropriate all the $F(h0\bar{h}l)$ must be positive because the atomic scattering factor of Pb far outweighs the sum of those of the other atoms. Also, due to the symmetry of the space group, it is sufficient to calculate the projection for x running from 0 to $\sqrt{3}\ a/2$ and z running from 0 to $c/6$.

On the projection the maxima at $(0.66_7;\ 0.06_7)$ and $(0.81_0;\ 0.08_3)$ may easily be identified as due to an S-atom, and two O-atoms projected on top of one another respectively. To fit into this picture the maximum at $(0.39_0;\ 0.08_3)$ then has to be interpreted as an oxygen maximum. The x- and z-coordinates so obtained comply excellently with the space group requirements (see Table 2). The space group symmetry further requires the remaining oxygen atom of the sulphate group, and the nitrogen, to be located on the triad axes through the S-atoms (i.e. $x = 0$; $\frac{1}{3}$ or $\frac{2}{3}$). Of the three maxima $(0.00_0;\ 0.06_7)$, $(0.66_7;\ 0.11_7)$ and $(0.33_3;\ 0.13_3)$ the first two are much too close to either the S-maximum or the Pb-maximum to be consistent with the ionic radius of NH_4^+ (1.43—1.49 Å) so that it seems reasonable to identify the last one as an N-maximum.

Unfortunately the oxygen maximum to be expected on the triad axes is very badly blurred by an extra maximum — presumably due to diffraction effects accompanying the Pb-maximum — and is in fact displaced a little away from the expected x-coordinate. But there seems no reason to doubt that $x = \frac{1}{3}$ is correct, and using $x = 0$ with $z = 0.330$ as a starting point for this

particular atom, all the parameters obtained from the Fourier projection have been finally adjusted by trial and error. The three extra maxima occurring in the projection are all likely to be due to diffraction effects and neglect of the absorption. The following also supports this conclusion: From the atomic parameters obtained above relative $F(hkil)$-values have been calculated and compared with the experimental $F(hkil)$, still neglecting absorption. The quotient $F(hkil)_{calc} / F(hkil)_{obs}$ for high sin ϑ-values is nearly one and shows only relatively small variations, whereas for small sin ϑ-values and strong reflections it can amount to about two. This effect is probably due to absorption because the reflections of low scattering angle are most influenced by absorption. In the worst cases (marked with an asterisk in Table 3) the F_{obs}'s have been corrected so as to give the same quotient as for high scattering angles and a correction to the first Fourier projection calculated on this basis. It then turns out that apart from the two blurred-out maxima which are practically unaffected, the extra maxima are reduced 20—30 % while the oxygen maxima are increased by a similar amount. The Pb- and S-maxima are also increased a little but the N-maximum is slightly reduced. The positions of the maxima, however, are not affected.

Although as explained above the quotient F_{calc}/F_{obs} is larger for small scattering angles than for higher ones, the calculated relative F-values show a variation very similar to that of the observed F's so that the parameters given in Table 2 may be considered fairly reliable.

Table 2. Atomic positions in $Pb(NH_4)_2(SO_4)_2$.

Atomic positions according to space group $R\bar{3}m$:	Values obtained for parameters
(0, 0, 0; $\frac{1}{3}$ $\frac{2}{3}$ $\frac{1}{3}$; $\frac{2}{3}$ $\frac{1}{3}$ $\frac{2}{3}$) ±	
3 Pb in 0, 0, 0	
6 S in 0, 0, z_1	$z_1 = 0.394$
6 N in 0, 0, z_2	$z_2 = 0.200$
6 O′ in 0, 0, z_3	$z_3 = 0.325$
18 O″ in x, $\bar{x}$, z; x, $2x$, z; $2x$, x, z	$x = 0.820$; $z = 0.083$

DESCRIPTION OF THE STRUCTURE

The main feature of the structure is that the lead atoms are arranged in planes perpendicular to the c-axis of the crystal; between successive planes of Pb-atoms there is a 'double layer' of sulphate ions kept together by the positive ammonium ions. This explains the rather strong birefringence.

The lead atoms are coordinated by 6 sulphate groups forming a regular octahedron about the lead. The nearest neighbours of the lead atoms are 6 oxygen atoms, the Pb-O distances being 2.5_2 Å.

The NH_4-ions are irregularly coordinated by 7 sulphate groups and have as nearest neighbours 10 oxygen atoms. Of these, the one lying on the same triad axis as the N-atom seems to be nearer than the others: 2.7_4 Å as against 3.0_5 Å, the latter being somewhat above the sum of the ionic radii: 2.8_0 Å.

Table 3. Calculated and observed relative F(hkil)-values.

Indices	F_{calc}	F_{obs}	Indices	F_{calc}	F_{obs}
0 0 0 3	38	(17) *	4 0 $\bar{4}$ 20	23	21
0 0 0 6	8	9	4 0 $\bar{4}$ 17	12	13
0 0 0 9	24	26	4 0 $\bar{4}$ 14	7	5
0 0 0 12	35	35	4 0 $\bar{4}$ 11	14	11
0 0 0 15	26	34	4 0 $\bar{4}$ 8	24	23
0 0 0 18	20	21	4 0 $\bar{4}$ 5	24	31
			4 0 $\bar{4}$ 2	12	15
1 0 $\bar{1}$ 20	20	21	4 0 $\bar{4}$ $\bar{1}$	14	14
1 0 $\bar{1}$ 17	15	18	4 0 $\bar{4}$ $\bar{4}$	19	18
1 0 $\bar{1}$ 14	15	15	4 0 $\bar{4}$ $\bar{7}$	17	16
1 0 $\bar{1}$ 11	16	12	4 0 $\bar{4}$ $\bar{10}$	20	20
1 0 $\bar{1}$ 8	19	11 *	4 0 $\bar{4}$ $\bar{13}$	20	17
1 0 $\bar{1}$ 5	38	24 *	4 0 $\bar{4}$ $\bar{16}$	20	16
1 0 $\bar{1}$ 2	29	18 *			
1 0 $\bar{1}$ $\bar{1}$	18	11 *	5 0 $\bar{5}$ 13	21	17
1 0 $\bar{1}$ $\bar{4}$	14	8 *	5 0 $\bar{5}$ 10	24	23
1 0 $\bar{1}$ $\bar{7}$	24	15 *	5 0 $\bar{5}$ 7	13	9
1 0 $\bar{1}$ $\bar{10}$	37	20 *	5 0 $\bar{5}$ 4	9	5
1 0 $\bar{1}$ $\bar{13}$	24	17	5 0 $\bar{5}$ 1	15	14
1 0 $\bar{1}$ $\bar{16}$	17	15	5 0 $\bar{5}$ $\bar{2}$	18	17
1 0 $\bar{1}$ $\bar{19}$	15	14	5 0 $\bar{5}$ 5	19	18
2 0 $\bar{2}$ $\bar{20}$	25	19	1 1 $\bar{2}$ 0	38	>18
2 0 $\bar{2}$ $\bar{17}$	13	14	2 2 $\bar{4}$ 0	39	31
2 0 $\bar{2}$ $\bar{14}$	6	3	3 0 $\bar{3}$ 0	31	22
2 0 $\bar{2}$ $\bar{11}$	17	8 *	6 0 $\bar{6}$ 0	25	18
2 0 $\bar{2}$ $\bar{8}$	32	20 *	5 1 $\bar{4}$ 0	24	17
2 0 $\bar{2}$ $\bar{5}$	32	27	6 3 $\bar{3}$ 0	22	23
2 0 $\bar{2}$ $\bar{2}$	11	12			
2 0 $\bar{2}$ 1	18	11 *	Intensities from powder photograph:		
2 0 $\bar{2}$ 4	29	20			
2 0 $\bar{2}$ 7	22	16	Indices	I_{calc}	I_{obs}
2 0 $\bar{2}$ 10	22	21			
2 0 $\bar{2}$ 13	24	20	2 1 $\bar{3}$ 2	26	*m-w*
2 0 $\bar{2}$ 16	24	19	2 1 $\bar{3}$ $\bar{4}$	8	0
2 0 $\bar{2}$ 19	12	8	2 1 $\bar{3}$ 5	30	*m-w*
			2 1 $\bar{3}$ 8	11	*m*
3 0 $\bar{3}$ 18	23	20	2 1 $\bar{3}$ $\bar{10}$	26	*m-w*
3 0 $\bar{3}$ 15	24	23	2 2 $\bar{4}$ 3	8	*vw*
3 0 $\bar{3}$ 12	17	16	2 2 $\bar{4}$ 6	3	0
3 0 $\bar{3}$ 9	20	18	3 1 $\bar{4}$ $\bar{2}$	6	*vw*
3 0 $\bar{3}$ 6	22	18	3 1 $\bar{4}$ $\bar{5}$	15	*w*
3 0 $\bar{3}$ 3	23	17	3 1 $\bar{4}$ $\bar{10}$	17	*w*
3 0 $\bar{3}$ 0	31	33	3 2 $\bar{5}$ $\bar{1}$	3	*vw*?
3 0 $\bar{3}$ $\bar{3}$	28	35			
3 0 $\bar{3}$ $\bar{6}$	23	22			
3 0 $\bar{3}$ $\bar{9}$	15	10			
3 0 $\bar{3}$ $\bar{12}$	17	18			
3 0 $\bar{3}$ $\bar{15}$	27	23			
3 0 $\bar{3}$ $\bar{18}$	23	19			

The sulphate groups are all in pairs lying on triad axes; the plane $(1\bar{2}10)$ bisects the O—S—O angle and passes through the other two O-atoms belonging to the same group. The O—S—O angle can therefore be measured directly on the Fourier projection as the angle between the triad axis through the S-atom

and the S—O″ line; it is found to be 110° ±3°. Within the limits of uncertainty the S—O distances are all the same: 1.5_5 Å.

A list of interatomic distances is given below together with the distances expected from ionic radii or bond lenghts [5]; the uncertainty is estimated to be ± 0.10 Å or less.

Table 4. List of interatomic distances.

Distance	From this investigation	From ionic radii or bond lengths
S—O	1.55 Å	1.51 Å
Pb—S	3.49 Å	
Pb—O	2.52 Å	2.64 Å
O—O	2.55 Å	2.6 Å
N—O′	2.74 Å	2.8 Å
N—O″	3.05 Å	

The double salts containing Rb^+ or Tl^+ instead of NH_4^+ are likely to have the same structure as the NH_4-compound because the ionic radii are nearly identical although the much higher polarizability of the Tl^+-ion may be of some importance in this connection. The structure of $PbK_2(SO_4)_2$ as proposed by Bellanca differs from the one found here for the corresponding NH_4-compound only in the arrangement of the K^+- and SO_4^{--}-ions along the *c*-axis, *i. e.*, the order of these ions on lines through the Pb-atoms and parrallel to the *c*-axis is changed. However, the arguments on which Bellanca has based his structure seem not completely convincing, and above all an electron projection on the $(1\bar{2}10)$-plane seems highly desirable to supplement the information obtained from the Patterson projection. Considering the structural similarities of these double salt crystals one would feel inclined to assume the same atomic arrangement in case of $PbK_2(SO_4)_2$ also, though it must be admitted that the ionic radius of K^+ is smaller than the radii of the other monovalent ions mentioned above.

COMPARISON OF MOLECULAR VOLUMES

Let us finally compare the molecular volumes of the $PbMe_2(SO_4)_2$-compounds obtained from our crystal analysis with the values given in the literature [6] for the single compounds $PbSO_4$ and Me_2SO_4. Although these values are rather old they are supposed to be accurate to ± 1 Å^3 except in the case of Tl_2SO_4 where the uncertainty presumably is larger.

Table 5. Comparison of crystal molecular volumes.

Me	Vol. of Me_2SO_4 V_1	Vol. of $PbSO_4$ V_2	Vol. of $PbMe_2(SO_4)_2$ V_3	$\Delta V = V_1 + V_2 - V_3$
K	108 Å^3	79 Å^3	181 Å^3	6 Å^3
NH_4	123 Å^3	79 Å^3	196 Å^3	6 Å^3
Rb	122 Å^3	79 Å^3	195 Å^3	6 Å^3
Tl	125 Å^3	79 Å^3	199 Å^3	5 Å^3

From Table 5 it is seen that in all four cases of double salt formation there is a significant decrease in volume. This shows that the ions are more densely packed in the double salt crystals than in the pure Me_2SO_4 and $PbSO_4$ separa-

tely and also gives the clue as to why these solid double salts are formed: The closer packing of the ions gives less electrostatic energy and hence greater stability.

SUMMARY

The unit cell dimensions and space group of some double salts of the type $PbMe_2(SO_4)_2$ have been determined by X-ray methods. In case of Me = NH_4 atomic parameters and interatomic distances have been obtained. It is shown that on formation of these double salts from the single compounds there is a decrease in molecular volumes.

The author wishes to thank the director of the laboratory, Professor Dr. phil. A. Tovborg Jensen for his interest in this work and for many helpful discussions.

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Investigations on Barley Malt Amylases and Related Proteins

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Much work has been done on the well known barley and malt amylases, one of the earliest known enzyme systems. Ever since Wijsman [1] showed that there were two different components and demonstrated their different solubilities in ethyl alcohol, there have been many attempts to isolate them: by ammonium sulphate fractionation [2–7], by ethyl alcohol [8,9,3], by tannine precipitation [10,11] and by different adsorption methods [5,11–15]. Important steps also used for the purification are the heating of the solutions to +70° C, which inactivates β-amylase and precipitates a lot of impurities [16], and the dialysis of solutions which removes the globulins [17–19].

Quite recently some investigators have reported crystalline barley malt amylase preparations: Schwimmer and Balls [5,15] have prepared crystalline α-amylase by a combined heating and chromatographic fractionation procedure and Fischer, Meyer, Noelting and Piguet [20] claim to have crystallized β-amylase by combined fractionation with ammonium sulphate and acetone. But these methods involve steps (heating, acetone precipitation) which in general are considered to cause modifications in the physico-chemical state of enzymes.

The aim of this paper is to elucidate the properties of native enzymes and related proteins. For this purpose we have avoided precipitations with organic solvents and heating of solutions. But on the other hand even salting-out may alter the physico-chemical properties of enzymes: consequently the enzymes obtained by these methods are strictly speaking not native [21]. Thus the term native is used only in a relative sense.

The terms, albumin, globulin and glutelin, are defined as follows: albumins are soluble in salt-free water, globulins in approximately neutral salt solutions (pH 5—8) and glutelins in alkaline solutions.

EXPERIMENTAL METHODS

1. *Enzyme assay:* a. *Dextrinizing activity* was determined by a modified Wohlgemuth procedure according to Sandstedt, Kneen and Blish [22]. Spectrophotometric control of colour change according to Hultin [23] was sometimes used. The substrate, limit dextrin, was spectrophotometrically controlled before use. The temperature was +30° C and the pH 5.0.

Specific activity unit: The number of grams of starch hydrolyzed to dextrins, in one second under conditions specified above, to give the standard colour according to Sand-

stedt *et al.*[22] on adding an amount of enzyme preparation containing one gram of nitrogen. The dextrinizing power was used as a measure of α-amylase activity.

Conversion factors: One of our units corresponds to about

14 400 units according to Danielsson and Sandegren [19]
575 » » » Kneen and Sandstedt [24]
6.5 » » » Hultin [23]
1.5 α-amylase units according to Schwimmer and Balls [5]

b. *Saccharifying activity* was determined by a modified Windisch-Kohlbach procedure [25] by titrating against Fehling's solution [26]. Assays were performed at +20° C and at pH 4.5 in 2 % Lintner starch solution.

Specific activity unit: The number of grams of maltose produced in one second under conditions specified above on adding an amount of enzyme preparation containing one gram of nitrogen.

When the saccharifying activity of α-amylase, found to be approximately 0.0190 (± 0.0007) times the dextrinizing activity, was subtracted from the total saccharifying activity, the value found could be used as a measure of the β-amylase activity.

Conversion factors: One of our units corresponds to about

29 000 degrees Lintner (new units: g maltose in 30 min. per 100 g preparation)
575 units according to Kneen and Sandstedt [24].

The conversion factors are only approximate owing to the fact that the units above are defined on the dry weight basis or under other experimental conditions. In the latter case the conversion factors are determined experimentally whilst in the former it is assumed that the nitrogen content is 16 % (really 12—18 %) of the salt-free dry weight.

2. *Determination of nitrogen content.* The nitrogen content of the preparations was estimated by a semimicro Kjeldahl procedure according to Blom and Schwarz [27]. The protein content was estimated by multiplying the nitrogen value by the usual factor 6.25, which however gives only rather approximate values. It can be calculated from the data in Table 3, that this factor really varies from 5.4 to 8.0.

Preparation of the amylase containing fraction of malt proteins

After several experiments it was found to be best to extract the finely crushed malt with distilled water containing 1 g calcium chloride and 0.5 g sodium bromate/l. The yield of α-amylase is said to be higher when the extraction is performed with 1 *M* sodium chloride [28]. The content of globulins in such extracts is however higher. As the purity was more important for our purpose than the quantity, we did not use extraction with sodium chloride. The following preparation procedure was found to be the best one in order to obtain a purified fraction containing maximum amounts of both α- and β-amylase.

1. Extraction: 1 kg malt is mixed with 3 l dist. water containing 0.1 % $CaCl_2$ and 0.05 % $NaBrO_3$ and stirred for 1 hour at 20° C. Centrifugation.

2. Supernatant liquid (yield: 2 l): 410 g ammonium sulphate/l is added at 0° C. (63 % sat.) Centrifugation after two hours. → Insoluble residue; discarded

3. Precipitate: suspended in 250 ml dist. water and dialyzed at 0° C for two days against dist. water containing 0.05 % $CaCl_2$ and $NaBrO_3$. Solution is changed at least three times. The suspension is then centrifugated. → Supernatant liquid: albumins, low molecular compounds, carbohydrates; discarded.

4. Supernatant liquid: the amylase-containing fraction. → Precipitate: globulins, 10—20 % of α-amylase; discarded.

Table 1. Activity yield in the first purification steps.

Preparation phase (see scheme at p. 89)	Volume ml	Total N g/l	α-Activity		β-Activity	
			spec.	yield %	spec.	yield %
Calcium chloride extracted, kilned malt (phase 1)	2 100	1.70	4.4	100	0.72	100
precipitated and dialyzed (phase 4)	680	1.30	14	79	2.3	81
Calcium chloride extracted, freeze dried green malt (phase 1)	3 100	1.57	1.8	100	0.35	100
precipitated and dialyzed (phase 4)	270	3.2	7.2	72	1.56	82

The preparations obtained by this method contained principally albumins but also considerable amounts of globulins soluble in low salt concentrations. They could be precipitated by prolonged dialysis but we did not use this method because α-amylase was also precipitated but not inactivated as stated by several authors [29,30,11].

Two examples of the effect of this procedure are shown in Table 1. The purification is three or four fold and the yield normally about 80 %. The carbohydrate content was about 1—3 % calculated on dry matter.

Precipitation of amylases with salt solutions

As it is known that the solubility of amylases in ammonium sulphate solutions varies with different preparations [31,6], we have made several fractionation experiments with the following types of amylase preparations: I) crude extracts of malt which had been cautiously dried and kilned at +55° C, II) freshly milled green malt, III) green malt dried from the frozen state and IV) commercial barley flour. Some purified amylase-containing fractions corresponding to phase 4 in the scheme on p. 89 have also been fractionated. The experiments were carried out at varying pH values, from 4.9 to 7.3. In some cases tertiary potassium citrate was used instead of ammonium sulphate [32].

The precipitation procedure was generally as follows: In a sufficient number of 25 ml volumetric flasks equal amounts of a known buffered amylase solution and increasing amounts of dry ammonium sulphate (or potassium citrate) were added at +2° C. The flasks were made up to volume with dist. water, the salt cautiously dissolved (foaming must be avoided) and kept in the cold room at +2° C for *ca* 16 hours. The precipitate was then centrifuged off and the enzyme activity in the supernatant was determined.

As the supernatant in some solutions contained so much salt that it interfered with the enzyme assays, a correction factor was calculated by measuring the inhibitory power of different salt concentrations on known enzyme samples of about equal activity and purity. The correction factor was in no cases greater than 20 % of the value.

It can be pointed out that citrate ion, which forms soluble complexes with calcium, did not show any inhibitory power towards the α-amylase. The calcium ion concentration during the fractionation was calculated according to Hastings *et al.*[33] to be not lower than 2×10^{-6} moles in any case. According to Hultin [34] this calcium ion concentration is enough to stabilize the α-amylase, at least at pH 7.3.

The saturated ammonium sulphate solution was considered to be 4.14 molar at +20° C and the saturated potassium citrate 2.92 molar at +20° C.

The results of the experiments are shown graphically in Fig. 1. Most remarkable are the properties of freshly milled green malt. We did not obtain reproducible results with this type of extract. Figures C and D correspond to extracts prepared in exactly the same manner but the precipitation curves are quite different. The curves are also irregular owing that the amylases in these solutions do not follow, not even approximately,

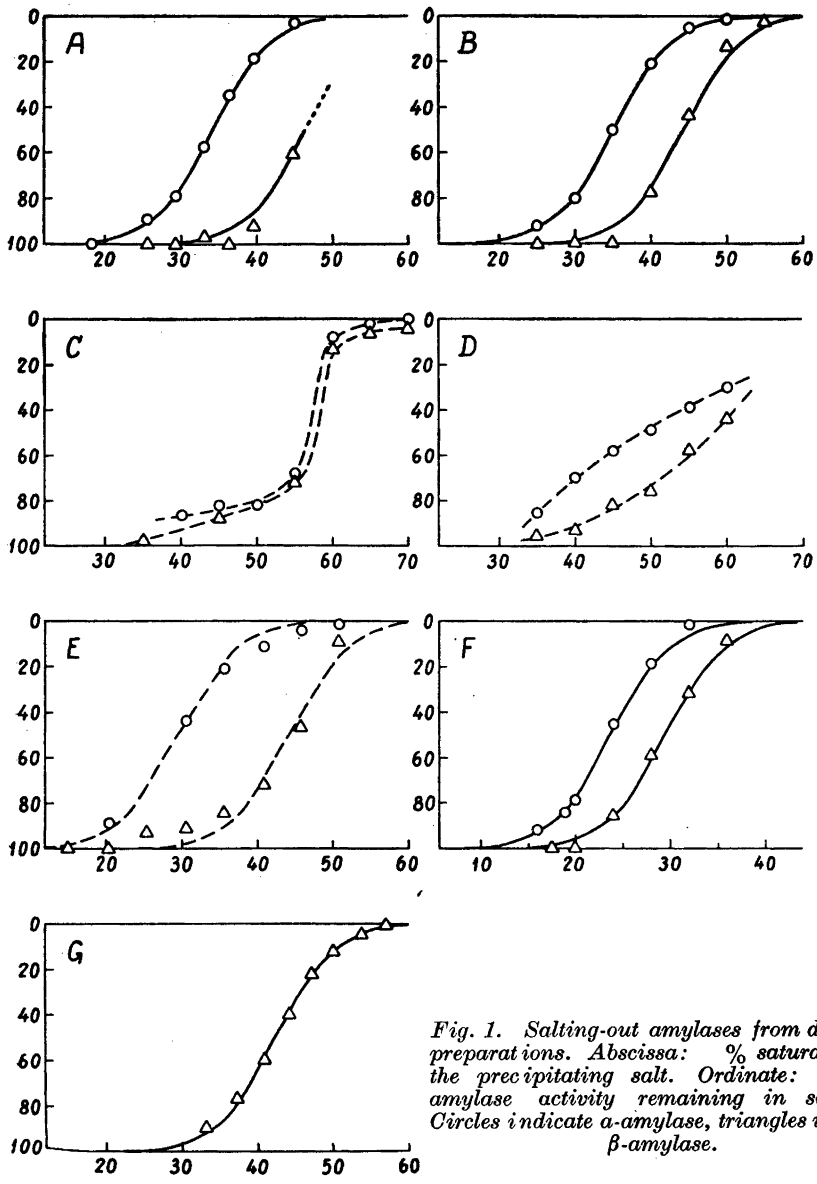

Fig. 1. Salting-out amylases from different preparations. Abscissa: % saturation of the precipitating salt. Ordinate: % of amylase activity remaining in solution. Circles indicate α-amylase, triangles indicate β-amylase.

A. Extract from malt kilned at +55° C, am. sulphate fractionation at pH 5.9—6.1.
B. Extract from freeze dried green malt, am.sulphate fractionation at pH 4.9.
C. Extract from freshly milled green malt, am.sulphate fractionation at pH 4.9.
D. Extract from freshly milled green malt, am.sulphate fractionation at pH 4.9.
E. Malt protein fraction enriched on amylases, am.sulphate fractionation at pH 5.6.
F. Malt protein fraction enriched on amylases, potass.citrate fractionation at pH 7.2—7.4.
G. Extract from commercial barley flour, am.sulphate fractionation at pH 5.6—6.1.

Cohn's solubility law [25]. The solubility also seems to be very high. Some tentative experiments showed, however, that, when the amylases have once been precipitated from these solutions, the solubility is lowered to the normal level.

Our hypothesis concerning these irregularities is as follows: the amylases in the living cells of green malt are in another state than in dried malt (possibly bound to other proteins because a considerable quantity of total protein seems to precipitate together with the amylases). This state is evidently not broken up by milling or crushing but only by drying, salt precipitation or any other kind of dehydratation.

The precipitation curves of the other preparations — those of freeze-dried malt as well as those of kilned malt — have all the normal S-shape showing that the amylases in these preparations follow approximately Cohn's solubility law[25]: $\log S = \beta - K_s \mu$ (where S is the solubility, μ the ionic strength and β and K_s characteristic constants). To lower the amylase content from 90 % to 5 % an increase of the ionic strength by 2.2—2.3 is needed, *i.e.* K_s is really approximately constant. Although in more concentrated solutions the amylases are precipitated by lower salt concentrations, the term β is however not a constant — quite naturally because it is dependent on the variable solid phase. Therefore it seems practically very difficult to obtain constants appropriate for further fractionation experiments.

The results show also that α- and β-amylase are separated most effectively at pH-values 5.6—6.1, *i.e.* near their isoelectric points [19]. The results are well in accordance with those of Kneen, Sandstedt and Hollenbeck [3].

Electrophoretic investigation of the malt protein fraction enriched on amylases

An electrophoretic investigation of the malt protein fraction enriched on amylases was performed in order to obtain knowledge of the character of impurities and of the absolute quantity of amylases in such preparations.

Malt from six-row barley, dried cautiously and then kilned at 55° C (K series) and green malt from two-row barley, dried from the frozen state and milled after drying (F series), were investigated. Fractions of both types of malt are prepared following the scheme on p. 89. The green malt preparation was, however, precipitated once more with 60 % ammonium sulphate in order to obtain a product more free from carbohydrates.

The amylase fraction of kiln-dried malt was further fractionated with potassium citrate. Potassium citrate was used in order to facilitate the determination of nitrogen content in the supernatant liquid. Since the green malt preparation could not be fractionated with potassium citrate because the colloidal precipitate was almost impossible to separate from the supernatant liquid, we used ammonium sulphate.

The fractionation was performed in the following way: the dialyzed amylase-containing solution was diluted to a protein content of 0.5 %. The amylase concentration was thus brought to about the same level as in the 1:3 extracts. Only water distilled in a glass apparatus was used. The sodium bromate concentration was held at 0.5 g/l.

Table 2. Specific and total activities of kiln-dried malt fractions investigated by electrophoresis.

No.	Saturation of potassium citrate	Fraction contains % of total-N	Specific activities		Total activities %		Molisch reaction
			α-Amylase	β-Amylase	α-Amylase	β-Amylase	
K I	21 %	6	66	0.06	39	0.1	weak
K II	28 »	9	50	3.7	42	11	»
K III	32 »	14.5	11	6.2	15	30	»
K IV	36 »	10.5	3.6	12.5	3.5	44.5	»
K V	40 »	13	—	2.4	—	11	»
K VI	58 »	32	—	0.3	—	3.4	strong
Rest	—	15	—	—	—	—	—

Table 3. Specific and total activities of freeze-dried green malt fractions investigated by electrophoresis.

No.	Saturation of ammonium sulphate	Fraction contains % of non-dialyzable dry weight	total N	Specific activities α-Amylase	Specific activities β-Amylase	Total activities % α-Amylase	Total activities % β-Amylase	Molisch reaction
F I	30 %	17.5	20.5	26	0.54	62.5	8.2	weak
F II	37.5 %	11.0	9.2	26	1.7	28.0	11.5	»
F III	42.5 »	13.4	13.0	5.8	2.6	8.6	25.1	very weak
F IV	47.5 »	19.1	18.2	0.42	2.8	0.8	36.2	» »
F V	52.5 »	13.8	13.4	0.09	1.7	0.1	17.5	weak
F VI	62.5 »	14.5	13.7	—	0.12	—	1.4	very weak
F VII	82.5 »	10.1	11.2	—	—	—	—	» »
Rest	—	0.6?	0.6	—	—	—	—	no reaction

Potassium citrate or ammonium sulphate was then successively added to the solutions. The ammonium sulphate fractionation was carried out at pH 4.9, the citrate fractionation at pH 7.2—7.4. The values of the concentration and the activities of fractions are shown in Tables 2 and 3. After each addition of salt the solution was left at +2° C for two days (potassium citrate) or for one day (ammonium sulphate), and then centrifuged. The precipitate was then dissolved in a convenient buffer solution, dialyzed against it and examined by electrophoresis.

It was observed, that the solutions remaining (58 % saturated potassium citrate and 82.5 % saturated ammonium sulphate) contained a lot of nitrogenous degradation products. Jensen [36] made similar observations when fractionating barley proteins. Only a small part of these products could be precipitated with trichloroacetic acid. This is explained by considering the action of the proteolytic enzymes during the slow fractionation procedure and assuming that sodium bromate is incapable of completely preventing their action. It seems however that this action, which during the fractionation in F series degraded nearly 30 % of proteins, does not considerably alter the relative amounts of the different components. The electrophoretic analysis of the initial fraction gave approximately the same result as could be calculated from analyses of all the fractions prepared from it.

The fractions of kiln-dried malt (K series) were examined in phosphate buffer at pH 7.6, with an ionic strength of 0.10 and a sodium chloride concentration of 0.08 *M*. The protein concentration varied from 0.6 to 1.2 %.

The fractions of freeze-dried green malt (F series) were examined in triethanolamine buffer [37] at pH 7.7, with ionic strength of 0.107 and sodium chloride concentration of 0.08 *M*. The protein concentration varied from 1.4 to 2.6 %.

The electrophoretic patterns are shown in Fig. 2 and 3. The composition of the fractions was calculated from the patterns of the negative limb. The ionic mobilities were calculated for cationic components from the positive limb and for anionic components from the negative limb, *i. e.* from ascending boundaries in both cases. This was done as mobility calculations from descending boundaries could not be done exactly due to anomalies caused by high total protein concentrations.

As shown in Fig. 3, there was a very marked anomalous boundary in the triethanolamine buffer in the positive limb (ε-boundary, Svensson [37]), except for the fraction F VII, where the corresponding anomaly was observed in the negative limb.

The results of electrophoretic analyses are shown in Tables 4 and 5.

RESULTS OF ELECTROPHORETIC INVESTIGATIONS

The ionic mobilities of the different components do not vary in a random manner but fall with small variations around certain mean values. The proteins investigated can thus be divided into five groups and each of these into

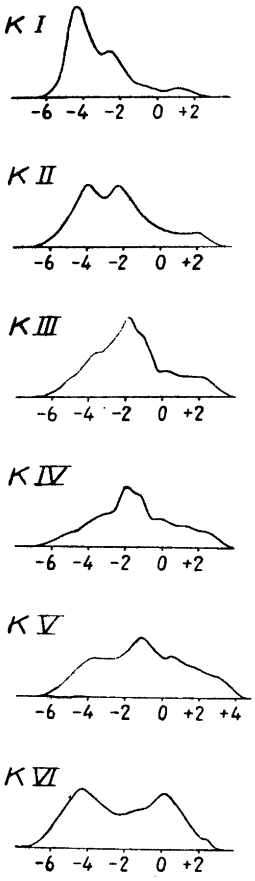

Fig. 2. Electrophoretic patterns of kilned malt fractions in phosphate buffer at pH 7.6. Figures correspond to exposures after 4 hours with a potential gradient of about 5—6 volt cm^{-1}. The abscissa values represent ionic mobilities (× 10^5).

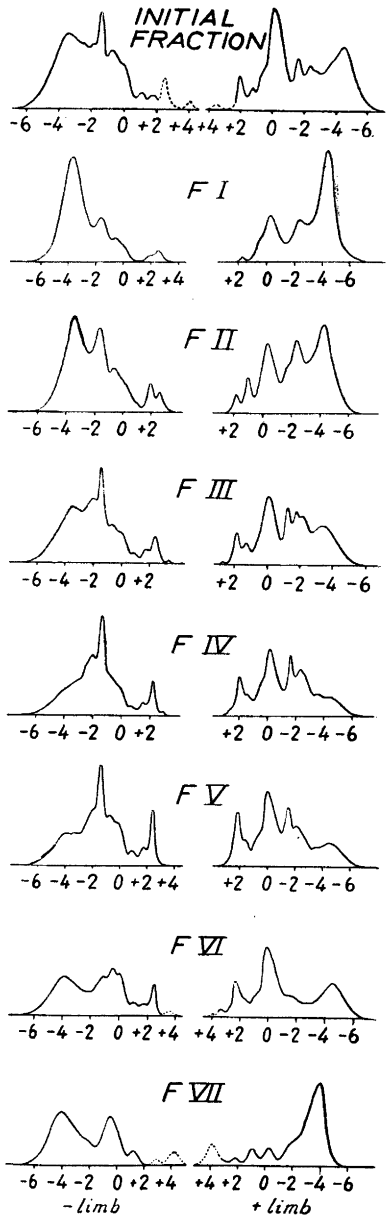

Fig. 3. Electrophoretic patterns of freeze-dried green malt fractions in triethanolamine buffer at pH 7.7. Exposures and ionic mobilities as in Fig. 2.

Table 4. Electrophoretic analyses of kilned malt fractions corresponding to Fig. 2. Ionic mobilities (× 10^5) in the column headings. Amounts of components in each fraction expressed as percentages of total N.

No. of fraction	% N in fraction	Group A		Group B		Group C		GroupD	GroupE
		a −(4.7 −3.8)	b −(3.6 −3.5)	a −(2.5 −2.2)	b −(2.0 −1.8)	a −(1.1 −0.6)	b −0.2 −+0.3	−(0.6 −2.0)	+(2.3 −3.0)
K I	6	4.1		1.3		0.2	0.4		
K II	8.7	3.8		2.5			1.3	1.1	
K III	14.3	1.3	3.4		5.2	0.4	2.1	1.9	
K IV	10.4		3.1		2.2	1.5	1.4	1.3	1.9
K V	13.1	3.9				3.9		4.5	0.8
K VI	32.2	23.2			1.5	1.9	4.7		1.0
Rest (not precip.)	15.3								
%of total N in each mobility group		36.3	6.5	3.8	8.9	7.9	9.9	8.8	2.7
%of precipitated N in each mobility group		43	7.5	4.5	10.5	9.5	11.5	10.5	3.0

two subgroups, as shown in Tables 4 and 5. These groups are also well distinguished in the pattern of the initial fraction in F series. The analysis of this fraction is also well in agreement with the sum of the analyses of all the fractions prepared from it as seen in Table 5.

None of these groups correspond, however, to any defined protein component. If the precipitation curves for all the groups are drawn there cannot

Table 5. Electrophoretic analyses of freeze-dried green malt fractions corresponding to Fig. 3. Ionic mobilities (× 10^5) in the column headings. Amoun s of components in each fraction expressed as percentages of non-dialyzable dry matter.

No. of fraction	% Dry weight in fraction	Group A		Group B		Group C		Group D		Group E	
		a −(4.7 −4.0)	b −(3.7 −3.4)	a −(2.8 −2.2)	b −(1.8 −1.5)	a −(0.8 −0.4)	b 0.0 −+0.2	a +(0.9 −1.3)	b +(1.6 −2.0)	a +(2.5 −2.7)	b +(3.0 −3.5)
Initial fraction	100	41	4	17	10	8	7	4.5	2	6	0.5
F I	17.4	12.4		2.4	0.1	0.8	0.8		0.2	0.3	
F II	11.0	6.5		2.4		0.9	0.5	0.1	0.4	0.2	
F III	13.4	6.4		2.0	1.7	0.7	1.1	0.2	0.5	0.7	
F IV	19.1	4.5	1.4	3.8	3.8	1.0	1.7	0.6	0.5	1.5	0.3
F V	13.8	3.4	1.0	2.3	1.9	1.5	1.4	0.5	0.5	1.3	0.1
F VI	14.5	5.7		1.2	1.9	1.9	1.8	0.6	0.3	1.2	
F VII	10.1	5.7		1.9		0.9		0.8		0.2	0.5
Rest (not precip.)	(0.6?)										
% of dry weight in each mobility group		44.6	2.4	16.0	9.4	7.7	7.3	2.8	2.4	5.4	0.8

be found any tendency towards regular S-shaped curves, as found for amylases (Fig. 1, p. 91) in similar conditions. Thus it can be concluded that the initial fraction comprised at least several tens of components of albumins and easily soluble globulins.

Group A comprises components with ionic mobilities from -4.7×10^{-5} to -3.4×10^{-5}. It is the largest group: 50 % in kilned malt, 45 % in freeze-dried malt. It contains both globulins precipitated at rather low salt concentration as well as albumins precipitated first at rather high concentrations.

The globulins of this group are carriers of the brownish colour which is intensified by oxidation. A part of them has a solubility very similar to that of α-amylase (*cf.* p. 98), and an ionic mobility and a sedimentation constant (6.1 *S*) resembling those of β-globulin as determined by Quensel [38]. They can be divided into several components by electrophoresis at a pH of 5.9.

The albumins of group A are rather ill-defined components. Some of them at least have apparently rather high diffusion constants, as evidenced by the relatively rapid spreading of the boundary (observed in electrophoresis apparatus before the connection of the current). Consequently it can be supposed that they represent (at least partially) relatively low molecular components. They are present also in unmalted barley.

The B group includes components within the mobility range, -2.8×10^{-5} to -1.5×10^{-5}. It contains mostly albumins but also globulins. Both of the amylases belong to this group. This group is present principally in fractions precipitated between 40 and 60 % saturated ammonium sulphate (30 and 40 % saturated potassium citrate). They seem to be better defined components than those of the A group. Their amount is about 15 % in kilned malt, and 20 % in freeze-dried green malt fractions.

The proteins of the C group with mobilities ranging from -1.1×10^{-5} to $+0.3 \times 10^{-5}$ are present in all the fractions. The true percentage of proteins of this group is probably much lower (about 10 %) as shown in Tables 4 and 5, owing to the fact that the false δ and carbohydrate boundaries (especially in fraction K VI) are immobile. At least in the Ca group there are however some true albumin components, one of them following the β-amylase in further fractionation.

D and E groups include proteins of basic character. Some of them with relatively high mobilities at pH 7.6 (as for example a very well defined component in group Ea) must have their isoelectric points rather far on the basic side. Their amount is 12—13 % in fractions from both types of malt and is

Table 6. Ionic mobilities of α- and β-amylases in some different fractions, expressed in cm volt^{-1} sec^{-1}.

Buffer and malt type	Mobility of α-amylase	Mobility of β-amylase
Phosphate buffer, pH 7.6, kilned malt fractions (K series)	2.5×10^{-5}	2.0×10^{-5}
Triethanolamine buffer, pH 7.7, freeze dried green malt fractions (F series)	2.2×10^{-5}	1.9×10^{-5}

not diminished by prolonged dialysis: consequently there are no globulins. They are completely and reversibly precipitated by salts, which excludes the possibility that they are an easily soluble glutelin fraction. They are also present in barley.

The ionic mobilities of α- and β-amylase in some fractions were determined according to the method of Danielsson and Sandegren [19]. The results are shown in Table 6.

DISCUSSION

The results show clearly the complex nature of malt albumin. The conclusion is valid even for barley albumins because it has been found that barley and malt albumin contain the same principal components (*cf.* p. 102 and Fig. 6). Barley albumin or leucosin, prepared according to Osborne [39] cannot be regarded as a more or less homogeneous fraction with an isoelectric point at pH 4.5, as has hitherto sometimes been supposed [40,41].

The results also show that the globulin fraction precipitated between 15 and 40 % saturated ammonium sulphate contains several globulins, some of which have a certain resemblance to the β-globulin of Quensel [38].

When comparing the electrophoretic patterns of kilned malt fractions with those from freeze-dried green malt it was observed that the peaks were much more distinct and characteristic in the latter, *i. e.* the properties of malt proteins are altered during heating to 55° C.

An analogous observation has been made by Briggs and Mann [42,43], who have found that the originally distinct peaks in patterns of soybean extract partially combine when the extract is heated. They suppose that this is due to interaction between components with different mobilities. This explanation seems not to be valid in our case. The peaks of kilned malt fractions are larger than those of green malt indicating greater inhomogeneity. But if we suppose that the rather high kilning temperatures give rise to denatured components which then are partially regenerated, there may be components with slightly higher and lower mobilities than the original component [44,21]. In this way the peaks become larger and lower.

The oxidation may also be a cause of the alteration of the electrophoretic mobilities. We have found that the mobility of Aa component in fraction F I is raised from -4.3×10^{-5} to -5.5×10^{-5} when the solution is exposed to air in an open beaker at +2° C for three weeks. Simultaneously the brownish colour is intensified.

We should not exclude, however, the possibility that there could be molecular associations. We have found that a fraction similar to K I when submitted to electrophoresis at pH 5.9 gives a more complicated pattern with several components. But the nature of the whole salt soluble barley protein fraction is evidently so complicated that more penetrating investigations are needed before more exact theories can be built up. The complexity of the salt soluble barley protein fraction has been recently emphasized by some authors [45–48].

ALPHA-AMYLASE

A. Some purification experiments

As already pointed out, crystalline α-amylase has been prepared by Schwimmer and Balls [5]. However this preparation as also most other purified preparations has been obtained by heating the solution to +70° C whilst there are in literature very few recent investigations of the properties of unheated, "native" α-amylase.

a) *Salting out:* Repeated fractionation with ammonium sulphate of fractions similar to K I and F I has not led to essentially purer preparations. A globulin of Aa-group seems to have a solubility very similar to that of the α-amylase, and even three times reprecipitated fractions contained 60 % of this protein.

b) *Isoelectric precipitation:* As the globulins of Aa-group have some properties similar to those of the β-globulin of Quensel [38], we tried dialysis against very dilute calcium acetate buffer ($\mu = 0.002$) at pH 4.9 (the isoelectric point of β-globulin). The fraction precipitated was however small and contained some α-amylase.

c) *Adsorption:* We could not use the adsorption in ethanol solutions [13,5] because the native α-amylase is precipitated even by 15—20 % ethanol. Some preliminary experiments (made when investigating the adsorption properties of β-amylase) showed that α-amylase is adsorbed almost completely by diatomaceous earth (Standard Super-Cel) in 5 % ammonium sulphate solution at pH 4.3. The yield by elution was however never higher than 60 %, which indicates that partial inactivation takes place. Thus the adsorption experiments were abandoned.

d) *Electrophoretic separation:* It seems probable that, using a suitable apparatus, the electrophoretic purification of fractions similar to K I might give highly active α-amylase preparations. We made an attempt at pH 5.9, but at this pH the proteins of the Aa-group migrated in both directions. The purification was only twofold. At pH 7—8 better results might however be obtainable.

B. Inactivation of α-amylase by dialysis

Several investigators [29,30,11] have found, that α-amylase is inactivated by dialysis. According to our experience, however, the dialysis is not inactivating if calcium ions are not removed completely and if the water used is of the highest purity. The inactivation observed by the cited authors may be due to the following factors:

1) The native, unheated α-amylase is precipitated by dialysis in the same manner as many other easily soluble globulins. The precipitate is partially soluble in 1 *M* sodium chloride solutions. But even the finely divided insoluble suspension particles retain their activity. They can be centrifuged and resuspended without any loss of the total activity.

2) α-Amylase is inactivated irreversibly when it is dialyzed in such a way that the calcium ions are completely removed [11,34]. The inactivation is observed when dialyzing against oxalate buffer, against distilled water which is often renewed or provided with a mixture of anionic and cationic exchange resins, or when electrodialyzing the solutions [29].

3) When purified α-amylase preparations are dialyzed against water distilled in a metallic apparatus, a loss of activity is often observed. This may be due to the action of heavy metal cations. The losses are avoided by using water distilled in glass.

We have never observed that α-amylase could pass through cellophane membranes during the dialysis as suggested by Hultin [11].

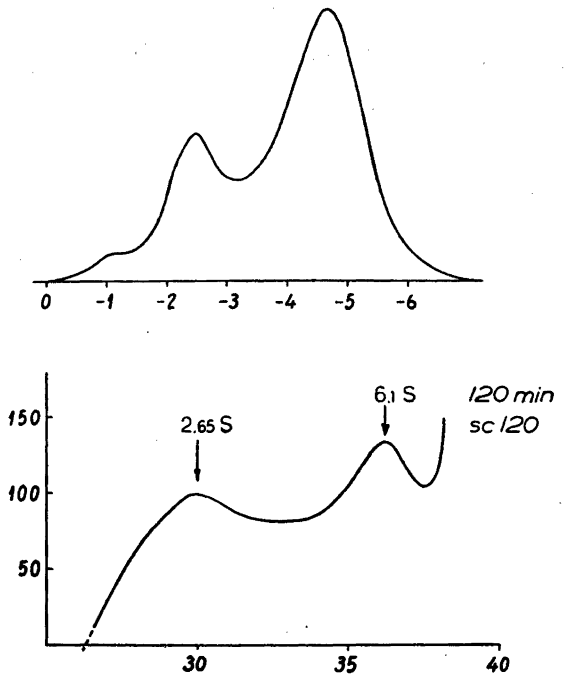

Fig. 4. Electrophoretic and ultracentrifugal patterns of the fraction K I. Electrophoresis was performed in phosphate buffer at pH 7.6. Ionic mobilities ($\times 10^5$) and corrected sedimentation constants (S_{20}) are marked in the figure.

C. On the chemical nature and absolute purity of α-amylase

Since the investigations of Osborne [17,18], α-amylase has been considered an albumin, although Osborne pointed out that the dextrinizing activity did not follow the albumin fraction as closely as did the saccharifying activity. But the experimental facts mentioned above indicate that the native α-amylase is more like a globulin: it is precipitated by dialysis (even in the presence of small amounts of calcium ions) as well as by ammonium sulphate and ethanol in low concentrations. Also the stability of α-amylase at relatively high temperatures is a property characteristic of globulins.

The electrophoretic pattern of fraction K I seemed rather simple: it showed a major fast-moving component and a minor component with the same mobility as α-amylase. When examined in the ultracentrifuge *, this fraction showed again two main components which were however not well defined, as is seen in Fig. 4. The major component had a sedimentation constant of 6.1 *S* and the minor, 2.65 *S*. We believe, however, that the similarity of electro-

* The ultracentrifugal runs were performed at the Institute of Physical Chemistry of Upsala University. For this help the authors wish to express their sincere thanks.

phoretic and ultracentrifugal patterns is accidental because it is hardly probable that the slower sedimentating minor component could be native α-amylase. Estimating from the sedimentation constant, the slower component must have a molecular weight much lower than 60 000, which value was found by Schwimmer and Balls [15] for heat-treated, crystallized α-amylase.

Hultin [41] has estimated that the pure α-amylase must have an activity greater than that of the crystallized α-amylase prepared by Schwimmer and Balls [15]. By calculating in a similar manner from the specific activity and the electrophoretic pattern of fraction K I, we have found a value which is also slightly higher than that of Schwimmer and Balls. But this value is based on the assumption that the slow moving component in this fraction is entirely α-amylase. Electrophoretic and ultracentrifugal experiments, however, have shown that this is probably not the case (see p. 98 and Fig. 4). Thus it should be possible to isolate a more active α-amylase than that prepared by Schwimmer and Balls. Using the experimentally determined conversion factors (p. 89) the specific activity of crystallized α-amylase was calculated to be 250 g sec^{-1}/g N, the corresponding value of pure α-amylase predicted by Hultin, should be 520 g sec^{-1}/g N, and the value based on our experiments, 310 g sec^{-1}/g N.

BETA-AMYLASE

A. Purification experiments

a) *Salting out.* The electrophoretic examination of malt albumin fractions has shown that there are several protein components with solubility and electrophoretic properties similar to those of β-amylase. Repeated salting out and subsequent electrophoretic control has shown that there is little chance of obtaining in this way essentially better β-amylase preparations than *e. g.* fractions K IV and F IV (*cf.* p. 94). These observations are in accordance with the findings of Meyer, Fischer and Piguet [7].

b) *Dialysis.* As the β-amylase is a typical albumin, the globulins can be eliminated by dialysis. This is effectively performed with the aid of ion exchange resins. After the main part of the salts has been removed by usual dialysis, a suitable mixture of cation and anion exchange resins is put into the dialysis vessel (one volume of IR-120 and three volumes of IRA-400, supplied by Rohm and Haas Co). The dialysis is then continued for 3—5 days and the precipitated globulins centrifuged off and discarded.

c) *Alcohol precipitation.* In order to eliminate some impurities, especially globulins of group A, we have found it advantageous to use ethanol in low concentrations, *i. e.* in 15—20 % solutions (these operations are carried out in a cold room, as also the other operations). Under these conditions the β-amylase is not precipitated and its properties are not altered.

d) *Adsorption.* A lot of work was made in order to find out a suitable selective adsorbent for β-amylase. The following substances were tried: wheat starch, paper pulp, silica gel, silicous earth, aluminium oxide (γ-modification), aluminium hydroxide, and freshly precipitated zinc sulphide. The most effective adsorbent was zinc sulphide, but it had also a strongly inactivating effect. Silicous earth (Standard Super-Cel) showed in the first experiments very promising results: the adsorption rate was sufficient and the inactivation of adsorbed preparations was almost negligible at first. However, when applying the chromatographic techniques the inactivation rate was increased, probably due to successive adsorptions and elutions. As the purifying effect of these methods was also found to be rather slight, they were abandoned. The salting-out adsorption technique of Shepard and Tiselius [49] showed a pronounced inactivation effect. Meyer, Fischer and Piguet [7] had similar experiences when trying to purify β-amylase by adsorption.

One of the β-amylase preparations, made by the chromatographic column technique using zinc sulphide precipitated on diatomaceous earth as adsorbent, was investigated by

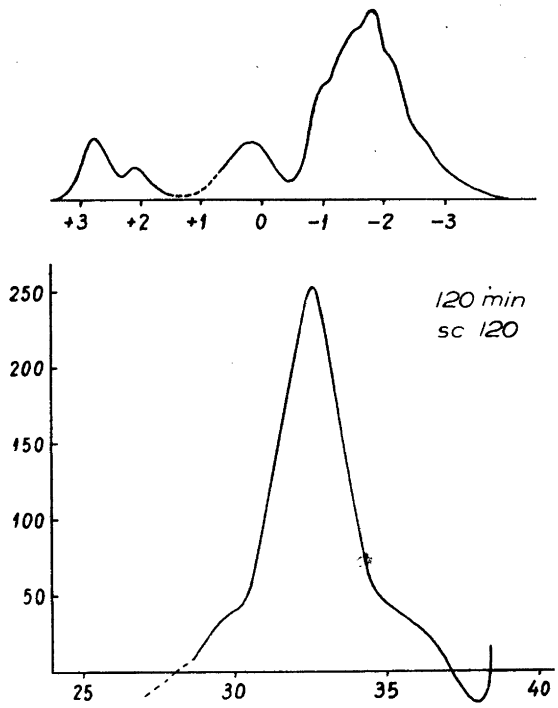

Fig. 5. Electrophoretic and ultracentrifugal patterns of a β-amylase-containing fraction prepared by adsorption technique (see text). Electrophoresis was performed in phosphate buffer at pH 7.6; exposure after 6 hours. Ionic mobilities (× 10^5) are marked in the figure. Sedimentation constant s_{20} = 4.5 S.

electrophoresis (at pH 7.6) and in the ultracentrifuge. Both patterns are shown in Fig. 5. The preparation had a specific activity of 12.5 g sec^{-1}/g N and was probably partially inactivated. The electrophoresis diagram shows several components but the ultracentrifuge pattern only one very sharp peak. The sedimentation constant 4.5 *S* is in accordance with the value of Danielsson [52] for a common malt albumin preparation. The highest peak in the electrophoretic pattern has the same mobility as that of β-amylase.

B. A stable β-amylase preparation from barley and malt

A useful β-amylase preparation (for the purpose of α-amylase determination) can be made from the water extract of barley flour by taking the fraction between 30 and 57 % saturated ammonium sulphate and dialyzing this product successively against dilute (0.01 *M*) oxalate buffer at pH 3.6 (2 hours), 0.01 *M* sodium acetate (2 hours) and then against distilled water which is changed 3—4 times and finally provided with a suitable mixture of cationic and anionic exchange resins (as mentioned above).

Small amounts of impurities (of group A) can be removed by precipitating with 20 vol % ethanol at 0° C before dialysis. However, the ammonium sul-

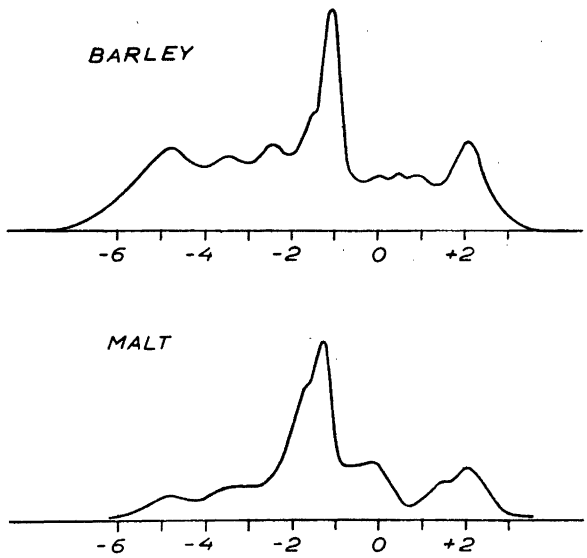

Fig. 6. Electrophoretic patterns of barley and malt albumin fractions (see text). Phosphate buffer, pH 7.6, potential gradient 5.5 volt cm^{-1}. Exposures after 4 hours.

phate must be removed by dialysis before the addition of ethanol in order to avoid denaturation.

The electrophoretic pattern of such a barley β-amylase preparation is shown in Fig. 6 and compared with that of a similar malt β-amylase preparation. (Ethanol precipitation was used in both cases). Fig. 6 clearly shows the similar character of barley and malt albumins (*cf.* p. 97). The differences observed may be due to slight differences in the preparation method.

The specific activities of the final barley and malt products shown in Fig. 6 were 5.0 and 12.5 g sec^{-1}/g N respectively. The malt β-amylase still contained slight traces of α-amylase but the barley preparation was completely free from it. We have found that the barley β-amylase is stable for at least a year (even in aqueous solution).

C. On the absolute purity of β-amylase

Fischer, Meyer, Noelting and Piguet [7,20] claim to have prepared crystalline, pure malt β-amylase. The purification was 15-fold, calculated on the basis of the starting material, a commercial malt extract which probably had an activity of the same order of magnitude as the usual extracts (estimated by a rough calculation from the data of Fischer, Meyer *et al.* [7]).

As we obtained by simple ammonium sulphate fractionation and subsequent dialysis about 20-fold purification (fraction K IV), their results seem somewhat doubtful. Further, because the component with the same mobility as β-amylase amounts to only 21 % of the total protein in this fraction, the specific activity of pure β-amylase must be at least 100 times higher than that

in crude extracts. We have calculated the minimum value to be 60 g sec^{-1}/g N but the true activity is probably still higher (*cf.* p. 96).

By fractionation with ammonium sulphate and ethanol, Caldwell and Doebbeling [9,50] have prepared a β-amylase with an activity 130 times higher than that of crude malt extracts. This cereal β-amylase seems to be the most pure hitherto prepared. A "malt amylase" of Holmbergh [51], containing also α-amylase, had also a remarkably higher specific activity than that of the β-amylase of Meyer, Fischer *et al.* and those of our preparations.

On the basis of our experiments we consider that the crystallized β-amylase of Meyer, Fischer *et al.* is a mixture of β-amylase and very similar albumins. As mentioned before (p. 100), the other albumins of Bb-group (and also some of Ca-group) are very difficult to separate from β-amylase. Further, we know, according to Danielsson [52] and our own experiments (Fig. 5), that a great part of these proteins have the same sedimentation constant (4.5 *S*) and probably also the same molecular weight as β-amylase. Thus, the formation of mixed crystals would readily occur. It is possible that there is some intimate correlation between β-amylase and these albumins (which form the main part of barley and malt albumin) because it has been found by Sandegren and Klang [53] that the contents of β-amylase and of barley albumin are closely related.

SUMMARY

In trying to elucidate the properties of native barley malt amylases we have investigated the protein fraction of malt having similar solubility properties, *i. e.* precipitated by 60 % saturation with ammonium sulphate and remaining in solution when dialyzed against water containing 0.1 % calcium chloride and 0.05 % sodium bromate ($\mu \sim 0.03$).

1) A partial separation of α- and β-amylases can be obtained by salting-out from different extracts and from partially purified fractions. The precipitation curves have in general a regular shape. The separation is most effective in pH region, 5.6—6.1.

2. The precipitation of freshly milled green malt extracts by ammonium sulphate seems to be rather variable and irregular. This is supposed to be due to the combination of amylases with other proteins in the living cells.

3. The amylase-containing fraction is very complex when examined electrophoretically. Several tens of components, albumins as well as globulins, were found. 12—13 % of basic albumin-like proteins are also present.

4. The proteins of kilned malt are partially denaturated judging by the electrophoretic patterns.

5. The solubility properties of the native α-amylase are similar to those of a globulin. It is precipitated by dialysis but not inactivated provided that calcium ions are not completely removed. A protein fraction somewhat ressembling β-globulin is coprecipitated with the native α-amylase.

6. The native β-amylase is a typical albumin. The fraction enriched on β-amylase contains several proteins with similar solubility properties to β-amylase and with almost identical sedimentation constants. The specific activity of pure β-amylase is estimated to be at least 100 times that of protein in water extracts from malt with high amylolytic activity.

7. The corresponding fraction of unmalted barley seems to have a very similar composition to that of malt.

The authors wish to express their thanks to the head of the laboratory, Dr. Sven Brohult, and to Dr. Evald Sandegren, head of the laboratory of the *Stockholm Brewing Company*, for their encouraging interest in our work and for valuable advice and discussions.

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On the Nature and Activation of the Phosphatase of *Torulopsis utilis*

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In a previous communication [1] the increase in the phosphatase activity of *Torulopsis utilis* cells during starvation in respect to phosphorus was described. This interesting phenomenon led us to a more detailed study of the nature of the *Torulopsis* phosphatase, as well as of the factors affecting its activation and inhibition.

EXPERIMENTAL

The cultivation of the yeast mass was carried out in Kluyver flasks at 29° C with aeration (200 l/hour). The pH was kept within the range 4.5—5.0 with sodium hydroxide. The *normal yeast* (yeast with normal phosphorus content) was cultivated in a solution containing 50 g glucose, 3 g ammonium hydrogen phosphate, 4 g ammonium sulfate, 1.5 g potassium sulfate, 1 g magnesium sulfate and 0.5 g calcium chloride per litre of tap water (*cf.* Roine [2]). The *low-phosphorus yeast* (phosphorus-starved yeast) was cultivated by placing washed normal yeast in a medium of the above composition but devoid of phosphate salt. Cultivation was continued until growth ceased owing to the exhaustion of the cells in respect to phosphorus.

In the experiments *lyophilized cells* and *cell-free extracts* of the cells were used. The extracts were obtained by disintegrating a 30 % suspension of fresh cells in distilled water 30 minutes in a Raytheon sonic vibrator at 9 kilocycles. After 10 minutes' centrifugation at 16 000 rpm in a refrigerated centrifuge, the clear supernatant was decanted.

Phosphatase activity was measured in veronal buffer, with disodium phenylphosphate as the substrate. The liberated phenol was determined with the Folin-Ciocalteau phenol reagent. When the effect of activators or inhibitors was to be studied, the enzyme preparation was incubated with these substances for 30 min. at 37° C before the substrate was added.

All results are given in Rae-Eastcott units: the amount of phenol in mg liberated per 10 mg of the dry preparation from a 0.01 *M* solution.

RESULTS AND DISCUSSION

The phosphomonoesterases are classified according to the dependence of their activity on the hydrogen ion concentration as well as on certain divalent metals, of which magnesium is the most important (*cf.* Roche [3]). In a prelimi-

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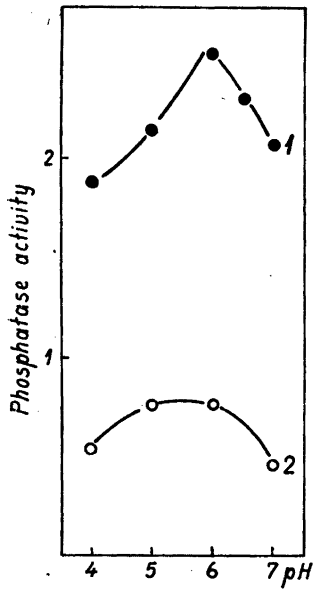

Fig. 1. Dependence of the phosphatase activity of low-phosphorus cells on hydrogen ion concentration in the presence and in the absence of magnesium.

Curve 1: With 10^{-2} M $MgCl_2$
Curve 2: Without magnesium.

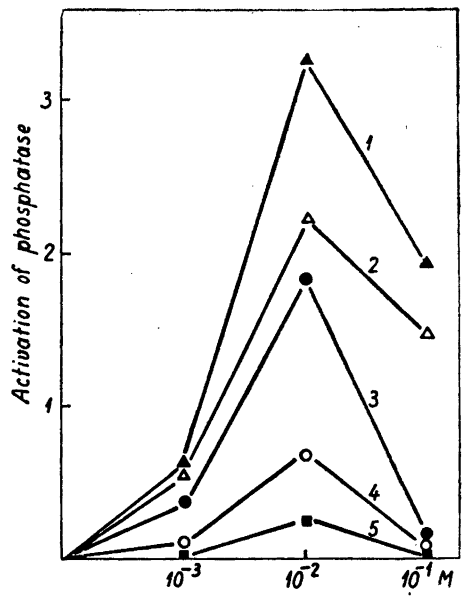

Fig. 2. Activation of the phosphatase of normal and low-phosphorus cells by manganese, magnesium and calcium at different concentrations.

Curve 1: Low-P cells with $MnSO_4$
Curve 2: Normal cells with $MnSO_4$
Curve 3: Low-P cells with $MgCl_2$
Curve 4: Normal cells with $MgCl_2$
Curve 5: Low-P cells with $CaCl_2$

nary test, it was found that magnesium has a stimulatory effect on the *Torulopsis* phosphatase. Owing to the minimal activity of the normal cells, the dependence of the phosphatase activity on the hydrogen ion concentration was studied with the low-phosphorus cells in the presence (10^{-2} *M*) and in the absence of magnesium (Fig. 1).

The pH optimum lies between 5 and 6, probably at about 6.

In addition to magnesium, some other divalent metals appeared to activate this phosphatase. The effects of these on the normal and low-phosphorus cells at different concentrations are illustrated in Fig. 2.

Manganese is the most potent activator. The activation of the almost inactive normal cells by manganese is considerably greater than the activation of the active low-P cells by magnesium. Calcium has a slight but distinct effect on the low-P cells, but no effect on the normal cells. All these cations cause the maximal activation at approximately the same concentration (10^{-2} *M* or a little higher). A negligible effect of zinc can be observed at a lower concentration (0.06 units at 10^{-4} *M* $ZnSO_4$) and a similar effect of cobalt at a higher concentration (0.05 units at 10^{-1} *M* $CoCl_2$).

Table 1. Dependence of the phosphatase activity of lyophilized cells and cell-free extracts on their phosphorus contents in the presence and in the absence of magnesium.

	Total P % of dry matter	Phosphatase activity No addition	10^{-2} *M* $MgCl_2$	Activation by Mg
Lyophilized cells				
Normal	1.97	0.09	0.81	0.72
Low-P	0.29	0.86	2.46	1.60
Cell-free extracts				
Normal	4.03	0.05	0.78	0.73
Low-P	0.73	1.48	5.31	4.53

According to Folley and Kay [4], four main types of isodynamic phosphomonoesterases may be distinguished on the basis of pH activity relationships and specific activation with the divalent metals. In regard to its pH optimum, between 5 and 6, and its activation by magnesium and manganese, the phosphatase of the yeast *Torulopsis* resembles the phosphatase found, for example, in bottom yeast and red blood cells, and belongs to the fourth group.

It was very interesting to compare the properties and the activation of the whole lyophilized cells and of the cell-free extracts of the same origin. Table 1 shows the dependence of the phosphatase activity on the phosphorus content of the preparations and the activation by magnesium at optimal concentration (10^{-2} *M*).

The enormous difference in the phosphorus contents of the whole cells of normal and low-phosphorus yeasts can also be seen in the phosphorus contents of the cell-free extracts of these cells. The phosphatase activity of both the lyophilized cells and the cell-free extract of the normal yeast is very low. The activity of the low-phosphorus cells is about ten-fold compared to that of the normal cells. However, the activity of the cell-free extract from the low-phosphorus yeast is about thirty-fold compared to the extract from the normal yeast. Accordingly, the destruction of the cell structure when preparing the extract with a sonic vibrator is followed by a coactivation of the enzyme of the low-phosphorus cells, but has no effect on the enzyme of the normal cells.

The activation by magnesium is another very interesting point of this experiment. In normal yeast the activation both of the lyophilized cells and of the cell-free extract is the same. The activation of the low-phosphorus preparations is much more marked than the activation of the preparations with normal phosphorus content, the activation of the low-P cells being twice that of the normal cells and the activation of the cell-free extract of the low-P cells about six times that of the extract of the normal cells. Accordingly in low-P preparations the activation of the cell-free extract is much greater than that of the whole cells. It is interesting to note that the activity of the cell-free extract from low-P cells activated by magnesium is more than 100-fold compared with the activity of the non-activated extract from normal cells.

In addition to the activators, the effect of some commonly known inhibitors of phosphatases have been investigated. Owing to the very low activity of the normal yeast, the inhibition can be studied only with low-P preparations. The results of the experiments with lyophilized cells and cell-free extract are given in Fig. 3 and Table 2.

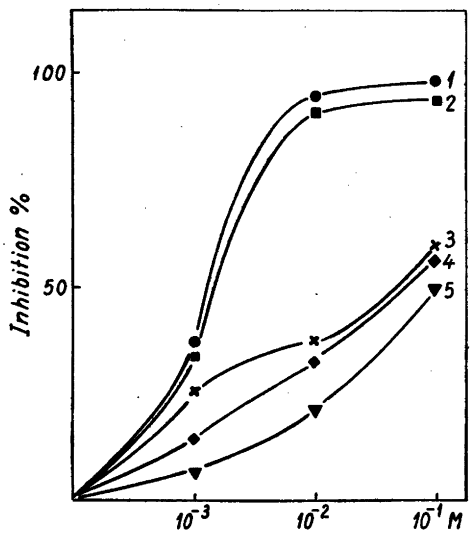

Fig 3. Effect of inhibitors at different concentrations on the phosphatase activity of low phosphorus cells.

Curve 1: Pyrophosphate
Curve 2: Molybdate
Curve 3: Fluoride
Curve 4: Orthophosphate
Curve 5: Oxalate

The very strong inhibition by pyrophosphate and molybdate is characteristic of this enzyme. Even fluoride, orthophosphate and, to a lesser extent, oxalate cause an inhibition. The phosphorus compounds, which are normal constituents of the cells, reduce the activity of the enzyme in varying degrees (Table 2). Although the values in this table are not in all respects comparable with each other and are not of quantitative importance, they give a qualitative picture of the inhibition.

The phosphorus content of the cell-free extracts can be lowered by removing the freely dialyzable phosphorus compounds by a short period of dialysis. Table 3 shows the effect of 24 hours' dialysis at 4° C against distilled water on the phosphorus and nitrogen contents as well as on the phosphatase activity of the cell-free extracts from normal and low-P cells.

The total phosphorus content of both preparations has decreased very considerably, but the ratio of the P content between normal and low-P preparations has not changed. The total nitrogen content has also decreased, but to a lesser degree. The decrease of the phosphorus contents has, however, no

Table 2. Inhibitory effect of some phosphorus compounds on the phosphatase activity of the cell-free extract of low-phosphorus cells.

		Phosphatase activity
No addition		1.65
Orthophosphate	10^{-3} *M*	1.65
»	10^{-2} *M*	1.29
Pyrophosphate	10^{-2} *M*	0.48
Polymetaphosphate	0.5 %	0.51
Ribonucleic acid	»	1.45
Desoxyribonucleic acid	»	1.31

Table 3. Effect of dialysis on the phosphorus and nitrogen contents and on the phosphatase activity cell-free extracts of normal and low-phosphorus cells.

Cell-free extract	Total phosphorus % of dry matter		Total nitrogen % of dry matter		Phosphatase activity	
	before dialysis	after dialysis	before dialysis	after dialysis	before dialysis	after dialysis
of normal cells	4.03	1.33	2.43	1.69	0.15	0.05
» low-P »	0.75	0.19	2.23	1.65	1.65	1.48

stimulatory effect on the phosphatase activity. On the contrary, the enzyme activity has in both cases clearly decreased. This decrease can be explained by the fact that the metal present in the enzyme or some other dissociable and dialyzable activators of the enzyme are partly removed, or that the protein is slightly inactivated.

Owing to the limitation of our knowledge concerning the nature of the influence of the factors affecting the phosphatase activity in general, it is very difficult to decide the real cause of the activation of this phosphatase during starvation of the cells in respect to phosphorus. The first supposition would be that the inorganic and organic phosphorus constituents of the cells are the natural inhibitors of phosphatase. The low activity of phosphatase in normal cells would partly be due to the high content of these compounds in the cells and to the formation of combinations of these anions with the metal naturally present in the enzyme. The activation of the enzyme during the starvation in respect to phosphorus would at least to some extent be caused by the considerable disappearance of the phosphorus constituents of the cells. The decrease in different phosphorus fractions of the cells during the starvation has been shown by Rautanen and Miikkulainen [5]. Accordingly the starvation is a very radical process and it would be possible to suppose that it is followed by an anomalous liberation of some active groups of the enzyme protein. Although the phosphorus-starved yeast is an artefact, the characteristics of the phosphatase of this yeast are in all respects comparable with the preparations of normal phosphorus content. Accordingly no change in the fundamental nature of the enzyme seems to have occurred during starvation.

SUMMARY

The properties of the phosphomonoesterase of *Torulopsis utilis* have been investigated with lyophilized cells and cell-free extracts obtained by sonic disintegration of the fresh cells. The pH optimum lies between 5 and 6, probably at about 6. The enzyme is activated especially by manganese and magnesium. It is also activated by starvation of the cells in respect to phosphorus, and a cell-free extract of very high activity has been obtained from the phosphorus-starved cells. The effect of inhibitors has been studied with the active phosphorus-starved yeast preparations. The enzyme is strongly inhibited by pyrophosphate and molybdate and, to a lesser extent, by other inorganic and organic phosphorus compounds.

The authors wish to express their thanks to Dr. K. Penttinen, State Serum Institute, for allowing us to use the Raytheon sonic vibrator, donated by the Juselius Foundation, and to Prof. E. Uroma, Director of the aforesaid institute, for the other equipment used in the preparation of the cell-free extracts.

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Potentiometric and Spectrophotometric Studies on 8-Quinolinol and Its Derivatives. IX. Stability of Some Metal Chelates of 8-Quinolinol-5-Sulphonic Acid in Aqueous Solution

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In a previous paper [1] from this laboratory the ionization of 8-quinolinol-5-sulphonic acid was studied. We have now completed this investigation by the determination of the concentration constants of this dibasic acid using cells without liquid junction:

H_2 or glass electrode / H_2A, HA^-, K^+, Cl^- / AgCl-Ag
H_2 or glass electrode / H_2A, HA^-, $A^=$, K^+, Cl^- / AgCl-Ag

The previous data for the mixed constants were checked, too, by the same method as before.

For the above cell we obtain

$$-\log [H^+] = (E^\circ - E) / 2.303\ RT + \log [Cl^-] + 2 \log f \pm \quad (1)$$

For E° the value 222.3 mV was used [2]. The activity coefficient $f\pm$ was considered to be that of the pure hydrochloric acid in potassium chloride solution and its values were obtained from a paper of Harned and Hamer [3]. The values of E° and $f \pm$ were checked occasionally. The medium effect of the acid studied was neglected because of its low concentration. After the hydrogen ion concentration has been determined, the ionization constant is obtained in the usual way.

When a glass electrode was used, the standard potential E° was determined before and after each measurement, using a dilute hydrochloric acid solution as reference.

The silver-silver chloride electrode was prepared by the method of Shedlovsky and MacInnes [4]. In other respects the experimental procedures were the same as before.

The stability constants were determined by the same method as before [5]. In the case of oxine, the ligand was a univalent anion, but in the present case the ligand is a divalent anion. Therefore the equations used for the calculation of the stability constants undergo a slight modification. The equation

$$k'_1 + \Sigma x k'_n\ (nc_M - c') / (c_M - c') - c' / x\ (c_M - c') = 0 \quad (2)$$

was used at higher pH's and the equation

$$k''_1 + \Sigma y k''_n\ (nc_M - c'') / (c_M - c'') - c''/x\ (c_M - c'') = 0 \quad (3)$$

at lower pH's. In these equations

$$k'_n = k_n K'^n_2 \text{ and } k''_n = k_n K'^n_1 K'^n_2. \quad (4)$$

Further,

$$x = [HA^-] / (H^+),\ y = [H_2A] / (H^+)^2, \quad (5)$$

$$c' = c - [HA^-]\ (1 + K'_2 / (H^+) + (H^+) / K'_1 \quad (6)$$

$$c'' = c - [H_2A]\ (1 + K'_1 / (H^+)) + K'_1 K'_2 / (H^+)^2) \quad (7)$$

$$[HA^-] = (2c - c_B - [H^+] + [OH^-]) / (1 + 2\ (H^+) / K'_1) \quad (8)$$

$$[H_2A] = (2c - c_B - [H^+] + [OH^-]) / (2 + K'_1 / (H^+)) \quad (9)$$

Here c is the stoichiometric concentration of the chelating agent, c_M that of the metal salt and c_B that of sodium hydroxide. The stability constants

$$k_n = [MA_n^{(2n-2)-}] / [M^{++}]\ [A^=]^n \quad (10)$$

are calculated by means of the relations (4) from the values of k'_n or k''_n. In the present case, $n = 2$.

RESULTS

In the measurement of the ionization constants, the silver-silver chloride electrode, the hydrogen electrode, the glass electrode and the calomel electrode (the last through a junction of saturated potassium chloride solution) were dipped in the same solution simultaneously. The potential differences $E_{H,S}$ (hydrogen and silver chloride), $E_{G,S}$ (glass and silver chloride), $E_{H,C}$ (hydrogen and calomel) and $E_{G,C}$ (glass and calomel) where then measured. The results are recorded in Tables 1 and 2, and can be represented by the following equations:

Table 1. Determination of the first ionization constant of 8-quinolinol-5-sulphonic acid at 25° C.

KCl 2.837 M, ml	$\sqrt{I}$	$E_{G,S}$ mV	$E_{H,S}$ mV	$E_{G,C}$ mV	pK_1 G, S	pK_1 H, S	pK'_1 G, C
1.00	0.195	87.2	−553.3	153.6	3.971	3.970	4.025
3.03	0.330	113.9	−526.6	154.6	3.912	3.910	4.001
5.06	0.418	125.5	−514.8	155.6	3.892	3.886	3.978
10.06	0.575	139.8	−500.7	155.9	3.878	3.887	3.969
15.06	0.684	147.7	−492.8	155.8	3.876	3.874	3.968
20.06	0.770	152.8	−487.8	155.7	3.880	3.880	3.970
30.09	0.895	159.4	−481.1	154.8	3.910	3.908	3.981
50.15	1.062	165.5	−474.9	152.9	3.979	3.976	4.012
70.20	1.166	169.5	−471.0	152.1	4.006	4.000	4.020

75.07 ml 0.002954 M H_2A.
1.125 ml 0.0985 M NaOH
$E°_{G,S}$ = 419.0 mV. $E°_{H,S}$ = −222.3 mV. $E°_{G,C}$ = 395.1 mV.
p_{H_2} = 743 mm Hg.

Table 2. Determination of the second dissociation constant of 8-quinolinol-5-sulphonic acid at 25° C.

KCl 2.837 *M*, ml	$\sqrt{I}$	$E_{G,S}$ mV	$E_{H,S}$ mV	$E_{G,C}$ mV	$E_{H,C}$ mV	pK_2 G,S	pK_2 H,S	pK_2 G,C	pK_2 H,C	pK'_2 G,C	pK'_2 H,C
1.00	0.242	−164.3	−804.8	−104.7	−744.1	8.415	8.413	8.383	8.365	8.450	8.433
3.03	0.401	−131.5	−771.5	− 97.5	−737.7	8.262	8.249	8.240	8.238	8.327	8.325
5.06	0.506	−116.7	−757.6	− 93.0	−733.4	8.186	8.191	8.166	8.166	8.252	8.252
10.06	0.680	− 99.4	−740.2	− 90.2	−730.6	8.126	8.128	8.128	8.129	8.205	8.205
15.06	0.799	− 90.4	−730.9	− 88.6	−727.2	8.105	8.103	8.113	8.082	8.178	8.147
20.06	0.890	− 83.4	−723.7	− 86.9	−727.0	8.093	8.087	8.099	8.094	8.149	8.144
30.09	1.021	− 74.6	−715.3	− 86.0	−726.5	8.083	8.089	8.110	8.112	8.134	8.136
50.15	1.178	− 66.3	−706.9	− 84.0	−724.6	8.098	8.097	8.117	8.120	8.101	8.104
90.25	1.342	− 59.2	−699.0	− 83.2	−723.2	8.121	8.107	8.150	8.139	8.086	8.075

75.07 ml 0.002954 *M* H_2A.
1.125 ml 0.0985 *M* NaOH.
$E^\circ_{G,S} = 419.0$ mV. $E^\circ_{H,S} = -222.3$ mV. $E_{G,C} = 395.1$ mV. $E_{H,C} = -246.0$ mV.
$p_{H_2} = 743.0$ mm Hg.

$$pK'_1 = 4.104 - \frac{0.509\sqrt{I}}{1 + 1.16\sqrt{I}} + 0.126\ I \tag{11}$$

$$pK_1 = 4.112 - \frac{1.018\sqrt{I}}{1 + 1.40\sqrt{I}} + 0.251\ I \tag{12}$$

$$pK'_2 = 8.750 - \frac{2.036\sqrt{I}}{1 + 1.41\sqrt{I}} + 0.168\ I \tag{13}$$

$$pK_2 = 8.757 - \frac{2.036\sqrt{I}}{1 + 1.41\sqrt{I}} + 0.168\ I \tag{14}$$

The parameters of these equations are calculated by the method of least squares from the data in Tables 1 and 2.

The results on the chelates of the alkaline earth metals are recorded in Table 3. The thermodynamic constants $k'_{1,0}$ were obtained by extrapolation by means of the Debye-Hückel equation

$$pk'_1 = pk'_{1,0} + \frac{2.545\sqrt{I}}{1 + a\sqrt{I}} - BI \tag{15}$$

The results on the chelates of some other metals are presented in Table 4.

Table 3. Determination of the stability of 8-quinolinol-5-sulphonic acid chelates of alkaline earth metals at 25° C.

Ba($BaCl_2$) $\sqrt{I}$	Ba pk'_1	Sr($SrCl_2$) $\sqrt{I}$	Sr pk'_1	Ca($CaCl_2$) $\sqrt{I}$	Ca pk'_1	Mg($MgCl_2$) $\sqrt{I}$	Mg pk'_1	Mg pk'_2
0.0940	6.598	0.089	6.195	0.082	5.415	0.061	~4.1	~9.3
0.145	6.762	0.143	6.261	0.125	5.493	0.072	4.108	~9.3
0.273	6.760	0.271	6.351	0.215	5.618	0.100	4.183	
0.695	7.037	0.585	6.523	0.413	5.761	0.170	4.209	
1.21	7.078	1.21	6.602			0.320	4.374	
1.54	7.001	1.54	6.515			0.570	4.498	
p$k'_{1,0}$ = 6.444		p$k'_{1,0}$ = 6.000		p$k'_{1,0}$ = 5.234		p$k'_{1,0}$ = 3.962		

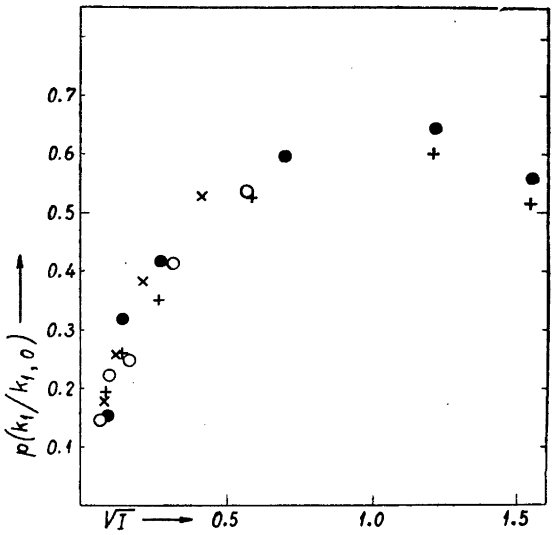

Fig. 1. The effect of ionic strength on the chelate stability. ● *Ba* ($BaCl_2$), + *Sr* ($SrCl_2$), × *Ca* ($CaCl_2$) *and* ○ *Mg* ($MgCl_2$).

The thermodynamic constants were now obtained by means of

$$pk''_1 = pk''_{1,0} + \frac{2.036\sqrt{I}}{1 + a\sqrt{I}} - BI \qquad (16)$$

The thermodynamic constants $k_{1,0}$ were obtained by means of the relations (4) from the above $k'_{1,0}$ and $k''_{1,0}$ values. These values are presented in Table 5. The values for the second stability constants in the table were calculated by means of the same equation and extrapolated to zero ionic strength by means

Table 4. Determination of the stability of 8-quinolinol-5-sulphonic acid chelates of some divalent metals at 25° C.

Mn($MnCl_2$)		Cd($Cd(ClO_4)_2$)			Pb($Pb(NO_3)_2$)			Zn($Zn(NO_3)_2$)		
$\sqrt{I}$	pk''_1	$\sqrt{I}$	pk''_1	pk''_2	$\sqrt{I}$	pk''_1	pk''_2	$\sqrt{I}$	pk''_1	pk''_2
0.067	5.97	0.045	5.24	~11.3	0.035	4.40	~9.7	0.035	4.25	~9.5
0.137	6.25	0.054	5.27	~11.8	0.050	4.42	~9.5	0.187	4.57	—
0.431	6.44	0.083	5.33	—				0.413	4.77	—
$pk''_{1,0}$ = 5.93		$pk''_{1,0}$ = 5.17			$pk''_{1,0}$ = 4.33			$pk''_{1,0}$ = 4.21		

Co($Co(NO_3)_2$)			Ni($NiCl_2$)			Cu($Cu(ClO_4)_2$)		
$\sqrt{I}$	pk''_1	pk''_2	$\sqrt{I}$	pk''_1	pk''_2	$\sqrt{I}$	pk''_1	pk''_2
0.050	4.15	~9.8	0.032	3.12	~7.2	0.059	1.42	~4.1
0.170	4.29	—	0.130	3.39	—	0.123	1.61	—
0.190	4.29	—	0.265	3.43	—			
$pk''_{1,0}$ = 4.04			$pk''_{1,0}$ = 3.11			$pk''_{1,0}$ = 1.33		

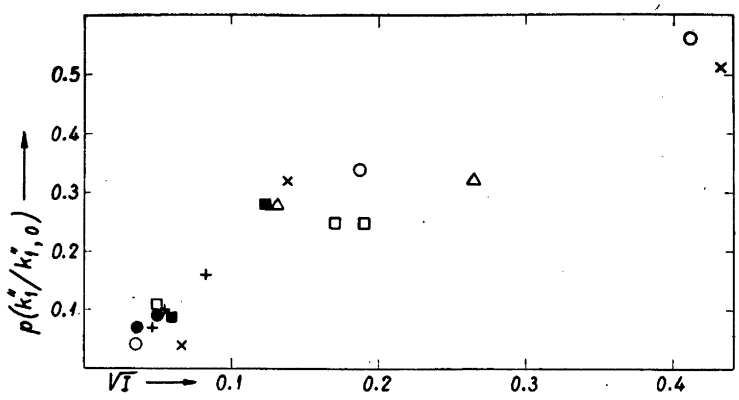

Fig. 2. The effect of ionic strength on the chelate stability. × *Mn* ($MnCl_2$), + *Cd* ($Cd(ClO_4)_2$), ● *Pb* ($Pb(NO_3)_2$), ○ *Zn* ($Zn(NO_3)_2$), □ *Co* ($Co(NO_3)_2$), △ *Ni* ($NiCl_2$), ■ *Cu* ($Cu(ClO_4)_2$).

of the Debye-Hückel limiting law.

Table 5. Stability constants of 8-quinolinol-5-sulphonic acid chelates of some divalent metals at 25° C.

	log $k_{1,0}$	log $k_{2,0}$		log $k_{1,0}$	log $k_{2,0}$
Ba	2.31	—	Pb	8.53	~16.1
Sr	2.75	—	Zn	8.65	~16.2
Ca	3.52	—	Co	8.82	~15.9
Mg	4.79	~ 8.2	Ni	9.75	~18.5
Mn	6.94	—	Cu	11.53	~21.6
Cd	7.70	~14.2			

DISCUSSION

The values obtained by us for the first ionization constant, using cells with ($pK_{1,0} = 4.104$) and without ($pK_{1,0} = 4.112$) liquid junction, are in very satisfactory agreement. The same may be said concerning the values for the second constants, $pK_{2,0} = 8.750$ and 8.757. The agreement is very satisfactory, too, with the values obtained by Näsänen and Ekman [1], $pK_{1,0} = 4.092$ and $pK_{2,0} = 8.776$. At higher ionic strengths ($I > 1$), however, a correction has been made to the previous values, because a glass electrode used in the previous work gave wrong values in concentrated potassium chloride solution, as was observed later on. From equations (11) and (12) we have calculated that

$$p(K_1/K'_1) = \frac{0.509\ \sqrt{I}}{1 + 1.95\ \sqrt{I}} - 0.118\ I \qquad (17)$$

In the case of 8-quinolinol [5], the result was

$$p(K_1/K'_1) = \frac{0.505\ \sqrt{I}}{1 + 2.20\ \sqrt{I}} - 0.129\ I \qquad (18)$$

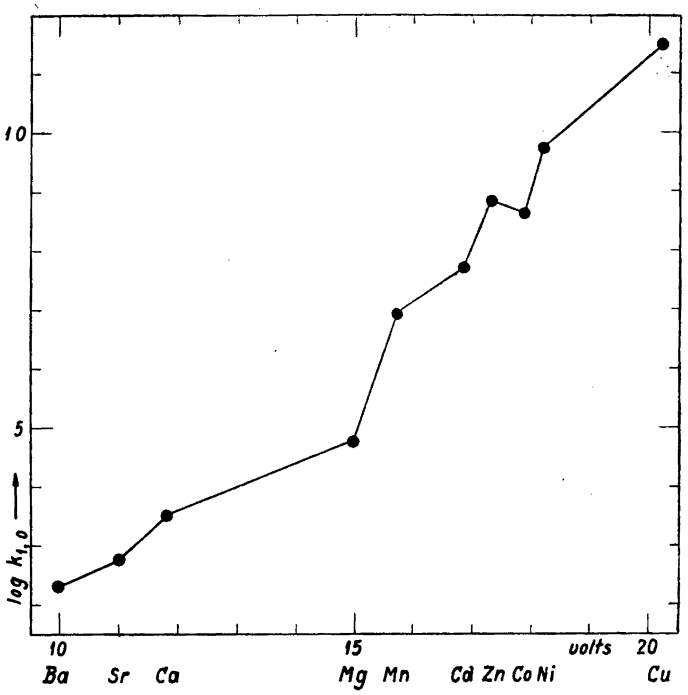

Fig. 3. The chelate stability and the second ionization potential of the gaseous metal atom.

The difference between these two results is 0.026 at $I = 1$. The agreement is thus satisfactory.

In Fig. 1 the quantity p $(k'_1/k'_{1,0})$ is represented as a function of ionic strength in the case of the alkaline earth metals. The stability constants have grouped themselves around the same curve. In Fig. 2, the p$(k''_1/k''_{1,0})$ of the other metal chelates studied is represented. The accuracy is somewhat smaller and the measurements are restricted to a narrower range of ionic strength. The values of the second stability constants are relatively inaccurate. Maley and Mellor [6] have determined the stability constants for zinc, cobalt and copper chelates. Their values for the first stability constants of zinc and copper chelates are in close agreement with our values, but in the case of cobalt a greater difference exists. The second constants differ more.

Recently the relation between the stability of complex compounds and of the ionization potentials of gaseous metal atoms has been discussed by many authors [7]. In Fig. 3 our results are summarized according of these ideas. The figure represents the logarithm of the stability constants as a function of the second ionization potential. The picture is similar to the case of 8-quinolinol [8].

SUMMARY

The stability constants of 8-quinolinol-5-sulphonic acid chelates of eleven divalent metals in aqueous solutions have been determined. The results reveal that the effect of the sulphonic acid group on the chelate stability is generally relatively slight. The ionization constants of 8-quinolinol-5-sulphonic acid have been determined using cells with and without liquid junction.

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Synthetic Plant Hormones

V *. Synthesis of α-3-Indole*iso*butyric Acid, an Antagonist of 3-Indoleacetic Acid, and of Some Other 1- and 3-Indole Acids

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In the preceding paper of this series the plant growth activity of some α-phenoxyacetic acids methylated in the side chain, and the working hypothesis which initiated our present line of research was discussed [1]. It was emphasized, that with one exception (phenoxyacetic acid itself, which is inactive at low concentrations but exhibits antiauxin activity at higher concentrations) all phenoxyacetic and α-phenoxypropionic acids discussed therein show strong auxin activity, whereas the α-phenoxy*iso*butyric acids are very potent auxin antagonists (antiauxins).

The present communication records an improved synthesis of α-3-indolepropionic acid (I) and the synthesis of α-3-indole*iso*butyric acid (II), compounds possessing special interest because of their close relation to 3-indoleacetic acid, one of the naturally occurring plant growth regulators.

Rather unexpectedly α-3-indole*iso*butyric acid (II) (m.p. 135°) could be obtained in a yield of about 30 % simply by the action of alkali, acetone and chloroform on indole, at room temperature. When the reaction was carried out at reflux temperature the yield of α-3-indole*iso*butyric acid decreased to about 10 %, but in addition a dibasic acid, $C_{16}H_{19}NO_4$ (IV) m.p. 228—230° (decomp.) was obtained.

From the known reactivity of the indole nucleus substitution could be expected to occur in position 3 or 1. Since the monobasic acid contains two "active" hydrogen atoms (Zerewitinoff), it must be a 3-indole derivative; it must possess an α- rather than a β-*iso*butyric acid side chain in view of the fact that it can also be obtained from acetonecyanhydrin and indole, a reaction completely analogous to the preparation of α-3-indolepropionic acid from indole and acetaldehydecyanhydrin (see below). Structures (III) and (IV) are possible for the dibasic acid, also containing two "active" hydrogen atoms. The acid (III) could, be prepared by addition of methacrylonitrile to ethyl α-indole-

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*iso*butyrate, a method analogous to the preparation of β-1-cyanoethylindole from acrylonitrile and indole [2], and subsequent alkaline hydrolysis of the resulting nitrile-ester. The acid (III) had m.p. 163—164°, and is thus not identical with the above dibasic acid, m.p. 228—230°, which consequently must be α,α'-1,3-indoledi*iso*butyric acid (IV).

As acetonechloroform, 2,2,2-trichloro-*tert*butanol, could be considered a possible intermediate in the synthesis of α-3-indole*iso*butyric acid, and as indole is known to react with formaldehyde and hydrogen cyanide with formation of 3-indoleacetonitrile [3], attempts were made to condense indole with acetonecyanhydrin in ethanolic sodium hydroxide solution. A neutral product $C_{18}H_{16}N_2$, containing two "active" hydrogen atoms and surprisingly found to be identical with Oddo and Toffoli's [4] α,α-bis-(3-indole) ethane (V), α-3-indole-*iso*butyric acid and an acid having the formula $C_{11}H_{11}NO_2$, m.p. 111—112°, were obtained. Like α-3-indole*iso*butyric acid the latter was found to contain two "active" hydrogen atoms, and it was found to be identical with DL-α-3-indolepropionic acid previously prepared by Ellinger [6], further studied by Kögl and Kostermans [6] and resolved by Kögl and Verkaaik [7]. The melting point of the picrate, the specific rotation of the quinine salt of the (+)-acid prepared by Kögl's and Verkaaik's method as well as the melting point and specific rotation of the (+)-acid agreed with the figures given by the above authors.

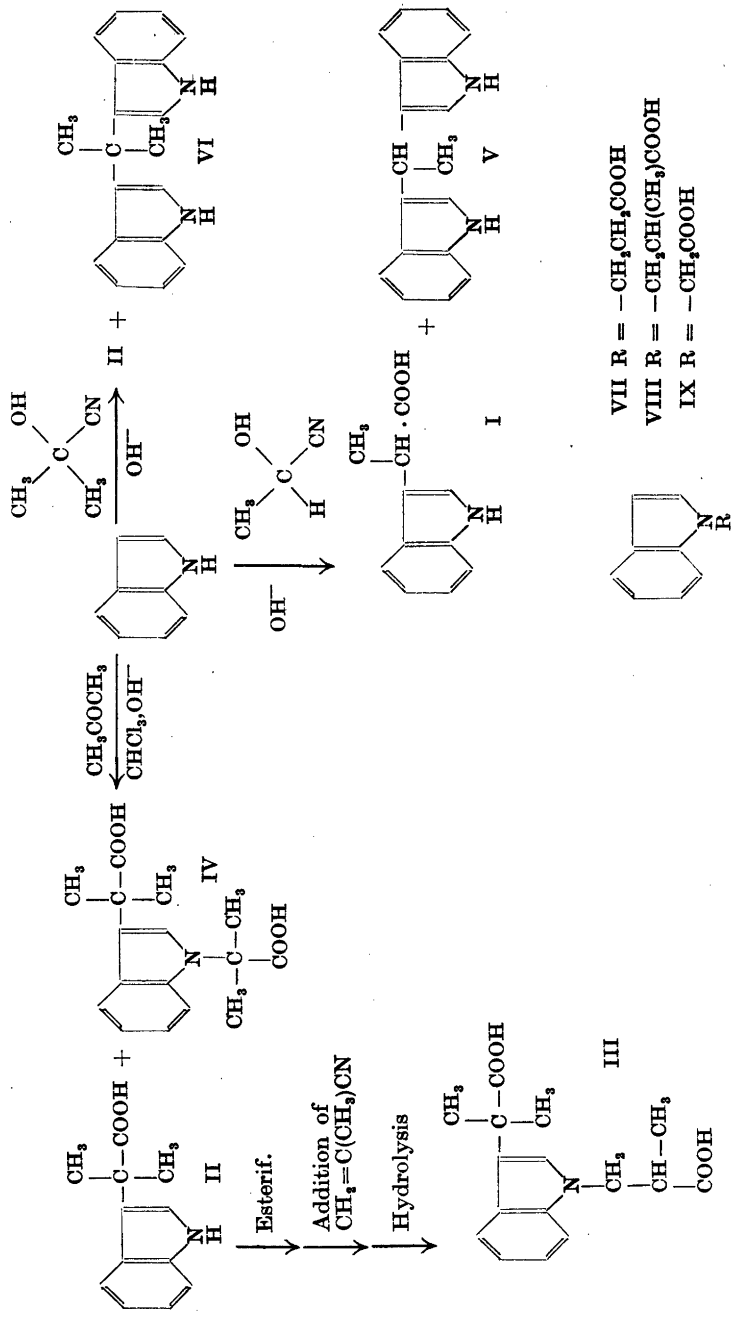
The production of these compounds is obviously due to a partial fission of the acetonecyanhydrin in the alkaline solution into acetone and cyanide ion, the acetone then undergoing a Meerwein-Ponndorf-Verley type of reaction with the ethanol present yielding *iso*propanol and acetaldehyde, the latter finally reacting with the indole.

This assumption was verified by carrying out the reaction in *iso*propanol instead of ethanol and α-3-indole*iso*butyric acid was now the only acid product, which could be isolated. In addition a neutral substance, $C_{19}H_{18}N_2$, m.p. 163°, identical with Scholtz's β,β-bis-(3-indole)propane [8] (VI) was obtained. When, on the other hand, the reaction was run with acetaldehydecyanhydrin in ethanol only α-3-indolepropionic acid (I) and α,α-bis-(3-indole)ethane were obtained.

For the preparation of α-3-indole*iso*butyric acid it was not necessary to use preformed acetonecyanhydrin. The same acid resulted, although in lower yield, when a mixture of acetone and potassium or sodium cyanide was used.

This reaction between indole and cyanhydrin is probably not restricted to formaldehyde-, acetaldehyde- and acetonecyanhydrin, but might be a rather general method for the preparation of side chain alkylated 3-indoleacetic acids.

β-1-Indolepropionic acid [9] (VII) was prepared *via* its nitrile by addition of acrylonitrile to indole by a method of I. G. Farbenindustrie [2]. The structure follows from the fact that it contains only one "active" hydrogen atom and no C—CH_3-group. When methacrylonitrile was used instead of acrylonitrile β-1-indole*iso*butyronitrile was obtained in high yield. Alkaline hydrolysis afforded the corresponding acid (VIII).


II
Esterif.
Addition of CH₂=C(CH₃)CN
Hydrolysis
III
IV
CH₃COCH₃
CHCl₃,OH⁻
II
OH⁻
VI
V
I
OH⁻
VII R = —CH₂CH₂COOH
VIII R = —CH₂CH(CH₃)COOH
IX R = —CH₂COOH

When the potassium salt of indole was heated with ethyl chloroacetate and the resulting mixture was hydrolysed with alkali 1-indoleacetic acid was obtained. This acid has recently been prepared by Smith and Moir [16] by a laborious route. Attempts to carry out this reaction with ethyl α-chloropropionate and ethyl α-bromo*iso*butyrate failed.

PHYSIOLOGICAL ACTIVITY

The plant growth activity of the substances has been tested by Prof.H. Burström *et al.*, Lund, employing their wheat root elongation technique [10,11] and is reported elsewhere by them. In accordance with the results of Kögl and Kostermans [6] D,L-α-3-indolepropionic acid was found to be a very potent auxin. The activity is, however, almost exclusively due to the dextrorotatory form of the acid (Ratio of the activity of the (+)-acid to that of the (±)-acid 2:1; Hansen [13]). This is in agreement with the results obtained by Kögl and Verkaaik [7,8] with the *Avena* curvature test, but not with the *Avena* straight growth test, in which both antipodes showed the same activity, and also agrees with the results obtained by Fredga, Matell and Åberg with optically active α-aryloxypropionic acids [14]. Smith and Moir's finding [15] that 1-indoleacetic acid possesses auxin activity was confirmed [13]. α-3-Indole*iso*butyric acid was found to be a very potent antiauxin [12]. β-1-Indolepropionic and β-1-indole*iso*butyric acid also possessed some antiauxin activity [13].

As 3-indoleacetic acid has been found to inhibit the growth of a number of microorganisms [16,17] it was of interest to study the effect of its branched homologues on various such organisms. Drs. N. Nielsen, A. B. Kabi, and G. Wallmark, Statens Bakteriologiska Laboratorium, have kindly carried out these tests. When tested in concentrations up to 0.1 mg per ml substrate α-3-indoleisobutyric acid had no significant effect on *Staph. aureus* and *albus*, *Enterococci*, *Ps. pyocyanea*, *Proteus vulg.*, *Salm. paratyphi*, *Pneumococci*, *α-Streptococci*, *β-Streptococci*, *M. tuberculosis*, some *Bacillus species*, *Escherichia coli*, *Candida albicans* and *Pullularia pullulans*. It showed an inhibitory effect on *Sarcina lutea* and *B. subtilis*.

D,L-α-3-Indolepropionic acid was tested in concentrations up to 1.0 mg per ml on *Sarcina lutea*, *Staph. aureus*, *B. subtilis*, *E. coli*, *Streptomyces griseus*, *Aspergillus niger*, *Penicillium chrysogenum* and *Pullularia pullulans*. In no case any significant effect was observed.

EXPERIMENTAL *

α-3-Indole*iso*butyric acid (II) from indole, acetone, chloroform and alkali

a) *At room temperature.* Indole (117 g), dry acetone (1.0 l) and sodium hydroxide (220 g, pellets) were stirred in a three-necked flask (3 l capacity) equipped with a reflux condenser, dropping funnel and thermometer, and cooled in an ice bath. Chloroform (156 g) was added dropwise at such a rate, that the temperature of the mixture did not exceed 15° (about 2 hours). The ice bath was removed, and the mixture stirred for one hour followed by a further four hours with warming on a water bath. Excess acetone was removed by distillation, and the salt cake dissolved in water (1.5 l). The solution was extracted four times with ether (49 g of unreacted indole was recovered from the extracts) and acidified with dilute sulphuric acid (1 : 1). The oil which separated was taken up in ether, washed with water, dried and the ether removed. The product was dissolved in anhydrous ethanol (250 ml) containing 5 per cent sulphuric acid, heated under reflux for four hours and poured into water (1 l). The ester was extracted with ether, washed with water, with sodium bicarbonate solution, again with water, and dried. Removal of the ether yielded an oil, which distilled between 180 to 205°/3 mm Hg (55 g) which crystallised on standing for some hours. One crystallisation from benzene-petrol (1 : 1) gave

* All boiling and melting points uncorrected. Petrol refers to the fraction b.p. 40—60°.

pure *ethyl α-3-indoleisobutyrate*, m.p. 106—107°. The yield was 43 g (32 per cent calculated on indole actually consumed (Found OC_2H_5 19.4 %. $C_{14}H_{17}NO_2$ requires OC_2H_5 19.5 %).

When refluxed for four hours with excess 40 % aqueous sodium hydroxide and sufficient ethanol to make the mixture homogenous the ester was hydrolysed to *α-3-indoleisobutyric acid* in almost quantitative yield. The acid separated from a chloroform-petrol mixture in coarse needles, m.p. 135°. (Found C 70.9; H 6.40; N 6.90; equiv. weight 204; active H 1.7; C—CH_3 89 % of theory. $C_{12}H_{13}NO_2$ requires C 70.9; H 6.45; N 6.90; equiv. weight 203.2; active H 2.00.)

The *cyclohexylammonium salt* was prepared in ether solution, and crystallised from an ethanol-ether mixture in needles, m.p. 162—163° (decomp.). (Found N 9.2. $C_{18}H_{26}N_2O_2$ requires N 9.3.)

b) *At reflux temperature.* The above reaction was carried out at reflux temperature using a third of the previous quantities. The acid products were not esterified but their etheral solution was repeatedly extracted with sodium bicarbonate solution. The combined extracts were acidified to pH 5—6 with acetic acid and the solution was extracted four times with ether. *α-3-Indoleisobutyric acid* could be obtained in 5 to 10 % overall yield from this extract.

The aqueous raffinate was acidified to pH 1—2 with dilute sulphuric acid and repeatedly extracted with ether. The combined extracts were thoroughly washed with water, concentrated to a small volume and filtered through a short column of aluminium oxide. Evaporation of the ether yielded an oil which crystallised in a few days. The product was triturated with benzene-petrol (1 : 1) and the solid *α,α'-1,3-indolediisobutyric acid* (IV) crystallised from aqueous dioxane and from aqueous methanol in faintly pink needles, m.p. 228—230° (decomp.). Yield 5 to 10 %. (Found C 66.8; H 6.60; N 4.81; equiv. weight 145; active H 1.8. $C_{16}H_{19}NO_4$ requires C 66.4; H 6.62; N 4.84; equiv. weight 144.6; active H 2.00.)

α-3-Indole*iso*butyric (II) and α-3-indolepropionic acid (I) from indole and cyanhydrins

a) *Acetaldehydecyanhydrin.* A mixture of indole (12 g), acetaldehydecyanhydrin (8.8 g), sodium hydroxide (10 g, pellets) and absolute ethanol (50 ml) was heated with stirring at 135° C for 18 to 24 hours in a stainless steel autoclave with silver packings. (Lead packings must be avoided as even small amounts of lead favour undesirable side reactions leading to resinous materials.) The light yellow reaction mixtures from three such runs were combined and poured into water. Neutral products were extracted with ether.

The aqueous solution containing the acid reaction products was acidified with dilute (1 : 1) sulphuric acid. The oil, which separated was dissolved in ether, thoroughly washed with water and dried. Removal of the ether afforded a light brown oil (24 g), which crystallised when stored in a refrigerator over night. Crystallisation from a benzene-petrol mixture (2 : 1) yielded an acid (19 g), m.p. 106—108°, which by repeated crystallisation was raised to 111—112°. (Found N 7.38, 7.41; equiv. weight 189; active H 1.8; C—CH_3 92 % of theory. Calc. for $C_{11}H_{11}NO_2$ N 7.40; equiv. weight 189.2; active H 2.00.)

The acid gave a picrate, m.p. 145—146°. Picrate of D,L-α-3-indolepropionic acid, Kögl and Kostermans [7], m.p. 146—147°. Following the method of Kögl and Verkaaik [7] for the resolution of D,L-α-3-indolepropionic acid a quinine salt, having $[\alpha]_D^{20} = -105°$ (95 % ethanol, $c = 1$) was obtained. The (+)-acid, m.p. 137—138°, $[\alpha]_D^{20} = +73°$ (95 % ethanol, $c = 0.4$) was obtained from the quinine salt. The acid m.p. 111—112° must therefore be D,L-*α-3- indolepropionic acid.* (Literature [5,6] m.p. 111°; the *dextro*rotatory form m.p. 138—139°, $[\alpha]_D = +76 \pm 2°$ (ethanol, $c = 1$). The quinine salt of the latter $[\alpha]_D = -104 \pm 2°$ (ethanol, $c = 1$).

The *cyclohexylammoniumsalt* of D,L-α-3-indolepropionic acid was prepared in ether and crystallised from acetone in needles. M.p. 183—185° (decomp.). (Found C 71.1; H 8.5; $C_{17}H_{24}N_2O_2$ requires C 70.8; H 8.39.)

The ether solution containing the neutral reaction products was evaporated yielding a brown oil, from which indole (5 g) was recovered by steam distillation. The nonvolatile residue was dissolved in ethanol and set aside for crystallisation. A colourless product

(13.5 g) was obtained from ethanol as crystals, m.p. 162—163°, undepressed on admixture with *α,α-bis-(3-indole)ethane* (V) m.p. 158—160° prepared by the method of Oddo and Toffoli [4]. (Found C 82.8; H 6.24; N 10.78; active H 1.9; C—CH_3 93 % of theory; mol. weight (Rast) 258, 262. Calc. for $C_{18}H_{16}N_2$ C 83.0; H 6.19; N 10.76; active H 2.00; mol. weight 260.4.)

b) *Acetonecyanhydrin.* Indole (12 g), acetonecyanhydrin (10.6 g), sodium hydroxide (10 g pellets) and anhydrous *iso*propanol (50 ml) were treated exactly as described for acetaldehydecyanhydrin. The yield of α-3-indole*iso*butyric acid from three such runs was 15 g. The m.p. was 135° alone and in admixture with acid prepared by the acetonechloroform reaction. Of a neutral compound, crystallising in colourless prisms from aqueous ethanol and melting at 163°, was obtained 14 g in addition to the acid. (Found N 10.1; 10.2; active H 2.0; C—CH_3 93 % of theory. Calc. for $C_{13}H_{18}N_2$; N 10.2; active H 2.00.) This compound was identical (mixed m.p.) with *β,β-bis-(3-indole)propane* (VI), obtained by Scholtz [6] from indole and acetone in acetic acid solution.

A mixture of α-3-indole*iso*butyric and α-3-indolepropionic acid resulted when the reaction was carried out in ethanol instead of *iso*propanol. Only unchanged indole and α,α-bis-(3-indole)ethane could be isolated from the neutral fraction. The main product was an uncrystallisable oil, which was not further investigated. It was probably a mixture of α,α-bis-(3-indole)ethane and β,β-bis-(3-indole)propane.

The separation of the two acids was effected after esterification with ethanol containing 5 % sulphuric acid. The esters distilled together at 150—210°/5 mm Hg giving a semisolid distillate, which when dissolved in hot petrol and kept over night in a refrigerator yielded ethyl α-3-indole-*iso*butyrate in an almost pure state. (M.p. 106—107° after one crystallisation.) The solvent was evaporated from the original mother liquor and the residual oil hydrolysed with excess 20 per cent aqueous sodium hydroxide. Acidification gave D,L-α-3-indolepropionic acid as an oil which crystallised on standing. Further crystallisation from benzene-petrol gave pure acid m.p. 111—112°. The yield of each acid was 10 to 15 %.

D,L-β-1-Indole*iso*butyronitrile

Indole (30 g), methacrylonitrile (35 g), ethanolic sodium ethoxide solution from sodium (0.5 g) and absolute ethanol (50 ml), and cupric acetate (0.2 g) were heated in a stainless steel autoclave at 120—130° for four hours. The reaction mixture was dissolved in ether, washed thoroughly with water, dried, the solvent removed and the residual oil distilled. The yield of D,L-*β-1-indoleisobutyronitrile* b.p. 160—164°/3 mm Hg was 35 g (74 %). (Found N 15.1; $C_{12}H_{12}N_2$ requires N 15.2.)

D,L-β-1-Indole*iso*butyric acid (VIII)

The above nitrile was refluxed for three hours with excess 40 per cent aqueous sodium hydroxide and sufficient ethanol to give a homogenous mixture. The liquid was poured into water and acidified; the resultant oil was taken up in ether, washed, dried and the solvent evaporated. The residual oil soon solidified. Crystallisation from benzene-petrol gave D,L-*β-1-indoleisobutyric acid*, m.p. 74°. Yield 70 %. (Found N 7.0; equiv. weight 203; active H 0.9; C—CH_3 76 % of theory. $C_{12}H_{13}NO_2$ requires N 6.9; Equiv.weight 203.2; active H 1.00.)

D,L-β-3-(1-α-Carboxypropyl)indole*iso*butyric acid (III)

Ethyl α-3-indole*iso*butyrate (8.0 g), methacrylonitrile (4.0 g) ethanolic sodium ethoxide from sodium (0.1 g) and absolute ethanol (25 ml), and cupric acetate (0.05 g) were mixed and heated as described for β-1-indole*iso*butyronitrile. The reaction mixture was heated under reflux for five hours with a solution of potassium hydroxide (20 g) in water (25 ml) and ethanol (100 ml), poured into water (500 ml), filtered and acidified. The acid products were extracted with ether, washed and dried. Removal of the ether gave a noncrystallisable oil (8.3 g) which was dissolved in ethanol (30 ml) and excess *cyclo*hexylamine added. After four hours the precipitated salt was collected and thoroughly extracted with hot ethanol. The yield of the insoluble *cyclohexylammonium salt of* D,L-α-3-

(1-α-carboxypropyl)indoleisobutyric acid m.p. 184—188° (decomp.) was 7.0 g. (Found N 8.65; $C_{28}H_{45}N_3O_4$ requires N 8.62.) The combined ethanol mother liquors were concentrated and α-3-indole*iso*butyric acid *cyclo*hexylammoniumsalt (4 g) was precipitated with acetone.

The D,L-*α-3-(1-α-carboxypropyl)indoleisobutyric* acid was obtained from its *cyclo*hexylammonium salt by treatment with dilute sulphuric acid. Crystallisation from chloroform-petrol gave colourless needles, m.p. 163—164°. (Found N 4.80; equiv.weight 145; active H 1.7; C—CH_3 78 % of theory. $C_{16}H_{19}NO_4$ requires N 4.84; equiv.weight 144.6; active H 2.00.)

1-Indoleacetic acid (IX)

Molten indole (22 g) was stirred with finely powdered potassium hydroxide (17 g) in a fractionating flask (200 ml capacity) inserted deeply in a salt bath kept at 200—225°, until no more water distilled (6 to 8 hours). The flask was removed from the bath and allowed to cool a little. Dry ethyl chloroacetate (36 g) was added and the flask was heated at 140—150° for four hours with occasional shaking, and cooled. A solution of potassium hydroxide (25 g) in water (75 ml) was added, and the mixture heated on a water bath for three hours and poured into water (250 ml). After some hours unreacted indole (7 g) was collected, and the filtrate was acidified. The crystalline product which separated, was collected, washed, dried, dissolved in ether, and the solution filtered through a short column of aluminium oxide and then evaporated to dryness. The crude *1-indoleacetic acid* (12 g) was dissolved in hot ethanol, and decolourised with animal charcoal. On cooling the acid was obtained as light brown needles of m.p. 168—170°, raised on crystallisation to 174—175° (decomp.). Smith and Moir report m.p. 178.4—179.4°. The acid is difficult to obtain in a colourless state, and rapidly deteriorates on storing. (Found C 68.4; H 5.19; equiv. weight 176; active H 0.8. Calc. for $C_{10}H_9NO_2$; C 68.6 H 5.18; equiv. weight 175.2; active H 1.00.)

Colour reactions

Fischer's fir chip test and the *p*-dimethylaminobenzaldehyde test are not very satisfactory in this series of indole compounds. In the fir chip test all give reddish colours, but compounds with free 3-position give an initial blueish coloration.

SUMMARY

The convenient synthesis of α-3-indole*iso*butyric acid (II) and D,L-α-3-indolepropionic acid (I) by novel methods, the condensation of indole with acetone and chloroform and condensation with cyanhydrins in the presence of alkali are described. D,L-β-1-indole*iso*butyric acid (VIII) has been prepared and an improved method for the synthesis of 1-indoleacetic acid (IX) is reported. The root growth activity of the acids is discussed as well as the activity of the first two on a number of microorganisms.

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Short Communications

The Resistance of Nisin to Penicillinase

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Storgårds and Anderson have stated that penicillinase has a "restraining" action on nisin[1]. Although penicillinase is highly specific (Abraham[2]) and the evidence presented by Storgårds and Anderson was merely suggestive, the implications of their findings if confirmed, would be so interesting that it was felt worth while to attempt confirmation. This was all the more important since some penicillin resistant staphylococci had been isolated, which were also resistant to nisin (Neave 1953[3]). However no action of penicillinase on nisin could be detected under a wide variety of conditions.

Nisin in all the solutions was assayed by the method of Berridge and Barrett.[4] In this method the increase in turbidity of broth containing growing cells of *Streptococcus agalactiae* and an unknown amount of nisin is compared with similar increases in samples containing known amounts of nisin, using a photo-electric absorptiometer. The penicillinase (Wellcome) was found to be of the activity specified. For the purpose of this note 1 unit of penicillinase is that amount which will destroy 1 IU of penicillin in 16 hours at 40°. One unit of nisin will inhibit the growth of *Str. agalactiae* in 1 ml of broth for 16 hours at 37° C.

The activity of penicillinase on nisin was tested by incubating mixtures at various pH values, and at 40° for 2 hours. All samples were then brought to pH 2.0 and heated for 5 min. at 100°, before assaying. The results are summarised in Table 1.

Table 1. Activity of nisin before and after the action of penicillinase.

40 000 units of nisin were incubated in 10 ml with 50 000 units of penicillinase and treated as described in the text.

Experiment 1		Experiment 2	
pH of incubation	Final activity units/ml	pH of incubation	Final activity units/ml
Control (no penicillinase)	4 000	Control (no penicillinase)	4 000
1.43	4 000	5.19	4 000
2.34	3 850	5.50	4 000
3.45	4 000	5.70	4 200
		6.95	4 200
		7.84	4 000
		8.42	4 000

Penicillinase alone at concentration of 50 000 units per tube did not cause any inhibition of growth.

Other experiments, using different proportions of nisin and penicillinase, altering the times of incubation and changing the pH values, gave the same result.

It would appear safe to conclude that this particular kind of penicillinase has no action on nisin.

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Studies on Arsenic Trichloride as a Solvent I. The Existence of Crystalline Solvates

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The first experimental studies of $AsCl_3$ as a solvent were carried out by P. Walden [1], who investigated the solubility of some salts. The pioneer work, however, was done by V. Gutmann [2]. His main results can be summarized thus:

1. Extensive knowledge of the solubility and reactions of inorganic compounds in $AsCl_3$ was obtained.
2. The existence of a number of reactions similar to the acid-base reactions in aqueous solutions was proved.
3. The usefulness of $AsCl_3$ as a medium for the preparation of complex chlorides was established.

From the point of view of an extended acid-base conception later suggested by Gutmann and Lindqvist [3], it seemed interesting to study the reactions in $AsCl_3$ further than had been done by Gutmann in his broad survey work. This is the aim of the present and some following papers.

$(CH_3)_4NAsCl_4 \cdot 2AsCl_3$.

Tetramethylammoniumchloride $(CH_3)_4NCl$ is one of the salts most soluble in $AsCl_3$. Gutmann obtained from its solution a compound $(CH_3)_4NAsCl_4$ by evaporation of the excess solvent followed by heating to constant weight *in vacuo* at 40° C. Using conductometric measurements he was also able to show that the compound contained $AsCl^-_4$ ions. Many other similar compounds, tetrachloroarsenites, are described by Gutmann. On the other hand, no solvates of these salts have been prepared analogous to the hydrates of the aqueous system, *i.e.* containing more loosely bound $AsCl_3$. Knowledge of the crystal chemistry of crystalline hydrates has been very useful, however, for the understanding of the hydration mechanisms (*cf. e. g.* Tovborg Jensen [4]). We have therefore tried to prepare such a solvate. It is obvious that milder evaporation must be used in this case. We were able to prepare a salt $(CH_3)_4NAsCl_4 \cdot 2AsCl_3$ by such a careful treatment. It crystallizes in well developed crystals which are unstable in air because they are attacked by water vapour (at a humidity of about 40 % at room temperature). We intend to try to study the structure of this compound by X-ray methods.

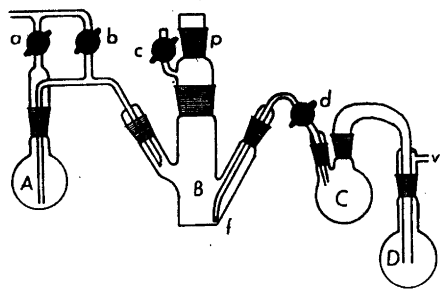

Fig. 1.

Chemicals used: Arsenic trichloride (Schuchardt) was allowed to stand for three days with sodium, and was then removed from the sodium and distilled. The main fraction (95 % of the crude product) has a boiling point of 130.0° C. *Tetramethylammonium chloride* (Eastman Kodak) was dried for 12 hours at 110° C.

Procedure. The apparatus used is sketched in Fig. 1. In *A* there was supply of $AsCl_3$. The $(CH_3)_4NCl$ was transferred to *B* through *P*, and a suitable amount of $AsCl_3$ was then pressed to *B* by a stream of dry nitrogen through *a*. When the salt had dissolved, dry N_2 was led through *b* and the stopcocks *c* and *d* opened. With *c* closed, some solution was transferred through *d* to *C* (filtration at *f*), after which *c* was opened again and *d* closed. The vessel *D* was cooled with a freezing mixture, and $AsCl_3$ was evaporated from *C* by evacuation at *v* (9 mm Hg). Crystals of $(CH_3)_4NAsCl_4 \cdot 2AsCl_3$ were formed in *C*.

Analyses. The crystals were dissolved in 1 *M* NaOH. The solution was slightly acidified with conc. HNO_3, and Cl determined by a potentiometric titration with 0.1 *M* $AgNO_3$ (silver electrode). As was determined by a potentiometric titration with 1/60 *M* $KBrO_3$ (platinum electrode). A silver/silver-chloride electrode was used as reference electrode. *Results:* Found, Cl 54.5, As 34.5; calc. Cl 54.3, As 34.4.

Desolvatisation. It was proved that two of the three $AsCl_3$ molecules are loosely bound in $(CH_3)_4NAsCl_4 \cdot 2AsCl_3$ by deter-

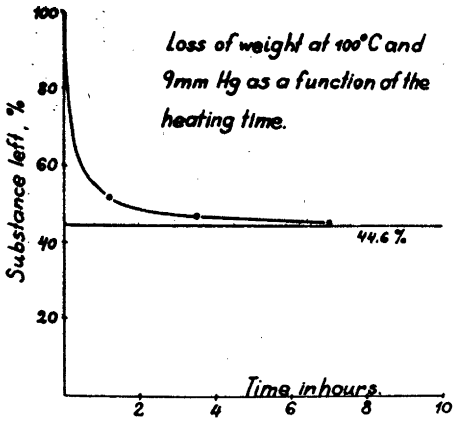

Fig. 2.

mining the loss of weight of the salt after heating at a pressure of 9 mm Hg to 100° C (Fig. 2). The calculated loss of weight is 55.5 %, that found 54.6 %.

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On the Synthesis of Methoxinine

J. A. CHRISTENSEN

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Methoxinine, the oxygen analog of methionine, has been prepared by Roblin [1] by alkylating ethyl phthalimidomalonate with β-methoxyethyl bromide, followed by hydrolysis and decarboxylation of the resultant ethyl (β-methoxyethyl)phthalimidomalonate.

In an attempt to repeat this synthesis, using ethyl acetamidomalonate and either β-methoxyethyl chloride or β-methoxyethyl *p*-toluenesulfonate it was found difficult to obtain the intermediate ethyl β-methoxyethyl acetamidomalonate in a pure state. The substance was therefore prepared by the method of Barry and Hartung [2] using γ-methoxy-α-oximino butyric acid as an intermediate. Hydrogenation of this oximino acid in aqueous ammonia gave methoxinine in a pure state.

Experimental. (Micro analyses are made by Mr. P. Hansen. All melting and boiling points are uncorrected.)

Ethyl-β-methoxyethylmalonate was prepared in conventional manner [3] from ethyl malonate, sodium ethoxide and β-methoxyethyl chloride in 69 % yield. B.p. 124—5°/10 mm; n_D^{25} 1.4228; $C_{10}H_{18}O_5$ (218.2): Calc. C 55.0; H 8.3. Found C 54.9; H 8.0.

γ-Methoxy-α-oximinobutyric acid was prepared according to the directions of Barry and Hartung [2] in 80 % yield. M.p. 112—3° (from ethyl acetate). $C_5H_9NO_4$ (147.1). Calc. C 40.8; H 6.2; N 9.5. Found C 41.0; H 6.1; N 9.2; Acid eqv. 147.

Methoxinine: A solution of 36 g of γ-methoxy-α-oximino butyric acid in 360 ml of 10 % aqueous ammonia was treated with hydrogen at 50° and 100 atm for 6 hours using 10 g of Raney-nickel as a catalyst. The catalyst was filtered off and the solution concentrated to 100 ml in vacuum, treated with hydrogen sulfide to remove some dissolved nickel salts, filtered, and the solution concentrated to approx. 50 g, 150 ml of 99 % ethanol added, and the solution left in the ice box for 15 hours. Filtration gave 18 g (55 %) of colorless leaves, decomposing at 220—250° without melting (Roblin [2] gives m.p. 253° (dec. with effervescence)). $C_5H_{11}NO_3$ (133.1). Calc. C 45.1; H 8.3; N 10.5. Found C 44.9; H 8.0; N 10.5; Formol titr. 134.

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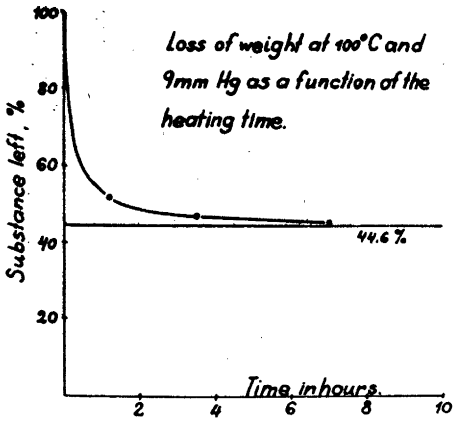

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Rac.-1,2-Dithiolane-3,5-dicarboxylic Acid

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Already in 1938 Fredga [1,2] pointed out the importance of the study of aliphatic α,α'-dimercapto dicarboxylic acids and the cyclic disulphides derived from them. Thus both the *racemic* and *meso* form of α,α'-dimercaptoadipic acid were investigated together with the 1,2-dithiane derivatives. Later on α,α'-dimercaptosuccinic acid was reported by Owen and Sultanbawa [3], although with no separation of the stereoisomeric forms.

It is the author's intention to continue the investigation of this series with particular reference to the glutaric and pimelic acids. As the monomeric oxidation products from these mercapto acids, the corresponding disulphides, are cyclic, important information might be obtained for the interpretation of redox processes of the mercaptan-disulphide type. As the compounds are water-soluble and the reactions reversible, it should be possible to study more thoroughly the formation and behaviour of the polymeric linear disulphides, which are obtained not only in oxidation processes under certain conditions but also in direct polymerization of the cyclic disulphides. The latter reaction may proceed either through radical formation or by a redox mechanism. — In addition the presence of two asymmetric centres makes it possible to study the influence of steric effects on the ring closure.

The above mentioned investigation is in progress, and this publication concerns the synthesis and preliminary resolution of *rac.*-1,2-dithiolane-3,5-dicarboxylic acid (*rac.*- 1,2-dithia*cyclo*pentane-3,5-dicarboxylic acid). Only a few substances with this ring system are known, but already from the existing material, the unsubstituted heterocycle [4] and its 4,4-dimethyl derivative [5], it can be seen that, probably due to considerable strain in the molecule, interesting properties characterize this group of compounds. It seems obvious that an examination of the dicarboxylic acid, now accessible, will essentially contribute to the knowledge of this group of heterocycles.

The *rac.*- 1,2-dithiolane-3,5-dicarboxylic acid (*rac.*-1,2-dithia*cyclo*pentane-3,5-dicarboxylic acid) has been prepared from the high melting form of α,α'-dibromo glutaric acid and sodium disulphide in water solution. It is very remarkable that the 1,2-dithiolane derivative obtained has a racemic structure as the high melting form of α,α'-dibromo glutaric acid is ascribed the meso-configuration [6,7] (the proof is, however, questionable). Two explanations can be offered. Either the hitherto accepted structure for the dibromo acid is erroneous or perhaps the reaction mechanism implies an intermediate formation of a γ-lactone with subsequent attack of the disulphide group within the molecule to form the cyclic disulphide dicarboxylic acid, the last replacement proceeding with inversion. Such a mechanism would involve two inversions at one of the asymmetric centres and one at the other, resulting in a transformation from *meso* to *racemic* structure. It is of course doubtful, whether a ring opening of a γ-lactone can occur as easily as has to be presumed, but it must be remembered that the reaction of alkyl mercaptides with γ-lactones gives rise to a cleavage of the ring with the formation of salts of γ-alkylmercapto butyric acids [8]. It is further of some importance to call attention to the fact that the ring opening step would in this case mean an intramolecular reaction, which must facilitate the disulphide ion attack to form the cyclic dicarboxylic acid. The investigations are being pursued.

Preliminary experiments on resolution with brucine and quinine have given clear evidence of the *racemic* structure of the 1,2-dithiolane-3,5-dicarboxylic acid prepared. The (+)-acid can be obtained from the acid brucine salt, crystallized from dilute alcohol, and the (−)-acid from the neutral brucine salt, best crystallized from the former mother liquor. As could be expected [9] a rather high rotatory power has been observed (see below), probably due to the combined effects of cyclic structure, the electronic conditions of a disulphide bond and the position of the disulphide group relative to the asymmetric centres. A complete and detailed description will be published later.

EXPERIMENTAL. Rac.-1,2-Dithiolane-3,5-dicarboxylic acid. 25.7 g (0.09 moles) of α,α'-dibromo glutaric acid (m.p. 169—170°) was rapidly neutralized with a water solution of 25.8 g (0.09 moles) of sodium carbonate de-

cahydrate. A solution of sodium disulphide in excess (0.11 moles), prepared about 48 hours before use from $Na_2S \cdot 9\ H_2O$ and sulphur in water as solvent, was added cautiously with cooling. The total volume was 200 ml, and the solution was allowed to stand for 8 hours with occasional shaking. Some sulphur, having separated, was filtered off and the still alkaline solution acidified with dilute sulphuric acid. After the removal of precipitated amounts of non-crystalline material, mainly sulphur, the aqueous part was extracted 10 times with 50 ml portions of ether. The ether was allowed to evaporate spontaneously, leaving a semi-solid, colourless mass. By treatment with a small volume of hot water, this product mostly dissolved, and after rapid filtration the solution was placed in a refrigerator over night. The dicarboxylic acid crystallized as beautifully formed prisms. Repeated recrystallizations yielded 1.9 g (11 %) of a pure substance with the melting point 197—199°. The melting point depends on the rate of heating; the value given was found by plunging the capillary tube into the preheated (about 190°) apparatus. (Found: Equiv.wt. 97.8; S 33.33; Mol.wt. 191, 186, 188. Calc. for $C_5H_6O_4S_2$ (194.2)· Equiv. wt. 97.1; S 33.02; Mol. wt. 194.2).

Preliminary experiments on resolution. (+)-1,2-Dithiolane-3,5-dicarboxylic acid. 0.39 g (0.002 moles) of the *racemic* acid and 0.94 g (0.002 moles) of brucine were dissolved in a hot mixture of water (10 ml) and ethanol (6 ml). Crystallization over night at room temperature yielded 0.60 g (45 %) of salt. The acid was set free from its salt by acidifying with dilute sulphuric acid and extraction with ether in the usual manner. The rotatory power was measured in alcohol. M.p. 176—180°.

$$[\alpha]_D^{25} = + 414°$$

(—)-1,2-Dithiolane-3,5-dicarboxylic acid. 0.47 g (0.001 mole) of brucine was added to the mother liquor from above together with 5 ml of ethanol. Crystallization as before yielded 0.60 g of salt. The measurement of the rotatory power was performed as described above. M.p. 177—181°: $[\alpha]_D^{25} = - 564°$, Equiv. wt. Calc. 97.1. Found 97.3.

The author is indebted to Professor Arne Fredga for valuable discussions and stimulating help. A grant from the *Swedish Natural Science Research Council* is gratefully acknowledged.

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Some Sulphur Compounds Related to Pimelic Acid

LENNART SCHOTTE

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In the preceding paper [1] attention is drawn to the study of α,α'-dimercapto dicarboxylic acids and the important results, that might be obtained by investigations already shortly outlined. No such derivatives related to pimelic acid have until now been published, and only those of adipic acid have been more thoroughly investigated [2,3].

The corresponding dibromo acids are usually employed as starting materials, but for α,α'-dibromo pimelic acid the isolation of one of the stereoisomeric forms still remains. Fehnel and Oppenlander [4] report and discuss the preparation of the high melting form in connection with an examination of some symmetrical 2,6-disubstituted tetrahydrothiapyrans. In a reaction with sodium sulphide they obtained *cis-(meso)*-2,6-tetrahydrothiapyrandicarboxylic acid, and they thus concluded that the high melting isomer of α,α'-dibromo pimelic acid possesses the *meso* configuration. Already before these results were published, the same compounds had been prepared in a slightly different way by the present author. As his observations are in good agreement with those of Fehnel and Oppenlander, it seems unnecessary to publish the experimental details.

The present publication deals with some further sulphur derivatives obtained from the high melting dibromo acid, while attempts to isolate the other stereoisomer are in progress.

For the preparation of mercapto-substituted carboxylic acids two methods are

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For the preparation of mercapto-substituted carboxylic acids two methods are

mainly taken into consideration, namely the reduction of the corresponding disulphides and the treatment of the xanthogen-substituted acids with ammonia [5,6].

The monomeric disulphide related to α,α'-dimercapto pimelic acid has a seven-membered ring structure and can be obtained from the dibromo acid and sodium disulphide, although in a rather low yield. As could be expected considerable amounts of polymeric linear disulphides are formed. It is further interesting to note that as a by-product the above mentioned *cis*-2,6-tetrahydrothiapyrandicarboxylic acid has been isolated. When α,α'-dibromo *adipic* acid is treated with potassium disulphide, both the cyclic sulphide and disulphide are formed, the former with separation of sulphur [7]. The difference between the homologues thus only implies that the dibromo pimelic acid gives also polymeric products, obviously due to the unfavourable conditions for cyclization to a ring of the size in question. — The structure of the monomeric disulphide is definitely settled by reduction with zinc powder in ammoniacal solution, which yielded dimercapto pimelic acid, identified by titration with sodium hydroxide and with iodine. Owing to its solubility properties the mercapto acid has, however, not yet been obtained quite pure. — The formation of a dithiol from the cyclic disulphide shows that the structure $>S=S$ for the disulphide group is excluded. On the other side, a substance of this type might possibly act as an intermediate in the formation of the cyclic monosulphide dicarboxylic acid, which is obtained with separation of sulphur. — The relative difficulties in obtaining the seven-membered ring system in question, dithiacycloheptane, of which only the unsubstituted heterocycle [8] is known previously, are further elucidated by experiments on the oxidation of the dimercapto acid with hydrogen peroxide. Preliminary results show that only polymeric products are formed as has also been found for α,α'-dimercapto suberic acid [9]. The lower homologue, dimercapto adipic acid, yields the six-membered cyclic disulphide without complications [2,3]. The influence of the dilution on the course of such oxidation reactions is being investigated.

From the high melting form of α,α'-dibromo pimelic acid the corresponding *bis*-ethylxanthogen acid has been prepared in the usual way. By treatment with ammonia the dimercapto derivative is obtained in a good yield, the report of which, however, will be given later, when the purification operations are quite finished. The definite evidence of the structure of these compounds from stereochemical point of view will be discussed later.

EXPERIMENTAL. 1,2-Dithiacycloheptane-3,7-dicarboxylic acid. A solution of sodium disulphide (0.058 moles) in water, prepared in the usual manner [1] 48 hours before use, was added to an aqueous solution of the neutral sodium salt of α,α'-dibromo pimelic acid, obtained by rapid neutralization of 15.9 g (0.05 moles) of the acid (m.p. 150—151°) with sodium carbonate decahydrate. The total volume was 120 ml. After 27 hours, separated sulphur (0.4 g) was filtered off and the solution was acidified with dilute sulphuric acid. Polymeric products immediately precipitated as a viscous oil, and the aqueous solution was extracted repeatedly with 35 ml portions of ether. On spontaneous evaporation the ether solution left a semi-solid, colourless mass. Recrystallizations (twice) from water gave 1.2 g (11 % yield) of the dicarboxylic acid as hard prisms. The melting point is 193—194° but depends somewhat on the rate of heating; the value given was found by plunging the capillary tube into the preheated (about 188°) apparatus. The melting seems to occur without any decomposition. (Found: Eq.wt. 112.3; S 29.12; Mol.wt. 223, 222, 224, 224; Calc. for $C_7H_{10}O_4S_2$ (222.3): Eq. wt. 111.1; S 28.85; Mol. wt. 222.3).

It is possible that the yield above of monomeric substance can be somewhat improved, if the reaction is performed in more dilute solution.

The mother liquor from the first recrystallization was extracted with ether, and the crude product obtained by evaporation of the solvent was purified by fractionated crystallization from small amounts of water. 0.4 g of a pure substance, m.p. 208—210°, (more soluble in water than the disulphide) was isolated and identified as *cis*-2,6-tetrahydrothiapyrandicarboxylic acid (mixed m.p. 208—209°).

α,α'-Bis-ethylxanthogen pimelic acid. 15.9 g (0.05 moles) of α,α'-dibromo pimelic acid (m.p. 150—151°) was neutralized in water solution with 14.3 g (0.05 moles) of $Na_2CO_3\,10H_2O$, the total volume being 100 ml. 18.4 g (15 % excess) of solid potassium ethyl xanthate was added immediately and the mixture shaken until homogeneous. After standing at room temperature for three days, the solution was acidified with dilute sulphuric acid. The *bis*-xanthogen acid separated as an oil, which soon crystallized (if the reaction time is shorter

a lower yield is obtained, and the product crystallizes less readily). The crude product was filtered off and treated with water in order to remove any dibromo acid left. The yield of acid thus obtained was 18 g (calc. 20.2 g), and the melting point was about 130°. After five recrystallizations from formic acid the product was pure and showed the m.p. 135—136°. (Found: Eq.wt. 199.6; S 31.93; Calc. for $C_{13}H_{20}O_6S_4$ (400.4): Equiv. wt. 200.2; S 32.02).

The author wants to express his gratitude to Professor Arne Fredga for valuable discussions. A grant from the *Swedish Natural Science Research Council* is gratefully acknowledged.

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On the Growth-Inhibition of *Lactobacillus bifidus* by Certain Fatty Acids

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Recent investigations [1,2] have shown that free long-chain (C>6) saturated fatty acids as well as unsaturated oleic and linoleic acids inhibit the growth of *Lactobacillus bifidus in vitro*. According to Barbero *et al.*[3] the same is true *in vivo*; in their experiments the number of *L. bifidus* in the intestinal tract of infants increased when the inhibitory saturated acids were removed from the cow's milk used in the feeding. A comparison of the composition of human milk and cow's milk shows that their content of the most strongly inhibiting saturated acids (lauric and myristic acids) is approximately equal [4]. Since, however, it is known that in practice human milk promotes the growth of *L. bifidus* in the intestinal tract of infants, it has been concluded that human milk must contain substances which can reverse the effect of the inhibitory acids. Some evidence has been cited [1,5] to show that proteins, and lactalbumin in particular, act as the detoxifying agents. The stronger detoxifying effect of human milk compared with cow's milk could, accordingly, be explained by the relatively greater content of whey proteins in human milk.

The *L. bifidus* strains used in our investigation were isolated from the faeces of breast-fed infants. The basal medium was that proposed by Hassinen *et al.*[6], supplemented with tryptic digest of casein as a source of strepogenin, found by us [7,8] to be essential for *L. bifidus*. The growth was measured titrimetrically after incubation at 37° C for 72 hours. The fatty acids investigated were added to the media as potassium salts, obtained by saponification of the corresponding acids with KOH in ethanol.

Our results on the inhibition of *L. bifidus* by different fatty acids agree in general with the data given by Tomarelli *et al.*[1] and Hassinen *et al.*[2] Propionic and butyric acids were found to have no effect, caproic acid was slightly inhibitory, and with increasing length of the carbon chain the inhibitory effect increased until lauric acid, which had the strongest effect. With the chain-length increasing further the toxicity decreased, the effect of myristic acid being nearly identical with that of capric acid. According to Hassinen *et al.*[2] palmitic, stearic and oleic acids do not inhibit *L. bifidus;* in our experiments, however, these acids also showed inhibiting, although distinctly weaker, effects than those of linoleic, lauric, capric and myristic acids. It is interesting to note in this connection that ricinoleic acid had an effect that was about 5 times as strong as that of oleic acid.

In order to overcome the inhibitory effect of fatty acids we added different proteins and yeast extract to the media containing lauric or linoleic acids. The results are given in Table 1. It will be seen that the additions of egg albumin, casein and blood albumin have permitted a fairly good growth of the bacteria, even in the presence of the inhibiting acids. Gelatin had a somewhat weaker effect whereas

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Table 1. Effect of certain proteins and yeast extract on the growth-inhibition of L. b i f i d u s by lauric and linoleic acids. (Consumption of 0.1 N NaOH, ml/10 ml medium.)

Addition to the medium, 5 mg/ml	Medium		
	Basal	Basal + 200 γ lauric acid/ml	Basal + 200 γ linoleic acid/ml
None	7.5	0.0	0.0
Egg albumin	6.9	2.5	7.1
Casein	6.7	4.0	5.5
Blood albumin	7.1	3.2	4.9
Gelatin	7.1	1.6	3.3
Trypsin	8.1	1.3	1.3
Pepsin	7.4	1.0	0.2
Yeast extract	8.3	0.3	0.4

trypsin, pepsin and yeast extract had a very weak or practically no effect. In general the inhibitory effect of linoleic acid was easier to overcome than that of lauric acid. It is noticeable that casein was as effective as the albumins tested, indicating that the detoxifying ability *in vivo* may not be limited specifically to the albumin of milk.

Results of experiments conducted to show the detoxifying effects of skimmed human and cow's milk are given in Table 2. It can be seen that both milks possess the detoxifying ability, increasing amounts of milk leading to a more complete effect. It is also evident that skimmed cow's milk has a stronger detoxifying potency than skimmed human milk. The results with tryptic digests of milk show that in both cases detoxification is due to the proteins and not to their degradation products.

The results arrived at by earlier authors investigating the inhibitory effects of certain saturated and unsaturated fatty acids on the growth of *L. bifidus* thus were confirmed. The inhibitory effect could be overcome by certain pure proteins and by skimmed cow's or human milk. Since human milk and cow's milk are very similar in their content of inhibitory fatty acids and since the proteins of human milk have a weaker detoxifying potency than those of cow's milk, it can be concluded that the different effects of human and cow's milk on *L. bifidus in vivo* must be ascribed to other factors than an inhibition of this bacterium by certain fatty acids present in milk fat.

Table 2. Effect of skimmed human milk and skimmed cow's milk on the growth-inhibition of L. b i f i d u s by lauric, linoleic, and oleic acids. (Consumption of 0.1 N NaOH, ml/10 ml medium).

Additions to 10 ml medium	Medium			
	Basal	Basal + 200 γ lauric acid/ml	Basal + 200 γ linoleic acid/ml	Basal + 200 γ lauric + 500 γ oleic acid/ml
None	6.8	0.5	0.4	0.6
Skimmed human milk				
0.2 ml		1.0	0.5	
0.5 ml		2.5	5.0	2.6
1.0 ml		4.2	5.8	
Tryptic digest of skimmed human milk				
0.5 ml		1.4		0.7
Skimmed cow's milk				
0.2 ml		3.8	5.0	
0.5 ml		4.9	6.5	5.2
1.0 ml		6.4	6.9	
Tryptic digest of skimmed cow's milk				
0,5 ml		2.8		1.9

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Rate Constants and Reaction Products of the Alkaline Hydrolysis of Ethylene and Trimethylene Chlorohydrins with Alkyl Substituents

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The hydrolysis of the two series of alkyl-substituted ethylene chlorohydrins of the types

$RCHOHCH_2Cl$ and $RCHClCH_2OH$

have been kinetically investigated at 20.00 °C in alkaline aqueous solution. As with other ethylene chlorohydrins that have been investigated, these gave good second-order constants, and the reaction products were found to be the corresponding oxides. The following constants (k_{20} mole litre^{-1} min^{-1}) were obtained in the two series.

CH_2OHCH_2Cl	$k_{20} = 0.332$
$CH_3CHOHCH_2Cl$	$k_{20} = 7.85$
$C_2H_5CHOHCH_2Cl$	$k_{20} = 9.60$
n-$C_3H_7CHOHCH_2Cl$	$k_{20} = 9.50$
i-$C_3H_7CHOHCH_2Cl$	$k_{20} = 12.3$
$CH_2CHClCH_2OH$	$k_{20} = 2.65$
$C_2H_5CHClCH_2OH$	$k_{20} = 2.41$
n-$C_3H_7CHClCH_2OH$	$k_{20} = 2.71$
i-$C_3H_7CHClCH_2OH$	$k_{20} = 1.14$

The chlorohydrins of the type $RCHOHCH_2Cl$ were prepared by Grignard syntheses from chloroacetaldehyde and alkyl bromides. The chlorohydrins of the type $RCHClCH_2OH$ were prepared from the corresponding chlorosubstituted acid chlorides by reduction with lithium aluminium hydride.

In order to investigate the corresponding reactions of the trimethylene chlorohydrins, compounds of the types

$RCHOHCH_2CH_2Cl$ and $RCHClCH_2CH_2OH$

were synthesized using the same methods as in the case of the ethylene chlorohydrins. — The compounds $RCHOHCH_2CH_2Cl$ from alkylmagnesium bromides and β-chloropropionaldehyde and the compounds $RCHClCH_2CH_2OH$ by lithium aluminium hydride and β-chlorosubstituted acid chlorides.

They were also found to give good second-order constants, but the rates of the alkaline hydrolysis were much slower than for the corresponding ethylene chlorohydrins. The rate constants were therefore measured at 80.07° C (k_{80} mole litre^{-1} min^{-1}). At that temperature there is also a first-order reaction with water (hydrolysis, yielding hydrochloric acid) that must be measured in pure water in order to calculate the second-order constants from the measurements in the alkaline solution.

$CH_2OHCH_2CH_2Cl$	$k_{80} = 0.0212$
$CH_3CHOHCH_2CH_2Cl$	$k_{80} = 0.049$
$C_2H_5CHOHCH_2CH_2Cl$	$k_{80} = 0.071$
n-$C_3H_7CHOHCH_2CH_2Cl$	$k_{80} = 0.069$
i-$C_3H_7CHOHCH_2CH_2Cl$	$k_{80} = 0.12$
$CH_3CHClCH_2CH_2OH$	$k_{80} = 0.45$
$C_2H_5CHClCH_2CH_2OH$	$k_{80} = 0.79$
n-$C_3H_7CHClCH_2CH_2OH$	$k_{80} = 0.79$
i-$C_3H_7CHClCH_2CH_2OH$	$k_{80} \sim 1.5$

As in the case of the ethylene chlorohydrins, the unsubstituted trimethylene chlorohydrin and the chlorohydrins of the type $RCHOHCH_2CH_2Cl$ gave the corresponding trimethylene oxides in alkaline aqueous solution. The chlorohydrins of the type $RCHClCH_2CH_2OH$ gave no oxides but were decomposed with the formation of formaldehyde and an alkene as shown in the equation.

$$RCHClCH_2CH_2OH + OH^- \longrightarrow RCH{=}CH_2 + CH_2O + H_2O + Cl^-$$

Thus in alkaline solution the carbon chain of the compounds $RCHClCH_2CH_2OH$ is cleaved. The alkenes were identified through their boiling-points (in the case of propylene, also through the bromo addition-compound), and the formaldehyde through the color reactions with orcinol [1] and resorcinol [2]. The compounds of the type $RCHOHCH_2CH_2Cl$ gave no alkenes and no reaction of formaldehyde.

After the termination of this investigation a publication by Searles and Gortatowski [3] appeared in which the alkaline hydrolysis of 3-bromo-2,2-dimethyl-propanol was discussed. According to their investigations this compound gave oxide and simultaneously *iso*butylene and formaldehyde in alkaline solution. I have found also that 3-chloro-2,2-dimethyl-propanol gave formaldehyde when it was treated with alkali.

A more detailed report on these reactions will be published in the near future.

1. Sumner, J. B. *J. Am. Chem. Soc.* **45** (1923) 2378.
2. Walker, J. F. *Formaldehyde*, New York, 1953, p. 370.
3. Searles, S., and Gortatowski, M. J. *J. Am. Chem. Soc.* **75** (1953) 3031.

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Chemical Analysis of a New Mineral, Bøggildite, from Ivigtut, Greenland

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In 1950 the late Richard Bøgvad, chief geologist to "Kryolitselskabet Øresund A/S", discovered a hitherto unknown mineral in the cryolite mine at Ivigtut. Bøgvad published a provisional notice [1] of this mineral of which he had prepared a thorough investigation. His plans were brought to an end by his sudden death on August 7, 1952; but not before Bøgvad had named the new mineral "bøggildite" in honour of O. B. Bøggild, professor emer. of the University of Copenhagen.

In January 1951 Bøgvad handed over to me 4.4 g of pulverised bøggildite. Of this material a sample was sent to the Central Laboratory of "Bolidens Gruvaktiebolag", Sweden, for spectrographic estimations. The results of these were in reasonable agreement with the chemical-analytical data. — 0.5 g were reserved for X-ray and crystaloptic investigations. — The rest of the material I have used for quantitative analyses, the results of which are given below.

As regards the purity of the material Bøgvad stated that "the material for analysis only contains a very slight trace of quartz and "ivigtite". Further, traces were found of presumed sphalerite in particles about 1 μ large. Otherwise it is very pure".

Chlorine, zinc, and cerium were sought for by microanalytical methods, but were not detected. The alkalies were determined by the method of Wells and Stevens [2].

Formula for bøggildite: $Na_2Sr_2Al_2(PO_4)F_9$. Mol.wt. calculated from the analysis = 543.3.

On heating in a glass tube over a Bunsen burner the mineral melts and deposits a small quantity of white sublimate.

Chemical Analysis of Bøggildite from Ivigtut.

	Weight %	Ionic ratios		Number of ions
Al	10.04	0.372	0.373	2.01
Fe	0.06	0.001		
Sr	31.89	0.364	0.379	2.04
Ba	0.35	0.003		
Mg	0.18	0.007		
Ca	0.20	0.005		
Mn	trace	—		
Na	8.60	0.374	0.378	2.03
K	0.15	0.004		
Li	trace	—		
PO_4	17.63	0.186	0.186	1.00
F	31.70	1.668	1.668	8.97
$H_2O\pm$	none	—		
Total	100.80			

1. Bøgvad, R. *Medd. Dansk Geol. Foren.* **12** (1951) 109; Bøggild, O. B. *The Mineralogy of Greenland. Medd. Grønland* **149** No. 3 (1953) 439.
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The Complexity of Uranium(IV) Chloride, Bromide and Thiocyanate

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The complex chemistry of solutions of U(IV) has not been treated until recently and it must still be considered as rather undeveloped. It is significant that such a fundamental fact as the formula U^{4+} of the ion existing in strongly acid solutions was unequivocally established only a few years ago. As U(IV) no doubt merits the greatest attention, especially on account of its close resemblance to the four-valent states of the other actinides, a wider knowledge of its complex chemistry seems highly desirable. Towards this end the experimental difficulties which have largely caused the slow development up till now must be overcome. The solutions of U(IV) are thus easily oxidized by the air [1], they are heavily hydrolyzed even at very low pH [2,3], and even if the resultant difficulties could be mastered, it is not so easy to find a suitable method of measurement for the investigation of the complex systems.

SOME PREVIOUS INVESTIGATIONS ON U(IV) SOLUTIONS

Kraus and Nelson [2] have given the first decisive proof of the formula U^{4+} by measuring with the glass electrode the increase of acidity when known amounts of anhydrous UCl_4 are dissolved in acids of known concentration. It is found that considerably less than one mole of acid is liberated per mole of U(IV) dissolved. Thus a great part of U(IV) must remain in the unhydrolyzed state even in solution.

The formula U^{4+} could also be verified by measuring the influence of acidity on the potential of the U(VI)/U(IV) couple, as the species of U(VI) existing in acid solutions is certainly known [4] to be UO_2^{2+}. Several attempts have been performed to determine this effect of acidity. The earlier investigators [5–7] all used sulfate solutions, now known to be rather strongly complex [8–10] and measured, moreover, at varying ionic strength, employing very uncertain "degrees of dissociation" in their subsequent calculations. Their results are therefore not conclusive. A recent investigation [11] was performed using non-

complex perchlorate solutions of constant ionic strength but the range of pH used was too narrow to allow a definite conclusion.

For the reasons mentioned the "normal potential" calculated from the measurements of sulfate solutions is a formal quantity, which is not identical with the real normal potential of the couple UO_2^{2+}/U^{4+}. This quantity has in fact quite recently been determined for the first time [12]. A value of 334 mV was obtained, which is ≈ 70 mV lower than that of the sulfate measurements, a consequence of the strong complexity of the sulfate system of U^{4+} in comparison with that of UO_2^{2+}.

As U^{4+} itself can exist only in strongly acid solutions, at least a preliminary knowledge of its hydrolysis is necessary for the investigation of any complex system of U(IV). The subject has first been studied by Kraus and Nelson [2] who measured extinctiometrically the concentrations of species of U(IV) in solutions of known $[H^+]$. They were able to explain all their data for $[H^+] > 20$ mC by one single equation of equilibrium, *viz.* $K_M = [H^+][UOH^{3+}]/[U^{4+}]$ where $K_M = 0.0278$ C at the ionic strength $I = 1.01$ C and the temperature 25° C. At lower $[H^+]$ the existence of higher, probably polynuclear, complexes must be postulated. In such solutions slow reactions are also observed undoubtly indicating a proceeding polymerization.

The number of experiments performed at a given I were, however, rather few, and the concentration of U(IV) was not varied greatly. The measurements, therefore, certainly do not permit a very elaborate theory, especially as the experimental errors are great.

A very thorough investigation has since been performed by Sillén and Hietanen [3]. Only measurements of $[H^+]$ were carried out, but this concentration as well as that of U(IV) were varied within very wide limits. The result is that the hydrolysis must involve merely polynuclear complexes, presumably of the general formula $U(UOOH)_n^{n+4}$ which may mean a chain complex resembling the chains proved [13] in crystals of basic uranium(IV) sulfate *.

In addition to the hydrolysis, only two complex systems of U^{4+} seem to have been investigated before. From a comparison between the hydrolysis of perchlorate and chloride solutions, Kraus and Nelson [2] estimated a thermodynamic complexity constant = 7.0 C^{-1} for UCl^{3+}, provided the given form of the Debye-Hückel law is obeyed. This value would correspond to 0.5 C^{-1} at $I = 1$ C. Such a calculation is, of course, very uncertain, but in any case the chloride system must be very weakly complex.

Betts and Leigh [9] have investigated the sulfate system, using the extraction method of Connick and Mc Vey [15]. As the concentration of U(IV) extracted into the benzene phase is approximately a function of only one unknown variable, *viz.* $[U^{4+}]$, the method is equivalent to a determination of the concentration of the free central ion. Methods for the evaluation of complexity

* According to Sillén, the hydrolytic reactions of a great number of metal ions are of this exclusively polynuclear type. Also the measurements of one of the present authors [14] on UO_2^{2+} can be surprisingly well fitted into this scheme. As the interpretation tentatively presented before was dependent on rather uncertain extrapolations leading to limiting values which could hardly be reasonably explained, this new approach is likely to hold a greater part of the truth than the previous one.

constants from such measurements have been given by Bjerrum [16], Leden [17] and Fronaeus [18]. By applying these general methods, Sullivan and Hindman [10] were able to correct the erroneous values of the constants calculated by the original authors.

PLAN OF THE PRESENT INVESTIGATION

The method most readily available for a determination of the complexity of an U(IV) system is undoubtly measurements of the redox potential of the UO_2^{2+}/U^{4+} couple in solutions of known acidity and known total concentrations of U(VI) and U(IV), containing varying amounts of the ligand. If the complexity of the U(VI) system is known, all complexity constants of the U(IV) system may be calculated from the differences between the potentials of complex and non-complex solutions as will be shown below.

The U(VI)/U(IV) redox potential is sensitive to light in the presence of certain anions, *e. g.* sulfate [6,19], while other ions, *e. g.* chloride [19], have no effect. It is therefore advisable to investigate for every system measured whether its redoxpotentials are affected by illumination.

A couple of complications may arise owing to the existence of two states of oxidation in the solutions. Thus mixed polynuclear complexes may be formed containing both UO_2^{2+} and U^{4+} (*cf. e. g.* Mc Connell and Davidson [20]). The possibility must in any case be considered by our method of calculation.

Further, a mixture of U(VI) and U(IV) can react forming U(V)[21]. If this happens the total concentrations of U(VI) and U(IV) are no longer known and the new complex-forming species UO_2^+ is, moreover, formed. The equilibrium between the reacting components is, however, highly dependent on $[H^+]$, and in strongly acid solutions U(V) is formed only in quite negligible amounts [21]. A complex formation ought to diminish the relative amount of U(V) further, as UO_2^+ can be anticipated to have a lower complex-forming power than UO_2^{2+} and U^{4+} on account of its lower charge. As to the hydrolysis, this anticipation has also been experimentally verified [2,21,14].

As is often the case in redox measurements, the potential of the present couple reaches its value of equilibrium very slowly if no special measures are taken. As a great number of measurements are desired here in a reasonable period of time, a way of accelerating the adjustment of the potential must be found. This problem has often been successfully solved by the addition of a mediator system.

Generally all substances may serve as mediators which rapidly give a stable redox potential, even if present in very low concentrations. In order that a mediator may be able to adjust the potential of a given system some additional conditions must, however, be fulfilled, which in practice highly reduce the number of mediators effective in a certain case (Luther [22]). First, the two systems present must be able to react mutually so that a real redox equilibrium is rapidly established. It must further be possible to choose the concentrations of the components of the main system so much higher than those of the mediator that they are only slightly altered by the redox reaction. Preferably this alteration should be so slight that the differences of potential used in our calculations are not at all affected by the mediator present, but in every case it

must be possible to arrive at such a value of the differences by measuring at several concentrations of the mediator, subsequently extrapolating to zero. In this way the mediator only communicates a potential to an immersed inert electrode without affecting it. In order that a well-defined potential shall really be transferred it is, however, also necessary that the ratio between the concentrations of the components of the mediator system does not assume too extreme values in the solutions measured. For a given redox potential, this ratio is determined by the normal potential and pH-dependence of the mediator, the values of which must, therefore, be considered in the first place in the search for a suitable system.

All the present measurements are performed in a strongly acid medium, having $[H^+] = 600$ mC throughout, in order to prevent the disturbance of the complex formation of U^{4+} by hydrolytic reactions. According to the investigations [2,3] mentioned above such a $[H^+]$ implies a degree of hydrolysis of ≈ 4 % in a perchlorate medium of the ionic strength $I = 1$ and the temperature 20° C, *i. e.* under the conditions of the present measurements. Perhaps a still lower value would have been desirable, but an increase of $[H^+]$ would presuppose an increase of I too. This is out of the question, as the determinations [23,24] of the corresponding uranyl systems utilized here all refer to $I = 1$. For the same reason, the temperature must be retained at 20° C.

It is true that the present medium is, nevertheless, not identical with that of the uranyl measurements, as a considerable part of the sodium ions have been exchanged for hydrogen ions. The constants of the uranyl systems determined previously are, therefore, only approximately valid here. But as may be anticipated on account of the difference of charge, U^{4+} forms much stronger complexes than UO_2^{2+} with a certain ligand. The complexity of UO_2^{2+} is, therefore, in fact only a correction applied in the calculation of the corresponding system of U^{4+}. For this purpose the values at hand are considered as sufficiently correct. The truth of this assumption is moreover tested as described later on.

The redox potential E_H of a perchlorate solution having $[UO_2^{2+}]/[U^{4+}] = 1$ and $[H^+] = 600$ mC is presumably ≈ 310 mV. Among mediators known, only the systems indigosulfonate / leukoindigosulfonate [25] seem to have suitable values of the ratio between their oxidized and reduced forms in such solutions. As to the system selected for closer examination, *viz.* indigotetrasulfonate(-5,7,5′,7′) / leukoindigotetrasulfonate, this ratio is indeed only ≈ 0.03 at $E_H \approx 310$ mV, but on account of the general tendency of U^{4+} to form complexes stronger than those of UO_2^{2+}, E_H will rise in connection with the complex formation and hence the ratio too. Its optimal value of 1 corresponds to $E_H \approx 355$ mV.

The mediator selected possesses two —NH— groups which probably means that it has a strong complex-forming ability. As its two components have the same coordinating groups they ought, however, to give complexes of about the same strength. Then the ratio between their concentrations remains about the same and the mediator may thus still be used in the potential range previously calculated. On the other hand, complex formation of the mediator must of course alter the ratio $[UO_2^{2+}]/[U^{4+}]$, as UO_2^{2+} and U^{4+} certainly have

very different affinities also towards this ligand. The effect is, however, removed by the extrapolation to zero concentration of the mediator earlier described (p. 140).

It must be emphasized that redox potentials obtained by the use of a mediator must, nevertheless, be proved to be really identical with those found without a mediator. This may sometimes be ascertained by direct comparison if the adjustment is not too slow without a mediator. Another good criterion is that the potential must vary according to Nernst's law when the ratio $[UO_2^{2+}] / [U^{4+}]$ is varied in a known manner as can be brought about in non-complex solutions.

For the sake of simplicity, only anions of strong acids are selected as ligands for the present investigation, *viz.* chloride, bromide and thiocyanate ion. Only in this way the acidity can be kept quite constant which facilitates both experimental work and calculations. Moreover the free concentration [A] of such ligands may be raised to high values even in acid solutions, and hence it is possible to follow the complex formation within a wider concentration range.

CALCULATION OF THE COMPLEXITY CONSTANTS FROM THE POTENTIALS MEASURED

Experimentally the emf E of cells of the following type is measured:

+ Au	10.06 mC $HClO_4$ 990 mC $NaClO_4$ quinhydrone(s.)	1 C $NaClO_4$	C_M mC U(VI) C_L mC U(IV) C_A mC ligand C_I mC mediator $[H^+]=600$ mC; $I=1$	Au −

where C_M, C_L, C_A and C_I thus are the total concentrations of U(VI), U(IV), ligand and mediator respectively.

E is composed according to the following formula:

$$E = E_{RE} + E_d - E^\circ - \frac{2RT}{F} \ln [H^+] - \frac{RT}{2F} \ln \frac{f_M f_H^4}{f_L} - \frac{RT}{2F} \ln \frac{[M]}{[L]} \qquad (1)$$

where

E_{RE} = potential of the reference electrode
E_d = sum of liquid junction potentials
E^0 = normal potential of the UO_2^{2+}/U^{4+} couple; $[L] = [U^{4+}]$; $[M] = [UO_2^{2+}]$
f_H, *etc.* = activity coefficient of the ion indicated by the subscript.

As $[H^+]$ and I are kept constant, it is assumed that E_d and the coefficients f are constant too, and thus (1) may be written as

$$E = E_k - \frac{RT}{2F} \ln \frac{[M]}{[L]} \qquad (2a)$$

where E_k is the (unknown) sum of the constant terms. It is eliminated by measuring E $(= E')$ of a cell having $C_A = 0$ in the right half-cell. Then [M]

and [L] are identical with the known stoicheiometric concentrations of U(VI) and U(IV) which for this particular solution are denoted by C'_M and C'_L. Hence

$$E' = E_k - \frac{RT}{2F} \ln \frac{C'_M}{C'_L} \tag{2b}$$

and

$$E' - E = E_{LM} = \frac{RT}{2F} \ln \frac{[M]}{[L]} \cdot \frac{C'_L}{C'_M} \tag{3}$$

The ratio between the total concentrations of U(VI) and U(IV) is always kept constant during a series of measurement, *i. e.* $C'_L/C'_M = C_L/C_M$.

There do not exist any polynuclear complexes $M_m A_n$, but the possible existence of mixed complexes has to be considered. They may be represented in the sequel by their simplest type MLA_q. Thus it is valid for the total concentration of U(VI):

$$C_M = [M](1 + \sum_{n=1}^{N} \beta_n [A]^n + [L] \sum_{q=1}^{Q} \delta_q [A]^q) \tag{4}$$

where

$$\beta_n = [MA_n] / [M][A]^n \text{ and } \delta_q = [MLA_q] / [M][L][A]^q$$

As to U(IV) there may exist polynuclear complexes $L_l A_p$ in addition to the mononuclear and mixed series. In the following formula of the total concentration of U(IV) this possibility is taken into account by the introduction of terms belonging to the dinuclear complexes L_2A_p:

$$C_L = [L](1 + \sum_{p=1}^{P'} \gamma'_p [A]^p + [L] \sum_{p=1}^{P''} \gamma''_p [A]^p + [M] \sum_{q=1}^{Q} \delta_q [A]^q) \tag{5}$$

where

$$\gamma'_p = [LA_p] / [L][A]^p \text{ and } \gamma''_p = [L_2A_p] / [L]^2[A]^p$$

The expression of C_L/C_M $(= C'_L/C'_M)$ obtained from (4) and (5) gives when inserted in (3):

$$E_{LM} = \frac{RT}{2F} \ln \frac{1 + \sum_{p=1}^{P'} \gamma'_p [A]^p + [L] \sum_{p=1}^{P''} \gamma''_p [A]^p + [M] \sum_{q=1}^{Q} \delta_q [A]^q}{1 + \sum_{n=1}^{N} \beta_n [A]^n + [L] \sum_{q=1}^{Q} \delta_q [A]^q} \tag{6}$$

From (6) it is concluded:

a. E_{LM} is a function of [A] only, if only mononuclear complexes exist.
b. E_{LM} is a function of [A] and [L] if polynuclear complexes of U(IV) are present.
c. If mixed complexes are formed, E_{LM} is a function of [A], [L] and [M].

For the sake of simplicity, the following notation is now introduced:

$$1 + \sum_{n=1}^{N} \beta_n [\mathrm{A}]^n = X_M \quad (7a) \qquad 1 + \sum_{p=1}^{P'} \gamma'_p [\mathrm{A}]^p = X_L \quad (7b)$$

$$\sum_{p=1}^{P'} \gamma'_p [\mathrm{A}]^p = Y_L \quad (7c) \qquad \sum_{q=1}^{Q} \delta_q [\mathrm{A}]^q = Z \quad (7d)$$

Even if polynuclear complexes exist, *i. e.* $Z \neq 0$ and / or $Y_L \neq 0$, the constants γ'_p of the mononuclear series would still be computed according to a method of extrapolation, analogous to that advanced by Fronæus [18] for solutions containing one central ion. One would then measure E_{LM} as a function of C_A at decreasing values of C_M and C_L keeping the ratio between them constant. By cutting the functions thus found at constant E_{LM}, C_A is obtained as a function of C_L (or C_M). By extrapolation to $C_M = C_L = 0$, $\lim C_A = [\mathrm{A}]$ is found, while E_{LM} simultaneously takes the form

$$E_{LM} = \frac{RT}{2F} \ln \frac{X_L}{X_M} \quad (8)$$

where X_M is known from the uranyl measurements. From (8) X_L corresponding to the found [A] may thus be calculated and hence γ'_p according to the usual method (*cf.*[26 p. 787]).

Unfortunately this straightforward method cannot be well applied here as the redox potentials, even in the presence of a mediator, do not adjust themselves properly at the lowest values of C_L and C_M which must be used to secure a good extrapolation. Moreover, the mediator then affects the ratio C_L/C_M rather seriously.

Therefore the method must be so modified that first only $C_L \to 0$ whereas C_M is kept at a high value. An extrapolation to $C_L = 0$ at constant E_{LM} then gives

$$\lim_{C_L \to 0} C_A = [\mathrm{A}] + \bar{n} \cdot C_M \quad (9)$$

where $\bar{n}$ is the ligand number of the uranyl system at the prevailing [A]. These quantities are easily found from (9) and the known complex formation function of the uranyl system. The computation is conveniently performed graphically, the corresponding values of ($\bar{n}$, [A]) sought for being obtained as the point of section between the straight line (9) and the formation curve.

By this extrapolation, E_{LM} takes the form

$$E_{LM} = \frac{RT}{2F} \ln \frac{X_L + [\mathrm{M}] \cdot Z}{X_M} \quad (10)$$

which does not permit a direct calculation of X_L if $Z \neq 0$. If it is preliminarily assumed however that $Z = 0$, then corresponding values of X_L and [A] are known and hence γ'_p may be calculated as before.

The assumption $Z = 0$ is then immediately put to an experimental test by measuring E_{LM} as a function of C_A at decreasing C_M whereas C_L is kept at a high value. An extrapolation analogous to the preceding one gives

$$\lim_{C_M \to 0} C_A = [\mathrm{A}] + \overline{m} \cdot C_L \tag{11}$$

where $\overline{m}$ is the ligand number of the uranium(IV) system preliminarily known as a function of [A] from the constants γ'_p just determined. From (11), [A] may thus be calculated.

In this case E_{LM} has the form

$$E_{LM} = \frac{RT}{2F} \ln \frac{X_L + [\mathrm{L}] \cdot Y_L}{X_M + [\mathrm{L}] \cdot Z} \tag{12}$$

As seen, a finite value of Z influences (10) and (12) in quite different ways, and (12) is, moreover, also dependent on Y_L.

If now the values of X_L (or possibly $X_L + [\mathrm{M}] \cdot Z$) calculated from (10) are inserted for the numerator of the fraction of (12) and the denominator then computed and found to be the same function X_M as determined before in the uranyl measurements, the only reasonable explanation is that the functions (10) and (12) are in reality identical. This implies that $Z = Y_L = 0$, *i. e.* no polynuclear complexes exist whatsoever. In addition, it must then also be concluded that the function X_M remains unchanged at the qualitative change of medium made between the two determinations of it.

This method of determination compulsorily chosen thus affords a good test of the existence of polynuclear complexes as well as of the effect of the medium change. Its weakness lies in that it cannot be used at all if the test really indicates that polynuclear complexes are presumably formed.

Finally it should be pointed out that this method of calculation may be greatly simplified when a system is only weakly complex. In such a case, $C_A \approx [\mathrm{A}]$ in all solutions and no extrapolations are thus necessary to obtain the values of [A]. Then the criterions a—c p. 142 may be at once applied. If only mononuclear complexes are indicated, X_L may be directly calculated from (8).

EXPERIMENTAL

Chemicals used: Uranium (IV) perchlorate was prepared by cathodic reduction of a strongly acid solution of uranyl perchlorate, a method originally worked out by Rosenheim and Loebel [27]. A mercury pool, about 6 cm in diameter, covering the bottom of a Pyrex beaker served as cathode. It is connected with the outer circuit by a platinum wire, the part of which passing through the solution surrounded by a plastic tube. The anode compartment, containing $\approx$1 C perchloric acid, consisted of a glass tube ending in a sintered glass disc. In the tube a platinum wire was inserted as the anode.

The cathodic solutions contained a concentration of uranium $\approx$0.1 C, and a $[\mathrm{H}^+]$ $\approx$1.5 C. If then a voltage of 12 V was applied a current $\approx$0.7 A resulted. The completion of the reaction was indicated by an incipient formation of U(III), whose deep red colour when mixed with the green one of U(IV) turned the solution almost black. The simultaneous formation of hydrogen was rather slight on account of its great overvoltage towards the mercury electrode.

All U(III) formed was oxidized by aeration of the solution for one minute. It was then immediately transferred into a bottle where it could be stored under a permanent

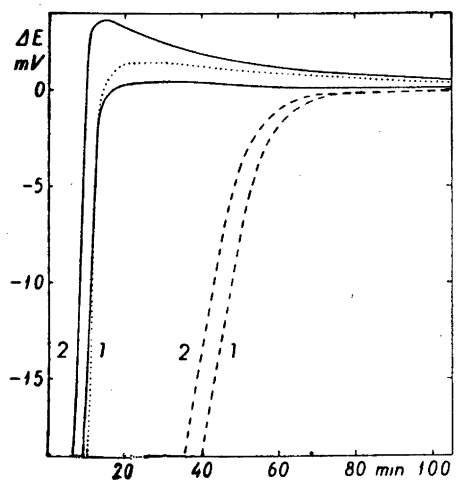

Fig. 1. The difference ΔE between the emf measured and its final value of equilibrium E' as a function of the time elapsed from the mixing of S. Fulldrawn, dotted and dashed curves refer to $C_M : C_L = 30 : 30$, $10 : 30$ and $30 : 10$ respectively. The values of C_I of the curves are given by the numbers attached to them.

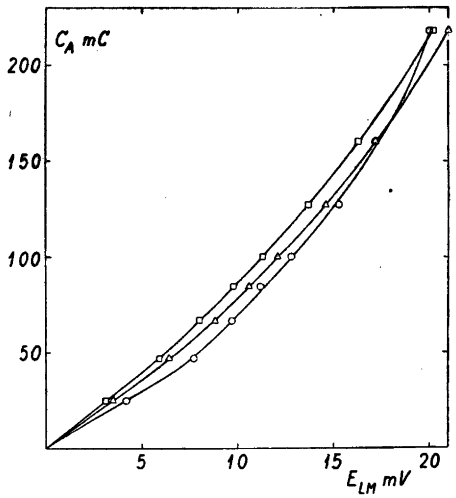

Fig. 2. The connection between E_{LM} and C_A in the thiocyanate system. The experimental points of the series of $C_M : C_L = 30 : 30$, $10 : 30$ and $30 : 10$ are denoted by squares, triangles and circles respectively.

overpressure of electrolytically prepared hydrogen, freed from oxygen according to Meyer and Ronge [28]. The bottle was equipped with a burette which permitted withdrawal of known portions of the solution without letting air into the stock.

This stock solution first had to be analysed for U(IV) and U(VI), the latter formed by the oxidation of U(IV) which could be hardly avoided in the long run. In fact there was no point in endeavouring to eliminate this oxidation completely, as a small contamination of U(VI) in the solution is quite harmless, provided its concentration is known.

C_L of the stock solution was determined by direct cerimetric titration, using ferroin (tri-*o*-phenantroline iron(II) sulfate) as an indicator. A similar titration on another sample which had first been reduced by the cadmium reductor gave $C_L + C_M$. The difference between the titrations thus yielded C_M. It was found that the solution keeps very well under the arrangement described even if portions are often withdrawn. C_L decreased by only by ≈ 0.8 % in one month while $C_L + C_M$ then remained constant.

Finally, the solution of U(IV) also had to be analyzed for $[H^+]$. This cannot be performed by direct alkalimetric titration on account of the acid properties of U^{4+}. Nor is it possible to measure $[H^+]$ potentiometrically with the high accuracy of ≈ 0.5 %, necessary owing to the strong influence of $[H^+]$ on E (*cf.* (1)). The method successfully employed here utilizes cation exchange. A sample was passed through a column of Amberlite in its hydrogen form. All U^{4+} and UO_2^{2+} were then exchanged for H^+. The solution leaving the column may therefore be titrated in the usual way. If a total concentration of acid $= [H^+]_t$ is found then the original $[H^+] = [H]_t - 4\,C_L - 2\,C_M$.

The uranyl perchlorate was prepared as decribed previously [14]. The present preparation did not contain any excess or deficit of perchloric acid (*cf* [14] p. 384). A stock solution of $C_M \approx 1/3$ C was prepared, whose exact concentration was cerimetrically determined = 337 mC.

Tab. 1. The chloride system, C_I = 1 mC

$\gamma_1 = 2.0 \pm 0.5$ C^{-1}

C'_M mC →	30	30	10	mean			
C'_L mC →	30	10	30	E_{LM}	X_M	X_L	X_{IL}
C_A mC	E_{LM} mV			mV			C^{-1}
67.5	0.9	0.9	0.8	0.9	1.054	1.132	1.95
117	1.4	1.5	1.4	1.4	1.094	1.222	1.90
166	1.9	1.9	1.8	1.9	1.133	1.317	1.91
210	2.3	2.3	2.3	2.3	1.168	1.402	1.92
250	2.7	2.6	2.8	2.7	1.200	1.486	1.95
315	3.5	3.2	3.7	3.5	1.252	1.651	2.07
400	4.5	4.0	4.7	4.4	1.320	1.870	2.18
500	5.3	4.8	5.9	5.3	1.400	2.130	2.26

The *perchloric, hydrochloric* and *hydrobromic acids* were of analytical grade, as well as the *sodium salts* used. The concentrations of the stock solutions of the last-mentioned were checked by ion exchange and subsequent alkalimetric titration.

The *sodium indigotetrasulfonate* used had been obtained from I. G. Farben. A solution of C_I = 25 mC appeared to be quite stable, whereas a precipitate was gradually formed when C_I = 100 mC.

Procedure. At the outset, the right half-cell of p. 141 contained a known volume of a solution S having C_L = 10 or 30 mC, C_M = 10 or 30 mC and C_I = 1 or 2 mC, according to the schemes given in the Tables 1—3. S was prepared directly in the electrode vessel from the stock solutions of U(IV), U(VI) and the mediator, and then perchloric acid and sodium perchlorate were added so that [H+] = 600 mC and I = 1 C. Immediately after the preparation of S, a bubbling of purified [28] nitrogen gas through the solution was started and then maintained during the whole series of measurement.

Tab. 2. The bromide system. C_I = 1 mC

$\gamma_1 = 1.5 \pm 0.5$ C^{-1}

C'_M mC →	30	30	10	mean			
C'_L mC →	30	10	30	E_{LM}	X_M	X_L	X_{IL}
C_A mC	E_{LM} mV			mV			C^{-1}
67.5	0.9	0.7	0.8	0.8	1.033	1.100	1.48
117	1.4	1.3	1.4	1.4	1.059	1.183	1.56
166	1.8	1.9	2.0	1.9	1.083	1.259	1.56
210	2.3	2.5	2.4	2.4	1.105	1.336	1.60
250	2.8	3.0	2.9	2.9	1.125	1.416	1.67
315	3.6	3.6	3.5	3.6	1.158	1.540	1.72
400	4.6	4.3	4.5	4.5	1.200	1.714	1.78
500	5.6	5.5	5.7	5.6	1.250	1.947	1.90

Tab. 3. The thiocyanate system. — Determination of E_{LM} as a function of C_A including the extrapolation to $C_I = 0$.

$C'_M \rightarrow$ mC	30			30			10		
$C'_L \rightarrow$ mC	30			10			30		
$C_I \rightarrow$ mC	2	1	0	2	1	0	2	1	0
$E' \rightarrow$ mV	301.7	304.2	306.7	285.0	287.9	290.8	314.0	317.5	321.0
C_A mC	E_{LM} mV								
25.1	3.0	3.1	3.1	4.0	4.1	4.2	3.4	3.5	3.5
47.0	5.9	5.8	5.9	6.7	7.2	7.7	6.5	6.4	6.4
66.7	8.0	8.0	8.0	8.8	9.3	9.7	8.8	8.8	8.8
84.2	9.9	9.7	9.8	10.4	10.8	11.2	10.5	10.7	10.6
100	11.4	11.2	11.3	11.8	12.3	12.8	12.1	12.1	12.1
127	13.9	13.5	13.7	13.8	14.6	15.3	14.6	14.6	14.6
160	16.6	16.1	16.3	16.0	16.6	17.2	17.2	17.2	17.2
218	20.4	20.0	20.2	19.5	19.8	20.0	20.9	21.0	21.0

The course of the emf of the cell formed was then observed. As the mediator was added in its oxidized form, S exhibited a high redox potential, and the emf therefore started at a low value (*cf* (1)). It rose, however, very quickly indicating the rapid progress of the redox reaction in the solution, also manifested by the bleaching of the indigo colour. After 1.5—4 hours, the length of time being dependent on C_L, C_M and C_I of S, the emf asymptotically reached the steady value E' of the redox equilibrium. If the reaction first proceeded very rapidly the emf described a damped oscillation about E' as seen from Fig. 1. In such a case a longer time was required for the definitive attainment of E' than if the reaction started at a lower rate. A certain E' could, as a rule, be reproduced within 1 mV.

After E' had been reached portions of a ligand solution T were added from a burette. In the chloride and bromide measurements T had $[H^+] = 600$ mC. As to thiocyanate, strongly acid solutions are not stable [29] and, therefore, ligand and acid were in this case added separately so that the resulting cell solution still retained $[H^+] = 600$ mC.

In the beginning of each series the emfs E adjusted themselves within $\approx$10 minutes, but with increasing C_A a longer time was required, at last up to more than half an hour. Simultaneously the reproducibility of the difference E_{LM} decreased from $\approx$0.2 mV to 0.5—1.0 mV. As a rule two parallel runs were made for every series.

Once reached, a value of E' or E, and thus E_{LM} too, showed no drift, indicating that the arrangements made were really able to prevent an appreciable oxidation by the air during the time of measurement. Nor was E' or E in any case affected by a change of the illumination, *e. g.* from darkness to ordinary daylight. None of the systems investigated here are thus sensitive to light.

The values of E' given in Table 3 are obviously dependent on C_I. The comparison intended between E' determined at different values of C_M/C_L must, therefore, be made with values of E' valid at $C_I = 0$. The extrapolation is performed in Table 3. The resulting difference of E' between $C_M/C_L = 1$ and 3 is 15.9 mV, between 1 and 1/3 14.3 mV which within the limits of experimental error agree with the value of 13.9 mV expected

Tab. 4. Determination of the complexity of the thiocyanate system of uranium(IV).

$\gamma_1 = 31 \pm 2$ C^{-1} $\gamma_2 = 90 \pm 30$ C^{-2} $\gamma_3 \approx 150$ C^{-3}

C'_L mC	30		10		0					
E_{LM} mV	C_A mC	C_L mC	C_A mC	C_L mC	C_A mC	C_M mC	[A] mC	X_M	X_L	X_{1L} C^{-1}
4.0	32.5	27.6	23.3	9.44	18.5	28.6	16.0	1.092	1.499	31.2
7.0	56.8	25.7	41.9	8.95	33.9	27.5	29.6	1.174	2.044	35.3
10.0	86.2	23.6	69.3	8.28	60.2	25.5	53.6	1.324	2.922	35.9
13.0	119.0	21.1	101.9	7.45	92.6	23.1	83.8	1.525	4.270	39.0
15.0	143.0	19.3	125.4	6.88	115.6	21.4	105.7	1.680	5.510	42.8
17.0	169.3	17.3	155.5	6.10	148.0	18.9	137.1	1.923	7.387	46.6
20.0	213		213		213		213	2.606	12.71	55

according to (2b). E' calculated in this way thus seems to be determined solely by the uranium system, which is also confirmed by some measurements performed without the mediator, giving approximately the same E' as above.

As expected from a comparison between (2b) and (3), E_{LM} proves to be much less dependent on C_I than E' is. From Table 3 it is seen that C_I has a pronounced influence only in the series of $C_L = 10$ mC, whereas in the other series no systematic deviation occurs when C_I is changed from 1 mC to 2 mC. Therefore an extrapolation to $C_I = 0$ has to be performed only for the series of $C_L = 10$ mC, while for the other series E_{LM} at $C_I = 0$ is taken as the mean between the values determined at the two C_I used.

Even for the series of $C_L = 10$ mC, the influence of C_I is very slight at the lowest C_A. As to the chloride and bromide systems E_{LM} reaches only very low values, Tables 1 and 2. It is then concluded that a variation of C_I is quite unnecessary in the determination of these systems.

Tab. 5. Determination of corresponding values of X_M and [A] of the uranyl thiocyanate system from the results obtained for the uranium(IV) system. — Comparison between the X_{1M} hence calculated and those found previously [24].

C'_M mC	30		10		0						
E_{LM} mV	C_A mC	C_M mC	C_A mC	C_M mC	C_A mC	C_L mC	[A] mC	X_L	X_M	X_{1M} C^{-1}	ext. X_M C^{-1}
3.0	24.9	28.1	21.5	9.47	19.8	28.5	11.8	1.379	1.088	7.4	5.8
6.0	48.2	26.4	43.6	8.91	41.3	26.9	27.4	1.920	1.195	7.1	5.9
8.5	71.5	24.6	64.0	8.40	60.1	25.5	42.8	2.504	1.278	6.5	6.0
10.0	86.2	23.5	78.6	8.04	74.7	24.4	55.7	3.034	1.374	6.7	6.0
12.0	107.8	21.9	99.0	7.53	94.4	22.9	73.8	3.826	1.480	6.5	6.2
14.0	130.7	20.2	120.7	6.98	115.4	21.4	93.8	4.820	1.592	6.3	6.4
17.0	169.3	17.3	158.0	6.05	151.9	18.6	130	6.880	1.791	6.1	6.7
20.0	214.0	14.0	202.0	4.95	195.4	15.3	176	10.06	2.065	6.1	7.2

RESULTS

As seen from Tables 1 and 2, the low values of E_{LM} in the chloride and bromide systems are, moreover, independent of C_L and C_M. This implies a very weak and solely mononuclear complex formation. The calculations could therefore be performed according to the simplified method given above, postulating $C_A \approx$ [A]. The functions X_{1L} thus found increase very slowly for both systems. Only the first complex can, therefore, be considered as definitely proved, while the slight increase of X_{1L} may be attributed to a change of the activity coefficients as well as to a formation of a weak second complex. This difficulty is common to all determinations of constants of complexes higher than the first one, as it is then necessary to exchange a considerable part of the perchlorate ions of the medium for ligand ions. The values of γ_1, with estimated experimental errors are given in Tables 1 and 2.

It is evident from Table 3 and Fig. 2 that the complexity of the thiocyanate system is so strong that the complete method of computation must be applied. Thus corresponding values of X_L and [A] are first calculated according to (9) and (10) on the presumption that no polynuclear complexes exist, Table 4. For this the curves of C_M:C_L = 30 : 30 and 30 : 10 of Fig. 2 are used. At the highest C_A, the curves seem to meet though the difference between C_A and [A] must still be rather considerable judging from the previous values of Table 4. One cause of this anomalous behaviour is, no doubt, the great unreliability of the highest E_{LM}, as illustrated by their poor reproducibility, but the full explanation is still obscure. For the last row of Table 4, the approximation $C_A \approx$ [A] is therefore used.

The course of X_{1L} indicates the existence of at least the first two mononuclear complexes, but a still closer agreement with the experimental points is obtained if also the third complex is postulated. Its constant could be only very roughly calculated and the computation is, of course, very uncertain on account of the changes of activity discussed above. The values of γ_p are found in Table 4.

The test of the assumptions made is then performed, using the curves of C_M:C_L = 30 : 30 and 10 : 30 of Fig. 2. The resulting values of [A], X_M and hence X_{1M} are found in Table 5, the last column of which also contains the values of X_{1M} extinctiometrically determined before [24]. A surprisingly good agreement exists between the two sets of X_{1M}, especially if the considerable differences in medium as well as in experimental method are considered. A value of $\beta_1 = 6.5 \pm 1$ C^{-1} is determined here which should be compared with $\beta_1 = 5.7 \pm 0.3$ C^{-1} obtained before. The higher complexes of the extinctiometric investigation are on the other hand not found here. Their concentrations at the present values of [A] are however rather slight, and the calculation of these higher constants are, as repeatedly pointed out, much more uncertain than that of the first one. Taken as a whole, the test must therefore be considered to verify our assumptions that only mononuclear complexes are formed and that the values of the previous uranyl measurements may be used even in the present medium.

SUMMARY

The complexities of the chloride, bromide and thiocyanate systems of uranium (IV) are determined from measurements of the UO_2^{2+}/U^{4+} redox couple utilizing the known values of complexity of the corresponding uranyl systems. The measurements are performed at 20° C in a perchlorate medium of the ionic strength 1 C.

A constant acidity is maintained which is chosen so high ($[H^+] = 600$ mC) that no hydrolysis exists. In order to bring about the necessary acceleration of the potential adjustment, an addition of indigotetrasulfonate is found suitable. The conditions necessary for a proper use of such mediators are discussed.

Only mononuclear complexes seem to exist in the systems investigated. For the chloride and bromide systems only the first complex can be certainly proved. The constants found are 2 C^{-1} and 1.5 C^{-1} respectively. In the thiocyanate system, the first two complexes are certainly proved and possibly the third one also exists. The following constants are computed $\gamma_1 = 31$ C^{-1}, $\gamma_2 = 90$ C^{-2} and $\gamma_3 \approx 150$ C^{-3}.

We are indebted to Professor Sven Bodforss who has in several respects highly promoted our work.

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Studies on Aspartase

III. On the Specificity of Aspartase

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Aspartase has been shown to be strictly specific to fumaric and L-aspartic acid. No other amino-acid tested (glycine [1], alanine [2], leucine [3], phenylalanine [4], tyrosine [4], dioxyphenylalanine [4], histidine [4] and glutamic acid [1]) are deaminated by the enzyme. Nor was D-aspartic acid attacked by aspartase [5,6]. It has been shown that with maleic [1], glutaconic [1], crotonic [7], pyruvic [7], mesaconic [3], aconitic [3] and sorbic acid [3] no addition of ammonia occurred. A change in the carboxyl groups of fumaric acid seems to be decisive for the action of aspartase, *e. g.* the diamide [3] and mono- and diethyl ester [8] of this acid were not acted on by aspartase.

The present investigation has been undertaken to elucidate the structural requirements of the substrate and of the competitive inhibitors of aspartase. Aspartase was obtained from propionic acid bacteria, and its action was studied using compounds with different chemical structure competing with L-aspartic acid.

EXPERIMENTAL

Enzyme material: A finely ground dry preparation of *Propionibacterium peterssonii* (strain of this laboratory) was used as the enzyme preparation. The culture procedure was the same as that used earlier [9].

Activity measurements: The activity of the preparations was determined by the rate at which aspartic acid was deaminated. The experiments were performed in exactly the same way as before [10].

Competition experiments: Before adding the competitors to the enzyme, their pH was adjusted to 7.0—7.5 with sodium hydroxide. The dry bacterial mass and 5 ml of 0.1 *M* solution of competitor were incubated at 37° C for 30 minutes, after which the reaction was started by adding aspartic acid, phosphate buffer and distilled water.

When testing the ability of the enzyme to deaminate D-aspartic, L-cysteic and α,β-diamino-succinic acid, concentrations similar to that used in the activity determinations of L-aspartic acid, *i. e.* 0.02 *M* solutions, were used.

L-α-hydroxy-β-sulphopropionic acid was prepared by deamination of L-cysteic acid, which was treated with nitrous fumes. The fumes were prepared from sodium nitrite and diluted hydrochloric acid [11]. After deamination, the solution was boiled in a water bath with a small amount of concentrated hydrochloric acid (0.3 ml) till the nitrous gases were removed, after which the solution was concentrated to a small volume, neutralized with sodium hydroxide to pH 7 (glass electrode) and made up to a 0.1 *M* solution of L-α-hydroxy-β-sulphopropionate.

RESULTS

Preliminary experiments were performed to test the ability of this enzyme preparation to attack D-aspartic acid, L-cysteic acid and α,β-diamino-succinic acid (Fig. 1). Practically no deamination was observed with cysteic acid and α,β-diamino-succinic acid, whereas a very weak deamination of D-aspartic acid was noticed (*cf.* later).

Changes in the carboxyl groups of fumaric acid have been found to determine the synthetic action of aspartase. In order to find out how the carboxyl groups and their interatomic distance in compounds may determine their affinity for aspartase, experiments were made with carboxylic acids competing with L-aspartic acid (Fig. 2). The strongest competitive effect was noticed with oxalic acid. The difference in competing ability between citric acid and tricarballylic acid is of interest, and emphasizes the importance of the hydroxyl group (tricarballylic acid differs from citric acid in having a hydrogen atom in the place of the hydroxyl group). The presence of the hydroxyl group markedly enhances the competing effect of citric acid. Succinic acid exhibited a weak competition whereas malonic acid displayed no competitive ability at all. Nor was any competition observed with glutaric and adipic acids. In view of the weak effect produced by succinic acid it was decided to investigate in detail the four-carbon dicarboxylic acids. The competing effect of the *trans*- and *cis*-isomeric forms were investigated (Fig. 3). Maleic acid showed no affinity for aspartase, whereas mesaconic acid had a noticeable, and fumaric acid a strong affinity.

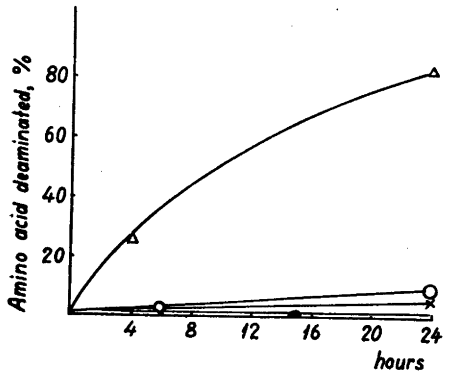

Fig. 1. Ability of the aspartase preparations to deaminate:

△ L-*aspartic acid*
○ D-*aspartic acid*
× L-*cysteic acid*
● α,β-*diamino-succinic acid*

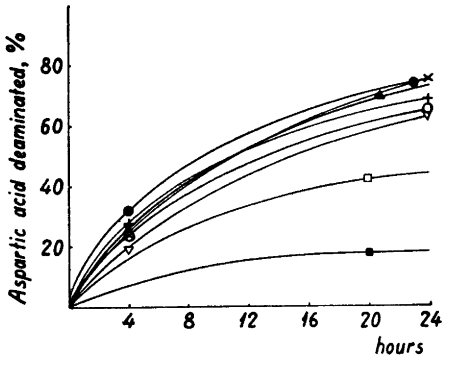

Fig. 2. Inhibiting effect of different carboxylic acids on the action of aspartase:

● *0.1* M *malonic acid*
○ *0.1* M *succinic acid*
\+ *0.1* M *glutaric acid*
× *0.1* M *adipic acid*
■ *0.1* M *oxalic acid*
▽ *0.1* M *tricarballylic acid*
□ *0.1* M *citric acid*
▲ *control without addition of inhibitors*

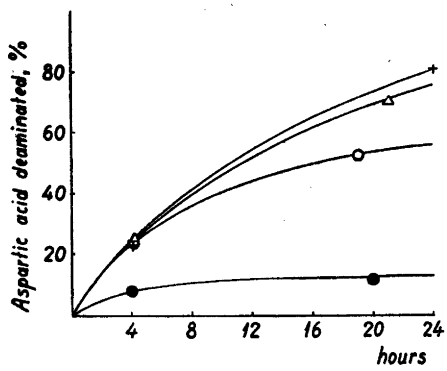

Fig. 3. Inhibiting effect of isomeric four carbon dicarboxylic acids on the action of aspartase:

○ *0.1* M *mesaconic acid*
\+ *0.1* M *maleic acid*
● *0.1* M *fumaric acid*
△ *control without addition of inhibitors*

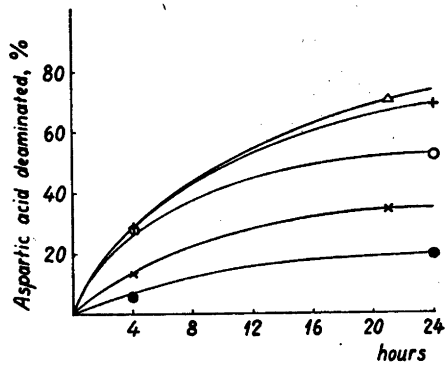

Fig. 4. Inhibiting effect of different sulphonic acids on the action of aspartase:

● *0.1* M *1,2-ethane disulphonic acid*
○ *0.1* M *sulphoacetic acid*
\+ *0.1* M p-*sulphobenzoic acid*
× *0.1* M L-*α-hydroxy-β-propionic acid*
△ *control without addition of inhibitor*

To show to what extent the carboxyl groups can be replaced by sulphonic acid groups *etc.*, compounds were tested in which one or both carboxyl groups are replaced by sulphonic acid groups (Fig. 4). The effect of 1,2-ethane disulphonic acid is almost as great as that of oxalic acid and much stronger than the effect of the corresponding dicarboxylic acid *i. e.* succinic acid. The affinity of L- *α*-hydroxy-*β*-sulphopropionic acid is strong compared with the affinity of succinic acid. L-*α*-hydroxy-*β*-sulphopropionic acid is identical with

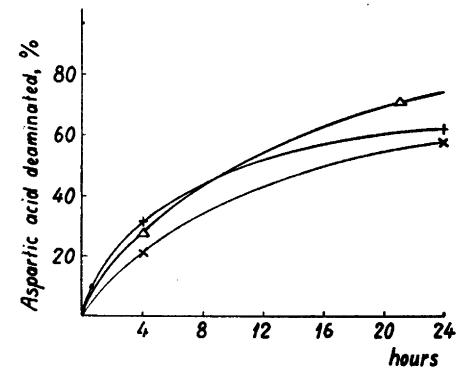

Fig. 5. Inhibiting effect of gluconic and salicylic acid on the action of aspartase,

\+ *0.1* M *gluconic acid*
× *0.1* M *salicylic acid*
△ *control without addition of inhibitor*

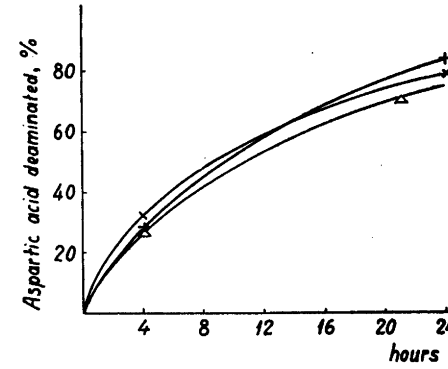

Fig. 6. Inhibiting effect of L-*cysteic and* D-*aspartic acid on the action of aspartase,*

× *0.1* M L-*cysteic acid*
\+ *0.1* M D-*aspartic acid*
△ *control without addition of inhibitor*

L-malic acid except that the carboxyl group further away from the hydroxyl group is replaced by a sulphonic acid group. If one of the two carboxyl groups in malonic acid is replaced by a sulphonic acid group, its affinity for aspartase increases compared with the original affinity of malonic acid.

Tests were made on the degree to which one of the strongly acid groups is replaceable by a phenolic group or by several alcoholic groups (Fig. 5). Salicylic acid and gluconic acid show a rather weak competing effect. Of these two acids, salicylic acid seems to possess the strongest affinity for aspartase. To ascertain whether L-cysteic acid and D-aspartic acid have any affinity for aspartase, although they do not play the role of substrates, their competing ability with L-aspartic acid was investigated (Fig. 6). In both cases no competition was noticed. Hence the weak evolution of ammonia observed earlier in the deamination experiments with cysteic acid and D-aspartic acid can hardly be due to a deamination of these acids, but ammonia is evidently liberated from other sources.

DISCUSSION

The results taken as a whole indicate that the combination of enzyme and substrate or enzyme and competing inhibitor is largely ionic in nature. Thus it appears that a two-point attraction of the substrate or competing inhibitor to the active centre is necessary for the occurrence of a combination.

The importance of the ionic attraction of the enzyme and substrate in the formation of a complex is shown by the strong inhibition produced by 1,2-ethane disulphonic acid. This molecule is identical with succinic acid except that the carboxyl groups are replaced by sulphonic acid groups. The fact that L-cysteic acid does not function as a substrate of aspartase is evidence of the great specificity of this enzyme, and is indicative of the ionic balance that must be required for the catalytic action of the enzyme.

The difference in affinity observed between citric acid and tricarballylic acid may also be explained on the basis of a difference in ionic attraction. All polar substituents almost invariably increase the acidity of an ionizing group.[12] The complexing properties of citric acid for Ca and Sr have been shown to be several times more powerful than those of tricarballylic acid, showing the importance of a hydroxyl group in this case, too [13].

The residual negative charges in a molecule should play an important role in this respect. These charges may be derived from the amount of residual charge on certain groups in a molecule due to the attractive forces of unequal kernel charges. An approximate calculation according to Latimer and Porter [14] shows that the charge on an oxygen atom in a hydroxyl group, for instance, actually possesses a residual negative charge equal to —0.20 [15]. The uncharged amino-group of amino-acids has a negative charge equal to —0.11 [15], when the charged group has a positive charge equal to +0.89 [15].

The most obvious ionic attraction would be between the ionized carboxyl groups of the substrate and the positively charged center in the enzyme. An explanation of the nature of this center may be that aspartase is a metal-protein [10] containing one of the alkaline-earth metals as activator. These

metals are known to form essentially ionic bonds with the more non-metallic elements [16].

The alkaline-earth metals tend to form rings, oxalic acid forming a five-membered, malonic acid a six-membered and succinic acid a seven-membered ring. Of these compounds, the five- and six-membered rings exhibit the greatest stability. The stability of these ring-compounds decreases steadily with increasing members in the ring [17].

Compounds unable to form rings probably have a low affinity for the enzyme. Hence the monocarboxylic amino-acids should have a low affinity for aspartase, although it may be assumed that the carboxyl group is combined with the metal as in the usual substrate, but since no ring formation is possible the affinity is low. The amino-dicarboxylic acids, aspartic and glutamic acid, may be expected to have an affinity of the same order as that of succinic and glutaric acid. However, the positively charged amino-group (positive residual charge = +0.89) can be expected to repel a positively charged ion from the adjacent carboxyl group. This should have an inhibiting effect on the combination with the enzyme. Convincing evidence to justify this suggestion is given in the competition experiments with L-cysteic acid and its deamination product L-α-hydroxy-β-propionic acid. In the case of cysteic acid no affinity to aspartase could be demonstrated but when deaminated the compound showed a strong affinity for the enzyme. The fact that no association has been found to take place between aspartic acid and calcium [18] and magnesium [19] in neutral solution confirmed these observations. Only in alkaline solution, where the amino-group possesses a negative residual charge ($pK = 9.60$ [12]) has a strong association between magnesium and aspartic acid been observed [19]. Accordingly, it is quite evident that a combination between an amino-acid and the enzyme is possible only if the charged amino-group is neutralized or blocked.

On the basis of all this the existence of a third point of attraction in the aspartase should be possible. Combining with the positively charged amino-group of the substrate the repulsion effect of the uncombined charged group may be prevented. In D-aspartic acid no combination takes place and the repulsion between the positive charges prevents the complexformation between the amino-acid and the enzyme. This should explaine why no competition between L-aspartic acid and D-aspartic acid was observed.

No reliable information on the nature of the third point of attraction can be given. There is, however, reason to assume that this point consist of a thiol group, since it has earlier been shown that thiol groups are necessary for the activity of aspartase [10].

To explain the stereochemical specificity, it is suggested that there should be at least three points of specific interaction between enzyme and substrate. In the case of aspartase, these three points seem to be made up of the two valencies of the metal assumed to bind the two essential carboxyl groups, and a third, possibly a thiol group, which combines with the positively charged amino-group, neutralizing it and so making possible the formation of the complex with the enzyme, which is necessary for the catalytic action.

SUMMARY

Aspartase has been shown to have no action on α,β-diamino-succinic acid, L-cysteic acid and D-aspartic acid. Nor did L-cysteic acid and D-aspartic acid show any affinity for aspartase. Strong affinity for the enzyme has been observed in the sodium salts of oxalic, citric, 1,2-ethane disulphonic and L-α-hydroxy-β-sulphopropionic acid. Succinic, mesaconic, sulphoacetic and salicylic acid show a weak affinity for this enzyme, whereas no affinity could be observed in the case of malonic, maleic, glutaric, adipic, *p*-sulphobenzoic and gluconic acid.

The author wishes to express his gratitude to Professor A. I. Virtanen for the kind interest he has taken in this work.

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On the Quantitative Determination and Qualitative Characterization of Organic Compounds through their Salts or Addition Compounds with Picric Acid

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Two methods for preparing picrates of unknown substances are given, the first to be used when the picrate is stable and the solubilities of the components are almost equal, the second to be used when the picrate is unstable and the solubilities of the components are different.

It is shown that equivalent weights of picrates may be determined by acid titration.

Of anisole and *p*-cresyl-methylether picrates were obtained which do not seem to have been previously reported. Of anthracene picrate a new modification was obtained.

It is well known that some organic substances can be characterized through their compounds with picric acid (picrates)[1–3]. It is less widely known that the equivalent weights of these compounds may be determined by acid titration of the picrates after decomposition with an excess of sodium hydroxide.

The limitations of the method are that it is sometimes difficult to prepare picrates — salts as well as addition compounds — of unknown substances. The common methods [1,3] of preparation are not always ideal and postulate that the molecular weights are known. When the latter are unknown these methods cannot be used as described. The acid titration of picrates has been used in a special course in organic identification for graduate students in this laboratory. The methods described below have been employed.

EXPERIMENTAL

Preparation of picrates. Many substances form picrates but a number of picrates have a very limitid stability range and may not be congruently soluble in many solvents. The stability increases with the size of the aromatic ring system in the partner component, and with the number of methyl, methoxyl, hydroxyl, and amino groups. The stability is diminished by nitro groups [4].

If the inequality below is satisfied, a picrate will — according to Dimroth and Bamberger [5] — be formed when equimolar quantities are dissolved in the necessary amount of boiling solvent, and then allowed to crystallize:

$$\frac{\text{Solubility of the more soluble component}}{\text{Solubility of the less soluble component}} < G. \qquad (1)$$

G is a constant related only to the affinity of the reaction

Picric Acid + X ⇌ Addition Compound or Picrate

Solubility means solubility at room temperature. If (1) is not satisfied it is necessary to use an excess of the more soluble component. When an excess is not used the less soluble component will contaminate the picrate.

When the picrate is stable, and the molar solubilities of the components are sufficiently close together the following method of preparation may be used. 0.75 g of the unknown component and 1.15 g (5 millimoles) of picric acid are dissolved in the necessary amount of boiling solvent and then allowed to crystallize. These quantities are chosen on the assumption that the molecular weight of the unknown substance is 150.

If the solubilities of the components are not sufficiently close together an excess of the more soluble component is taken, equal to the difference between the solubilities, in the amount of solvent used.

If the picrate is not very stable or if the solubilities of the components in the pure solvents are far from equal, a mixture of two solvents must be used. In the latter the components must have almost the same solubilities or at least have limited solubilities. The following procedure may be used.

A mixture of solvents (for instance ethanol-water) is prepared. The mixture should dissolve almost the same amounts of the components (if 5 ml of ethanol-water mixture is used, a mixture dissolving *ca.* 0.35 g of the unknown component will fit, since 0.35 g is the average solubility of picric acid in 5 ml of ethanol-water mixtures). The mixture can be prepared by the following method.

A convenient amount of the unknown component (in the case of ethanol-water, 0.35 g) is dissolved by adding increments of the two solvents, in turn, until a convenient volume is reached (if ethanol and water are used, 5 ml), and then adding the solvent with poorer solvent action drop by drop until the solute precipitates. — To this saturated solution there is added 0.75 g of the unknown component and 1.15 g (5 millimoles) of picric acid plus an amount of picric acid corresponding to the solubility in the mixed solvent. The substances are dissolved by heating and then allowed to crystallize. In this way equimolar amounts plus an excess of the more soluble component — corresponding to the difference between the solubilities of the components — are used, assuming the molecular weight of the unknown substance to be 150.

The picrates are separated from the solvent by suction on a suction funnel and then dried at room temperature between sheets of filter paper. Since the picrates rarely are congruently soluble washing and recrystallization are not advisable.

The Acid Titration of Picrates. It is important to have the picrate completely decomposed in the titration. The following method may be used.

To a weighed amount (*ca.* 0.25 g) of picrate there is added 20.00 ml 0.1 N sodium hydroxide and the mixture is heated to boiling. The mixture is then cooled, a soda-lime tube being attached to the flask to keep out carbon dioxide. Finally the excess of sodium hydroxide is determined by titration with 0.1 N hydrochloric acid and bromothymol blue indicator.

If the unknown substance X is an acid attention should be drawn to the fact that it is not always possible to titrate the picrate. When bromothymol blue indicator is used in the titration the acid dissociation constant of X must be less than $10^{-8.5}$. If the unknown substance is a base it is also not always possible to titrate the picrate. If bromothymol blue indicator is used in the titration the dissociation constant of the acid conjugate to X must be greater than $10^{-3.5}$.

RESULTS

In Table 1 are recorded the melting points and the titration results for picrates prepared from pure solvents. It may be added that in two cases *viz.*, anthracene picrate and guaiacol picrate, there were complications.

Table 1. Melting points and equivalent weights of picrates prepared from pure solvents.

	Solvent	Excess of	Melting points. °C Litt.*	exp.	Equivalent weights calc.	exp.
Naphthalene	ethanol		149 149.5 150.2 151.5	146	357	356
Phenanthrene	chloroform	picric acid	132.8 143 145	143–145	407	402
Anthracene	ethanol	picric acid	138 139 151.8 152.5	139	407	399
Guaiacol	chloroform	guaiacol	80 86 88.6 88 89 90	87	353	349
Guaiacol	water		80 86 88.6 88 89 90	85	353	352
p-Chloro-aniline	chloroform	*p*-chloro-aniline	178 187	185–186	357	355
Pyridine	»	pyridine	162 164 165 167 168	161	308	309

* The melting points quoted are taken from Beilsteins Handbuch der organischen Chemie, and from 3, and 6.

Table 2. Melting points and equivalent weights of picrates prepared from alcohol water mixtures.

	Melting points. °C Litt. **	exp.	Equivalent weights calc.	exp.
1-Bromo-naphthalene	134–135 129.6	134	436	437
2-Naphthole	157 156	158	373	372
Anisole *		99	566	570
p-cresyl-methylether *		103	580	582
Anethole	70	69	377	380
o-Chloro-phenol	81	80	358	360
o-Toluidine	212–215 213 185	213	336	336
Aniline	175–180 181 182	181	322	325

* These picrates contain 2 moles picric acid to 1 mole of the other component.

** The melting points quoted are taken from Beilsteins Handbuch der organischen Chemie, and from 3, and 6.

When first prepared from chloroform the anthracene picrate had a melting range of 148—180° C. After recrystallization from chloroform it melted at 183° C. Anthracene picrate prepared from ethanol melted at 134° C which is in agreement with the literature. A batch of anthracene picrate prepared this time from chloroform as solvent gave the same melting point, 134° C. Several months later, another batch prepared from chloroform showed the wide melting range of the first lot, and after recrystallization from chloroform melted at 184°. The "equivalent weight" of this new picrate was 378 before and 429 after the recrystallization ($C_6H_3O_7N_3 + C_{14}H_{10} = 407$). It was shown by X-ray diagrams that the high- and the low-melting picrates prepared from ethanol and chloroform were two definite modifications. Two modifications of guaiacol picrate were simultaneously obtained from chloroform: a red one consisting of six-sided prisms, and a yellow consisting of long needles. Both the picrates melted at 87° C and the equivalent weight was 349 for both.

In Table 2 are recorded the melting points and the titration results for some picrates prepared from ethanol-water mixtures. It should be mentioned that the picrates of anisole and of *p*-cresyl-methyl ether contain 1 mole of ether component to 2 moles of picric acid. In the preparation of these picrates it was necessary to change the amount of picric acid from 5 to 10 millimoles. The last two picrates do not seem to have been previously reported.

Acknowledgement. I wish to express my best thanks to professor A. Tovborg Jensen for suggesting this problem and for instructive discussions.

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Determination of "Strepogenin" with *Lactobacillus bifidus*

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In 1944 Sprince and Woolley [1] suggested the name "strepogenin" for a growth factor required by certain hemolytic streptococci. This factor was originally [2] found to be present in liver but later work [3,4] has shown that also enzymic hydrolysates of certain proteins possess similar activity. In addition to hemolytic streptococci various other lactic acid bacteria have been shown to be dependent on this factor.

The Group A streptococci to which strepogenin is an essential growth factor cannot be used for its assay since the growth obtained is too weak. *Lactobacillus casei*, first employed as the assay organism by Sprince and Woolley [1], gives better results but the growth response has been found to be not specific to strepogenin. Under certain conditions asparagine and glutamine and many synthetic peptides also show "strepogenin"-activity [5-9]. To avoid these defects Wright *et al.*[10] have developed an assay method based on the essential nature of strepogenin for a strain of *Lactobacillus bulgaricus*. A great number of peptides tested as well as asparagine and glutamine had no growth-promoting properties for this organism under the conditions employed. Recently Kodicek and Mistry [11] have again studied the reliability of the *L. casei*-method. Using an improved basal medium of Clegg *et al.*[12] they obtained satisfactory results. Under the conditions of this method, however, glutamine, asparagine and aspartic acid showed a slight strepogenin-like activity.

Since strepogenin has not yet been isolated and identified the selection of a suitable standard for microbiological assays is an important question. Sprince and Woolley [1] in their original studies used an arbitrary liver standard and later workers have selected one or another of their preparations as arbitrary standards. Kodicek and Mistry [11] in their recent work proposed the use of a partial hydrolysate of insulin as the standard, since insulin was known to be a rich source of strepogenin. The insulin standard was prepared by a short-time acid hydrolysis at a low temperature and under carefully controlled conditions.

Studies in our laboratory [13,14] have shown that strepogenin is an essential growth factor for certain strains of *Lactobacillus bifidus*. Its effect on this organism is specific at least in that asparagine and glutamine have no activity. Since the growth response of *L. bifidus* to strepogenin is very pronounced and

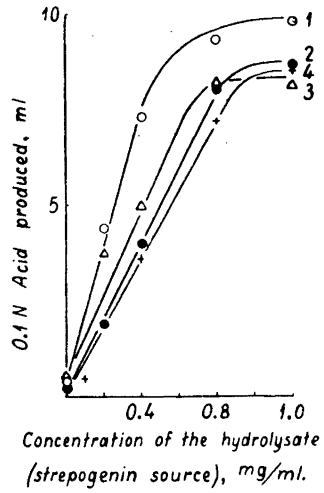

Fig. 1. Growth of strain THH 5 under different cultural conditions. 1. Tubes plugged with paraffin; inoculum 0.1 ml, 2. 0.1 % Sodium thioglycollate; no paraffin plug; inoculum 0.1 ml, 3. Incubated in CO_2 -atmosphere, no paraffin plug; inoculum 0.1 ml, 4. Incubated in ordinary atmosphere, no paraffin plug; inoculum 1 ml.

since this organism can be grown on a very simple medium it seemed to us that a method of determining strepogenin with *L. bifidus* should possess some advantages over the other methods in use. Several experiments were conducted to discover the optimal conditions and test the reliability of such a method.

EXPERIMENTAL

Test organism. Three strains of *L. bifidus* (labelled THH 3, THH 5, and KPH 16) isolated from the faeces of breast-fed infants were used in the experiments. Two of them, THH 3 and THH 5, were from the same child and might thus be considered identical. The isolation was carried out using the medium of Hassinen *et al.*[15], described below, supplemented with 15 g of agar and diluted from 500 to 1 000 ml. Only typical *bifidus* colonies grew on this medium.

A fresh stock culture was prepared every other week in the above medium further supplemented with 0.8 mg/ml of tryptic digest of casein (in terms of the original weight of casein). Incubation was carried out at 37° C for 3 days and the culture was then stored in a refrigerator.

All the strains of *L. bifidus* used by us have shown changes in morphology accompanied with a gradual loss in the ability to develop active growth. This degeneration has usually taken place within 4 to 6 months after isolation. Therefore, when *L. bifidus* is used for the assays fresh strains must be isolated at intervals of 4 to 6 months.

Anaerobiosis. To maintain the anaerobic conditions essential for the growth of *L. bifidus* the test tubes in the first experiments were plugged with paraffin. Although this procedure gave a good growth the method was found to be too laborious because the plugs had to be removed before titration; they also tended to increase the titration errors. Other methods for attaining anaerobic conditions were therefore also tested. They included an addition of 0.1 % sodium thioglycollate to the medium, incubation of the tubes in a CO_2-atmosphere, or using a fairly large inoculum to obtain a rapid fall in the redox potential of the medium. The results of these experiments are given in Fig. 1. It can be seen that strongest growth took place in the paraffin-plugged tubes. Incubation in CO_2-atmosphere and the addition of sodium thioglycollate gave somewhat lower figures. The growth was still weaker in the tubes where the only measure for attaining anaerobic

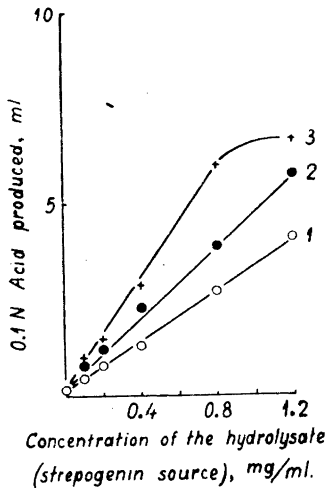

Fig. 2. Effect of the incubation time on the growth of strain THH 5. 1. Incubation time 48 hours, 2. 72 hours, 3. 96 hours. Inoculum in all tubes 0.4 ml.

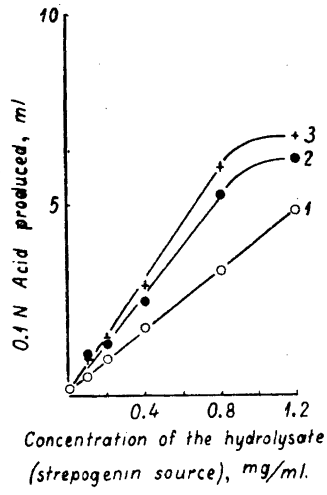

Fig. 3. Effect of the size of inoculum on the growth of strain THH 5. Incubation time 72 hours. 1. Inoculum 0.1 ml, 2. 0.4 ml, 3. 1 ml.

conditions had been the use of large inocula. Since, however, the growth curve even in this case had a good shape and covered a suitable range, this simplest procedure was selected for further experiments.

Fig. 2. shows the influence of the time of incubation and Fig. 3 that of the size of inoculum on the growth curves. It is noteworthy that the blanks are always very low, even in the cases where large inocula have been employed. On the basis of these results an incubation time of 72 hours and inocula of 1 ml were employed in the final procedure.

Procedure. The basal double-strength medium had the following composition (*cf.* Hassinen *et al.*[15]):

Lactose	35	g
Sodium acetate, anhydrous	25	»
Ammonium acetate	2	»
K_2HPO_4	2.5	»
Cysteine	0.1	»
Ca-pantothenate	0.4	mg
Biotin	0.004	»
Salt solution B ($MgSO_4 . 7 H_2O$ 10 g, $FeSO_4 . 7 H_2O$ 0.5 g, NaCl 0.5 g, $MnSO_4 . 4 H_2O$ 0.337 g in 250 ml of water)	10	ml
Distilled water	to 500	»
pH of the medium was 6.8		

Inocula for seeding the tubes were grown in the above medium supplemented with a tryptic digest of casein at a level corresponding to 0.8 mg of casein per ml of the medium. The cells of a 3-day, 10-ml culture were collected by centrifuging and suspended in 50 ml of sterile saline. 1 ml of this suspension per tube was used for inoculation.

The preparations to be investigated were dissolved in distilled water in a concentration of 4 mg/ml (tryptic hydrolysates were obtained by hydrolysis of the corresponding solutions with trypsin under toluene for 24 hours at 37°) and these solutions were then measured in test tubes at 5 different levels. 5 ml of the double-strength basal medium was added and the volume made up to 10 ml with distilled water.

After autoclaving for 20 min. at 105° C the tubes were inoculated and incubated at 37° C for 72 hours. The growth was measured titrimetrically using 0.1 *N* NaOH with bromothymol blue as the indicator.

In the selection of a suitable strepogenin standard we finally decided on peptonized milk (Bacto Difco). Available commercially and requiring no further hydrolysis, it possesses definite advantages compared with the enzymatic or acid hydrolysates of different proteins so far used as standards. The activity of peptonized milk was taken as 100 and relative activity values were calculated for the other materials from the standard curve obtained with peptonized milk.

RESULTS AND DISCUSSION

The strepogenin-activities of the following preparations were determined with the method described above: Peptonized milk, yeast extract, trypsin 1 : 250, pepsin 1 : 10 000, egg albumin, blood albumin, casein, gelatin, and tryptic hydrolysates of pepsin, egg albumin, blood albumin, casein, and gelatin. Suitable insulin preparations could, unfortunately, not be obtained.

The results of the determinations expressed as mean values of 8 or 9 separate experiments together with the standard mean deviations are given in Table 1. It can be seen that the mean deviation is between 5 and 10 % except in the cases where the activity has been very low. The accuracy of the method is thus of the order of magnitude generally occurring in microbiological assays. The results obtained with different strains of the test organism agree in general quite satisfactorily. In some cases, however, particularly where the tryptic hydrolysates are concerned, strain KPH 16 has given values distinctly higher than those obtained with the other strains.

A comparison of our results with those reported by other authors is very difficult because of the different techniques used. It seems that the *L. bifidus*-method gives higher activity values for the hydrolysates of albumins and lower values for the hydrolysates of casein than the *L. bulgaricus*-method (Wright *et al.*[11]). On the other hand, our values seem to be in good agreement with those of Kodicek and Mistry[12] obtained by the *L. casei* method. The differences between the results obtained by various methods is understandable

Table 1. The strepogenin-activities of different preparations determined with three strains of L. bifidus.

Preparation	Strain employed for determination		
	THH 3	THH 5	KPH 16
Peptonized milk (Bacto-Difco)	100	100	100
Yeast extract (Bacto-Difco)	625 ± 29.0	600 ± 33.0	470 ± 34.0
Trypsin, 1 : 250 (Difco)	76 ± 3.3	95 ± 6.3	69 ± 3.7
Pepsin, 1 : 10 000 (Difco)	31 ± 2.6	36 ± 2.7	52 ± 2.8
Pepsin, tryptic hydrolysate	36 ± 3.6	36 ± 1.8	88 ± 7.7
Egg albumin, soluble, (Difco)	14 ± 1.8	13 ± 1.9	14 ± 2.9
Egg albumin, tryptic hydrolysate	72 ± 4.6	64 ± 2.8	90 ± 7.0
Blood albumin, (B.D.H.)	17 ± 1.7	14 ± 2.1	14 ± 3.0
Blood albumin, tryptic hydrolysate	155 ± 11.5	150 ± 8.4	165 ± 14.3
Casein, vitamin-free, (Difco)	23 ± 3.1	19 ± 2.3	25 ± 3.9
Casein, tryptic hydrolysate	44 ± 5.2	49 ± 4.7	76 ± 7.3
Gelatin, (Gurr)	8 ± 0.6	8 ± 0.6	7 ± 0.5
Gelatin, tryptic hydrolysate	16 ± 2.8	19 ± 2.3	28 ± 4.3

considering the fact that there might be a variety of "strepogenins" with somewhat different combinations of amino acids. Little wonder, then, that various strepogenin-requiring species and strains of bacteria seem to respond somewhat differently to these different "strepogenins".

SUMMARY

An assay method for strepogenin based on the essential nature of this substance to *Lactobacillus bifidus* is described. The use of *L. bifidus* as the test organism has the following advantages: the blank values are always very small, the growth response is pronounced, and the basal medium is very simple containing in addition to lactose, sodium acetate, dipotassium phosphate and the salts B only ammonium acetate, cysteine, biotin and pantothenic acid. The anaerobic conditions required by *L. bifidus* are established by using a large inoculum. Figures for the activity of 13 different preparations are given, using peptonized milk as the strepogenin standard.

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Studies on the Electrochemistry of Silicate Melts. I

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The validity of Faraday's Law has been studied for 2 FeO · SiO_2, 2.5 CoO · SiO_2, 2.75 CoO · SiO_2 and 3 CoO · SiO_2. It has been shown that the law holds for 2 FeO · SiO_2, very closely for 2.5 CoO · SiO_2 and that, in contradistinction, 2.75 CoO · SiO_2 is a mixed conductor and 3 CoO · SiO_2 predominantly a semiconductor.

A method to determine transport numbers in silicate melts has been worked out. Cobalt silicates possess cation conductance if the basicity is below a certain value at which semiconductance comes into play.

From the observed data, two rules for the conductance of silicate melts have been deduced. The first one gives a connexion between conductivity and composition and the second states that the ionic mobility is inversely proportional to the square of the ionic radius.

The studies on the electrochemistry of silicate melts, so far carried out, have mostly been concerned with conductance measurements and only to a lesser degree with the applicability of Faraday's Law [1]. Certain difficulties are connected with determinations of transport numbers in melts, as there is not, unlike solids and liquid solutions, any frame of reference to which changes in concentration or boundary can be referred [2]. The purpose of this work has been to determine the applicability of Faraday's Law in molten silicates of different basicities and to obtain values for the transport numbers. From transport numbers and known conductance data it is possible to get a picture of what happens when an electric current passes through a silicate melt and to obtain a quantitative connexion between conductance and composition.

EXPERIMENTS AND RESULTS

The moving boundary method was regarded as the most convenient one for this investigation. Without a frame of reference, however, the only result is a determination of the degree of applicability of Faraday's Law. However, it was found to be possible to produce a frame of reference in an orthosilicate melt by introducing an excess of a basic oxide. The transport numbers can then be obtained by analysing the volume swept out by the boundary.

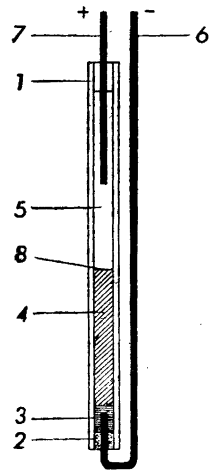

Fig. 1. 1. Alumina tube (Degussit AL23). 2. Alumina cement. 3. Silver cathode. 4. Bottom electrolyte (dark). 5. Top electrolyte (white). 6. Molybdenum wire. 7. Molybdenum anode. 8. Boundary.

The cell in the experiments was the arrangement shown in Fig. 1. After several different procedures for carrying out an experiment had been tried, the following one was found to be the best. The mixture intended as the lower electrolyte was melted in an alumina tube which was closed at one end. A narrower alumina tube of 10 cm length was pushed down to the bottom of the first tube. In this way a good meniscus was obtained. After cooling, the bottom was cut off and the solidified melt in the inner tube drilled away for a length of 1 cm. The silver with the molybdenum wire was then melted into the boring. Alumina cement was used to close the bottom end. The distance between the upper end of the tube and the meniscus was measured. The top electrolyte was introduced and the molybdenum anode inserted. The cell was placed in a furnace which was then heated to the temperature of the experiment and the electrolysing current switched on. After a certain amount of electricity had passed, the system was cooled. The tube was now cut along its length with a diamond saw and the movement of the boundary determined.

From the density and equivalent weight of the lower electrolyte, the radius of the tube and the distance travelled by the boundary (all of these quantities being measured at room temperature) it is possible to calculate the number of equivalents which the known quantity of electricity has deposited. We can formulate the following equation:

$$I \cdot \frac{f}{F} = \frac{\pi\, r^2\, h\, d}{E} \qquad (1)$$

where d = the density of the lower electrolyte
E = the equivalent weight = = the molecular weight/4
r = the radius of the tube
h = the distance which the boundary has travelled
f = quantity of electricity passed
F = 96 500 coulomb
I = degree of ionic conduction

The electrolysing current, taken from a 24 volts lead storage battery whose voltage could be varied by a variable resistance, was measured by a water coulometer.

An induction furnace with an isothermal field, 7—8 cm high, was used and the temperature was measured with a Pt : Pt—10 % Rh thermocouple.

The silicates studied in these experiments are given in Table 1.

Table 1. Silicates studied.

Bottom electrolyte, dark	Top electrolyte, white
2.00 $FeO \cdot SiO_2$	$Li_2O \cdot CaO \cdot SiO_2$
1.33 $FeO \cdot 0.67\ CaO \cdot SiO_2$	$Li_2O \cdot CaO \cdot SiO_2$
2.50 $CoO \cdot SiO_2$	$1.25\ CaO \cdot 1.25\ MgO \cdot SiO_2$
2.75 $CoO \cdot SiO_2$	$1.25\ CaO \cdot 1.50\ BaO \cdot SiO_2$
3.00 $CoO \cdot SiO_2$	$1.50\ CaO \cdot 1.50\ BaO \cdot SiO_2$

Only A. R. grade of the oxides and carbonates have been used for preparing the silicates.

The iron silicate was prepared from a carefully weighed mixture of Fe_2O_3 and SiO_2, which was melted and after cooling crushed and ground [2]. During the heating, oxygen is evolved and the Fe(II)-Fe(III)-equilibrium set up.

The cobaltous silicates were prepared from Co_3O_4 and SiO_2 in the same way as the iron silicate.

The top electrolytes have been prepared from carefully weighed mixtures of Li_2CO_3, CaO, MgO, $BaCO_3$ and SiO_2, which were heated, cooled and ground in the same way.

All experiments were performed in a purified nitrogen atmosphere.

The values for $2FeO \cdot SiO_2$ (density 4.30) are given in Table 2.

Table 2.

Temp. °C	Diameter of the tube mm	Change of boundary mm	Electricity passed coulomb	Ionic conductance
1 210	4.0	7.8	840	0.95
1 230	5.4	2.5	490	0.96
1 250	5.4	4.9	950	0.96
1 300	3.2	10.8	715	0.99
1 380	3.2	5.5	350	1.03
1 400	5.4	5.9	1 120	0.98

The degree of ionic conductance, which averaged 0.98, can in practice be put equal to 1.00 because in the computations all of the iron has been considered as ferrous (at 1 205° 2—3% of the iron is Fe(III) [3] and at higher temperatures even less).

It cannot be deduced from these experiments which of the ions is carrying the current because there is no frame of reference. However, in the experiments on Co-silicates, only the cations are carrying the current and this, in all probability, holds true also for the other silicates.

One thing worth mentioning is that the oxygen ions have nothing to do with the current transport because in such a case a deficit of oxygen would

occur at the cathode and this has not been observed either in the present work or by other authors [1].

At the cathode the iron is deposited as dendrites but is then to a greater part dissolved again in the melt (possibly as a colloid).

Around the anode oxygen and SiO_2 are set free. For this reason the resistance increases during the experiment and thus the current diminishes.

An experiment was also carried out with a mixture of 30 % by weight of $2CaO \cdot SiO_2$ and 70 % by weight of $2FeO \cdot SiO_2$, *i. e.* $1.33\,FeO \cdot 0.67\,CaO \cdot SiO_2$, density 3.83. The equivalent weight is computed in the usual way, Table 3.

Table 3.

Temp. °C	Diameter of the tube mm	Change of boundary mm	Electricity passed coulomb	Ionic conductance
1 250	5.0	4.6	660	1.04

Only iron is deposited, of course. It is seen that, in this case also, there is only ionic conductance.

Originally, the object of this investigation was to study the different mixtures of $FeO—SiO_2$. Unfortunately, it appeared that when the FeO content was higher than that of the orthosilicate, the corrosion of the alumina tube was too great. For this reason $CoO—SiO_2$ mixtures were tried and these melts were found not to attack the tube seriously provided that the temperature was not too high.

As previously mentioned, it is possible to determine transference numbers in melts if an excess of the basic oxide is taken as a frame of reference. If this excess is not too large, as in the mixture $2.5\,CoO \cdot SiO_2$, ionic conductance was found to prevail but only 2 Co^{2+} had migrated (Fig. 2). The analysis of the

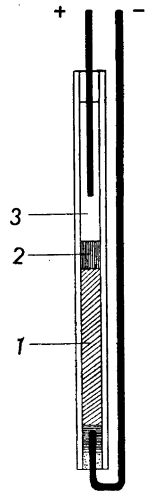

Fig. 2. 1. Bottom electrolyte. 2. 0.5 CoO + top electrolyte (the part that has migrated). 3. Top electrolyte.

volume (2 in Fig. 2) swept out by the boundary showed that 0.5 Co remained in it and had been mixed with the part of the top electrolyte that had migrated. This shows that the excess of cobalt in the bottom electrolyte must be dissolved in the orthosilicate as an unionised oxide. Possibly a slight semiconductivity occurs at this composition. The values 0.95 and 0.93 might differ from unity by more than the experimental error.

If the percentage of CoO in the melt is increased, the semiconductance will be greater until it becomes dominant in the mixture consisting of $3CoO \cdot SiO_2$.

The computations of the degree of ionic conductance have been carried out taking the equivalent weight as *e. g.* $2.5\ CoO \cdot SiO_2/4$ because only $2Co^{2+}$ are migrating.

Table 4.

No.	Substance	Temp. °C	Density	Diam. of tube mm	Change of bound. mm	Electr. passed coulomb	Ionic conductance
1	$2.5CoO \cdot SiO_2$	1440	4.36	2.0	4.7	106	0.95
2	$2.5CoO \cdot SiO_2$	1500	4.36	2.0	3.9	89.5	0.93
3	$2.75CoO \cdot SiO_2$	1450	4.52	2.0	6.0	171	0.72
4	$3.0CoO \cdot SiO_2$	1450	—	2.0	0.0	239	0.00

The analysis of the volume swept out by the boundary gave the following results:

No. 1. The gravimetric determination gave 23.2 % by weight CoO and 35.5 % by weight SiO_2, which is equivalent to a composition of $0.52\ CoO \cdot 1.00\ SiO_2$ (the rest being CaO and MgO). This means that only 1.98 Co^{2+} have migrated, which equals 2 within the experimental error. If only Mg^{2+} and Ca^{2+} ions have travelled from the top electrolyte and the CoO has been dissolved as oxide, the composition is considered to be $0.5\ CoO \cdot MgO \cdot CaO \cdot SiO_2$, which gives 19.3 % by weight CoO and 31.0 % by weight SiO_2. Now the analysis was made using a very small sample, 18.6 mg, and the determinations are therefore very uncertain. It is very probable that Al_2O_3 from the tube has dissolved in the melt and interferred with the determination of SiO_2, making the percentage too large.

To obtain the transport number of the cations in the bottom electrolyte, we must discuss both the movement of the boundary of the Co^{2+}-ions and the thickness of the layer containing 0.5 CoO between the top and bottom electrolyte (2 in Fig. 2). The movement of the first boundary, measured from the top when a given quantity of electricity has passed, is independent of the transport numbers, but the thickness of the layer 2 varies with the mobilities of the ions. If only the cations have moved ($t_+ = 1$), the thickness of the layer is equal to the movement of the boundary. But suppose only the anions had moved ($t_- = 1$), then there would be no layer containing cobaltous oxide between the top and the bottom electrolyte, both CoO and Co^{2+} being immobile. Between these two extremes ($t_+ = 1$ and $t_- = 1$) the thickness of the layer containing 0.5 CoO would be less than the movement of the boundary. In our case the transport number of the ionic conductance must be $t_+ = 1$.

No. 3. A very uncertain spectrographic determination of a very small sample gave 27 % Co, 22 % Ba and 12 % Ca (the rest being SiO_4^{4-}), which is equal to the composition $Co_{1.08} \cdot Ba_{0.38} \cdot Ca_{0.71} \cdot SiO_4$. The results of the analysis are too uncertain to allow any conclusions to be drawn concerning the transport numbers of the ionic conductance.

No. 4. The gravimetric determination gave 79 % by weight CoO for the bottom electrolyte immediately under the boundary, which means no change in the chemical composition at all (3.0 $CoO \cdot SiO_2$ equals 78.9 % CoO).

For pure CoO, Schrag [4] has shown that the melt is predominantly semiconductive with very little ionic conductance.

From these measurements of transport numbers and what is probable already from a geometrical point of view, *viz.* small cations and large anions it would appear that also in other cases apart from the orthosilicates there is unipolar conductance and $t_+ = 1$.

When the basicity is increased and free basic oxide is dissolved in the melt, we can then distinguish between two cases: 1) If the basic oxide has a high conductivity, it will increase with increasing content of the basic oxide; Ex: FeO—SiO_2 [5]; 2) If the basic oxide has a low conductivity, the curve will have a maximum, Ex: CaO—SiO_2, MgO—SiO_2 [5].

THE CONNEXION BETWEEN CONDUCTIVITY AND COMPOSITION OF MO-SiO_2-SILICATES.

It is well known that in solid ionic conductors the electrical conductance is due to defects in the lattice [6]. There are three explanations of ionic migration for solids; the two alternatives of Frenkel disorder: interstitial cations (AgCl) and interstitial anions ($PbCl_2$), and the Schottky disorder: ionic conductors deficient both in cations and anions (NaCl). It appears that Frenkel defects are likely to occur when the sizes of the cations and anions are very different, leaving appreciably large interstitial spaces.

According to the hole theory, the structure of a liquid is regarded as rather similar to that of the corresponding solid.

Now, a molten silicate is an ionic liquid with cation conductance (at any rate this is the case for Co-silicates) from which we infer that it has small cations and large anions. Here we may suppose that the electrical conductance is due to an extensive Frenkel disorder. The number of interstitial sites in the "lattice" of the melt will be dependent on the size of the anions. This size in its turn depends on the composition which can be described as follows [7].

Disilicates: Two-dimensional sheets with the composition $(Si_2O_5)^{2-}$, interstitial sites exist only between the sheets. Defect factor = 1.

Metasilicates: One-dimensional chains with the composition $(SiO_3)^{2-}$. The number of interstitial sites is twice that of the disilicates. Defect factor = 2.

Orthosilicates: Spatial restricted groups with the composition $(SiO_4)^{4-}$. The number of interstitial sites is three times that of the disilicates. Defect factor = 3.

This picture is admittedly a very crude one, but enough for our purpose.

As we have cation conductance, we will write in analogy with the customary expression for solids [8].

$$\varkappa = n \cdot z^2e^2 \cdot u \cdot S \qquad (2)$$

$\varkappa$ = specific conductance
n = number of cations/ml
ze = the charge of the ions
u = mobility of the migrating particles in the disilicates under unit force *
S = defect factor

If we know the conductivity for, let us say, a disilicate, it is possible to compute $\varkappa$ for a meta- or an orthosilicate and, of course, any other composition, if we know the defect factor. Thus

$$\varkappa_1 = \frac{n_1}{n_2} \cdot \varkappa_2 \cdot \frac{S_1}{S_2} \qquad (3)$$

To obtain the number of cations/ml the density at the temperature in question has to be known. The densities of the molten silicates below are unknown on account of which a linear dependence on the composition is assumed and these values are then reduced by 8, 10 and 12 % for the compositions $MO \cdot 2\,SiO_2$, $MO \cdot SiO_2$ and $2\,MO \cdot SiO_2$ respectively, according to Bockris *et al.* [9].

Table 5.

Composition	Defect factor	Number of cations/ml $\cdot N^{-1}$	Conductivity $ohm^{-1}\,cm^{-1}$ computed	observed	Melting point °C
$MgO \cdot 2SiO_2$	1	0.01565	—	0.23	1 750
$MgO \cdot SiO_2$	2	0.02635	0.77	0.72	1 524
$2MgO \cdot SiO_2$	3	0.03941	1.74	2.15	1 890
$CaO \cdot 2SiO_2$	1	0.01378	—	0.31	1 500
$CaO \cdot SiO_2$	2	0.02175	0.98	0.83 0.89	1 540
$2CaO \cdot SiO_2$	3	0.03044	2.05	1.15	2 130
$SrO \cdot 2SiO_2$	1	0.01275	—	0.21	1 370
$SrO \cdot SiO_2$	2	0.01925	0.63	0.63	1 580
$2SrO \cdot SiO_2$	3	0.02567	1.3	1.4	1 700
$BaO \cdot 2SiO_2$	1	0.01157	—	0.18	1 420
$BaO \cdot SiO_2$	2	0.01690	0.53	0.60	1 605
$2BaO \cdot SiO_2$	3	0.02192	1.0	1.32	1 755
$MnO \cdot 2SiO_2$	1	0.01603	—	0.55	1 700
$MnO \cdot SiO_2$	2	0.02645	1.8	1.8	1 274
$2MnO \cdot SiO_2$	3	0.03807	3.9	6.3 4.5 *	1 320
$FeO \cdot SiO_2$	2	0.02730	—	1.5 **	1 550
$2FeO \cdot SiO_2$	3	0.03946	3.2	3.25 **	1 205

All values for conductivities in Table 5 are taken from Bockris *et al.*[9], except *) and **), which are taken from Wejnarth [10], and Inouye *et al.*[11], respectively. The temperature is in all cases 1750° C except for the iron silicates for which it is 1400° C. The computations are based on those observed values for which there are no corresponding ones in the column, "computed".

* The mobility under a potential gradient of 1 volt/cm is equal to 300 *zeu* cm/sec.

Table 6. (1750° C).

Composition	Conductivity ohm^{-1} cm^{-1} computed	observed	Ionic radius
$CaO \cdot 2SiO_2$	—	0.31	0.99 Å
$CaO \cdot SiO_2$	0.98	0.89	0.99 Å
$SrO \cdot 2SiO_2$	0.22	0.21	1.13 Å
$SrO \cdot SiO_2$	0.66	0.63	1.13 Å
$2SrO \cdot SiO_2$	1.3	1.4	1.13 Å
$BaO \cdot 2SiO_2$	0.14	0.18	1.35 Å
$BaO \cdot SiO_2$	0.41	0.60	1.35 Å
$2BaO \cdot SiO_2$	0.80	1.32	1.35 Å
$MnO \cdot 2SiO_2$	0.55	0.55	0.80 Å
$MnO \cdot SiO_2$	1.8	1.8	0.80 Å
$2MnO \cdot SiO_2$	3.9	6.3 4.5 *	0.80 Å
$FeO \cdot SiO_2$	2.1	1.82	0.75 Å

* Wejnarth [10].

As is seen from Table 5, the results are in fair agreement. The deviations may be caused partly by our estimations of the densities and partly by the fact that not all silicates have been measured in the molten state.

It will now be shown that the following rule holds for the binary silicate systems studied in this paper with the exception of magnesium silicates.

The ionic mobility (u) is inversely proportional to the square of the ionic radius (r) of the cation.

$$u \sim \frac{1}{r^2} \tag{4}$$

This also means that under the same conditions of temperature and potential field different cations sweep out equal volumes.

For the comparison of two systems we can write

$$\frac{u_1}{u_2} = \left(\frac{r_2}{r_1}\right)^2 \tag{5}$$

Further

$$\begin{aligned} \varkappa_1 &= n_1 \cdot z^2 e^2 \cdot u_1 \cdot S_1 \\ \varkappa_2 &= n_2 \cdot z^2 e^2 \cdot u_2 \cdot S_2 \end{aligned} \tag{6}$$

(5) and (6) give

$$\varkappa_1 = \varkappa_2 \cdot \frac{n_1}{n_2} \cdot \left(\frac{r_2}{r_1}\right)^2 \cdot \frac{S_1}{S_2} \tag{7}$$

The computed values in Table 6 are all based on the $\varkappa$-value for $CaO \cdot 2SiO_2$ and compared with the experimental values of Bockris *et al.*[9] The ionic radii are all taken from Pauling [12].

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Aquo Ion Formation

II. The First Transition Group in Ethanol

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J. Bjerrum and Klixbüll Jørgensen [1] recently studied the consecutive formation of aquo neodymium ions in alcoholic solution *. Investigations of this kind have been continued, and the present paper reports results for some of the first transition group metal ions, enabling conclusions to be drawn on the relative affinity of water and ethanol molecules to the different metal ions. After some remarks on the experimental methods in general, there follow sections on each of the divalent ions of cobalt, nickel and copper together with the relevant experimental details. Most of the theoretical discussion and the analytical applications are given in the section on cobalt(II) nitrate. A preliminary study of the hydration of bis(ethylenediamine)copper(II) ion and of the slow reactions of chromium(III) salts in aqueous ethanol is also given.

EXPERIMENTAL

A Beckman DU spectrophotometer was used for the measurements. The absorption cell chamber was thermostated at 25° ± 2° C. With the exception of a few measurements at low concentrations, the 1 cm rectangular cells were used in the short chamber. This has the advantage that three measurements, on different solutions, can be carried out with the same adjustment instead of one as in the cylindrical cells. This is valuable for the demonstration of small relative differences in optical density at a given wavelength which is not exactly reproducible.

No special technique was used for excluding moisture from the air; the solutions were pipetted from glass-stoppered bottles, covered with latex foil, and water added from a microburette. Experiments showed that high humidities do not have significant effects. In the covered cells, the ethanol solutions concentrated by evaporation by about 2 % per hour. Effects of water have only been observed when one of the cells was filled with an aqueous solution. The comparison cell was filled with the absolute ethanol from De Danske Spritfabrikker. This ethanol was used for nearly all the solutions, and its water content determined by comparison with magnesiumdehydrated ethanol [2], using the optical densities of dilute $Co(NO_3)_2$ solutions in the 10 cm cells. It was found to contain 0.11 wt. % of water, *i. e.*, 0.05 *M* H_2O. Dust errors were computed by comparison with some accurate values (see p. 180) for the absorption of cobalt ions in water and found to be of the order of magnitude of 0.005 in optical density, and proportional to λ^{-2}, where λ is the wavelength.

* In the following this paper is referred to as *Aquo Ion Formation I.*

Extinction coefficients were not measured in the ultraviolet because nitrate ions absorb in this region. In the near infrared, the solvents showed characteristic absorption, which is given in the section on copper(II) nitrate. This also explained an early observation that strong solutions of praseodymium nitrate in water had a regular negative molar extinction coefficient in a double band near 1 200 mμ when the comparison cell was filled with pure water. The spectrophotometer gives reproducible results out to the end of scale, 2 000 mμ, but their physical significance is perhaps dubious. The narrowest possible slit-width was always used. This corresponds, according to information from the Beckman company, to an effective band-width of 5 mμ at 1 100 mμ, 20 mμ at 1 200 mμ and 60 mμ at 1 300 mμ, while in the visible range the band-width is about 0.5 mμ.

The cobaltous nitrate used was B.D.H. Analar $Co(NO_3)_2$ with 5.5 H_2O. This partly dehydrated, rose-red powder did not resemble the usual transparent deep red crystals with 6 H_2O or more. After standing over concentrated sulphuric acid in a desiccator at 100° C for 20 hours, it contained 3.7 H_2O. The solidified mass was dissolved in ethanol, and weighed portions of the approximately 1 *M* solution diluted in graduated flasks.

Some of the copper solutions were made by dissolving known amounts of pure copper in nitric acid, evaporation nearly to dryness at 120° C and diluting with ethanol after cooling. The bis(ethylenediamine) complex was made from Merck's cupric chloride dihydrate. All concentrations in this paper are given in moles per liter.

COBALT(II) NITRATE

The much greater absorption, and correspondingly deeper red colour, of this salt in ethanol than in ethanol containing a few percent of water has been observed by Katzin and Gebert [3]. They explained it by nitrato complex formation, but as pointed out in *Aquo Ion Formation I*[1], it is more likely caused by the exchange of alcohol with water in the solvated cobalt(II) ions.

The total water concentration, C_{H_2O}, of one of the solutions, which were used for these measurements can be found from

$$C_{H_2O} = \frac{0.05 + 3.7\ C_{Co} + \dfrac{V_w}{0.018\ V_0}}{\dfrac{V_0 + V_w}{V_0}}, \qquad (1)$$

where C_{Co} is the cobalt concentration, V_0 the original volume of the solution, V_w the volume of the added water, 0.018 the molar volume of H_2O, and 0.05 and 3.7 the quantities stated in the general experimental part. Corrections for the contraction of the ethanol by admixture of water are not, in general, necessary, since they are not more than 1 % for 10 wt. % of water, and 2 % for 40 % water, for salt free mixtures [4]. The free water concentration $[H_2O]$ is given as the difference between the total water concentration and the water bound to cobalt

$$[H_2O] = C_{H_2O} - \bar{n} C_{Co}, \qquad (2)$$

where $\bar{n}$ is the average number of water molecules bound per cobalt ion. It is assumed here, as in the previous paper [1] that the ratio $\frac{f_{H_2O}}{a_{alc}}$ between the activity coefficient of water ($f_{H_2O} = \frac{a_{H_2O}}{[H_2O]}$) and the activity of alcohol (both of which are defined as 1 in pure ethanol) remains nearly constant, even at high water concentrations. This is quite justified for salt-free solutions where

$C_{H_2O}<20$ for methanol and ethanol, as shown by the vapour-pressure measurements of Wrewsky [5]. Therefore it is permissible to substitute in the activity expression for the uptake of the n'th water molecule

$$K_n = \frac{a_{Co(H_2O)_n^{++}} \cdot a_{alc}}{a_{Co(H_2O)_{n-1}^{++}} \cdot f_{H_2O} \cdot [H_2O]} \tag{3}$$

concentrations for activities, since the relative ionic activities probably remain constant:

$$K_n \sim \frac{[Co(H_2O)_n^{++}]}{[Co(H_2O)_{n-1}^{++}][H_2O]} \tag{4}$$

In the formulae, $Co(H_2O)_n^{++}$ represents $Co(H_2O)_n(C_2H_5OH)_{N-n}^{++}$, where N is the coordination number.

From the N consecutive formation constants, K_n, we define an average formation constant

$$K_{av} = (K_1 \cdot K_2 \cdot \ldots \cdot K_N)^{\frac{1}{N}} \tag{5}$$

In systems where related ligands are exchanged, the average ligand number often corresponds closely to the statistical formula:

$$\bar{n} = \frac{N \cdot K_{av} \cdot [H_2O]}{1 + K_{av} \cdot [H_2O]}, \tag{6}$$

where $K_n = \frac{N-n+1}{n} K_{av}$. It should be noted that the average formation constant is the reciprocal water concentration when the system has reached its midpoint of formation (*viz.*, $\bar{n} = \frac{N}{2}$)

$$K_{av} = \left(\frac{1}{[H_2O]}\right)_{\bar{n}=\frac{N}{2}} \tag{7}$$

This relation is often a valid approximation, even when the assumption of statistical exchange is not correct.

In our special case, the correction $\bar{n}C_{Co}$ is quite small compared with C_{H_2O}. At small concentrations, for $\bar{n} < 1$,

$$\bar{n} \sim K_1 \, [H_2O],$$

and thus

$$[H_2O] \sim \frac{C_{H_2O}}{1 + K_1 C_{Co}}. \tag{8}$$

K_1 is about 6 liter/mole, and, therefore, at very small water concentrations, $[H_2O]$ is about 20 % lower than C_{H_2O} in a 0.05 M cobalt solution, and only 2 % lower than C_{H_2O} in a 0.004 M $Co(NO_3)_2$. The optical estimate of $\bar{n}$ as function of $[H_2O]$ (see Fig. 2) was used for the correction at greater water concentrations. In Fig. 1, several spectra of cobalt(II) nitrate in aqueous ethanol are shown. In the text to the figure, C_{H_2O}, C_{Co} and the calculated $\bar{n}$ and $[H_2O]$ are given for each solution.

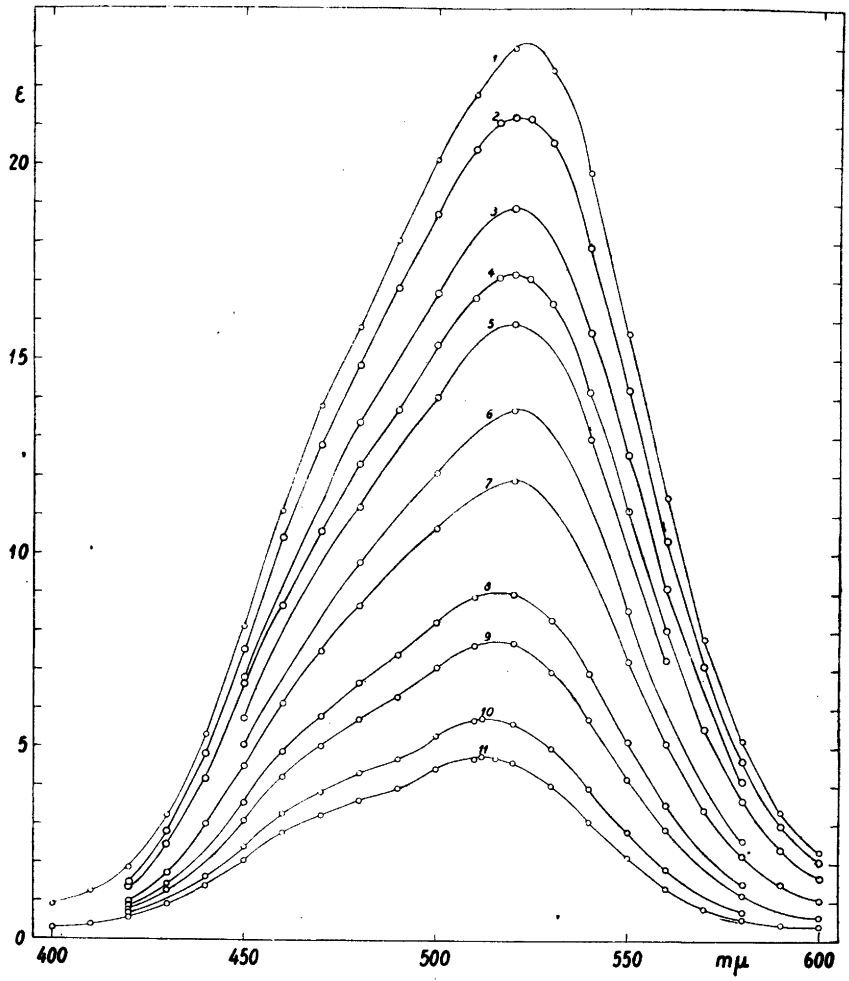

Fig. 1. Absorption spectra of cobalt (II) nitrate in aqueous ethanol at 25° C. $\bar{n}$ and $[H_2O]$ calculated from the statistical case for $\bar{N} = 6$ and $K_{av} = 1.08$.

Curve	C_{H_2O}	C_{Co}	$\bar{n}$	$[H_2O]$
1	*0.06*	*0.004*	*0.4*	*0.06*
2	*0.20*	*0.040*	*0.9*	*0.16*
3	*0.47*	*0.040*	*1.8*	*0.40*
4	*0.63*	*0.040*	*2.2*	*0.54*
5	*0.81*	*0.039*	*2.6*	*0.71*
6	*1.13*	*0.039*	*3.1*	*0.99*
7	*1.63*	*0.125*	*3.4*	*1.21*
8	*2.66*	*0.123*	*4.2*	*2.15*
9	*3.66*	*0.121*	*4.6*	*3.11*
10	*9.2*	*0.108*	*5.4*	*8.6*
11	*55.*		*6.0*	

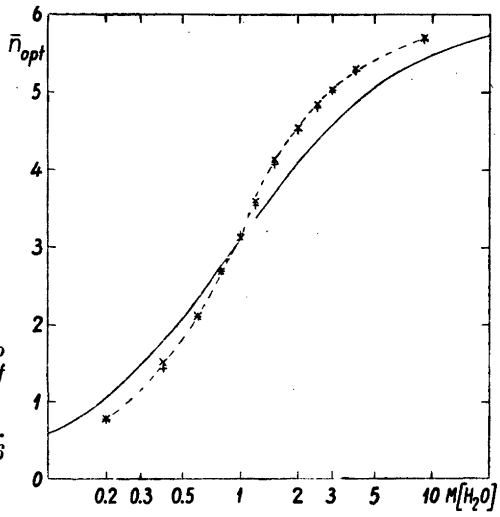

Fig. 2. Optical formation curve of aquo cobalt ions in ethanol. Logarithmic scale of free water concentration.

+ from wavelength 500 mμ, × from 520 mμ. Full drawn the statistical case for N = 6 and K_{av} = 1.08.

From these spectra, and especially from the interpolated data in Table 1 (see p. 180) the average ligand number $\bar{n}$, which is only a second order correction for the calculation of $[H_2O]$, can be found by a method similar to that used for the hypothetical points of Fig. 2 in *Aquo Ion Formation I*, assuming that the extinction coefficients at a given wavelength are a linear function of $\bar{n}$. This is quite reasonable, since the spectra, as seen from Fig. 1 and Table 1, are nearly geometrical transformations of each other. Thus, a quantity $\bar{n}_{opt}$ can be defined

$$\bar{n}_{opt} = \frac{\varepsilon_0 - \varepsilon}{\varepsilon_0 - \varepsilon_w} N, \qquad (9)$$

where, for the same wavelength, ε_0 is the molar extinction coefficient in pure ethanol, ε_w in pure water and ε in the given solution. For cobalt(II) ions N is assumed to be 6 (ref. 6, p. 80, as also discussed in *Aquo Ion Formation I*). Fig. 2 is the formation curve, *i. e.* $\bar{n}_{opt}$ as function of log $[H_2O]$, determined at two wavelengths in order to demonstrate that the assumption was reasonable. It follows quite closely the statistical curve with K_{av} = 1.08 liter/mole. The deviations may be due to activity effects or non-linear variation of ε. If ε varies, *e. g.*, in an exponential way, $\bar{n}_{opt}$ will be greater than the real $\bar{n}$, and the optical formation curve will be distorted. For the possible relation $\log \varepsilon = \log \varepsilon_0 - \frac{\bar{n}}{6} \log \frac{\varepsilon_0}{\varepsilon_w}$, $\bar{n} = 3$ would occur at $[H_2O] = 1.55$, corresponding to $K_{av} = \frac{1}{[H_2O]} = 0.64$ liter/mole.

Yates *et al.* have shown [7] by extraction experiments in octanol that perchlorates of divalent metals hydrate with over 20 moles H_2O per metal ion. This may suggest that $\bar{n}$ would have values well beyond 6, when a water activity comparable to that of pure H_2O is reached. It is interesting in this

connection to note that the spectra of cobalt and nickel salts in octanol are not changed by increasing water concentration above 6 times that of the metal concentration. This seems to show that only very small free water concentrations are necessary to stabilize the hexaquo ions. The excess water is very likely second-sphere-water without much influence on the spectra.

Table 1. Molar extinction coefficients of cobalt(II) nitrate in aqueous ethanol.

by given $[H_2O]$	450 $m\mu$	480 $m\mu$	500 $m\mu$	520 $m\mu$	540 $m\mu$	560 $m\mu$
0.00	8.3	16.1	20.7	23.7	20.4	11.8
0.20	7.5	14.7	18.6	21.1	17.7	10.3
0.40	6.7	13.4	16.8	18.9	15.7	9.1
0.60	6.1	12.1	15.0	17.0	13.9	7.9
0.80	5.5	10.9	13.4	15.2	12.4	6.7
1.00	5.0	9.8	12.1	13.7	10.9	5.8
1.20	4.6	8.9	11.1	12.3	9.7	5.2
1.50	4.1	7.8	9.7	10.6	8.3	4.5
2.00	3.7	6.9	8.5	9.3	7.2	3.7
2.50	3.3	6.2	7.7	8.3	6.4	3.2
3.00	3.1	5.8	7.1	7.7	5.8	2.9
4.00	2.8	5.2	6.4	6.8	5.1	2.5
55.	2.05	3.63	4.45	4.60	3.09	1.35

Table 1 contains the molar extinction coefficients for some free water concentrations at six selected wavelengths, obtained by interpolation of several spectra of the type in Fig. 1. The data given in Table 1 are useful for analytical purposes, since the free water concentration of a given solution can be found by addition of a known amount of a nearly anhydrous standard solution of cobalt(II) nitrate, and measuring the resulting extinction. If ε can be determined with accuracy to 1 %, the water concentration can be found, with a mean error of 0.02 M, provided $[H_2O] < 1$. If colourless constituents in solution (*e. g.* other metal ions) also hydrate, the decrease in $[H_2O]$ can be found from Table 1. This is the method of J. Bjerrum [8] for optical use of a coloured complex system. The temperature-sensitive chloro-complex system of cobalt (II) has recently been used by Ayres and Glanville [9] for determination of water in ethanol.

The last row of extinction coefficients in pure water (Table 1) was determined in this laboratory by Miss Lene Rasmussen, M. Sc., who took great care to exclude dust-errors. Her data also have analytical application, since by dilution to a large known volume with water, the cobalt content of an alcoholic solution can be spectrophotometrically determined. The agreement at different wavelengths is very good.

Media effects in solutions of low dielectric constant. To examine whether a given change in spectra by addition of water is due to the change of medium or to specific effects of water and ethanol the optical effects of addition of small quantities of water can be compared with a similar system with large amounts of an inert solvent which can be assumed not to solvate with metal ions, *e. g.* carbon tetrachloride. Since dielectric constants vary surprisingly linearly with the composition of mixtures [10], it cannot reasonably be maintained that the

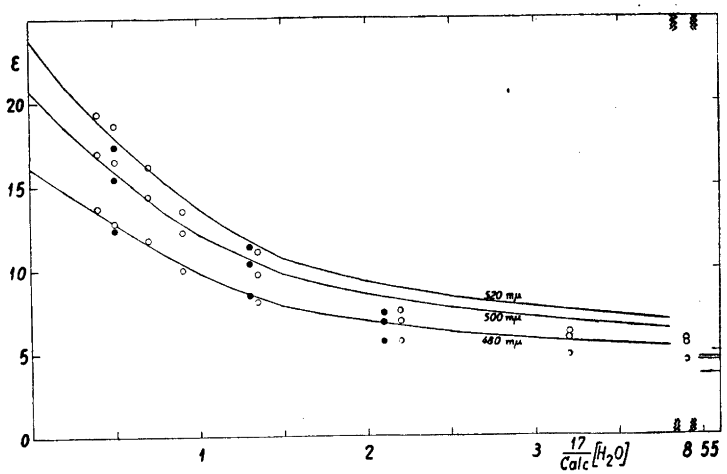

Fig. 3. Media effect of CCl_4 on ethanol-water cobalt (II) solutions. Extinction coefficients for three different wavelengths as function of $\frac{17}{C_{alc}}[H_2O]$. *Full drawn curve gives the results from Table 1 without inert solvent added. Open circles for 50 volume percent CCl_4 and full circles for 75 vol. percent. At the right hand side of the figure are given the three extinction coefficients in 50 vol. percent CCl_4, 4 M in* $H_2O\left(\frac{17}{C_{alc}}[H_2O] = 8\right)$ *as circles, and in pure water as levels.*

medium effects of a few vol. % of water can prevail at fifty or more vol. % carbon tetrachloride.

It was suggested by Professor J. Bjerrum that the relative affinity of water and ethanol to the metal ion is little changed by addition of an inert solvent. This means that the mass-action-determining factor $\frac{f_{H_2O}}{a_{alc}}[H_2O]$ in (3) can be represented by $\frac{17}{C_{alc}}[H_2O]$, where 17 is the molar concentration of pure ethanol.

In Fig. 3, ε is given for different wavelengths as a function of $\frac{17}{C_{alc}}[H_2O]$ in ethanol alone (full curve) and in 50 (circles) and 75 (filled circles) vol. % CCl_4. It is seen that at higher water concentrations, the activity of water seems to be slightly greater than calculated. See especially the extinction coefficients in 4 *M* H_2O, 50 % CCl_4, which is near to separating in two phases.

Salt-effects. In order to use Table 1 for determining free water concentrations, and thus for instance the uptake of water by colourless constituents of the solution, it is important to consider possible salt-effects on the cobalt spectrum which are not caused by solvate-exchange. As shown for instance by Scheffer and Hammaker [11] in a paper on cobalt(II) tetrafluoborate in water, there is often an increased absorption by approximately the same percentages throughout the whole spectrum, analogous to the solvation spectra in organic

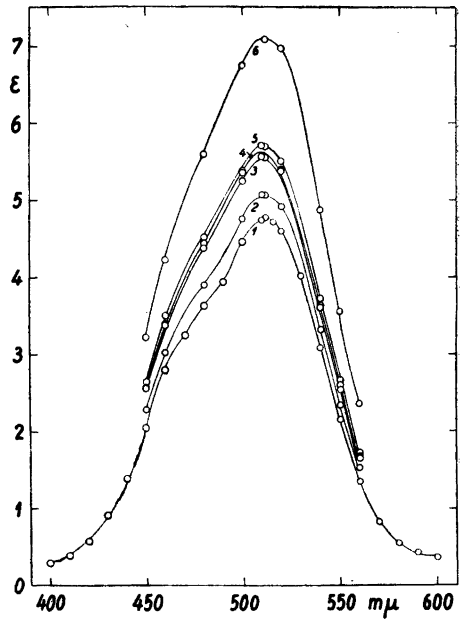

Fig. 4. Salt effects on spectrum of 0.2 M cobalt(II) nitrate in water.

Curve	*Added Substances*
1	*none*
2	*8 M $HClO_4$*
3	*3.5 M $Ca(NO_3)_2$*
4	*6 M HNO_3*
5	*8 M NH_4NO_3*
6	*12 M HNO_3*

solvents. Some spectra of solutions, 0.2 *M* in $Co(NO_3)_2$, are given in Fig. 4. It is seen that 6 *M* HNO_3, 8 *M* NH_4NO_3 and 3.5 *M* $Ca(NO_3)_2$ each increase the absorption about 20 %, and 12 *M* HNO_3 about 50 % against pure water. Perchloric acid has only about a third the effect of nitric acid per mole.

It is very difficult by optical means alone to distinguish between a salt-effect and a complex formation, which is far from being complete in the strongest possible solutions of the complexing agent. The salt-effect is usually linear in the salt concentration, to a first approximation, thus:

$$\varepsilon = \varepsilon_0 + \varepsilon_s C_s, \tag{10}$$

where ε_0 is the extinction coefficient in pure water, ε_s a constant and C_s the salt concentration. For a true complex formation:

$$\bar{n} = K_1 \cdot C_s \tag{11}$$

There may thus be postulated in complex whose extinction coefficient is

$$\varepsilon_c = \varepsilon_0 + \frac{\varepsilon_s}{K_1}. \tag{12}$$

Such dubious complexes are the result of many unfortunate applications of Job's principle and similar methods on systems where the activities were not constant.

In our particular case, the decision between formation of nitrato-complexes or a general salt effect, greater for nitrates than perchlorates, is not easily made. But it is interesting to note that media with low dielectric constants do

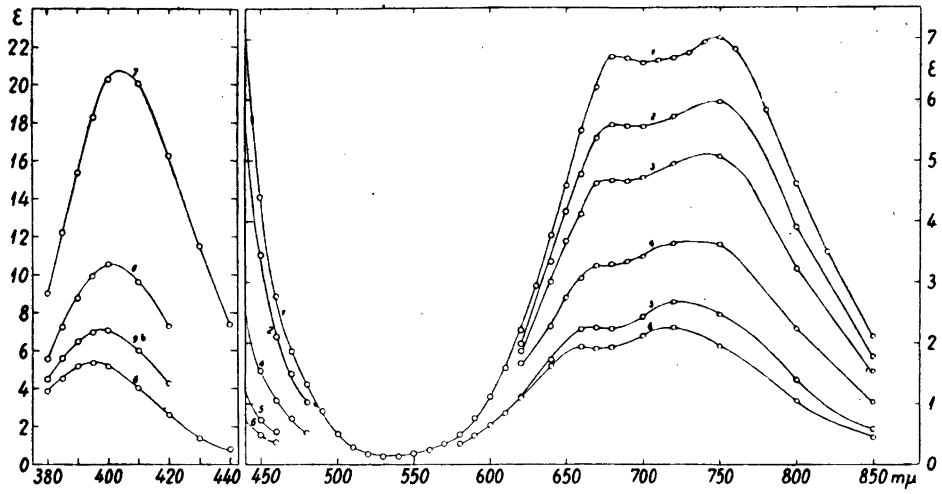

Fig. 5. Absorption spectra of nickel(II) nitrate in aqueous ethanol at 25°. $\bar{n}$ and $[H_2O]$ calculated from the statistical case for $N = 6$ and $K_{av} = 0.82$.

Curve	C_{H_2O}	C_{Ni}	$\bar{n}$	$[H_2O]$
1	*0.1*	*0.01*	*0.5*	*0.1*
2	*0.5*	*0.11*	*1.3*	*0.35*
3	*1.0*	*0.11*	*2.3*	*0.7*
4	*2.1*	*0.11*	*3.5*	*1.7*
5	*10.6*	*0.40*	*5.3*	*8.5*
6	*55.*	*0.40*	*6*	—
7	*0.2*	*0.04*	*0.7*	*0.17*
8	*1.8*	*0.04*	*3.4*	*1.6*
9	*5.2*	*0.04*	*4.9*	*5.0*

not alone produce optical effects (see Fig. 3) which resemble the salt effect, but that ionized salts in such media have even greater salt effects [1,12] on the spectra than in aqueous solutions. This makes it probable that the salt effect is not due to complex formation but rather to the Stark effect from the strong electric fields from ions, since the effect $\frac{\varepsilon_s}{\varepsilon_0}$ per mole is roughly proportional to the reciprocal dielectric constant.

NICKEL(II) NITRATE

In absolute ethanol, nickel nitrate dissolves with a distinctly more intense yellow-green colour than in water or in 90 % ethanol. This behaviour closely resembles that of cobalt(II) nitrate, but the intensity differences are not so great. Fig. 5 shows some of the spectra. It is divided into two parts, because the band at 400 mμ is essentially higher than the bands in the red. The flat minimum in the red is preserved in the spectra of ethanol-solvates in contradistinction to the ammine complexes [6]. From the spectra a reasonable mid-

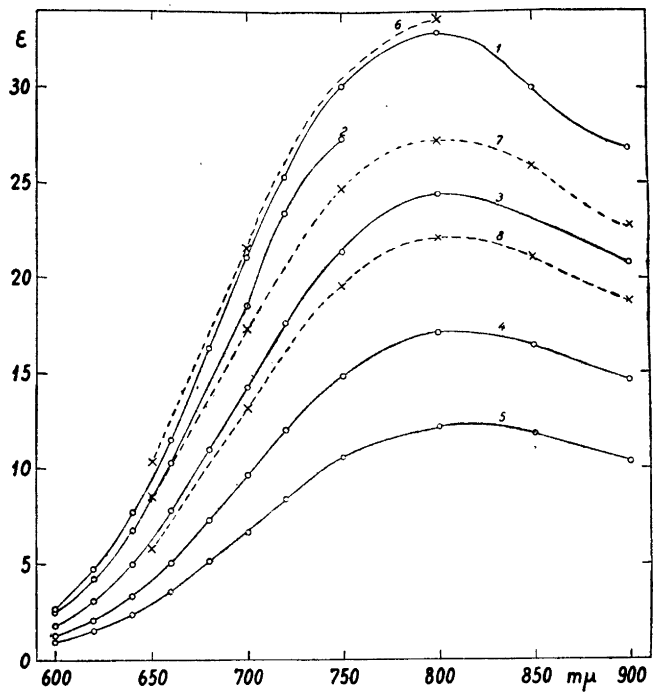

Fig. 6. Absorption spectra of copper(II) nitrate in aqueous ethanol at 25° C.

Curve	$[H_2O]$	C_{Cu}	*Volume % CCl_4*
1	*0.5*	*0.02*	*0*
2	*2.0*	*0.07*	*0*
3	*5.4*	*0.07*	*0*
4	*16.*	*0.06*	*0*
5	*55.*	*0.05*	*0*
6	*0.3*	*0.04*	*50*
7	*1.3*	*0.04*	*50*
8	*2.9*	*0.04*	*50*

point of change (*i. e.* $\bar{n}_{opt} = \frac{N}{2}$) can be found at $[H_2O] = 1.2$, *i. e.* $K_{av} = 0.82$ liter/mole, a slightly lower formation constant than found for cobalt ions. In 62.5 vol. % CCl_4, the variations of spectra due to water addition are similar, and the relative complexity constants seem to be slightly lower than in pure ethanol. Nitrato-complex formation, which would be promoted by low dielectric constant, is thus not the cause of the change in spectra.

COPPER(II) NITRATE

This salt dissolves in ethanol with a distinctly deeper blue colour, but the changes from water addition continue even at great water concentrations. Since the CCl_4-mixtures show similar behaviour, it can be concluded that there

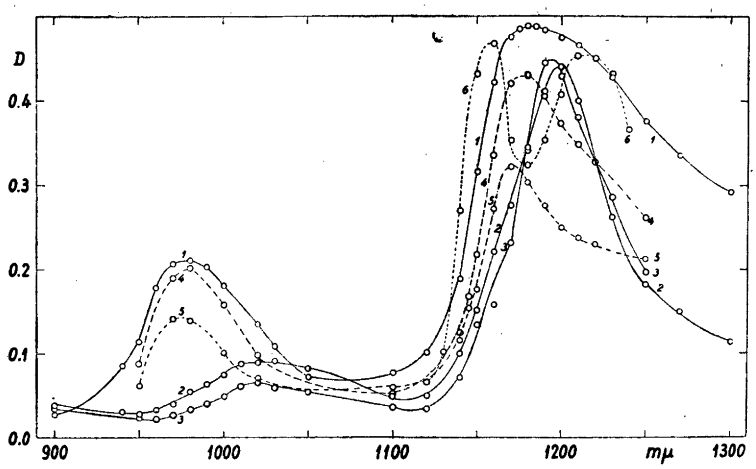

Fig. 7. Absorption spectra of solvents in the near infra-red.

Curve

1	*Water (55 M)*
2	*Methanol (25 M)*
3	*Ethanol (17 M)*
4	*4.2 M calcium nitrate (42 M H_2O)*
5	*14 M nitric acid (22 M H_2O)*
6	*Numerical value of negative absorption of praseodymium nitrate, compared with pure H_2O, calculated for a 10 M solution.*

is really aquo ion formation analogous to that of Co^{++} and Ni^{++}, but of lesser complexity.

The ethanolic solutions are unstable and on ageing deposit green-blue basic nitrates, while the free nitric acid esterifies to ethyl nitrate. Dilute copper solutions may, however, be stored for weeks without decomposition. The mixtures with CCl_4 turn bright yellow green in a few days, because the hydrolysis of CCl_4 involves chloro-complex formation.

Fig. 6 shows some spectra at different water concentrations. It is seen that the midpoint of change in spectra lies at $[H_2O] = 5.3$, or $K_{av} = \frac{1}{[H_2O]} = 0.19$. The extrapolated spectrum of anhydrous solution is similar to a 50 vol. % CCl_4 solution, and the cross-points in the figure show that the relative complexity is increased a little in the CCl_4-mixture.

The spectra of the solvents. It was found that Beer's law was not obeyed in the infrared by the copper solutions. This was explained as being due to absorption by the solvents themselves. Fig. 7 shows the spectra in the range 900—1 300 mμ of water, methanol, ethanol and 4.2 *M* calcium nitrate and 14 *M* nitric acid in water, all with carbon tetrachloride in the comparison cell. The bands are those which Mecke *et al.* have used [13] for the investigation of association of water, alcohols and other hydroxy-compounds in inert solvents.

It may be seen that the double band of water is distorted by salts, and the lowered water concentration has decreased the optical density in general. Salt effects on the absorption bands of water have been studied by Suhrmann, Breyer and Ganz [14]. The negative extinction coefficient of $Pr(NO_3)_3$, compared with water, as mentioned p. 176, is shown on the figure for a 10 *M* solution, which according to the molar volume would nearly correspond to pure salt. When benzene was compared with CCl_4, no difference in optical density was observed, except a very narrow band at 1 148 mμ, which had an optical density of 0.680 in the 1 cm cells.

BIS(ETHYLENEDIAMINE)COPPER(II) CHLORIDE

The complex ion $[Cuen_2]^{++}$ is soluble with bluish violet colour in ethanol and reddish lilac in water, and it shows the usual effects upon addition of small quantities of water. Fig. 8 shows some spectra at different water concentrations. The cross-points refer to measurements in 50 % CCl_4.

Bjerrum and Juhl Nielsen [15] investigated the copper(II) complexes with ethylenediamine in water, and found that the third amine molecule is much more weakly bound than the first two. Since the stability of ammine complexes is not increased greatly in ethanol (compare the measurements by Rosenblatt [16], which J. Bjerrum [17] used for calculation of the binding of the fifth ammonia to copper in methyl glycol) the colour change cannot be caused by formation of tris(ethylenediamine)copper(II) ions, the characteristics of which would otherwise resemble the observed spectra. Experiments also show that addition of a considerable excess of ethylenediamine has no influence on the spectrum of the solution in ethanol, which was about 0.008 *M* $CuCl_2$ and 0.02 *M* in ethylenediamine. It was necessary to use the chloride, since the nitrate was only slightly soluble in ethanol. The change in spectra cannot be due to chloro-complex formation, since anhydrous lithium chloride does not have a very great influence on the aqueous-ethanolic solutions.

Hantsch and Robertson [18] considered that $[Cu(NH_3)_4]^{++}$ contained no water of constitution, since the colour was similar in ethanol and water. This argument was partially reversed by Rosenblatt's measurements [16], where the tetrammine, but not the pentammine, complex showed changes similar to those in this paper for $[Cuen_2]^{++}$ in ethanol. This ion thus exchanges at least one molecule H_2O.

According to the theory of J. Bjerrum [12,15,17], the maximum coordination number of divalent copper is 5, so only one H_2O molecule should exchange with one C_2H_5OH molecule in the bis(ethylenediamine) complex. Because of the large extinction coefficients, it has not been possible to measure at a copper concentration high enough to utilize J. Bjerrum's principle of corresponding solutions [8]. However, the spectra in Fig. 8 are treated by the method used by J. Bjerrum [19] to prove the existence of penta- and tetra-ammine copper complexes only in strong ammonia solutions. If only two copper complexes, $[Cuen_2(C_2H_5OH)]^{++}$ and $[Cuen_2(H_2O)]^{++}$, are present in our case, the observed spectra should be linear combinations of the two pure spectra, *i. e.* $\overline{n}_{opt}$ (see eq. 9) should be the same for each wavelength of the spectrum, and equal to the real $\overline{n}$. When the three spectra of solutions 2, 3 and 4 in Fig. 8 are conside-

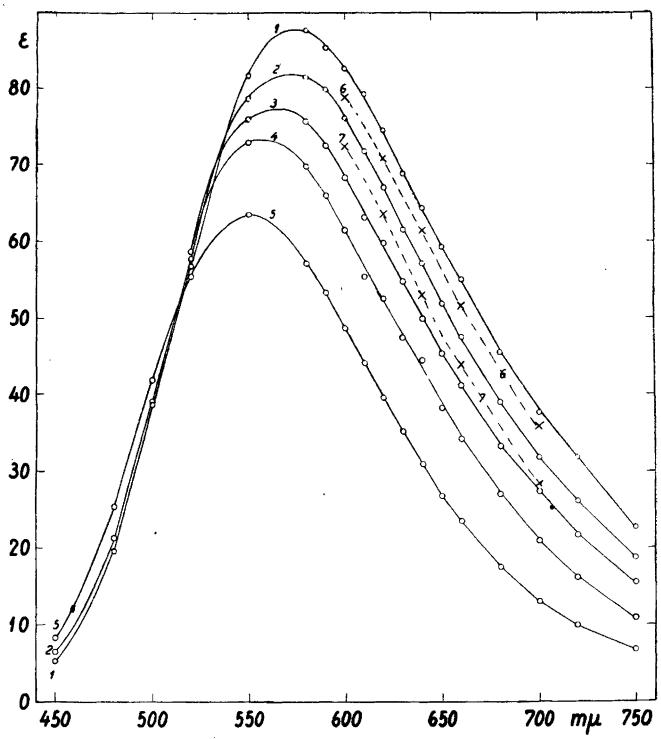

Fig. 8. Absorption spectra of bis(ethylenediamine)copper(II) chloride in aqueous ethanol at 25° C. In all cases $C_{Cu} = 0.008$ *and* $C_{en} = 0.02$.

Curve	$[H_2O]$	*Volume % CCl_4*
1	*0.1*	*0*
2	*1.7*	*0*
3	*4.2*	*0*
4	*9.2*	*0*
5	*55.*	*0*
6	*0.1*	*50*
7	*1.7*	*50*

red, we obtain $\bar{n} = 0.23$, 0.43 and 0.65, respectively, for $[H_2O] = 1.7$, 4.2 and 9.2. The numbers are nearly independent of wavelength when small media effects and experimental uncertainty are disregarded. It is remarkable that the formation constants K in the three cases are nearly equal, being 0.18, 0.18 and 0.20 liter/mole. This is strong evidence for the theory of pentacovalency, since an ion with coordination number of, *e. g.*, 6, must take the two water molecules up nearly statistically in order to explain the constant K, which then would be the real K_{av} for the two formation constants of aquo ions. In the ammonia system [17,19], if $[Cu(NH_3)_6]^{++}$ exists at all, it is formed at much higher concentrations of ammonia than necessary for the pentammine com-

plex, and there is no evidence for statistical behaviour after the fourth complex is formed in any ammine system of copper.

In 50 vol. % carbon tetrachloride, the anhydrous solutions seem to have a slightly lower extinction than in ethanol alone, and the relative complexity of aquo ions is slightly lower, *i. e.* $\frac{f_{H_2O}}{a_{alc}} < \frac{17}{c_{alc}}$. These solutions are unstable, since the HCl from hydrolyzed CCl_4 neutralizes the ethylenediamine.

CHROMIUM(III) SALTS

At ordinary temperatures, chromium(III) nitrate displays remarkable behaviour in ethanol, for the violet colour of the hexaaquo ions transforms to a deep green. If equal amounts of water are added to such green solutions, they turn violet again. Both processes take place over the course of several days.

Recently, Taube *et al.*[20] discovered that the water in $Cr(H_2O)_6^{+++}$ exchanges with oxygen-18-water, with half-times nearly proportional to the anion concentration and of a magnitude of two days at ordinary temperatures. Some preliminary experiments on the change in ethanol of hexaaquo ions to the green form show that a small spectrum change corresponding to $\bar{n}_{opt} = 5.6$ (see eq. 9) occurs 7 minutes after mixing of the strong chromium nitrate solution with ethanol at 27° C, while at 0° C it takes 360 minutes. Assuming ethanol to be the reacting agent, these data corresponds to an activation energy ~ 22 kcal/mole and a frequency factor $\sim 10^{12}$ sec^{-1} ($C_{C_2H_5OH} = 17\ M$). The activation energy is thus similar to that found by Taube *et al.*[20]: many chromium(III) complexes [21] have activation energies ~ 25 kcal/mole. The latter processes, leading to the green form, proceed more slowly; this is even more pronounced at low temperatures, corresponding to increasing activation energies for these processes. The curves of Fig. 9, Nos. 5—8, correspond to solutions of chromium(III) nitrate enneahydrate in ethanol with different water content, which have remained at 25° C for four days. Storage for six months does not change the spectra much.

According to the measurements of N. Bjerrum [22] and Brönsted and King [23], the first acid dissociation constant of $Cr(H_2O)_6^{+++}$ is about $10^{-4.0}$. The small difference in spectrum, which develops instantaneously (see curves 2 and 1) is due to the formation of monohydroxo-complexes, about 6 % and 0.07 %, respectively.

The green solutions in ethanol do not change their spectra much by addition of acids, as shown by curve 9 compared with curve 6. In ethanol, where nitric acid is a weak acid and an active oxidant, *p*-toluenesulphonic acid was used. This acid does not easily esterify with alcohols. The monohydrate of this acid was delivered chloride- and sulphate-free from L. Light & Co.

When chromium(III) salts are acidified before the reaction with ethanol, the change to the green form goes very slowly, and the problem is whether the same equilibrium solution is obtained as without acid. Such "negative hydrogen ion catalysis", *i. e.* a reaction mechanism with hydroxo-complexes, was described in the chloro-chromium(III) system by N. Bjerrum [24]. Curves 3 and 4 show spectra at high water concentrations and containing nitric acid,

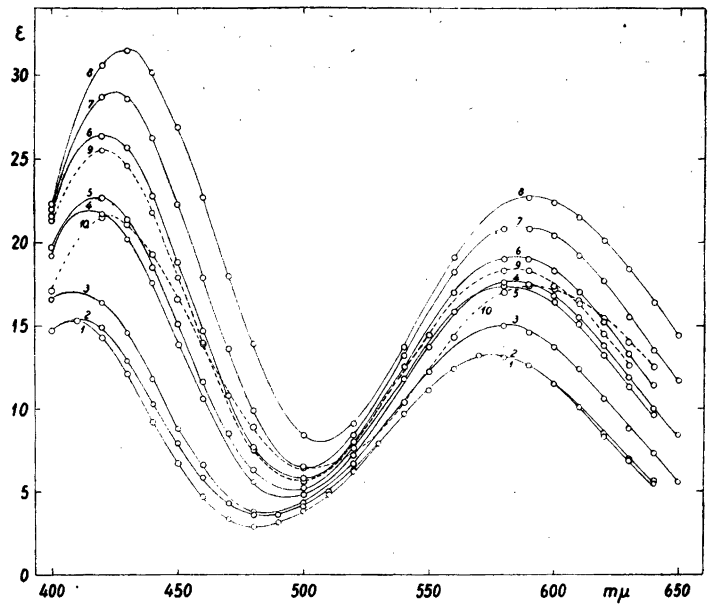

Fig. 9. Absorption spectra of chromium(III) salts in aqueous ethanol.

Curve	C_{H_2O}	C_{Cr}	*Remarks*
1	*55*	*0.025*	*0.15 M HNO_3, stable.*
2	*55*	*0.025*	*two hours after preparation, no acid added.*
3	*9.2*	*0.050*	*0.06 M HNO_3, remained 17 days.*
4	*2.7*	*0.050*	*0.06 M HNO_3, remained 17 days.*
5	*9.4*	*0.021*	*after 4 days.*
6	*4.2*	*0.023*	*after 4 days.*
7	*1.4*	*0.025*	*after 4 days.*
8	*0.3*	*0.025*	*after 4 days.*
9	*4.2*	*0.023*	*after 17 days added 0.1 M p-toluene sulphonic acid.*
10	*0.4*	*0.022*	*0.1 M p-toluenesulphonic acid, then reaction for 4 days.*

which after 17 days are in reasonable agreement with the solvation hypothesis. But curve 10, with *p*-toluenesulphonic acid, shows that after four days the spectra resemble ethanol solutions containing no acid, but with some water. Later, however, it shifts to higher wavelengths, as the spectrum of the acidified green forms also does after some days. Nevertheless, the formation of polynuclear hydroxo chromium complexes, which react slowly with acids (*i. e.* N. Bjerrum's "latently basic chromium") is not very probable as an explanation of the optical change. The green solutions react only three times faster with four parts of 4 *M* HNO_3 than with four parts of pure H_2O, and even slower with 0.1 *M* acid than with pure water. The formation of nitrato-complexes in our case seems to be excluded, since after several days 0.15 *M* NH_4NO_3 has no influence on the partially green solution with 3 *M* H_2O.

If the various spectra between the hexaaquo spectrum (curve 2) and the nearly pure green form (curve 8) are interpreted as solvate spectra, a K_{av} can be calculated, similarly to the other metal ions, to be 0.25 liter/mole, since $\bar{n}_{opt} = \frac{N}{2}$ at $C_{H_2O} \sim 4$.

Discussion of the average formation constants. In Table 2, the different K_{av} are given directly and multiplied by the ethanol concentration C_{alc}, when the aquo ion formation is half completed ($\bar{n}_{opt} = \frac{N}{2}$). The quantity $K_{av} \cdot C_{alc}$ is the numerical expression for the relative tendency of the metal ion to be bound to a water molecule rather than to an ethanol molecule. It is, therefore, independent of the concentration scale, and is used in the fifth column of the table for calculation of the standard free energy change $-\Delta G = RT \ln (K_{av} \cdot C_{alc})$ per mole H_2O.

Table 2. Average formation constants in ethanol at 25° C.

	$[H_2O]_{\bar{n}} = \frac{N}{2}$ mole/liter	K_{av} liter/mole	$K_{av} \cdot C_{alc}$	$-\Delta G$ kcal/mole H_2O
Co^{++}	0.93	1.08	18	1.7
Ni^{++}	1.2	0.82	13	1.5
Nd^{+++}	3 *	0.33	5	0.9
Cr^{+++}	4	0.25	4	0.8
Cu^{++}	5.3	0.19	2.9	0.65
$Cuen_2^{++}$	5.5	0.18	2.7	0.6

According to Irving and Williams [25], the stability of complexes of the divalent transition metal ions in general increases in the direction

$$Co < Ni < Cu.$$

The results from the aquo ion formation in ethanol seem distinctly to indicate the opposite order.

SUMMARY

The spectra of divalent cobalt, nickel and copper ions in mixed solutions of ethanol and water are studied, and they are interpreted analogously to the neodymium ion spectra as being caused by solvation with ethanol and water molecules. From the spectra, estimates can be made of the water concentration, when the aquo ion formation is half complete (*i. e.* $\bar{n} = \frac{N}{2}$). The average consecutive formation constants K_{av} are given in Table 2. Salt and medium effects are discussed, and the addition of inert solvents as carbon tetrachloride shown not to disturb the specific chemical effects of water and ethanol. For the bis(ethylenediamine)copper(II) ion, a similar uptake of water is found, and it may be assumed to take place on the fifth and last coordination place of

* in CH_3OH[1]. According to H. C. Jones the influence on the spectrum in methanol and ethanol is equal at the same water concentration.

divalent copper. For hydrated chromium(III) salts, reactions take place in ethanol, which seem to be reversible and reach equilibrium at room temperature only after some days.

I am very much indebted to the head of the laboratory, Professor Jannik Bjerrum, for his theoretical elucidations and great interest in the work.

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The Isolation of 2-Deoxy-D-ribose from Deoxypentosenucleic Acids

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The sugar component of deoxypentosenucleic acids (DNA) isolated from various sources is generally assumed to be 2-deoxy-D-ribose, and in recent years Chargaff and his collaborators [1] have provided evidence by their chromatography studies, to support this assumption. The most satisfactory way of establishing this point would be to develop a method for the isolation in good yield, of the sugar in crystalline form, from hydrolysates of various samples of DNA. In the past the isolation of 2-deoxy-D-ribose from DNA has proved to be very difficult. It was usual to degrade the nucleic acid by chemical methods to the constituent nucleosides, separate these, and then hydrolyse the sugar-base glycosidic linkage, and isolate the sugar portion. Separation of the nucleosides was a tedious procedure, and since acidic treatment for hydrolysis results in some conversion of the deoxypentose to levulinic acid [2], yields were poor. Following attempts by Thannhauser and Ottenstein [3], who employed picric acid for the hydrolysis of thymus nucleic acid and obtained diphosphoric esters of pyrimidine deoxyribosides, Levene and London [4] resorted to enzymic methods. A solution of thymus nucleic acid was passed through a segment of the gastro-intestinal tract of a dog and collected from an intestinal fistula. After incubation for several days the deoxyribonucleosides of guanine, hypoxanthine (arising from deamination of adenine), cytosine and thymine were isolated. Very mild hydrolysis of the guanine nucleoside gave the deoxy-sugar in crystalline form [5,6]. Similar mild acidic hydrolysis of hypoxanthine deoxyriboside gave a solution with the same optical rotation as an equivalent amount of 2-deoxy-D-ribose [4]. Attempts to isolate the sugar from the pyrimidine deoxyribonucleosides were unsuccessful, as the sugar was immediately converted into levulinic acid under the more drastic hydrolytic conditions necessary to cleave the linkage between the pyrimidine base and the deoxysugar [4].

Although methods of enzymic hydrolysis afforded the possibility of preparing with comparative ease, quantities of mixed nucleotides and nucleosides from DNA, the problem remained of obtaining the individual compounds in pure form by separation of the mixtures. The development of chromatographic

methods and of ion-exchange resins has solved this problem and it is now possible to isolate from enzymic digests of DNA's, deoxypentose-nucleotides and -nucleosides in greatly improved yield and in a high degree of purity. For example, by chromatographic separation on a column of alumina, Schindler [7] was able to obtain the deoxyribosides of guanine, hypoxanthine, thymine and cytosine, from an enzymic hydrolysate of sodium thymonucleate. Chromatography on Dowex 50 and starch was also effective [8]. Volkin, Khym and Cohn[9] have separated deoxyribonucleotides in good yield, on ion exchange resins and other workers [10], have described similar experiences with this method. Ion-exchange fractionation of enzymic digests of DNA isolated from thymus gland and wheat germ has resulted in the isolation of the deoxyribonucleoside [11] and -nucleotide [12] of 5-methylcytosine. Recently a method suitable for the large scale preparation of deoxyribonucleosides from DNA has been described [13]. In the present communication we describe a further extension of the use of ion-exchange resins, since we have been able to use acidic resins to cleave the glycosidic linkage in purine deoxyribosides.* The liberated deoxy-sugar has been freed from purine bases by the ion-exchange resin technique, and has been obtained in crystalline form and characterised. It is our belief that the procedure now reported is the most satisfactory method so far described for obtaining 2-deoxy-D-ribose from DNA.

EXPERIMENTAL

Materials

DNA from soft cod roe was prepared essentially according to the method already described by us [14] for the preparation of DNA from soft herring roe. The material was dialysed at +3° C against distilled water and was finally obtained by evaporation of its solution in the frozen state. [Found: P 8.76; N 14.63 %, *i. e.* N/P = 1.67 (After drying at 110° C/15 mm Hg over phosphoric oxide)].

The herring roe DNA used in this investigation was a purified preparation of a commercial product supplied by Isaac Spencer and Co. Ltd., Aberdeen. Purification was effected as follows: the commercial material (10 g) was dissolved in 1 *M* sodium chloride solution (800 ml) and "Teepol" (100 ml) and 50 % (by volume) acetic acid (800 ml) was added. The solution was treated twice with chloroform-amyl alcohol mixture (75 : 25 respectively). The nucleic acid was precipitated by the addition of ethanol (2 vols.) and the precipitate was collected at the centrifuge. The solid obtained was redissolved in water (200 ml) and the solution was dialysed against water for 48 hours and then dried in the frozen state. The solid obtained (4 g) was essentially free from protein material.

Deoxyribonuclease was prepared from ox-pancreas according to the procedure of McCarty [15] as modified by Overend and Webb [16].

Intestinal phosphatase was isolated by the method described by Klein [17]. Both enzymes were stored in the freeze-dried state.

Methods

Phosphorus analyses were carried out by the colorimetric method of Allen [18] as modified by Jones, Lee and Peacocke [19] and nitrogen values were estimated by means of a micro-Kjeldahl method using the apparatus described by Markham [20].

* When this work was completed it came to our notice that Khym *et al.* (*J. Am. Chem. Soc.* **75** (1953) 1262) have cleaved the glycosidic linkage in purine ribonucleotides with an acidic type of resin.

Enzymic Hydrolysis of Cod-roe DNA

Cod-roe DNA (3 g, undried), hydrated magnesium sulphate (2.35 g) and deoxyribonuclease (0.03 g) were dissolved in water (32.5 ml) and thereafter 10 % ammonium hydroxide was added cautiously to give a pH value of 7. Toluene (20 ml) was layered on the solution which was incubated at 37° C for 12 hours. The pH value was maintained constant by addition at intervals of ammonium hydroxide. The phosphorus content was determined on an aliquot (1 ml) of the digest suitably diluted (2 ml of the digest was diluted to 25 ml) and hence the total amount of phosphorus in the digest was calculated (0.165 g) and from this the dry weight (1.88 g) of nucleic acid used was determined. (Control analyses showed that the amount of phosphorus in the digest arising from the enzyme was negligible.)

Ammonium sulphate (2.0 g) and intestinal phosphatase (0.12 g) were added to the digest and the pH value of the mixture was adjusted to 9 by addition of 10 % ammonium hydroxide. Digestion was continued at 37° C for a further 48 hours. At intervals the pH value was checked and maintained at a constant value (9). The solid precipitate (magnesium ammonium phosphate) was removed by filtration and analyses showed that dephosphorylation of the nucleic acid was essentially quantitative (98 %). A solution in hot water (100 ml) of the theoretical amount of barium hydroxide (4.8 g) required to precipitate quantitatively the sulphate ions in solution, was added to the digest and the barium sulphate which separated was removed by filtration and well washed with water (4 × 50 ml). The washings and filtrate were combined and concentrated to small volume (A) (10 ml) by heating at 40° C under diminished pressure.

Passage of the DNA-Hydrolysate Through a Bed of Ion-exchange Resins

The resins employed were Amberlite IR—120 (H) and IR—4B (OH) (40—60 mesh). The former was prepared for use by treatment with 10 % hydrochloric acid (520 ml per 100 g of resin) followed by washing with water (2—3 litres) until free from chloride ions, whereas the latter was treated with 2 % sodium carbonate solution (3.6 litres per 100 g of resin) and subsequent washing with water until free from carbonate ions.

The regenerated resins (57 ml of each in the wet state) were shaken together and then packed into a glass column (4 cm diameter). The concentrated mixture of nucleosides (A) (10 ml) was poured onto the column and washed through with water (8 ml per minute), until the effluent contained negligible amounts of material exhibiting a positive Dische [21] test or absorption at 2 600 Å. [The Dische test was carried out as follows: Effluent (0.5 ml), water (1.5 ml) and Dische diphenylamine reagent (5 ml) were heated together at 100° for 15 minutes, thereafter immediately cooled to room temperature, and the solution examined in the Colman spectrophotometer at 5 900 Å]. When 600 ml of effluent had been collected, negative results were obtained in the above tests. As will be seen from the subsequent account the effluent contained 2-deoxy-D-ribose and thymidine and Table 1 shows the approximate amounts of the deoxy-sugar in various fractions collected from the resincolumn. It is obvious that the major portion of the sugar is concentrated in fraction "b" (*i. e.* 100—300 ml of effluent).

Table 1

Fraction	*Vol. of effluent (ml)*	*Amount of 2-deoxy-D-ribose (mg)*
a	0—100	0
b	100—300	256
c	300—400	17
d	400—500	11
e	500—600	0.8
	Total weight of 2- deoxy-D-ribose	284.8

(The amounts of deoxy-sugar in the fractions of effluent were determined by the Dische method using synthetic 2-deoxy-D-ribose as standard. It was necessary to dilute the aliquot of fraction "b" before carrying out the analysis.)

The presence of thymidine affects the determination of 2- deoxy-D-ribose only very slightly. For example it was demonstrated that the presence of 100 μg of thymidine in the Dische mixture during a determination of 2-deoxy-D-ribose gave a "Spekker" reading corresponding to only 3 μg of the sugar, compared with control tests. Since the maximum amount of thymidine present in a Dische analysis was of the order of 100 μg, and the corresponding amount of 2- deoxy-D-ribose was 40 μg it was clear that the presence of thymidine could be ignored in comparing the results in Table 1.

The ultra-violet absorption spectrum of the combined fractions of the effluent (a neutral solution) after suitable dilution (2 ml diluted to 100 ml) was examined in the Beckmann spectrophotometer. Results obtained were as follows:

Wavelength ($m\mu$)	240	250	260	267	270	280	290
Optical density of the effluate (expressed relative to the maximum value measured)	0.28	0.57	0.92	1.00	0.98	0.66	0.20
Optical density of a solution of the thymidine in water (expressed relative to the maximum value measured) as reported by Hotchkiss [22]	0.30	0.60	0.91	1.00	0.97	0.64	0.20

It is clear that the ultra-violet absorption spectrum of the effluate closely ressembled that reported by Hotchkiss [22] for thymidine. When an aliquot of the solution was subjected to chromatography on a paper strip [the solvent system employed was prepared by shaking together *n*-butanol, ethanol, water and concentrated aqueous ammonium hydroxide (40 : 10 : 49 : 1, *v/v/v/v*) and separating for use the upper phase] only one fast-moving spot was detected by photography in ultra-violet light [14,23].

Isolation of 2-Deoxy-D-ribose and Thymidine from the Effluent

The combined fractions (a—e) were concentrated at 40° C under diminished pressure to small volume and then finally dried in the frozen state. Absolute acetone (10 ml) was added to the residue and the mixture was stored at 0° C for 24 hours. A crystalline precipitate (0.27 g) was formed and was collected by filtration. (The filtrate [B] was set aside for further use.) The solid showed m. p. 170—183° and its extinction coefficient at 2 600 Å in aqueous solution (1 mg/ml) was 33.5. Hotchkiss [22] reports a value of 36 for thymidine, when measured under these conditions. Recrystallisation from acetone afforded pure thymidine (0.2 g), m. p. 183—185°, $[\alpha]_D^{20}$ + 62.5° (*c*, 1.54 in water) (Found: C 49.8; H 6.2. Calc. for $C_{10}H_{14}O_5N_2$: C 49.6; H 5.8. Brady [24] reports m. p. 185—186° for thymidine and Todd *et al.* [13] state m. p. 182—183°. The acetone-filtrate (B) obtained after removal of the precipitated thymidine, and which contained 2- deoxy-D-ribose and some residual thymidine was evaporated to dryness under diminished pressure and the residue was redissolved in water (10 ml). The amount of residual thymidine in solution was estimated by ultra-violet absorption measurements to amount approximately to a 10 % contamination of the 2- deoxy-D-ribose. The solution was reconcentrated and the syrupy residue (0.27 g) obtained was induced to crystallise. It showed m. p. 70—80° C [this m. p. was not depressed when the substance was mixed with synthetic 2- deoxy-D-ribose having m. p. 78—80° C] and $[\alpha]_D^{20}$ —56° (equilibrium; *c*, 0.44 in H_2O) (corrected for 10 % impurity of thymidine with $[\alpha]_D^{20}$ + 62.5°). It was demonstrated by chromatographic methods that the substance contained no other sugars than 2- deoxy-D-ribose, and that the R_F value of the sugar isolated was identical with that of authentic 2- deoxy-D-ribose. The sugar (0.025 g) isolated, was added to a solution of freshly distilled aniline (0.08 ml) in absolute ethanol (5 ml) and the mixture was heated for 2 hours at 100° C. Thereafter the solution was concentrated until crystals appeared which were collected and recrystallised from

ethanol. Shining crystals of aniline deoxy-D-riboside (0.014 g) were obtained, m.p. 163–164° alone or in admixture with an authentic specimen, $[\alpha]_D^{20}$ +19° (*c*, 0.525 in EtOH). Deriaz *et al.*[25] report m.p. 168° and $[\alpha]_D^{20}$ + 19.5° (in EtOH) and Kent, Stacey and Wiggins[26] quote m.p. 165–166° for this compound.

An Alternative Method for the Isolation of Thymidine and 2-Deoxy-D-ribose

A solution (10 ml) of a mixture of nucleosides from an enzymic digest of DNA (prepared as described above) was diluted with water (40 ml) and shaken for 30 minutes with Amberlite resin IR–120 (H) (20 ml of wet regenerated resin). The resin was filtered off and washed with water (100 ml). The filtrate and washings were combined and shaken for a further 30 minutes with Amberlite resin IR–4B (OH), (20 ml of wet regenerated resin). This resin was removed and washed as before. By methods already described the combined filtrate and washings were shown to contain 2- deoxy-D-ribose and thymidine, and these were separated as previously outlined. The yield of thymidine was 0.26 g (*cf.* Todd *et al.*[13] who obtained 4.2 g of thymidine from 36 g of nucleic acid). The impure 2-deoxy-D-ribose was recrystallised from ethyl acetate according to the procedure of Deriaz *et al.*[13] and small nodules of crystals (0.2 g) were obtained, m.p. 78–80° C alone or on admixture with an authentic specimen.

A control experiment was carried out to determine whether any 2- deoxy-D-ribose had been retained by the resins. A column (5 cm × 4 cm diam.) of mixed resins was prepared as already described and a solution of 2- deoxy-D-ribose (0.0027 g) in water (5 ml) was poured on the column and washed through with water. The amount of deoxy-sugar in the effluent was determined by the aforementioned Dische method. When 100 ml of effluent had been collected no more 2-deoxyribose appeared to be eluted from the column, and recovery of the sugar was quantitative.

Isolation of 2-Deoxy-D-ribose from the DNA Isolated from Soft Herring Roes

By application of the above methods to DNA from soft herring roes, 2- deoxy-D-ribose and thymidine were obtained in crystalline form in comparable yields with those reported when using DNA from cod roes. In earlier experiments an alternative procedure had been used, involving acidic hydrolysis with dilute hydrochloric acid of the isolated purine nucleosides from enzymic digests of DNA from herring sperms, followed by removal of the purine bases from solution by absorption on resin columns.

Purified herring roe DNA (4.5 g) in solution in water (450 ml) was digested in the usual manner with deoxyribonuclease and alkaline phosphatase (see digestion of cod roe DNA). The precipitated solid ($MgNH_4PO_4$) was filtered off and found to correspond to 85 % dephosphorylation of the nucleic acid. Barium hydroxide (13 g) in hot water (100 ml) was added and the resultant precipitate removed by centrifuging. The solid residue was washed with hot water (3 × 100 ml) and the combined washings and supernatent liquid were evaporated under diminished pressure to smaller volume (100 ml). Solid carbon dioxide was added to precipitate excess barium ions and the solid formed was removed. After addition of acetic acid to bring the pH value to 6 the solution was evaporated under diminished pressure to small volume (25 ml) and then dried in the frozen state (residue 2.7 g). Chromatographic analysis of the mixture revealed that the following bases were present: Guanine, hypoxanthine (trace), adenine, cytosine, thymine, and uracil (formed by deamination of cytosine). The detection of uracil deoxyriboside in enzymic hydrolysates of DNA from various sources has been reported by other workers also [11,13,27]. The nucleoside mixture (2.7 g) dissolved in ammonium acetate solution [8] (0.1 *M*, 20 ml) was treated with acetic acid to give a solution of pH value 4.9. The solution was filtered onto a column (20 cm × 4 cm diameter) of zeocarb 225 resin, which had been prepared by washing the resin with 4 *N* hydrochloric acid (500 ml) and then distilled water (2.5 litres): thereafter it was treated with 5 % ammonium hydroxide (500 ml) and again with water (2.5 litres) and finally the column was treated with ammonium acetate buffer (0.1 *M*,

pH 3.9) until the eluate showed a pH value of 3.9. The column was developed with ammonium acetate buffer and the eluate (350 ml) contained thymidine and uracil deoxyriboside. The column was then eluted with 5 % ammonium hydroxide (500 ml) which gave a nucleoside mixture of the deoxyribosides of guanine, adenine and cytosine. This eluate was evaporated to dryness under reduced pressure and any ammonium acetate present was removed by heating at 40°/0.01 mm Hg. Finally, a white powdered residue (0.915 g) was obtained. This material was dissolved in water (15 ml) and 0.1 *N* hydrochloric acid (15 ml) was added. The solution was heated at 100° for 10 minutes, then cooled and filtered onto a mixed bed of Amberlite resins IR—120 (H) (100 g) and IR—4B (OH) (100 g). The resin column was eluted with water (500 ml) until the eluate gave a negative diphenylamine test. The eluate was concentrated to dryness by evaporation under reduced pressure followed by drying in the frozen state. The colourless residue (0.2 g) was induced to crystallise and was identified by chromatography as 2-deoxy-D-ribose. On treatment with freshly distilled aniline (31 mg) in absolute ethanol, by heating under reflux for 3.5 hours, the solid residue (40 mg) afforded shining crystals (15 mg) of aniline 2-deoxy-D-riboside, m.p. 163—164° (after recrystallisation twice from ethanol), alone or in admixture with an authentic specimen. (Found: C 63.3; H 7.3 N 6.8. Calc. for $C_{11}H_{15}O_3N$: C 63.1; H 7.2; N 6.7).

RESULTS AND DISCUSSION

In recent years one of us (W.G.O.) with other colleagues have described numerous attempts to synthesise 2-deoxy-D-ribose in good yield, by chemical methods from easily accessible initial materials [28,29]. Although worthwhile improvements have been achieved, highly pure 2-deoxy-D-ribose is still difficult to prepare in large quantities. Our original intention for the work described in this paper, was to try and improve the yield of the deoxy-sugar obtainable from the acidic hydrolysis of purine deoxy-ribonucleosides. We proposed to use ion-exchange resins to separate successively the enzymic digest of DNA into purine and pyrimidine deoxyribonucleosides, and then to free the sugar from the purine bases, in the acidic hydrolysate.

Initial experiments using this two-stage procedure with nucleoside mixtures obtained by treating DNA from soft herring roes with deoxyribonuclease [15,16] and alkaline phosphatase [17] were not encouraging and from hydrolysates of purine nucleosides with dilute hydrochloric acid only relatively poor yields of the crystalline 2-deoxy-D-ribose were obtained. We soon discovered that passage of a mixture of deoxyribonucleosides through a column consisting of a strongly acidic resin and a weakly basic resin, resulted in hydrolysis of the purine nucleosides by the strongly acidic resin, and simultaneously the free purine-bases so formed were retained on the resin column, as also were cytosine deoxyriboside and inorganic impurities. The eluate contained 2-deoxy-D-ribose and thymidine, since the latter is not sufficiently acidic to be retained by the resin. Acetone fractionation of this mixture afforded pure crystalline samples of both substances.

Due to the similarity in density of the resins used in the column of mixed resins it was not possible to separate them by the usual flotation method and so the column could not be regenerated. It was shown, however, that by successively shaking a solution of nucleosides with the acidic resin and then the weakly basic resin, a solution containing only 2-deoxy-D-ribose and thymidine resulted. The amounts of these substances isolated in crystalline form were approximately the same as obtained when a column of mixed resins

was used. The method should be applicable to the preparation of 2-deoxy-D-ribose in relatively large amounts. Although the overall yield of the sugar (0.27 g) from DNA (1.88 g) might not appear too good, it should be borne in mind that at the same time thymidine is obtained in excellent yield, and also that it is not necessary to use highly polymerised samples of DNA, since samples of commercial origin are quite adequate.

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SUMMARY

A method is described for the isolation in crystalline form from enzymic digests of DNA, of both 2-deoxy-D-ribose and thymidine. The method has been applied to DNA prepared from soft herring roes and cod roes.

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An X-Ray Study of the Tantalum-Nitrogen System

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The preparation and properties of a compound TaN were described by Joly [1], who also maintained, that Ta_3N_5 and TaN_2 exist. However, later investigations have shown that nitrides with a higher nitrogen content than that corresponding to the stoichiometric composition 1 : 1 are most unlikely to occur. Van Arkel [2] suggested a hexagonal structure probably of wurzite type for TaN. Achermann, Friedrich, Justi and Kramer [3], however, found that this nitride did not correspond to the composition 1 : 1, but was analogous to and had almost the same unit dimensions as the compound Nb_2N prepared by Brauer [4]. Besides this hexagonal close-packed Ta_2N, a tantalum nitride of complex nature has been described by, among others, van Arkel [2], Becker and Ebert [5], Horn and Ziegler [6]. The latter gave d/n values and relative intensities for the strongest reflections in the diffraction pattern of a nitride with 50.2 atom-% of nitrogen. But they pointed out that "the complexity of the diffraction spectrum is consistent with the results of other investigations, but there is no general agreement in d/n values or relative intensities".

EXPERIMENTAL

As starting materials in the preparation of the nitrides powdered tantalum metal (Fansteel) and tantalum hydride (Metal Hydrides Inc.) were used. Ammonia was used instead of nitrogen for the nitriding, partly because it is considerably more effective for this purpose and partly because of the difficulty of purifying commercial nitrogen. After drying with calcium oxide a constant and relatively fast stream of the gas was passed through a silica tube furnace, in which the specimen was placed in an alumina boat. The tube was packed with asbestos pellets containing zirconium powder in order to prevent possible traces of oxygen and water reacting with the tantalum and thus producing oxides or ternary phases.

The nitriding process was performed at various temperatures between 700° C and 1 100° C and for times varying between an hour and two weeks. In order to homogenise the product and to detect the possible presence of high temperature modifications of the nitrides, the samples were heated in evacuated and sealed silica tubes at temperatures between 600° C and 1 100° C and then quenched in cold water.

The phase analysis and structure determinations were made from powder photographs taken with Cu-$K\alpha$ radiation in Guinier type cameras using bent quartz and aluminium monochromators. For measurements of the high-angle reflections additional photographs were taken with Hägg-Phragmén type focusing cameras.

It was not possible to obtain single crystals of any of the intermediate phases.

The nitrogen content of the specimens was determined by means of the Kjeldahl method.

THE STRUCTURE OF THE PHASES

The interpretation of powder photographs and the nitrogen content determination of samples resulting from nitriding of tantalum metal and tantalum hydride with ammonia indicated four intermediate phases.

β phase: $TaN_{\sim 0.05}$

The tantalum atoms of this phase occupy a cubic lattice which is only slightly different from the body-centred cubic lattice of pure tantalum (α).

For pure tantalum the cube edge was found to be $a = 3.311$ Å ($V = 36.30$ Å^3). The body-centred cubic sublattice of the β phase invariably gives an edge with $a = 3.369$ Å.

Besides the strong lines of the sublattice a series of weak superlattice lines occur. These can be explained by a cubic lattice having an edge three times as long as that of the sublattice, *i. e.*, $a = 10.11$ Å.

The occurence of the superlattice is probably related to the fact that the nitrogen atoms can hardly be accomodated in the largest interstices of the undeformed body-centred cubic lattice. The introduction of a few nitrogen atoms (about 5 atomic %) can only take place after a slight deformation of the metal lattice.

In this connection it may be of interest that, in order to investigate metallic tantalum oxides [7], tantalum hydride was oxidized with steam in an excess of hydrogen at about 600° C. In this way oxygen atoms were introduced into the body-centered tantalum lattice, causing a deformation. The dimensions of the resulting rhombic unit cell were found to vary slightly around the values $a = 3.61$ Å, $b = 3.27$ Å, $c = 3.21$ Å ($V = 37.89$ Å^3), as compared with the cube edge of tantalum $a = 3.311$ Å ($V = 36.30$ Å^3). The upper composition limit of this bluish black oxide corresponds to the formula Ta_4O.

γ phase: $TaN_{\sim 0.40}$—$TaN_{\sim 0.45}$

In agreement with earlier investigations [3], this nitride was found to be of the interstitial type common among the carbides and nitrides of the transition metals with the metal atoms in a close-packed hexagon and the nonmetal atom in one of the two octahedron centres of the unit cell. The unit cell dimensions were found to vary between $a = 3.041$ Å, $c = 4.907$ Å, $c/a = 1.614$ ($V = 39.29$ Å^3) and $a = 3.048$ Å, $c = 4.918$ Å, $c/a = 1.614$ ($V = 39.57$ Å^3). This phase could not be obtained in a pure state, and the nitrogen analyses therefore give only roughly approximate values for the homogeneity limits. The formula may be written as Ta_3N or as Ta_2N, but by analogy with the great number of related carbide and nitride phases the proposed formula may be convenient. The following are mean interatomic distances: Ta — 6Ta 3.02 Å, Ta — 6Ta 3.05 Å, Ta — 3N 2.14 Å, N — 6Ta 2.14 Å.

δ phase: $TaN_{\sim 0.80}$—$TaN_{\sim 0.90}$

The powder photographs of a pure phase with the composition $TaN_{0.83}$ (45.4 atom-% of N) indicated a simple hexagonal cell with the stacking sequence AAA . . . of the metal atoms. The only nitride of this type earlier

known is MoN [8], but recently the following isomorphous phases have been found: $NbN_{0.8}$, WN, $(Ti_{0.7}Co_{0.3})N$, $(Ti_{0.7}Ni_{0.3})N$, $(Mo_{0.8}Co_{0.2})N_{0.9}$ and $(Mo_{0.8}Ni_{0.2})N_{0.9}$ [7]. From space considerations the most likely position of the nitrogen atom must be in one of the centres of the two trigonal metal atom prisms in the unit cell. The space-group is D_{3h}^1—$P\bar{6}m2$ with 1 Ta in 1(a) 000 and ~0.85 N in 1(d)1/3 2/3 1/2 or 1(f) 2/3 1/3 1/2.

The phase analysis indicated a homogeneity range from about $TaN_{0.80}$ to about $TaN_{0.90}$, corresponding to the cell dimensions $a = 2.925$ Å, $c = 2.876$ Å, $c/a = 0.983$ ($V = 21.31$ Å^3) and $a = 2.938$ Å, $c = 2.883$ Å, $c/a = 0.981$ ($V = 21.55$ Å^3) respectively. The mean interatomic distances are: Ta — 2Ta 2.88 Å, Ta — 6Ta 2.93 Å, Ta — ~ 5N 2.22 Å, N — 6Ta 2.22 Å (as compared with the corresponding distances in Ta_2N, 2.14 Å).

The calculated density for the composition $TaN_{0.83}$ is 14.95. The observed value was 14.1.

ε phase: TaN

The end product of the nitriding was found to be homogeneous at the stoichiometric composition TaN. It was easily obtained in a pure state for instance, at a temperature of 1 000° C over three days — if sufficient care was taken to exclude oxygen from the reaction. Traces of oxygen, however, caused the formation of ternary Ta-N-O phases and/or tantalum oxides. The diffraction pattern of the compound TaN published by Horn and Ziegler [6] is not in any way related to the patterns of the δ or ε phases. But it agrees rather well with the observed positions and relative intensities of the diffraction lines of two ternary Ta-N-O phases. The present author has prepared at least four such phases, which will be described elsewhere. All the tantalum nitrides have a greyish black metallic lustre but the colours of these nitride — oxides varies from greyish brown to dark red, and three of them have been found to have metallic properties.

The photographs indicate a hexagonal cell for TaN, related to the unit cell of the δ phase as well as to that of the γ phase. The dimensions were invariably found to be $a = 5.185$ Å, $c = 2.908$ Å, $c/a = 0.561$, ($V = 67.70$ Å^3). The similarity to the lattice of the δ phase is seen if the latter is described with an axial system of type H. The dimensions are then $a = 5.08$ Å, $c = 2.88$ Å, $c/a = 0.57$ ($V = 64.34$ Å^3) and the coordinates of the metal atoms 000; 1/3 2/3 0, 2/3 1/3 0. As reflections with h—$k \neq 3$ n and $l = 2$ n are absent, the three tantalum atoms in TaN have to be placed in the positions 000; 1/3 2/3 1/2; 2/3 1/3 1/2. This atomic grouping can perhaps be regarded as a mixture between the "simple" hexagonal stacking AAA . . . and the hexagonal close-packing ABAB . . ., and seems to be unique. There is only one possibility for the positions of the three nitrogen atoms, namely, at the centres of three deformed metal atom octahedra.

The space-group is thus D_{6h}^1—$P\,6/m\;m\;m$ with 1 Ta in 1(*a*) 000, 2 Ta in 2 (*d*) 1/3 2/3 1/2; 2/3 1/3 1/2, and 3 N in 3(*f*) ½ 0 0; 0 ½ 0; ½ ½ 0. TaN is thus of the B 35-type. Calculated and observed relative intensities are compared in Table 1, and the values of interatomic distances are given in Table 2. The calculated density is 14.34, and the observed value was 13.6.

Table 1. Observed and calculated intensities for TaN (ε).

h k l	Intensities	
	Calc.	Obs.
001	9	10
110	175	175
101	133	115
111	18	15
201	60	50
300	48	50
211	81	75
002	15	15
301	9	10
220	40	50
112	80	75
221	8	10
311	65	75
401	24	25
302	54	50
410	41	35
321	36	35
003	1	0
222	41	35
103	18	15
411	9	10
113	4	0
203	13	15
330	18	15
501	20	15
213	37	35
331	6	0
421	50	50
303	6	0
412	112	115

SUMMARY

Tantalum nitrides have been prepared by ammonia nitriding of tantalum powder and tantalum hydride at 700—1 100° C. The phase analysis was performed by X-ray methods, and the nitrogen content of the samples determined by the Kjeldahl method. Four intermediate nitrides have been found to exist.

Nitrogen is practically insoluble in tantalum.

The β phase has the approximate composition $TaN_{0.05}$, and a body-centered cubic sublattice of tantalum atoms is indicated. The diffraction data can be explained by a cubic superlattice having a cube edge three times as long as that of the sublattice. The exact structure has not been determined.

The γ phase has a composition closely corresponding to the formula Ta_2N, and the metal atoms are arranged in a close-packed hexagon.

The δ phase has a homogeneity range of about three atomic percent of nitrogen within the limits $TaN_{0.80}$—$TaN_{0.90}$. The metal atoms form a simple hexagonal lattice.

Table 2. Interatomic distances in TaN (ε).

Atom	Surrounding atoms	Interatomic distance, Å
Ta in 1(*a*)	2 Ta in 1(*a*)	2.908
	12 Ta in 2(*d*)	3.402
	6 N in 3(*f*)	2.593
Ta in 2(*d*)	2 Ta in 2(*d*)	2.908
	3 Ta in 2(*d*)	2.994
	6 Ta in 1(*a*)	3.402
	6 N in 3(*f*)	2.204
N in 3(*f*)	4 Ta in 2(*d*)	2.204
	2 Ta in 1(*a*)	2.593

The ε phase is homogeneous at the stoichiometric composition TaN. The hexagonal unit cell contains three formula units TaN in the space-group D_{6h}^1—$P\,6/m\;m\;m$ with 1 Ta in 1(*a*) 000, 2 Ta in 2(*d*) 1/3 2/3 1/2, 2/3 1/3 1/2 and 3 N in 3(*f*) $\frac{1}{2}$ 0 0; 0 $\frac{1}{2}$ 0; $\frac{1}{2}$ $\frac{1}{2}$ 0 (B 35-type).

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Contributions to the Knowledge of the Molybdenum-Nitrogen and the Tungsten-Nitrogen Systems

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Schematic phase diagrams for the Mo—N and the W—N systems have been constructed by Hägg [1] on the basis of the X-ray diffraction study of his preparations. He nitrided fine and very pure metal powders with ammonia in a porcelain-tube furnace for up to two days. The reaction temperature was varied between 400° C and 725° C for the molybdenum nitrides and between 700° C and 800° C for the tungsten nitrides.

The following phases are recorded by Hägg [1]:

1. *The Mo—N system.*
   α phase (Mo). The solubility of nitrogen in molybdenum is very small.
   β phase. Stable only above 600° and containing about 28 atom-% of N. The metal atoms form a face-centered tetragonal lattice.
   γ phase. This phase has a narrow homogeneity range in the vicinity of 33 atom-% of N and hence corresponds to the formula Mo_2N. The metal atoms form a face-centered cubic lattice.
   δ phase. Exists at about 50 atom-% of N and hence corresponds to the formula MoN. The metal atoms form a simple hexagonal lattice but weak extra lines indicate the existence of a superlattice.

2. *The W—N system.*
   α phase (W). Nitrogen is practically insoluble in tungsten.
   β phase. Isomorphous with Mo_2N (γ phase).
   Kiessling and Liu [2] found a second W_2N-modification — a γ phase — which is formed by nitriding at 825°—875° C. It is cubic, probably with a distorted face-centered lattice.

The present author has confirmed the results of Hägg in all respects but could not verify the existence of the γ tungsten nitride reported by Kiessling and Liu. Here, therefore, it is only necessary to report some new facts about the two nitride systems.

The new information was obtained from preparations with a maximum nitrogen content. These were prepared by heating two porcelain boats con-

taining molybdenum (fine powder; Söderfors 99.9 %) and tungsten (fine powder; Lindberg, Stockholm 99.6 %) respectively in a constant stream of dry ammonia up to 800 °C for a few hours. The temperature was then lowered at a constant rate down to 300° C over a period of four weeks. The nitrogen content was determined by the Kjeldahl method, and was found to be 49.6 atom-% of N in the molybdenum sample and 41.7 atom-% of N in the tungsten sample. For the X-ray analysis a Guinier-type camera constructed at this Institute was used, with a ground and bent quartz crystal monochromator.

MoN (δ phase)

The diffraction pattern of this molybdenum nitride contained strong lines corresponding to a simple hexagonal sublattice, and a series of comparatively weak superlattice reflections. The latter reflections were generally stronger for large deviation angles than for small angles. An ordered distribution of the nitrogen atoms in the interstices of a "perfect" sublattice should give extra reflections hardly visible in the powder photograph, so in this case it seems necessary to assume a displacement of the metal atoms from the normal positions of the sublattice.

If both the a axis and the c axis of the sublattice are doubled, all the reflections can be interpreted. The unit cell dimensions will then be $a = 5.725$ Å, $c = 5.608$ Å; $c/a = 0.980$ ($V = 159.2$ Å^3). The Kjeldahl determination of nitrogen (49.6 atom-%) makes the ideal formula MoN very probable. The unit cell then contains eight formula units. The calculated density is 9.18, and the observed value was 8.60.

Only hkl reflections with $l \neq 2n$ were found to be absent in the diffraction pattern of the δ phase. This, together with space considerations, makes the space-groups D_{3h}^4, C_{6v}^4, D_6^6, and D_{6h}^4 the most probable ones. As the displacement of the metal atoms from the positions of the hexagonal sublattice is obviously very small, the only possible positions of the eight molybdenum atoms are:

in D_{3h}^4: $2(b)$ $00\frac{1}{4} + 6(h)$ x y $\frac{1}{4}$;
in C_{6v}^4: $2(a)$ $00z + 6(c)$ x $2z$ z;
in D_6^6 : $2(b)$ $00\frac{1}{4} + 6(h)$ x $2x$ $\frac{1}{4}$;
in D_{6h}^4: $2(b)$ $00\frac{1}{4} + 6(h)$ x $2x$ $\frac{1}{4}$;

The space-group D_{6h}^4 is chosen as it possesses the maximum symmetry common to the four space-groups.

The parameter x must then have a value close to $\frac{1}{2}$. It was therefore systematically varied between the values 0.480 and 0.500 in steps of 0.002. It is obvious from Table 1 that the agreement between calculated and observed intensities for the reflections are good if the value $x = 0.489$ is chosen.

Intermetallic distances are listed in Table 2.

The positions of the nitrogen atoms in molybdenum nitrides cannot be determined from X-ray data. However, space considerations suggest, that the nonmetal atoms in the δ phase are situated at the centres of trigonal prisms of metal atoms, as previously indicated by Hägg [1].

Table 1. Observed and calculated intensities for MoN.

h k l	Intensities Calculated	Observed	h k l	Intensities Calculated	Observed
100	0	0	224	32	25
101	4	5	500	0	0
110	0	0	314	0	0
002	65	75	501	4	5
200	121	125	323	5	5
102	0	0	215	1	2
201	2	2	330	0	0
112	0	0	420	30	25
210	0	0	413	3	2
202	125	125	502	0	0
211	4	5	006	6	5
103	1	2	404	32	25
300	0	0	305	2	2
301	4	5	421	0	0
212	0	0	106	0	0
203	1	2	332	0	0
220	35	45	510	0	0
302	0	0	422	56	45
004	13	15	116	0	0
310	0	0	324	0	0
104	0	0	511	1	0
311	1	2	503	4	5
213	3	5	206	34	25
222	65	75	315	0	0
114	0	0	414	0	0
400	26	25	512	1	0
303	3	2	423	0	0
312	0	0	216	0	0
204	54	45	405	0	0
401	0	0	600	13	15
320	0	0	601	0	0
402	41	45	430	2	2
214	0	0	306	0	0
321	7	5	504	0	0
313	0	0	431	9	10
105	0	0	513	1	0
410	0	0	325	7	10
304	0	0	520	4	5
411	3	5	602	43	45
322	0	0	107	0	0
403	0	0	334	1	0
205	0	0	521	17	25
412	0	0			

WN (δ phase)

The X-ray photograph of the tungsten sample containing 41.6 atom-% of N besides W_2N(β phase) indicated the presence of a phase not previously known (δ phase). The metal atoms are arranged in a simple hexagonal lattice with the lattice constants $a = 2.893$ Å, $c = 2.826$ Å; $c/a = 0.977$ ($V = 20.48$ Å^3). The composition of this nitride is not known, because it was not possible to prepare it in a pure state; the nitrogen content may be somewhat lower than

Table 2. Intermetallic distances in MoN.

Molybdenum atom in	Surrounding atom	Interatomic distance, Å	
2(*b*)	2 Mo in 2(*b*)	2.804	2.845
	6 Mo in 6(*h*)	2.865	
6(*h*)	2 Mo in 2(*b*)	2.865	2.851
	2 Mo in 6(*h*)	2.674	
	2 Mo in 6(*h*)	2.813	
	2 Mo in 6(*h*)	3.052	

Table 3. Interatomic distances in WN.

Atom	Surrounding atoms	Interatomic distance, Å
W	2 W	2.826
	6 W	2.893
	6 N	2.188
N	6 W	2.188

50 atom-%. If the nitrogen atom is situated at the centre of a trigonal prism of tungsten atoms, the space-group will be D_{3h}^1—$P\bar{6}m2$ with 1 W in 1(*a*) 000 and 1 N in 1 (*d*) 1/3 2/3 1/2 or (*f*) 2/3 1/3 1/2.

The decomposition of WN in vacuum at 600° C according to the reaction $2\,WN \rightarrow W_2N + \frac{1}{2}\,N_2$ was found to occur at a much faster rate than the corresponding decomposition of MoN (δ). This indicates a lower stability for the tungsten nitrides than for the molybdenum nitrides.

Interatomic distances for WN (δ) are listed in Table 3.

SUMMARY

The previously known simple hexagonal lattice of MoN (δ) exhibits a hexagonal superstructure formed by doubling the axes of the substructure. The new unit cell contains eight units MoN. The space-group is D_{6h}^4—$P6_3/m\,m\,c$ and the atomic coordinates are 2 Mo in 2(*b*) $00\frac{1}{4}$ and 6 Mo in 6(*h*) x, $2x$, $\frac{1}{4}$ with $x = 0.489$.

In the tungsten-nitrogen system a new phase (δ) has been found which has a composition close to the formula WN. It is isomorphous with the tungsten carbide WC. It is more unstable than MoN at high temperatures.

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Some Features of the Nb-N and Nb-N-O Systems

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I. THE Nb-N SYSTEM

In 1952 Brauer and Jander [1] published a paper on the phases in the niobium-nitrogen system. In order to facilitate comparison of their results and those attained in the present investigation, the following data are recorded from their paper:

Nb dissolves up to about 2 at.-% of N, the lattice constant increasing from $a = 3.301$ Å to $a = 3.303$ Å.

The $NbN_{0.40-0.50}$ phase is of the common "interstitial" structure type with the metal atoms hexagonally close-packed. The unit cell dimensions vary from $a = 3.056$ Å, $c = 4.956$ Å, $c/a = 1.622$ ($NbN_{0.40}$) to $a = 3.048$ Å, $c = 4.995$ Å, $c/a = 1.639$ ($NbN_{0.50}$).

The $NbN_{0.75-0.79}$ phase has a tetragonally deformed NaCl structure. The a-axis increases from 4.385 Å to 4.387 Å, and the c-axis from 4.312 Å to 4.330 Å ($c/a = 0.983$—0.987).

The $NbN_{\sim 0.87-\sim 0.94}$ phase — NbN III — is of the NaCl type with the cubic axial length $a = 4.39$ Å.

The metal atoms in the $NbN_{\sim 0.95}$ phase — NbN II — form a hexagonal close-packed unit cell with the dimensions $a = 2.94$ Å, $c = 5.46$ Å, $c/a = 1.87$.

The phase most rich in nitrogen has the composition NbN — NbN I — and contains four formula units NbN. The lattice constant of the hexagonal unit cell are: $a = 2.956$ Å, $c = 11.275$ Å, $c/a = 3.815$. The metal positions are given as: 0, 0, 0; 0, 0, ¼; 2/3, 1/3, 1/2; 2/3, 1/3, 3/4.

Brauer and Jander point out that the phases mentioned above are pure nitrides but report many specimens containing a small amount of oxygen. For instance, two samples with the composition $NbN_{0.76}O_{0.09}$ and $NbN_{0.88}O_{0.10}$ have the NaCl structure, and a sample with the composition $NbN_{0.75}O_{0.03}$ has the tetragonally deformed version of this structure.

Experimental

The nitrides were prepared by nitriding niobium metal powder (Fansteel) or niobium hydride (Metal Hydrides Inc.) with dry oxygen-free ammonia at various temperatures between 700° C and 1 100° C and for periods varying between one hour and two weeks.

The phase analysis and structure determinations were made from powder photographs taken with Cu*Ka* radiation in cameras of Guinier type using bent quartz and aluminium monochromators. For measurements of the high-angle reflections additional photographs were taken with focusing cameras of Hägg-Phragmén type.

The nitrogen content of the samples was determined by means of the Kjeldahl method.

α phase: Nb(N)

The solubility of nitrogen in the niobium lattice is very small, and could not be determined with any accuracy. The increase in the lattice constant agrees with the results given by Brauer and Jander.

β phase: $NbN_{0.40-0.50}(Nb_2N)$

The results obtained in the present investigation confirm the data given by Brauer and Jander.

γ phase: $NbN_{\sim 0.80-\sim 0.90}$

Powder photographs of nitrides with the composition varying between $NbN_{0.50}$ and $NbN_{0.80}$ showed besides the pattern of the β phase, diffraction lines of a new phase (γ). A sample with the composition $NbN_{0.86}$ contained this phase in a pure state. The γ phase was found to be isomorphous with the δ phase in the Ta-N system [2], *i.e.* of the WC type [3]. The unit cell dimensions vary between $a = 2.950$ Å, $c = 2.772$ Å, $c/a = 0.940$ ($V = 20.89$ Å^3) and $a = 2.958$ Å, $c = 2.779$ Å, $c/a = 0.939$ ($V = 21.06$ Å^3). The approximate phase limits were found to be $NbN_{0.80}$ and $NbN_{0.90}$. From space considerations the most likely position of the nitrogen atom must be at one of the centres of the two trigonal metal atom prisms in the unit cell. The space-group is D^1_{3h}—$P\bar{6}m2$ with 1 Nb in 1(*a*) 0, 0, 0 and 1 N in 1(*d*) 1/3, 2/3, 1/2 or 1(*f*) 2/3, 1/3, 1/2.

In the region $NbN_{0.75-0.94}$ Brauer and Jander report the existence of two phases, *viz.* $NbN_{0.75-0.79}$ with a tetragonally deformed NaCl structure and $NbN_{\sim 0.87-\sim 0.94}$ with NaCl structure. No traces of these phases were visible in the photographs. Oxidation of nitrides, however, caused the formation of these compounds, so in all probability they belong to the ternary Nb—N—O system (see below).

δ phase: $NbN_{\sim 0.95}$

This phase is identical with the compound NbN II reported by Brauer and Jander.

The unit cell dimensions were found to be: $a = 2.968$ Å, $c = 5.535$ Å, $c/a = 1.865$ ($V = 42.22$ Å^3). The nitrogen atoms are probably located at the centres of metal atom octahedra. The space-group is thus D^4_{6h}—$P6_3/mmc$, with the atoms in the positions: 2 Nb in 2(*c*) 1/3, 2/3, 1/4; 2/3, 1/3, 3/4 and 2 N in 2(*a*) 0, 0, 0; 0, 0, 1/2, *i.e.* the "anti-NiAs" configuration.

ε phase: $NbN_{1.00}$

The present investigation confirmed the metal atom positions reported by Brauer and Jander.

The hexagonal lattice constants were invariably found to be $a = 2.952$ Å, $c = 11.25$ Å, $c/a = 3.811$ ($V = 85.09$ Å^3). If the nitrogen atoms are assumed

to be located at the centres of metal atom octahedra giving the largest N-N distances, the space-group is D_{6h}^4—$P6_3/mmc$, with the atomic grouping: 4 Nb in 4(*f*) 1/3, 2/3, *z*; 2/3, 1/3, $\bar{z}$; 2/3, 1/3, $\frac{1}{2}+z$; 1/3, 2/3, $\frac{1}{2}-z$ with $z = 1/8$, 2 N in 2(*a*) 0, 0, 0; 0, 0, 1/2, and 2 N in 2(*d*) 1/3, 2/3, 3/4; 2/3, 1/3, 1/4. Three analogous compounds have been prepared earlier, *viz.* MoC(γ') [4], TiP [2], and β-ZrP [2]. It should be particularly noted that the present author was able to determine the above positions for the nonmetal atoms in the two phosphide phases.

If the projections of the positions of the atoms on the basal planes of the trigonal or hexagonal γ, δ, and ε phases are designated by A, B, and C, the niobium lattice in the three compounds can be symbolized by the sequences AA . . ., ABAB . . ., and AABBAABB . . . respectively. If the nitrogen atoms also are taken into consideration the resulting stacking sequences are $A_{Nb}B_N$. . ., $A_{Nb}C_NB_{Nb}C_N$ . . ., and $A_{Nb}B_NA_{Nb}C_NB_{Nb}A_NB_{Nb}C_N$ . . . It is remarkable that no contraction takes place along the *c*-axis when the contact AA in the γ-phase changes to the contact AB in the δ and ε phases ($c_\gamma \sim c_\delta/2 \sim c_\varepsilon/4$). The length of the *a*-axis also varies little for the three nitrides — between 2.950 Å and 2.968 Å. The value of the axial ratio for the δ phase is $c/a = 1.87$, while the corresponding value for the β phase is $c/a = 1.63$ ($= \sqrt{8/3}$). The last value is characteristic for a hexagonal close-packed arrangement of metal atoms. The large deviation must be due to the presence of approximately two nitrogen atoms in the unit cell of the δ phase instead of one in the β phase. The N-N distances always exceed a value of 2.6 Å in metallic nitrides, which can be explained by a displacement of electrons from the transition metal to the nonmetal atom, so that the metal-nitrogen bonds have a partial ionic character.

The mean interatomic distances are listed in Table 1.

Table 1. Mean interatomic distances in niobium nitrides.

Phase	Atom	Surrounding atoms	Interatomic distance, Å	
β	Nb	12 Nb	3.05	
		3 N	2.16	
	N	6 Nb	2.16	
γ	Nb	2 Nb	2.78	2.87
		6 Nb	2.95	
		6 N	2.20	
	N	6 Nb	2.20	
δ	Nb	6 Nb	2.97	3.11
		6 Nb	3.26	
		6 N	2.20	
	N	6 Nb	2.20	
ε	Nb	1 Nb	2.81	3.04
		6 Nb	2.95	
		3 Nb	3.29	
		6 N	2.21	
	N	6 Nb	2.21	

The nitrides of the group Va metals

The Me-N systems with Me = a transition element in group Va (V, Nb, Ta) exhibit great similarities for a low nitrogen content, but discrepancies for a high one. The solubility of nitrogen in the metal lattices is of the order 2 at.-%. The "interstitial" structure type Me_2N with the metal atoms hexagonally close-packed and the nitrogen atom in one of the two metal atom octahedra of the unit cell (L'3 type) is common for the three Me-N systems *. This phase has a comparatively broad homogeneity range, with the upper phase limit closely corresponding to the composition Me_2N. There exist with the greatest probability a continuous solid solution series V_2N—Nb_2N—Ta_2N. — The $NbN_{0.8-0.9}$ and $TaN_{0.8-0.9}$ phases are isomorphous, while no analogous V-N phase exists. — The atomic grouping for the ε phase in the NbN system can be regarded as a transitionary state between the arrangement of atoms in the γ and δ phases. — VN and NbN are of the NaCl and γ'-MoC [4] types respectively. The structure of TaN [2] is of the B 35-type. The hexagonal unit cell contains three formula units TaN in the space-group D^1_h—$P6/mmm$ with 1 Ta in 1(*a*) 0, 0, 0, 2 Ta in 2(*d*) 1/3, 2/3, 1/2; 2/3, 1/3, 1/2 and 3 N in 3(*f*) 1/2, 0, 0; 0,1/2, 0; 1/2, 1/2, 0.

II. THE Nb—N—O SYSTEM

Ammonia nitriding of niobium oxides or ammonium niobate lead to the formation of a phase of NaCl type. The nitrogen content was determined by the Kjeldahl method, the niobium content by weighing the Nb_2O_5 formed after oxidation by oxygen, and the oxygen content as the weight remainder. The composition of this phase was found to vary slightly about the approximate composition $NbN_{0.9}O_{0.1}$. The length of the cube edge varies between $a = 4.373$ Å ($V = 83.62$ Å^3) and $a = 4.390$ Å ($V = 84.60$ Å^3). The same compound was

Table 2. Mean interatomic distances in metallic niobium oxide-nitrides.

Phase	Atom	Surrounding atoms	Interatomic distance, Å	
$NbN_{\sim 0.9}O_{\sim 0.1}$	Nb	12 Nb	3.10	
		6 (N, O)	2.19	
	(N, O)	6 Nb	2.19	
$NbN_{\sim 0.6}O_{\sim 0.3}$	Nb	6 Nb	3.08	3.09
		6 Nb	3.10	
		3 (N, O)	2.16	2.17
		3 (N, O)	2.19	
	(N, O)	3 Nb	2.16	2.17
		3 Nb	2.19	

* A report on the V—N system was published by Hahn [5] in 1949. An X-ray investigation of the same system carried out by the present author confirmed in all respects the data given by Hahn. Recently, Rostoker and Yamamoto [6] reported the existence of a nitride isomorphous with the tetragonal $VO_{\sim 0.15-\sim 0.25}$ phase [7]. The preparation methods used by these authors indicate that they obtained oxides instead of nitrides, however.

also obtained from the oxidation of NbN (δ or ε) with steam in the presence of hydrogen in great excess at a temperature of about 700° C. This oxidation process also gave rise to a phase with a tetragonally deformed NaCl structure. The same compound was obtained if $Nb_{0.8-0.9}$ (γ) was used as starting material. The composition of this oxide-nitride was found to vary between the approximate limits $NbN_{0.6}O_{0.2}$ and $NbN_{0.6}O_{0.3}$, with the unit cell dimensions $a = 4.370$ Å, $c = 4.295$ Å, $c/a = 0.983$ ($V = 82.03$ Å^3) and $a = 4.386$ Å, $c = 4.325$ Å, $c/a = 0.986$ ($V = 83.21$ Å^3) respectively.

No other ternary metallic phases seem to exist in the Nb-N-O system, as oxidation of nitrides with steam for prolonged periods only lead to the formation of already known niobium oxides [8].

Interatomic distances are listed in Table 2.

As was mentioned earlier, it was not possible to prepare the two phases with NaCl and tetragonally deformed NaCl structure in an oxygen-free state.

SUMMARY

The Nb-N system and the part of the Nb-N-O system with the (N,O)/Nb ratio ≤ 1 have been examined by X-ray methods. The following phases have been found to exist:

I. *The Nb-N system*

α phase. The solubility of nitrogen in the niobium metal lattice is probably of the order 2 at.-%.

β phase. Homogeneity range $NbN_{0.40-0.50}$. Metal atoms hexagonally close-packed.

γ phase. Homogeneity range $NbN_{0.80-0.90}$. WC type.

δ phase. $NbN_{\sim 0.95}$. Metal atoms hexagonally closepacked but with $c/a = 1.87$.

ε phase. NbN of γ'-MoC type.

II. *The Nb-N-O system*

Two metallic ternary phases have been observed: $NbN_{\sim 0.9}O_{\sim 0.1}$ with NaCl structure and $NbN_{\sim 0.6}O_{\sim 0.3}$ with a tetragonally deformed NaCl structure.

The Me-N systems with Me = a group Va metal have been compared.

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An X-Ray Investigation on Ternary Phases in the Ta-Me-N Systems (Me = Ti, Cr, Mn, Fe, Co, Ni)

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During the last three decades the nitrides of the metals Ti, V, Cr, Mn, Fe, Co, Ni have been investigated by X-ray methods, and the binary metal-nitrogen systems are well established. In most of these compounds the metal atoms are situated in a close-packed arrangement, and the nitrogen atoms are interstitially placed at the centres of octahedra of metal atoms. The general formula of the so-called normal interstitial phases are Me_2N and MeN, the former having a hexagonal close-packing (ABAB . . . .) of the metal atoms, and the latter a cubic close-packing (ABCABC . . .). The structures and approximate compositions of the intermediate phases in the Ta-N system have recently been determined by the present author [1]. $TaN_{0.40-0.45}$ (γ) is of the common Me_2N type. The metal atoms form a simple hexagonal lattice in the $TaN_{0.80-0.90}$ (δ) phase. The hexagonal unit cell of the TaN (ε) phase contains three formula units TaN in the space-group D_{6h}^1—$P\ 6/m\ m\ m$ with 1Ta in 1(a) 000, 2Ta in 2(d) 1/3 2/3 1/2; 2/3 1/3 1/2, and 3 N in 3(f) 1/2 0 0; 0 1/2 0; 1/2 1/2 0. This atomic grouping may in some respects be regarded as a mixture of the simple hexagonal stacking AA . . . and the hexagonal close-packing ABAB . . .

EXPERIMENTAL

Transition metals of fairly high purity (about 99.5 %) were used as starting materials for the preparation of intermetallic compounds of different compositions (Table 1). The finely powdered metals were carefully mixed, pressed into pastilles, and sintered (or melted) in zirconia crucibles in a high frequency vacuum induction furnace [2] at a temperature of about 1 500° C. The alloys were then powdered in a steel mortar and placed in alumina boats in a porcelain-tube furnace. The nitriding was performed with dry ammonia at temperatures between 650° C and 950° C, the times varying between two hours and two weeks. The specimens were then cooled in ammonia to room temperature.

The phase analysis and structure determination were made from powder photographs taken with Cu-$K\alpha$ and Cr-$K\alpha$ radiation in Guinier type cameras using bent quartz and aluminium monochromators. For measurements of the high-angle reflections additional photographs were taken with Hägg-Phragmén type focussing cameras.

The nitrogen content of the samples was determined by means of the Kjeldahl method.

It was not possible to obtain single crystals of any of the intermediate phases.

The sintering of different nitrides in a high frequency vacuum induction furnace at about 1 350° C was not successful because of the instability of many of the nitride phases.

RESULTS

The phases observed in samples obtained by ammonia nitriding of binary alloys, together with a few ternary, at a temperature of 900° C for two days are listed in Table 1.

Ternary phases were found to exist in Ta-Me-N systems, where Me = Cr, Mn, Fe, Co, and Ni but not with Me = Ti and V. No quarternary phases were observed.

In all specimens, the dimensions of the TaN (ε) phase, where it existed, were invariably found to be $a = 5.185$ Å, $c = 2.908$ Å, $c/a = 0.561$ ($V = 67.70$ Å^3), indicating a very small solubility of other nitrides in this phase.

Table 1. Phases obtained after ammonia nitriding of binary and ternary Ta, Me alloys (Me = Ti, V, Cr, Mn, Fe, Co, Ni) at a temperature of 900° C for 50 hours.

Alloy	Formed phases
$Ta_{0.50}Ti_{0.50}$	Ti(Ta)N + TaN
$Ta_{0.25}Ti_{0.75}$	Ti(Ta)N + TaN
$Ta_{0.10}Ti_{0.90}$	Ti(Ta)N + traces of TaN
$Ta_{0.50}V_{0.50}$	V(Ta)N + TaN
$Ta_{0.25}V_{0.75}$	V(Ta)N
$Ta_{0.75}Cr_{0.25}$	X + TaN
$Ta_{0.50}Cr_{0.50}$	X + traces of unknown phase
$Ta_{0.25}Cr_{0.75}$	Cr(Ta)N + traces of the X-phase
$Ta_{0.90}Mn_{0.10}$	$Ta(Mn)N_{0.8}$ + traces of TaN
$Ta_{0.75}Mn_{0.25}$	Ta_3MnN_4
$Ta_{0.50}Mn_{0.50}$	X + Ta_3MnN_4
$Ta_{0.25}Mn_{0.75}$	X + Mn-nitrides
$Ta_{0.80}Fe_{0.20}$	Ta_2FeN_3 + TaN
$Ta_{0.67}Fe_{0.33}$	Ta_2FeN_3
$Ta_{0.50}Fe_{0.50}$	Ta_2FeN_3 + Fe-nitrides
$Ta_{0.80}Co_{0.20}$	Ta_2CoN_3 + TaN
$Ta_{0.67}Co_{0.33}$	Ta_2CoN_3
$Ta_{0.50}Co_{0.50}$	Ta_2CoN_3 + α-Co
$Ta_{0.80}Ni_{0.20}$	Ta_2NiN_3 + TaN
$Ta_{0.67}Ni_{0.33}$	Ta_2NiN_3
$Ta_{0.50}Ni_{0.50}$	Ta_2NiN_3 + Ni
($Ta_{0.50}Cu_{0.50}$)	(TaN + Cu)
$Ta_{0.33}Ti_{0.33}Mn_{0.33}$	Ti(Ta)N + Ta_3MnN_4 + x + η-MnN
$Ta_{0.33}Ti_{0.33}Co_{0.33}$	Ti(Ta)N + $(Ti, Ta)_2N$ + α-Co
$Ta_{0.33}V_{0.33}Cr_{0.33}$	(V, Cr, Ta)N + x + y *
$Ta_{0.33}V_{0.33}Co_{0.33}$	V(Ta)N + TaN + α-Co
$Ta_{0.33}V_{0.33}Ni_{0.33}$	V(Ta)N + TaN + Ni
$Ta_{0.33}Cr_{0.33}Co_{0.33}$	Cr(Ta)N + x + α-Co
$Ta_{0.33}Mn_{0.33}Co_{0.33}$	Ta_3MnN_4 + Mn-nitrides + α-Co
$Ta_{0.33}Fe_{0.33}Co_{0.33}$	$Ta_2(Fe, Co)N_3$ + Fe-nitrides + α-Co
$Ta_{0.33}Co_{0.33}Ni_{0.33}$	$Ta_2(Co, Ni)N_3$ + α-Co + Ni
$Ta_{0.67}Fe_{0.11}Co_{0.11}Ni_{0.11}$	$Ta_2(Fe, Co, Ni)N_3$

* The phase designated by y was found to have a tetragonal unit cell with the dimensions $a = 4.244$ Å, $c = 4.120$ Å, $c/a = 0.971$. As the corresponding alloy was contaminated by a small amount of oxygen this phase is most likely an oxide or an oxide-nitride.

The Ta-Ti-N system

No ternary phase seems to exist. The solubility of Ta in TiN is probably about 10 atom %. For pure TiN (50 atom % of N) the cube edge was found to be 4.238 Å, and for a nitride of the approximate composition $(Ti_{0.90}Ta_{0.10})$ N the cube edge was 4.247 Å. This compound was not pure, however, but contained traces of TaN.

The Ta-V-N system

Only phases which were solid solutions in binary phases were found. The VN phase dissolves approximately 25 atom % of Ta, the cube edge increasing from $a = 4.169$ Å ($VN_{1.00}$) to $a = 4.291$ Å.

The Ta-Cr-N system

One ternary phase was found to exist (designated by X in Table 1). As it was not possible to obtain it in a pure state the composition is unknown. The pattern indicates a complicated structure. The lattice constant of the CrN phase increases from $a = 4.149$ Å ($CrN_{1.00}$) to $a = 4.293$ Å, the latter value being obtained at the point of the maximum solubility of Ta in the cubic close-packed lattice (about 25 atom %).

The Ta-Mn-N system

Two ternary phases were found to exist, *viz.* one phase isomorphous with the corresponding Ta-Cr-N phase (X in Table 1) and one phase with a composition close to the formula Ta_3MnN_4. The structure of the latter has been determined. No solubility of Ta in $MnN_{0.61-0.63}$ (η phase) or of Mn in ε-TaN could be detected. But Mn atoms can replace about 10 % of the Ta atoms in δ-TaN, causing an expansion along the a axis of the simple hexagonal unit cell, and a contraction along the c axis. For $TaN_{0.80-0.90}$ (δ phase) the following dimensions were found: $a = 2.925$—2.938 Å, $c = 2.876$—2.883 Å, $c/a = 0.983$—0.981 ($V = 21.31$—21.55 Å^3). The dimensions of the δ phase in a sample of the composition $(Ta_{0.90}Mn_{0.80})N_{0.84}$ were found to be $a = 3.057$ Å, $c = 2.819$ Å, $c/a = 0.922$ ($V = 22.81$ Å^3). This specimen contained traces of TaN as an impurity. It is remarkable that the substitution of Mn atoms for Ta atoms causes an increase of the unit volume in spite of the fact that the atomic radius for Mn is smaller than that for Ta.

The structure of the Ta_3MnN_4 phase

Powder photographs of the Ta_3MnN_4 phase showed lines with $\sin^2\Theta$ values closely proportional to rather small integers. In spite of this, a cubic symmetry was improbable because of a great number of absent reflections. If small amounts of titanium were introduced into the lattice, the phase $(Ta_{0.9}Ti_{0.1})_3MnN_4$ was found to be homogeneous and slightly deformed, compared with the titanium free phase. This deformation was large enough to make a unit cell determination possible from the $\sin^2\Theta$ values of the diffraction lines. The phase was found to have hexagonal symmetry with the following dimensions:

$(Ta_{0.9}Ti_{0.1})_3MnN_4$	$a = 3.027$ Å,	$c = 10.57$ Å,	$c/a = 3.492$	($V = 83.87$ Å^3)
Ta_3MnN_4	$a = 3.023$ Å,	$c = 10.49$ Å,	$c/a = 3.470$	($V = 82.99$ Å^3).

The fact that c/a is very close to the value $2\sqrt{3} = 3.464$ for the phase containing only the metals Ta and Mn explains the above-mentioned difficulty in interpreting the powder photographs of this phase.

The nitrogen content is probably slightly lower than is indicated by the formula Ta_3MnN_4. Analyses corresponded to the composition $Ta_3MnN_{3.85}$. This formula is not quite certain, however, as the samples may have contained other phases not discernible in the powder photographs. The density calculated for a unit cell content of three Ta atoms and one Mn atom is 13.04. The observed value was 12.4.

Reflections with $h\text{-}k = 3n$ and $l = 2n$ are absent in the diffraction pattern. This absence is characteristic of atomic positions in the space-groups D_{6h}^4—$P\,6_3/mmc$, C_{6v}^4—$P\,6_3\,mc$, D_{3h}^4—$P\bar{6}\,2c$, C_{6h}^2—$P\,6_3/m$, C_{6d}^6—$P\,6_3$, D_{3d}^2—$P\,\bar{3}\,1c$ and C_{3v}^4—$P\,3\,1c$. From space considerations, the metal atoms must be in a close-packed arrangement. The only possible grouping in this case is a packing sequence designated by ABACABAC . . ., *i. e.* the mixture of the hexagonal close-packing ABAB . . . and the cubic close-packing ABCABC . . ., which is characteristic of the position of the anions in topaz. If the metal atoms are assumed to be distributed at random, the calculated and observed intensity values differ to some extent, especially for comparatively small diffraction angles. For this reason the manganese atoms have been assumed to have ordered positions. The agreement between calculated and observed intensities is good for an ordered distribution with the following atomic positions in the

Table 2. Calculated and observed intensities for Ta_3MnN_4.

$h\,k\,l$	$I_{calc.}$	$I_{obs.}$	$h\,k\,l$	$I_{calc.}$	$I_{obs.}$
0 0 2	20	25	0 0 10	1	0
0 0 4	68	70	2 0 6	28	25
1 0 0	1		1 1 8	58	50
1 0 1	102	100	2 1 0	0	0
1 0 2	144	150	2 0 7	17	50
1 0 3	64	70	2 1 1	33	
1 0 4	1	0	1 0 10	25	25
0 0 6	1	0	2 1 2	50	50
1 0 5	39	35	2 1 3	30	25
1 1 0	62	70	2 0 8	0	0
1 0 6	53	70	2 1 4	1	0
1 1 2	8		1 0 11	14	15
0 0 8	18	15	2 1 5	27	25
1 1 4	106	100	0 0 12	7	10
2 0 0	0		3 0 0	22	25
1 0 7	31	70	2 0 9	13	15
2 0 1	31		1 1 10	3	0
2 0 2	42	35	2 1 6	0	0
2 0 3	26	25	3 0 2	3	0
1 0 8	0	0	1 0 12	0	0
2 0 4	0	0	3 0 4	53	50
1 1 6	4	0	2 1 7	32	25
2 0 5	20	15	2 0 10	28	25
1 0 9	18	15	2 1 8	0	0

Table 3. Interatomic distances in Ta_3MnN_4.

Atom	Surrounding atoms	Interatomic distance, Å
(Ta, Mn) in 2(*a*)	6 (Ta, Mn)	3.02
	6 Ta	3.15
	6 N	2.18
Ta in 2(*d*)	6 Ta	3.02
	6 (Ta, Mn)	3.15
	6 N	2.18
N in 4(*f*)	3 (Ta, Mn)	2.18
	3 Ta	2.18
	1 N	2.62

space-group D_{6h}^4—$P\ 6_3/m\ m\ c$; 1 Ta + 1Mn in 2(*a*) 000; 001/2 and 2 Ta in 2(*d*) 1/3 2/3 3/4; 2/3 1/3 1/4. The corresponding intensity values are listed in Table 2. The most likely positions of the nitrogen atoms are 4(*f*) 1/3, 2/3, *z*; 2/3, 1/3 *z*; 2/3 1/3 1/2 + *z*; 1/3 2/3 1/2—$z \sim 1/8$. The parameter *z* probably has a value slightly different from 1/8, because the nitrogen atoms are octahedrally surrounded by metal atoms of two different kinds ($1\frac{1}{2}$ Ta + $1\frac{1}{2}$ Mn in 2(*a*) and 3Ta in 2(*d*)).

Interatomic distances are listed in Table 3, under the assumption that $z_{(f)} = 1/8$.

The Ta-Fe-N, Ta-Co-N and Ta-Ni-N systems

The Ta-Me-N systems with Me = Fe, Co and Ni are very similar. No solubility of Ta in Fe-nitrides was observed. One hexagonal ternary phase, closely related to the Ta_3MnN_4 phase and with a composition close to the formula $Ta_2MeN_{2.5}$ (Me = Fe, Co, Ni), was found in the three systems. In this phase the metals Fe, Co and Ni can replace each other in all proportions. No other ternary phase seems to exist. The unit cell dimensions as well as the calculated and observed densities are listed in Table 4.

A comparison of the diffraction patterns of the phases Ta_3MnN_4 and $Ta_2MeN_{2.5}$ (Me = Fe, Co, Ni) showed such a similarity that the metal atoms

Table 4. Unit cell dimensions, calculated and observed densities for $Ta_2MeN_{2.5}$ (Me = Fe, Co, Ni).

Phase	*a*, Å	*c*, Å	*c* / *a*	Density calc.	Density obs.
$Ta_2FeN_{2.60}$	5.156	10.31	2.000	12.71	11.9
$Ta_2CoN_{2.50}$	5.161	10.30	1.996	12.74	12.3
$Ta_2NiN_{2.45}$	5.168	10.36	2.005	12.60	12.2
$Ta_2(Fe_{0.33}Co_{0.33}Ni_{0.33})N_{2.50}$	5.162	10.33	2.001	12.67	12.1

Table 5. Calculated and observed intensities for $Ta_2NiN_{2.45}$.

$h\,k\,l$	$I_{calc.}$	I_{obs}	$h\,k\,l$	$I_{calc.}$	I_{obs}
002	24	25	214	4	5
100	12	10	206	2	0
102	14	15	008	14	15
004	58	60	304	86	100
110	0		220	0	
111	92	100	117	24	25
112	116	100	221	24	25
104	4	5	222	34	40
200	2		108	2	0
113	57	60	310	2	0
202	3	5	223	23	25
114	0	0	216	3	0
006	2	0	312	3	0
204	2	5	118	0	0
210	2		224	0	0
115	34	40	306	0	0
106	2	0	208	1	0
212	5	5	314	2	0
300	53	60	400	1	
116	46	40	225	17	15
302	8	10	402	1	0

must have the same packing sequence (ABACABAC . . .) in both compounds. A series of comparatively weak extra reflections seems to indicate that the Me atoms possess an ordered distribution. The intensities of these superlattice lines commonly grow weaker at increasing diffraction angles, and this is characteristic of ordered atomic positions, not of a displacement of the atomic positions from the "ideal" lattice points. The c axes of the two phases have almost the same values, and the a axis of the latter phase is approximately $\sqrt{3}$ times greater than that of the Ta_3MnN_4 phase. The $00l$ reflections with $l = 2n + 1$ and the $h\ k\ l$ reflections with $h \neq k$ and $l = 2n + 1$ are absent in the diffraction pattern. A systematic interpretation of the possible metal atom positions resulted in the following atomic arrangement in the space-group D_{3h}^3—P $\bar{6}$ m 2:

2 Ta in 2(*c*) 0 0 z; 0 0 z with $z = 1/4$
3 Ta in 3(*f*) x 0 0; 0 x 0; $\bar{x}$ $\bar{x}$ 0 with $x = 1/3$
3 Ta in 3(*g*) x 0 1/2; 0 x 1/2; $\bar{x}$ $\bar{x}$ 1/2 with $x = 2/3$
4 Me in 4(*h*) 1/3 2/3 z; 1/3 2/3 $\bar{z}$; 2/3 1/3 z; 2/3 1/3 $\bar{z}$ with $z = 1/4$

The calculated and observed intensities for the $Ta_2NiN_{2.45}$ phase are compared in Table 5.

It is not possible to determine the positions of the nitrogen atoms from X-ray data, but they are most likely to be situated at the centres of metal atom octahedra. Furthermore they are probably placed in the octahedral "holes" having the highest N-Ta coordination numbers.

Table 6. Interatomic distances in $Ta_2MeN_{2.50}$ ($Me = Fe_{0.33}Co_{0.33}Ni_{0.33}$).

Atom	Surrounding atoms	Interatomic distance, Å
Ta in 2(*e*)	3 Ta in 3(*f*)	3.10
	3 Ta in 3(*g*)	3.10
	6 Me in 4(*h*)	2.98
Ta in 3(*f*)	1 Ta in 2(*e*)	3.10
	6 Ta in 3(*f*)	2.98
	3 Ta in 3(*g*)	3.10
	2 Me in 4(*h*)	3.10
Ta in 3(*g*)	1 Ta in 2(*e*)	3.10
	3 Ta in 3(*f*)	3.10
	6 Ta in 3(*g*)	2.98
	2 Me in 4(*h*)	3.10
Me in 4(*h*)	3 Ta in 2(*e*)	3.10
	3 Ta in 3(*f*)	2.98
	3 Ta in 3(*g*)	3.10
	3 Me in 4(*h*)	2.98

The intermetallic distances in the $Ta_2(Fe_{0.33}Co_{0.33}Ni_{0.33})\ N_{2.50}$ phase are recorded in Table 6.

The homogeneity ranges of the compounds Ta_3MnN_4 and $Ta_2MeN_{2.5}$ (Me = Fe, Co, Ni) seem to be rather narrow, as indicated by the very small variations of the unit cell dimensions.

SUMMARY

Binary and ternary Ta, Me alloys (Me = Ti, V, Cr, Mn, Fe, Co, Ni) have been nitrided with dry ammonia at temperatures varying between 650° C and 950° C. The following ternary nitride phases have been found to exist: Ta_3MnN_4 is hexagonal with four metal atoms in the unit cell. The space-group is D_{6h}^4—$P\ 6_3/m\ m\ c$. $Ta_2MeN_{2.5}$ (Me = Fe, Co, Ni) has a similar structure but shows a superstructure containing twelve metal atoms in the cell. The space-group is D_{3h}^3—$P\ \bar{6}\ m\ 2$. The metals Fe, Co and Ni can replace each other in all proportions.

The metal atoms in the phases Ta_3MnN_4 and $Ta_2(Fe, Co, Ni)N_{2.5}$ occupy a close-packed arrangement designated by the sequence ABACABAC . . .

(Ta, Cr)N_x and (Ta, Mn)N_x with the ratios Ta : Cr and Ta : Mn probably close to unity are isomorphous, but the structure and composition are unknown.

No solubility of Me nitrides in TaN (ε phase) has been detected. Up to about 10 % of the Ta atoms in $TaN_{0.80-0.90}$ (δ phase) can be replaced by Mn atoms, thus causing a comparatively strong deformation of the simple hexagonal lattice. The TiN, VN and CrN phases of NaCl type have been found to

form solid solutions with the TaN phase up to the approximate limits 10, 25 and 25 atom % of TaN respectively. No solubility of Ta in the most nitrogen-rich nitrides of Mn and Fe has been observed.

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X-Ray Studies on Vanadium and Chromium Oxides with Low Oxygen Content

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METALLIC VANADIUM OXIDES

Samples of vanadium oxides with oxygen contents up to the composition $VO_{1.30}$ were obtained by heating vanadium metal powder (Vanadium Corporation of America, 99.7 % Grade) and dried vanadium pentoxide (Schuchardt, reinst. ca. 100 %) or vanadium trioxide *in vacuo* at a temperature of 1 000° C. V_2O_3 was prepared by the reduction of V_2O_5 in oxygen-free hydrogen at a temperature of 800° C for about three hours. The powder mixtures of metal and oxide were poured into alumina tubes, placed in silica tubes, which were then evacuated, sealed and heated at 1 000° C for about one weak. As it proved difficult to achieve equilibrium most of the samples from these reactions were powdered again, moulded into small brickettes and heated in zirconia crucibles in a high frequency vacuum induction furnace [1] up to about 1 600° C. In order to detect the possible presence of high temperature modifications of the oxides, some samples were heated *in vacuo* at temperatures between 600° C and 1 200° C and then quenched in cold water.

X-ray powder photographs of the samples taken in a Guinier focusing camera with monochromatized Cr-K_α radiation revealed the existence of three phases, *viz.* vanadium, the vanadium oxide with the metal atoms in a tetragonally deformed vanadium lattice [2] and the approximate homogeneity range $VO_{0.15}$—$VO_{0.25}$, and the vanadium oxide ("VO phase")[3,4] with the sodium chloride structure. The VO phase was found to have a wide range of homogeneity, with the limits $VO_{0.80}$ and $VO_{1.20}$.

As the present investigation confirmed in all respects the recently published results about the vanadium phase and the phase $VO_{0.15}$—$VO_{0.25}$ obtained by Seybolt and Sumsion [2], there is no need to repeat the actual data.

The X-ray patterns of the specimens with the compositions $VO_{0.80}$ and $VO_{1.20}$ showed only VO-reflections. But additional lines of the $VO_{\sim 0.2}$ phase were visible in the photograph when the composition of the oxide was $VO_{0.75}$, and the pattern of the V_2O_3 phase appeared for the composition $VO_{1.25}$.

The lattice constant was found to increase from $a = 4.042$ Å ($VO_{0.80}$) to $a = 4.126$ Å ($VO_{1.20}$). This result is not in agreement with the statement of

Table 1. Calculated and observed densities for the VO phase.

Phase composition	Density (d) calculated	observed	$d_{calc.} - d_{obs.}$
$VO_{0.80}$	6.41	6.12	0.29
$VO_{0.90}$	6.39	6.01	0.38
$VO_{1.00}$	6.49	5.92	0.37
$VO_{1.10}$	6.29	5.66	0.63
$VO_{1.20}$	6.08	5.50	0.58

Klemm and Grimm [3], that the cube edge decreases from $a = 4.11$ Å at the composition $VO_{0.9}$ to $a = 4.10$ Å at the composition $VO_{1.3}$. Also, these authors suggest that the cubic VO phase exists only at high temperatures, and that it decomposes at low temperatures into pure vanadium or a solid solution of oxygen in vanadium and an "X-phase" of undetermined composition and structure (not the V_2O_3 phase). This is contradicted by the fact that samples with the compositions $VO_{0.80}$ and $VO_{1.00}$ did not change their structure on being cooled *in vacuo* from 800° C to room temperature over a period of eight weeks, and the fact that a sample with the composition $VO_{1.30}$ proved to be a mixture of the VO and V_2O_3 phases after the same cooling procedure.

Mathewson, Spire and Samans [4] and Anderson [5] report that the unit cell dimensions increase with increasing oxygen content, which is in agreement with the present result.

For a sample with the composition $VO_{1.00}$ the lattice constant was found to be $a = 4.093$ Å. The V-V distances (12-coordination) are 2.89 Å and the O-V distances (6-coordination) 2.05 Å. If the oxygen content is greater than 50 atom-%, this must be due to vanadium atom vacancies in the close-packed metal lattice. It is not possible for oxygen to replace vanadium atoms because the resulting O-O distance would be 2.05 Å. The shortest O-O distances observed in oxides are of the order 2.6 Å, this value being characteristic of oxides with ionic as well as metallic properties.

Table 1 lists the densities calculated on the assumption that all vanadium sites are occupied in samples with an oxygen content up to 50 atom-% and that the vanadium lattice is defective in samples with a greater oxygen content. The accuracy of the density determinations is not great enough to give quantitative information about the percentage of unoccupied atomic positions in the unit cell. But the experimental results seem to indicate a defective lattice even for the composition $VO_{1.00}$.

It may be of interest to compare the VO phase with the analogous TiO phase. Ehrlich [6] reports the homogeneity range $TiO_{0.58}$—$TiO_{1.33}$ for this metallic oxide with the lattice constant varying between 4.184 Å and 4.152 Å. He further states that 15 % of the atomic sites are unoccupied at the composition $TiO_{1.00}$.

THE METALLIC CHROMIUM OXIDE Cr_3O

In connection with an investigation of metallic double-oxides containing two transition elements, Cr_2O_3 (Baker's Analyzed) was reduced with titanium, vanadium and zirconium in a high frequency vacuum induction furnace [1] at

Table 2. Observed and calculated intensities for the Cr_3O phase.

hkl	$I_{calc.}$	$I_{obs.}$
1,1,0	260	200
2,0,0	46	50
2,1,0	532	500
2,1,1	113	130
2,2,0	43	50
3,1,0	75	80
2,2,2	195	200
3,2,0	233	200
3,2,1	107	130
4,0,0	93	80
4,1,1	41	50
3,3,0	21	20
4,2,0	41	50
4,2,1	279	300
3,3,2	32	30
4,2,2	30	30
5,1,0	30	30
4,3,1	61	50
5,2,0	118	100
4,3,2	238	200
5,2,1	65	80
4,4,0	216	200

about 1 500° C. Besides oxides of titanium, vanadium and zirconium, a new phase was obtained with the atoms in a comparatively small cubic unit cell. As the same compound was formed in small amounts in the oxidation of chromium metal powder (Ferrolegeringar, Stockholm, 99.4 %) by steam in a large excess of hydrogen at a temperature of 600° C, this new phase must be a chromium oxide with an oxygen content smaller than that of Cr_2O_3. In spite of several experiments it was not possible to obtain this oxide in a pure state. The reaction between chromium metal and Cr_2O_3 in an induction furnace only yielded traces of the low-oxygen oxide.

A comparison of the powder photographs of the oxide phase and of Cr_3Si indicated that these two compounds were isomorphous, both having the β-tungsten structure (A 15 type). The probable formula of the oxide is therefore Cr_3O. The cubic lattice constant was found to be 4.544 Å ($V = 93.83$ Å^3). The previously known compounds with the β-tungsten structure are V_3Si[7] ($a = 4.71$ Å), Cr_3Si[8] ($a = 4.55$ Å), Mo_3Si[9] ($a = 4.81$ Å), V_3Ge[10] ($a = 4.76$ Å), Cr_3Ge[10] ($a = 4.61$ Å) and Mo_3Zr[11] ($a = 4.94$ Å) *.

The space-group is O_h^3—$Pm3n$ with 6 Cr in 6(*c*): $\frac{1}{4}$, 0, $\frac{1}{2}$; $\frac{1}{2}$, $\frac{1}{4}$, 0; 0, $\frac{1}{2}$, $\frac{1}{4}$; $\frac{3}{4}$, 0, $\frac{1}{2}$; $\frac{1}{2}$, $\frac{3}{4}$, 0; 0, $\frac{1}{2}$, $\frac{3}{4}$ and 2 O in 2(*a*): 0, 0, 0; $\frac{1}{2}$, $\frac{1}{2}$, $\frac{1}{2}$.

Calculated and observed relative intensities are compared in Table 2, and interatomic distances are listed in Table 3.

* The molybdenum-zirconium system has been investigated by the author, and the only intermediate phase existing in this system was found to be Mo_2Zr of the Laves C15 type. The cubic lattice constant $a = 7.563$ Å. The compound Mo_3Zr of A15 type reported by Wallbaum [11] is probably an oxide analogous to Cr_3O.

Table 3. Interatomic distances in the Cr_3O phase.

Atom	Surrounding Atoms	Interatomic Distance, Å
Cr	2 Cr	2.272
	8 Cr	2.779
	4 O	2.520
0	12 Cr	2.520

It is evident from Table 3 that the mean intermetallic distance is 2.68 Å (10-coordination). It is not possible to calculate the accurate oxygen positions from X-ray investigations, but $p|F|^2$ values corresponding to the distribution of two or three oxygen atoms in ordered or disordered 6(*c*) positions show poor agreement with the observed intensities. Hence the atomic positions given above are the most likely ones.

It is remarkable that the oxygen atoms are surrounded by twelve chromium atoms at abnormally large distances. All metallic oxides previously known have the oxygen atoms at the centres of metal atom octahedra with the interatomic oxygen-metal distance equal to or slightly greater than the sum of the metal atom radius and the oxygen atom radius. In Cr_3O the corresponding distance may be formally written as: chromium atom + oxygen ion.

The existence of an oxide CrO, probably of the wurtzite type, assumed by Lux and Proeschel [12] has not been confirmed.

SUMMARY

The vanadium-oxygen system has been investigated by X-ray methods up to an oxygen content corresponding to the composition $VO_{1.30}$ (56.5 atom-% of O). Three phases were found to occur, *viz.* the vanadium phase (α), the vanadium oxide with the metal atoms in a tetragonally deformed vanadium lattice and the approximate homogeneity range $VO_{0.15}$—$VO_{0.25}$ (β), and the VO phase with the sodium chloride structure (γ). The results reported by Seybolt and Sumsion [2] for the α and the β phases have been confirmed in all respects. The homogeneity limits of the VO phase have been found to be $VO_{0.80}$ (44.5 atom-% of O) and $VO_{1.20}$ (54.5 atom-% of O), with the lattice constants $a = 4.042$ Å and $a = 4.126$ Å respectively.

A chromium oxide of the β-tungsten structure has been prepared. The most likely formula is Cr_3O.

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An X-Ray Investigation of Transition Metal Phosphides

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The investigation reported in the following paper treats phosphides of transition metals with a low phosphorus content. The metals belong to the groups IV—VI of the periodic system, *viz.* Ti, Zr, V, Nb, Ta, Cr, Mo, W. The ratio phosphorus/metal is ≤ 1 ("subphosphides" and "monophosphides").

EXPERIMENTAL

Powdered metals of fairly high purities (about 99.5 %) and red phosphorus (Kahlbaum *pro analysi*) were used as starting materials. The phosphides were prepared by the following two methods (*A* and *B*):

A. The metal powder and red phosphorus, to a total amount of about 1 g, were mixed in various proportions, and heated in evacuated and sealed silica tubes. The heating time was varied between 1 hour and 1 month, and the temperature between 550° C and 1 100° C. The best results seemed to be reached under the following reaction conditions: uniform temperature rise from room temperature up to about 800° C over a period of 2 days, heating at a constant temperature of about 800° C for about 2 days, and cooling to room temperature over a period of 1 day. Below about 650° C the rate of reaction is very slow. Above about 850° C there is a certain tendency towards reactions between the silica tube and the materials used. On account of the high vapour pressure of phosphorus at elevated temperatures, explosions are liable to take place if the silica tubes have too thin walls or are improperly sealed.

B. PH_3, obtained from white phosphorus and potassium hydroxide solution, was dried over P_2O_5 and passed together with dry oxygen-free hydrogen over the metal powders placed in alumina boats in a porcelain-tube heated in an electric furnace. The temperatures were varied between 700° and 900° C, and the times between 2 and 10 hours. The air in the apparatus was first driven out by means of carbon dioxide, whereupon a steady stream of hydrogen was passed through. Since it proved very difficult to obtain the mono- and subphosphides in a pure state by this method, it was used for the preparation of diphosphides (800° C, 6 hours). A mixture of metal powder and diphosphides was then heated in evacuated and sealed silica tubes (method A) for the preparation of specimens with the desired compositions.

In order to homogenize the product and to detect the possible presence of high-temperature modifications of the phosphides, some samples were heated in sealed evacuated silica tubes at temperatures between 600° C and 1 100° C, and then quenched in cold water.

The phase analysis and structure determinations were made from powder photographs taken with Cu-$K\alpha$ and Cr-$K\alpha$ radiation in Guinier-type cameras. The monochromator was either a curved quartz monochromator or an aluminium monochromator with double curvature [1]. For measurements of the high-angle reflections additional photographs were taken with Hägg-Phragmén type focusing cameras.

THE STRUCTURES OF THE PHASES

The existence of the diphosphides of all the actual metals (Ti, Zr, V, Nb, Ta, Cr, Mo, W) was confirmed, but no attempt was made to determine the structures.

The structures and approximate compositions of all phases with the phosphorus/metal ratio close to unity were determined.

A synopsis of the phosphides' structure types, unit cell dimensions and densities, both calculated and observed, are given in Table 5. The interatomic distances are listed in Table 6.

1. The Ti—P System

According to Biltz, Rink and Wiechmann [2], two titanium phosphides exist, one of them being homogeneous in the interval $TiP_{0.92-0.94}$ and the other having the approximate composition Ti_2P.

The existence of a "subphosphide" was confirmed in the present work, although its structure and composition is unknown.

TiP

The powder photograph reflections of a phase which was homogeneous at the composition TiP indicated hexagonal symmetry. The observed density value, 4.08, corresponds to four formula units TiP in the unit cell. There were no reflections with $h-k = 3n$ and $l \neq 2n$ simultaneously. The space-group with the highest symmetry fulfilling these conditions is D_{6h}^4—$P6_3/mmc$ with the atoms in combinations of the positions 2(*a*) 0, 0, 0; 0, 0, $\frac{1}{2}$, 2(*b*) 0, 0, $\frac{1}{4}$; 0, 0, $\frac{3}{4}$; 2(*c*) 1/3, 2/3, $\frac{1}{4}$; 2/3, 1/3, $\frac{3}{4}$, 2(*d*) 1/3, 2/3, $\frac{3}{4}$; 2/3, 1/3, $\frac{1}{4}$, and 4(*f*) 1/3, 2/3, z; 2/3, 1/3, $\bar{z}$; 2/3, 1/3, $\frac{1}{2}+z$; 1/3, 2/3, $\frac{1}{2}-z$ with $z = 1/8$. As is evident from Table 1 good agreement between calculated and observed intensities is obtained for the following atomic grouping:

4 Ti in 4 (*g*) 1/3, 2/3, z with $z = 1/8$,
2 P in 2 (*a*) 0, 0, 0; 0, 0, $\frac{1}{2}$,
2 P in 2 (*d*) 1/3, 2/3, $\frac{3}{4}$; 2/3, 1/3, $\frac{1}{4}$

If the projections of the positions on the basal plane are designated by A, B, and C, the titanium lattice can be symbolized by the sequence AABBAABB . . . The corresponding phosphorus atom sequence is ACBCACBC . . ., a mixture of cubic and hexagonal close-packing. The resulting stacking of atoms is $A_{Ti}B_PA_{Ti}C_PB_{Ti}A_PB_{Ti}C_P$. . .

The phosphorus atoms are thus placed at the centres of metal atom octahedra. The lattice can be considered as a superstructure of the NiAs (B 8) lattice. The same arrangement of metal atoms occurs in MoC (γ'-phase) [3], NbN (I)[4], W_2B_5[5] and Ti_2B_5[6]. In the boride phases the parameter z is slightly differed from 1/8. From space considerations, the nonmetal atoms in MoC (γ') and NbN (I) ought to be placed in the same positions as the phosphorus atoms in TiP. These three compounds are therefore probably isomorphous. The atomic arrangement gives an explanation of the fact that the parameter z does not differ from the value 1/8. The "anti-form" of the TiP structure has recently been found for the ternary nitride Ta_3MnN_4[7].

Table 1. Calculated and observed intensities for the TiP phase.

hkl	I_{calc}	I_{obs}	*hkl*	I_{calc}	I_{obs}
0,0,2	0	0	2,0,8	71	80
1,0,0	41	35	2,1,5	83	80
1,0,1	83	75	3,0,0	15	10
0,0,4	8	10	1,0,11	37	35
1,0,2	108	115	3,0,2	0	0
1,0,3	231	250	2,1,6	23	25
1,0,4	96	80	2,0,9	5	0
0,0,6	0	0	0,0,12	1	0
1,0,5	127	115	1,1,10	0	0
1,1,0	176	175	3,0,4	5	0
1,1,2	0	0	2,1,7	11	10
1,0,6	28	25	1,0,12	16	10
2,0,0	6	5	2,0,10	7	0
2,0,1	14	35	2,1,8	10	10
1,1,4	19		3,0,6	0	0
2,0,2	21	25	2,2,0	43	35
1,0,7	14	50	2,0,11	25	25
0,0,8	46		2,2,2	0	0
2,0,3	81	80	1,0,13	27	25
2,0,4	40	25	2,1,9	7	0
1,0,8	11	10	1,1,12	5	0
1,1,6	0	0	3,1,0	5	0
2,0,5	62	50	3,1,1	8	10
2,0,6	17	15	2,2,4	5	
1,0,9	74	75	0,0,14	0	0
0,0,10	0	0	3,1,2	18	15
2,1,0	71	75	3,0,8	111	115
2,1,1	14	10	3,1,3	71	80
2,1,2	23	25	2,0,12	21	15
2,0,7	71	75	2,1,10	22	15
1,1,8	147	175	3,1,4	51	50
2,1,3	83	80	1,0,14	14	15
1,0,10	21	10	2,2,6	0	0
2,1,4	49	50			

2. The Zr—P system

Strotzer, Biltz, and Meisel [8] reported three intermediate phases, *viz.* Zr_3P, ZrP, and ZrP_2.

The present work confirmed the existence of a phosphide of unknown composition and structure within the interval $ZrP_{0.3-0.5}$.

α-ZrP

In the vicinity of the composition $ZrP_{0.9}$, a phosphide occurred which had a comparatively small homogeneity range. The phase is of NaCl type, and the length of the cube edge was found to vary between 5.261 Å and 5.278 Å. The zinc blende structure (B 3 type) could be rejected, both because of disagreement between calculated and observed intensities and from space considerations.

Table 2. Calculated and observed intensities for the VP phase assuming, I: "Anti-NiAs" structure, or II: NiAs structure.

hkl	I_{calc} I	I_{calc} II	I_{obs}
0,0,2	6	6	5
1,0,0	1	35	35
1,0,1	84	29	25
1,0,2	74	101	100
1,0,3	28	8	10
1,1,0	44	44	50

The NaCl structure gives ZrP as the ideal formula for this phase. A small fraction of the P positions is evidently empty. It therefore seems practical to call the phase ZrP and to denote it by α-ZrP in order to distinguish it from the following phase.

β-ZrP

This phase appeared to be homogeneous at the composition ZrP, and is isomorphous with the TiP phase.

3. The V—P system

The vanadium-phosphorus system was examined by Zumbusch and Biltz[9]. Their investigation indicated the existence of the compounds V_3P, VP and VP_2. The formula V_2P was given for the subphosphide by Chêne[10,11] and Andrieux [12].

Powder photographs of samples with the compositions $VP_{0.2}$, $VP_{0.4}$, $VP_{0.6}$, $VP_{0.8}$, $VP_{1.0}$, and $VP_{1.2}$ showed that the only compound existing in the V—VP interval is the VP phase.

From Table 2 is evident that VP has the NiAs structure (B 8 type). The space-group is thus D_{6h}^4—$P6_3/mmc$ with 2 V in 2(*a*) 0, 0, 0; 0, 0, $\frac{1}{2}$ and 2 P in 2(*c*) 1/3, 2/3, $\frac{1}{4}$; 2/3, 1/3, $\frac{3}{4}$. The homogeneity range is small. The *c*/*a* value is remarkably high: 1.96. For other crystals with well established NiAs structure *c*/*a* has been found to range between 1.2 and 1.7. The VP phase seems to be the only existing phosphide of B 8 type.

4. The Nb—P and Ta—P systems

According to Zumbusch and Biltz[13,14], the niobium and tantalum phosphides are probably isomorphous. They point out that the monophosphides occur in two modifications, none of them related to the vanadium monophosphide, and that the diphosphides give a diffraction pattern similar to that of the vanadium diphosphide.

The present work confirmed the results given by these authors.

α-NbP

A homogeneous phase was obtained at the approximate composition $NbP_{0.95}$. The powder pattern of this phase corresponds to a body-centered tetragonal cell containing one unit $NbP_{0.95}$. In order to facilitate the comparison

with the following phase the corresponding face-centered cell containing two units $NbP_{0.95}$ has been chosen here.

Consequently, the atoms are distributed over the positions 0, 0, 0; 0, $\frac{1}{2}$, $\frac{1}{2}$; $\frac{1}{2}$, 0, $\frac{1}{2}$; $\frac{1}{2}$, $\frac{1}{2}$, 0. As compared with the cubic close-packing ($c/a = 1$), the tetragonal deformation is very strong, $c/a = 1.71$. The lattice can be considered to be built up of two-dimensional layers parallel to (0, 0, 1). The strong bonds exist within each NbP layer and only comparatively weak bonds between the layers. Since the interatomic distance in a layer reaches the remarkably low value of 2.35 Å (4-coordination), the only possible atomic arrangement is a partly ordered grouping. Within a layer each Nb atom is always surrounded by four P atoms. For it is most unlikely that the Nb—Nb distances are less than 2.6 Å, and the observed P—P distances of all the examined phosphides exceed the value of 3.1 Å. But it is evident from the diffraction pattern (no lines with mixed indices are visible) that the NbP layers are irregularly displaced in relation to each other. The atomic distribution in the unit cell can therefore be written as: 1 Nb + 1 P in 0, 0, 0; $\frac{1}{2}$, $\frac{1}{2}$, 0 and 1 Nb + 1 P in 0, $\frac{1}{2}$, $\frac{1}{2}$; $\frac{1}{2}$, 0, $\frac{1}{2}$, with ordered positions of the atoms within each 001-plane.

β-NbP

This phase was found to be homogeneous at the composition 1 : 1. Besides the strong diffraction lines characteristic for the face-centered arrangement of atoms, the powder photograph showed a series of extra reflections from a tetragonal lattice having the value of the c-axis twice that of the α-NbP phase. Reflections with $h + k + l = 2n$, $h00$ with $h = 2n$, 0, 0, l with $l = 4n$, $2h + l = 2n + 1$ or $4n$ appeared in the photographs. This is characteristic for the space-group C_4^6—$I4$ and D_4^{10}—$I4_12$. According to the density determinations, the unit cell contains 4 Nb and 4 P atoms. The only possible atomic position in the space-group C_4^6—$I4$ is 4(a) (0, 0, 0; $\frac{1}{2}$, $\frac{1}{2}$, $\frac{1}{2}$) + 0,0,z; 0, $\frac{1}{2}$, $\frac{1}{4}$ + z. Both the metal and nonmetal atoms have to be placed in this position. From $p|F|^2$ calculations it was found that $|z_{Nb}—z_P| = \frac{1}{2}$, meaning a fourfold screw-axis 4_1 (or 4_3) in the |0, 0, 1|-direction. The space group is thus D_4^{10}—$I4_12$ with 4 Nb in 4(a) (0, 0, 0; $\frac{1}{2}$, $\frac{1}{2}$, $\frac{1}{2}$) + 0, 0, 0; 0, $\frac{1}{2}$, $\frac{1}{4}$ and 4 P in 4(b) (0, 0, 0; $\frac{1}{2}$, $\frac{1}{2}$, $\frac{1}{2}$) + 0, 0, $\frac{1}{2}$; 0, $\frac{1}{2}$, $\frac{3}{4}$.

While the NbP layers of the α-NbP phase occupy disordered positions in relation to each other, the β-NbP lattice has an ordered atomic arrangement.

Intensities for the β-NbP phase, both calculated and observed, are compared in Table 3.

α-TaP

α-TaP is isomorphous with α-NbP, and probably has the corresponding approximate composition $TaP_{0.95}$. It was not possible to obtain this phase in a pure state.

β-TaP

The β-TaP phase is isomorphous with β-NbP, and was found to be homogeneous at the composition 1 : 1.

Table 3. Calculated and observed intensities for the β-NbP phase.

hkl	I_{calc}	I_{obs}	*hkl*	I_{calc}	I_{obs}
1,0,1	51	40	1,1,10	28	25
0,0,4	65	85	2,0,9	4	0
1,0,3	26	25	3,0,5	4	0
1,1,2	117	125	1,0,11	3	0
1,0,5	12	10	2,1,9	6	0
2,0,0	31	40	0,0,12	5	5
1,1,6	52	60	3,1,6	39	40
2,1,1	17		3,2,1	6	
1,0,7	8	5	3,0,7	3	0
2,0,4	52	60	2,2,8	18	15
0,0,8	13	15	3,2,3	6	0
2,1,3	6	5	3,2,5	6	0
2,1,5	12	10	2,1,11	5	0
1,0,9	5	5	1,0,13	3	0
2,2,0	16	25	3,0,9	3	0
3,0,1	6	5	4,0,0	10	10
2,1,7	9	5	4,1,1	9	10
2,2,4	28	25	3,2,7	9	10
2,0,8	28	25	4,0,4	34	40
3,0,3	4	0	4,1,3	10	10
3,1,2	56	60			

5. The Cr—P system

The existence of the Cr_3P and CrP phases was proved by Nowotny and Henglein [15] by X-ray methods. Cr_3P is isomorphous with Mn_3P [16], Fe_3P[17,18], Ni_3P[13] and $(Fe, Ni)_3P$[20] with the atoms in the probable space-group S_4^2—$I\bar{4}$[18,21]. CrP is of the B 31 type [22], isomorphous with MnP, FeP, and CoP[23].

CrP

The CrP phase has 4 Cr and 4 P atoms in the positions $4(c) \pm (x, y, \frac{1}{4}; \frac{1}{2}-x, \frac{1}{2}+y, \frac{1}{4})$ in the space-group D_{2h}^{16}—*Pbnm*. Nowotny and Henglein reported the same parameter values for CrP as was earlier given for MnP[23], *i.e.* $x_{Cr} = 0.20$; $y_{Cr} = 0.005$; $x_P = 0.57$; $y_P = 0.19$. The present author, however, found the best agreement between calculated and observed intensities for the following values: $x_{Cr} = 0.16$; $y_{Cr} = 0.00$; $x_P = 0.63$; $y_P = 0.20$.

The B 31 type (MnP structure) can be regarded as an orthorhombic deformed B 8 type (NiAs structure). Though the NiAs structure is hexagonal, it may be described as an arrangement with the symmetry of D_{2h}^{16} in the special case of the axial ratio $\sqrt{3} : b/c : 1$ and the atoms located in two four-fold positions $4(c)$ $x, y, \frac{1}{4}$, with the parameters for the metal atoms $x = 0.25$; $y = 0$ and for the phosphorus atoms $x = 0.58$; $y = 0.25$. As is evident from the parameter values given above, those obtained in the present investigation signify a comparatively large deviation from the "ideal" atomic positions.

But on the other hand, the resulting interatomic distances are in good agreement with analogous distances observed for the monophosphides of the other transition metals studied here (Table 6).

6. The Mo—P system

The phases Mo_3P and MoP were reported by Andrieux and Chêne[12,24] and according to Faller and Biltz [25] Mo_3P is isomorphous with Cr_3P [15].

MoP

The MoP phase was found to have the WC structure. Very small variations of the lattice constants were measured, and the formula was found to correspond to the composition 1 : 1. No extra reflections appeared in the powder photographs. This excludes an arrangement of the P atoms corresponding to the NiAs type.

7. The W—P system

Whereas the formula W_4P and W_2P were given for the tungsten subphosphides by Hartmann and Orban [26], Faller and Biltz [27] showed that W_2P is a mixture of W and WP of MnP type. But the latter authors did not publish any parameter values. They further pointed out that WP_2 is isomorphous with MoP_2, but not with CrP_2.

The present author found that powder photographs of samples with the compositions $WP_{0.2}$, $WP_{0.4}$, $WP_{0.6}$, and $WP_{0.8}$, only showed diffraction lines of the W and WP phases, confirming the results obtained by Faller and Biltz [27].

WP

As pointed out above, no unit cell dimensions or parameter values for the atomic positions have hitherto been given for the WP phase. In the diffraction pattern there appeared $0kl$ reflections with $k = 2n$ and $h0l$ reflections with $h + l = 2n$, characteristic for the space-group D_{2h}^{16}—*Pbnm* (compare the isomorphous CrP phase). The lines, which did not fulfill the conditions: $h + k + l = 2n$, $0kl$ with $k = 2n$ and $l = 2n$, were found to be very weak and only appeared at relatively small diffraction angles. It was therefore assumed that the phosphorus gave rise to these weak reflections. This gives $y_W = 0$. A systematic variation of x_W within the limits of closest approach between the tungsten atoms (about 2.6 Å) resulted in a value of $x_W = 0.175$ for the best agreement between calculated and observed intensities. Because of the great difference between the atomic factors of tungsten and phosphorus, it is very difficult to determine the position of the nonmetal atoms by means of X-ray data. The parameters x and y for the phosphorus atoms have thus been determined with a view to the best space distributions $x_P = 0.62$ and $y_P = 0.20$.

WP thus has 4 W and 4 P atoms located in two four-fold positions 4(*c*) $\pm$ (x, y, $\frac{1}{4}$; $\frac{1}{2}-x$, $\frac{1}{2}+y$, $\frac{1}{4}$) in the space-group D_{2h}^{16}—*Pbnm*, with the following parameter values: $x_W = 0.175$; $y_W = 0$; $x_P = 0.62$; $y_P = 0.20$.

Calculated and observed intensities are compared in Table 4.

Table 4. Calculated and observed intensities for the WP phase.

hkl	I_{calc}	I_{obs}	*hkl*	I_{calc}	I_{obs}
1,0,1	125	75	0,2,4	7	5
0,0,2	56	75	4,1,3	2	0
0,1,1	182	175	1,2,4	0	0
2,0,0	103	110	2,1,5	36	35
1,0,2	4	5	5,0,2	0	0
2,0,1	1	5	4,2,0	36	35
1,1,1	6	5	0,3,1	28	35
2,1,0	1	5	5,1,1	0	0
2,0,2	39	40	3,2,3	67	70
1,1,2	175	175	4,2,1	0	0
2,1,1	211	175	4,0,4	4	0
1,0,3	94	110	1,3,1	0	0
3,0,1	25	25	2,2,4	7	5
2,1,2	0	0	3,0,5	17	15
0,1,3	7	10	0,0,6	16	15
2,0,3	0	0	1,0,6	0	0
1,1,3	1	0	5,1,2	43	35
3,0,2	0	0	4,2,2	22	25
0,2,0	46	50	2,3,0	0	0
3,1,1	1	0	1,3,2	43	35
0,0,4	6	5	5,0,3	31	35
1,2,1	22	25	4,1,4	0	0
1,0,4	0	0	2,3,1	50	50
2,1,3	0	0	3,1,5	0	0
3,1,2	61	50	2,0,6	28	110
0,2,2	21	25	1,1,6	58	
4,0,0	35	35	1,2,5	30	
2,2,0	48	50	3,2,4	0	0
3,0,3	67	75	2,3,2	0	0
1,2,2	2	5	0,3,3	1	0
4,0,1	0		5,1,3	0	0
2,2,1	0	0	6,0,0	13	10
2,0,4	3	0	4,2,3	0	0
1,1,4	102	110	1,3,3	0	0
4,1,0	1	0	6,0,1	0	0
4,0,2	19	15	4,0,5	0	0
3,1,3	0	0	2,1,6	0	0
2,2,2	36	35	2,2,5	0	0
4,1,1	73	75	3,3,1	0	0
2,1,4	1	0	5,2,1	11	10
1,2,3	90	110	5,0,4	0	0
1,0,5	27	25	6,1,0	0	0
3,2,1	28	25	6,0,2	8	10
4,1,2	0	0	3,0,6	0	0
3,0,4	0	0	2,3,3	2	0
4,0,3	1	0	6,1,1	45	50
2,2,3	0	0	4,1,5	25	25
0,1,5	17	15	3,3,2	34	25
3,2,2	0	0	5,2,2	0	0
2,0,5	0	0	5,1,4	44	35
1,1,5	1	0	4,2,4	4	0
3,1,4	74	75	1,0,7	1	0
5,0,1	5	0	6,1,2	0	0

Table 4. Cont.

hkl	I_{calc}	I_{obs}	*hkl*	I_{calc}	I_{obs}
3,1,6	4	0	6,2,0	33	75
1,3,4	46	75	2,1,7	66	
3,2,5	26		0,3,5	18	10
0,2,6	23	25	5,1,5	0	0
6,0,3	0	0	6,1,2	0	0
1,2,6	0	0	6,0,4	3	0
4,3,0	0	0	4,1,6	0	0
0,1,7	28	25	4,2,5	0	0
3,3,3	0	0	7,0,1	7	0
4,3,1	41	50	0,4,0	20	15
5,2,3	53	50	1,3,5	0	0
2,0,7	0	0	3,0,7	2	0
1,1,7	0	0	3,3,4	80	75
2,3,4	0	0	5,2,4	0	0
5,0,5	13	10	4,3,3	5	0
4,0,6	26	25	1,4,1	19	50
6,1,3	5	0	6,2,2	35	
2,2,6	52	50	3,2,6	0	0
4,3,2	0	0	7,0,2	0	0
			6,1,4	0	0

GENERAL DISCUSSION

All the phosphides listed in Table 5 have a greyish black metallic lustre, and are not attacked by water or dilute acid at room temperature. Samples of TiP, ZrP, and VP were not oxidized or decomposed when heated in air at 1 000° C for one hour.

The highest nonmetal content of carbides and nitrides of all transition metals, with the exception of the lanthanides and actinides, is about 50 atom-%. The nonmetals are interstitially situated at the centres of octahedra or trigonal prisms of metal atoms. The metal lattice commonly forms a hexagonal or cubic close-packing, in a few cases a simple hexagonal arrangement of atoms. The interatomic metal-metal distances are up to about 10 % larger than those of the pure metals, and the corresponding coordination numbers are high, varying between 8 and 12.

Hägg [28,29] has found empirically that the radius ratio nonmetal/metal is a determining factor for the structure of the phases and has an upper critical value of about 0.59. When the radius ratio exceeds 0.59 the structure is complicated, but the compounds are still metallic in character. This is characteristic of most borides and silicides. Space considerations account for the decrease in the coordination number of the metal atoms, but the coordination number for metal atom — nonmetal atom is still high. Furthermore, there exist "contact" bonds of non-ionic type between boron-boron and silicon-silicon atoms. Such nonmetal "contacts" have *not* been found to exist among the carbides and nitrides. For this reason, boron and silicon occurring in compounds with transition metals can be characterized as metals.

The radius value for the phosphorus atom has been found to be about 1.06 Å (Table 6). For the metals used here the radius ratio P/Me (Me = tran-

Table 5. Structures and unit cell dimensions of the phosphides of Ti, Zr, V, Nb, Ta, Cr, Mo, W and the phosphorus metal ratio ≤ 1.

Phase	Structure type	Space-group	Z	Cell dimensions in Å (Error: < ± 1 ‰)	Vol/metal-atom Å³	Density calc	Density obs
TiP		$P6_3/mmc$	4	$c=11.65$, $a=3.487$; $c/a=3.34$	30.67	4.27	4.08
α-ZrP	NaCl	$Fm3m$	4	$a=5.27$	36.62	5.43	5.10
β-ZrP	TiP	$P6_3/mmc$	4	$c=12.52$, $a=3.677$; $c/a=3.40$	36.65	5.57	5.35
VP	NiAs	$P6_3/mmc$	2	$c=6.22$, $a=3.18$; $c/a=1.96$	27.20	5.00	4.72
α-NbP		$F4/mmm$	4	$c=5.69$, $a=3.32$; $c/a=1.71$	31.35	6.40	
β-NbP		$I4_12$	8	$c=11.38$, $a=3.325$; $c/2a=1.71$	31.46	6.54	6.15
α-TaP	α-NbP	$F4/mmm$	4	$c=5.69$, $a=3.320$; $c/a=1.71$	31.35	11.04	
β-TaP	β-NbP	$I4_12$	8	$c=11.39$, $a=3.330$; $c/2a=1.71$	31.57	11.15	10.3
Cr_3P	Fe_3P	$(I\bar{4})$	8	$c=4.567$, $a=9.144$; $c/a=0.50$	15.91	6.51	6.25
CrP	MnP	$Pbnm$	4	$a=6.108$, $b=5.362$, $c=3.113$	25.11	5.49	5.25
Mo_3P	Fe_3P	$(I\bar{4})$	8	$c=4.923$, $a=9.729$; $c/a=0.51$	19.42	9.09	8.60
MoP	WC	$P\bar{6}m2$	1	$c=3.20$, $a=3.23$; $c/a=0.99$	28.64	7.50	7.20
WP	MnP	$Pbnm$	4	$a=6.219$, $b=5.717$, $c=3.238$	28.78	12.40	11.7

sition metal) exceeds the value 0.59 by between 10 and 30 %. From the structure determinations it is further evident that no P—P bonds of non-ionic type exist in the phosphides investigated in the present study. It is therefore of interest, that many of the monophosphides have structures analogous to those of the carbides and nitrides.

The metal atoms of the MoP phase form a simple hexagonal lattice. If we assume radii corresponding to neutral atoms, the available space permits another nonmetal atom to be placed in the metal lattice of the carbides and nitrides with the stacking AA . . . In this case both centres of the two trigonal metal atom prisms of the unit cell would be occupied. But as the observed phase composition is always close to the ratio 1 : 1, this case never occurs. This can be explained by an electron displacement from the metal towards the nonmetal atoms causing an increase in the radius of the latter. The corresponding bonds would then not have a pure metallic character but would be partially ionic.

The radius values for the carbon and nitrogen atoms are 0.77 Å and 0.71 Å respectively. Because of the comparatively large value for the phosphorus atoms, about 1.06 Å, the metal lattice in a phosphide of "interstitial" structure must be considerably expanded compared with that of analogous carbides and nitrides. The intermetallic distances in VP are 21 % and in MoP 18 % greater than in the corresponding metals, indicating a weakening of the metal bonds. The stability of the compound must be largely due to strong metal/phosphorus bonds (6-coordination) — probably of a partial ionic nature. The interatomic P—P distances follow the variation of the atomic radii of the metals. As a consequence, the nonmetal atoms are partially or entirely isolated from each other in the lattice, both in a geometrical and in a chemical (no covalent bonds) sense. This is also the case for the metallic carbides, nitrides, and oxides.

If the simple hexagonal metal lattice is "filled" with nonmetal atoms, the so-called AlB_2 structure (C 32 type) is formed, common for several diborides [30]: TiB_2, ZrB_2, HfB_2, VB_2, NbB_2, TaB_2, CrB_2, and MoB_2. As $r_B = 0.87$ Å

Table 6. Interatomic distances (in Å) of closest approach in the phosphides. The atomic radii of phosphorus have been calculated on the assumption of non-ionic bonds and metal coordination numbers = 8.

Phase			r_{Me}[34]/ 8-coord.	r_P(calc.)
TiP	Ti — 1Ti 2.91 6P 2.48	P — 6Ti 2.48 6P 3.49 6P 3.54 } 3.51	1.42	1.06
α-ZrP	Zr — 6P 2.64	P — 6Zr 2.64 12P 3.73	1.54	1.10
β-ZrP	Zr — 1Zr 3.13 6P 2.64	P — 6Zr 2.64 6P 3.68 6P 3.78 } 3.73	1.54	1.10
VP	V — 6P 2.41	P — 6V 2.41 6P 3.18 6P 3.61 } 3.39	1.31	1.10
α-NbP	Nb — 4P 2.35 (4Nb+4P) 3.29	P — 4Nb 2.35 4P 3.32 (4P+4Nb) 3.29	(1.43) * 1.28	1.07
β-NbP	Nb — 4P 2.35	P — 4Nb 2.35 4P 3.32 4P 3.30 } 3.31	(1.43) * 1.28	1.07
α-TaP	Ta — 4P 2.35 (4Ta+4P) 3.30	P — 4Ta 2.35 4P 3.32 (4Ta+4P) 3.30	(1.44) * 1.30	1.05
β-TaP	Ta — 4P 2.36	P — 4Ta 2.36 4P 3.33 4P 3.30 } 3.31	(1.44) * 1.30	1.06
CrP	Cr — 2Cr 2.48 2P 2.25 2P 2.27 1P 2.37	P — 2Cr 2.25 2Cr 2.27 } 2.28 1Cr 2.37 2P 3.08 2P 3.11 } 3.10	1.26	1.02
MoP	Mo — 6P 2.46	P — 6Mo 2.46 2P 3.20 6P 3.23 } 3.22	1.36	1.10
WP	W — 2W 2.71 2P 2.38 2P 2.43 1P 2.55	P — 2W 2.38 2W 2.43 } 2.43 1W 2.55 2P 3.18 2P 3.24 } 3.21	1.37	1.06

* As the niobium and the tantalum atoms have 4-coordination in the MeP planes the radii values for this coordination number have been calculated from Pauling's correction factors [34], taking the Born exponent n ~ 9.

and r_P = 1.06 Å the metal-metal distances in the phases VB_2[31] and MoB_2[32] are shorter than in VP and MoP, but still essentially greater than in the corresponding pure metals — for the diborides VB_2 and MoB_2 the increase is 15 % and 13 % respectively. A corresponding comparison between inter-

metallic distances in MoC [3] and Mo, VC [33] and V results in the values 6 % and 8 % respectively.

As has been pointed out above, TiP and β-ZrP phases are isomorphous with MoC (γ'-phase) [3] and NbN (I)[4]. The stacking of the metal atoms can be symbolized by the sequence AABBAABB . . . The phosphorus atoms are situated in the lattice holes so that the highest possible P—P distances are attained. A calculation of the mean intermetallic distances (10-coordination) in these two compounds gives values 17 % and 14 % greater than those observed for the corresponding pure metals. The Nb—Nb distances in NbN (I) are 6 % greater than those found in niobium metal.

Two borides with the same arrangement of metal atoms as in the TiP phase have been found to exist, *viz.* W_2B_5[5] and Ti_2B_5[6]. But in these compounds the boron atoms occupy ten lattice "holes" in the unit cell instead of four [TiP, β-ZrP, NbN (I), MoC (γ')], giving rise to hexagonal two-dimensional boron nets and slightly puckered boron nets, both with boron-boron bonds.

The structure of the NbP and TaP modifications seem to be unique, with two-dimensional metal-phosphorus layers. As metal-nonmetal bonds exist only within such layers and not between them, the separate layers must be bonded together by metallic forces. The interatomic P—P distances are so great as to isolate the phosphorus atoms from each other. The metal-metal distances both within the metal-phosphorus layers (4-coordination) and between them (4-coordination) are 15 % greater than in the pure metals.

The metal atoms in CrP, WP and isomorphous phases of the MnP structure (B 31 type)[23] are surrounded by two metal atoms at "contact" distances and six metal atoms at considerably larger distances. The structure can be regarded as a strongly deformed NiAs type, with the phosphorus atoms located at the centres of deformed metal atom octahedra. The calculated mean values of the intermetallic distances are between 15 and 20 % greater than in the corresponding pure metals.

The structures of the subphosphides of the metals Ti, Zr, Cr, and W are not known, but the symmetry, unit dimensions and cell contents are known for the Cr_3P and W_3P phases. An approximate calculation of the intermetallic distances in these compounds by means of the values obtained for unit cell values or densities of the pure metals, the subphosphides and the monophosphides, indicates a linear expansion of about 5 % (8-coordination) compared with the corresponding metals Cr and W. This corresponds to the observed value of the increase in the intermetallic distance for many metallic carbides, nitrides, and oxides. This agrees with the view that a decrease of the nonmetal content in a compound results in an increase of metallic properties. When the number of nonmetal atoms of a fixed kind is increased, or when the nonmetal atoms in a fixed composition are replaced by other nonmetal atoms with a greater atomic radius, the intermetallic distances will be enlarged and/or the metal-metal coordination number will decrease.

A continuous nonmetal-metal transition takes place in group Vb of the periodic system (N, P, As, Sb, and Bi), N being a nonmetal. As having the properties characteristic for a nonmetal as well as a metal, and Bi being a metal. Intermediate phases MeX_x of corresponding composition, containing a transition metal (Me) and any of the group Vb elements (X), must have a

minimum of metallic character when X = phosphorus. The strong metallic properties of the nitrides are explained by the favourable geometrical conditions for the formation of common "interstitial" compounds.

The increase in the radius of X on going from N to P results in an increase in metal-metal distances, and thus in a weakening of the metal-metal bond strength in lattices of analogous types, *i.e.* with as high coordination numbers as in the pure metals. With further increase of the radius value of X(As→Sb →Bi), it is highly probable that intermediate phases will occur with the Me—Me coordination number smaller than 8. But on the other hand, the Me—X bonds will show a continuous increase in metallic strength. The antimonides and bismuthides can be characterized as intermetallic compounds.

SUMMARY

The Ti—P, Zr—P, V—P, Nb—P, Ta—P, Cr—P, Mo—P, and W—P systems have been investigated by X-ray methods for phosphorus contents up to about 50 atom-%. The structures of all monophosphides have been determined and — with the exception of NbP and TaP — have been found to be of types analogous to those of metallic carbides and nitrides.

TiP and β—ZrP are isomorphous with MoC (γ'-phase) and NbN (I), the metal atoms being arranged in the stacking sequence AABBAABB . . .

α—ZrP ($ZrP_{0.9}$), VP, and MoP possess NaCl, NiAs, and WC structures respectively.

The phases $NbP_{\sim 0.95}$ (α—NbP) and NbP (β—NbP) have analogous tetragonal lattices, both built up of two-dimensional NbP layers, but in the first phase these are disordered relative to each other and in the latter they occupy ordered positions. The ordering leads to a doubling of the c-axis of the unit cell.

The two TaP modifications are isomorphous with the corresponding NbP phases.

The existence of the compounds Cr_3P and Mo_3P of the Fe_3P structure type has been confirmed.

The positions of the atoms in the CrP and WP phases of the MnP structure type have been redetermined.

All the phosphides have greyish black metallic lustre and are markedly resistant to chemical reagents.

The phosphides are compared with the metallic carbides, nitrides, borides, and silicides of the transition elements in a general discussion. They can be characterized as a peripheric group of these metallic compounds, having analogous structure configurations but weaker metal-metal bonds. The metal-phosphorus bonds are strong, probably of a partial ionic nature.

The radius of the phosphorus atom has been found to have a mean value of about 1.06 Å.

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An X-Ray Investigation of the Tantalum-Oxygen System

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The tantalum oxides TaO, Ta_2O_3, TaO_2, and Ta_2O_5 are described in literature. Among these compounds, however, the pentoxide is the only one whose existence has been firmly established. According to Zachariasen [1], the Ta_2O_5 phase is isostructural with the low temperature modification of Nb_2O_5 [2]. There exist two other modifications of the niobium pentoxide: β-Nb_2O_5 and γ-Nb_2O_5 [2]. In a recently published paper by Lagergren and Magnéli [3], these authors point out that Ta_2O_5 irreversibly transforms into a high temperature tantalum pentoxide at a temperature of 1 320° ± 20° C. The pronounced stability of Ta_2O_5 (low-temperature modification) was strikingly demonstrated by the negative result when attempts were made to reduce it in a stream of hydrogen at about 1 250° C.

EXPERIMENTAL WORK

From the data given above it is obvious that the preparation of intermediate tantalum oxides involves experimental difficulties.

Tantalum metal powder (Fansteel), tantalum pentoxide (Kahlbaum, *pro analysi*) and tantalum hydride (Metal Hydrides Inc.) were used as starting materials.

The oxides were prepared by the following methods: *

A. Tantalum metal powder or tantalum hydride was oxidized with steam in the presence of hydrogen in great excess at temperatures varying between 600° C and 900° C, and for periods between one hour and about two days.

B. Tantalum carbides (TaC, Ta_2C) or tantalum nitrides (TaN, Ta_2N) were oxidized with steam as in method A.

C. Tantalum carbide (TaC) was oxidized with steam in the presence of ammonia at 1 100° C for periods varying between one and six hours.

D. Tantalum metal powder was oxidized by means of zinc oxide in evacuated and sealed silica tubes at about 700° C for two weeks, whereupon the zinc metal and remaining zinc oxide were separated from the sample by means of dilute hydrochloric acid.

E. Tantalum pentoxide was reduced with carbon (soot) in graphite or zirconia crucibles in a graphite tube vacuum resistance furnace [4] or a high frequency vacuum induction furnace [5] at about 1 500° C for up to two hours.

* There certainly exist other more suitable methods for the preparation of intermediate oxides in the Ta—Ta_2O_5 interval, *e.g.* reduction of the pentoxide by means of potassium. Methods B, C, and E were originally used for the preparation of ternary Ta—N—O and Ta—C—O phases. This explains the use of these methods here, although they may not at first sight appear suitable for the preparation of oxides.

Some samples were homogenized, either in evacuated and sealed silica tubes at 800° C, or in zirconia crucibles in a vacuum induction furnace at 1 400° C.

The phase analysis and structure determinations were made from powder photographs taken with CuK_α radiation in a camera of the Guinier type, using a curved quartz monochromator. For measurements of the high-angle reflections, additional photographs were taken with focusing cameras of the Hägg-Phragmén type.

No single crystals of any of the new oxide phases could be obtained.

THE STRUCTURES AND COMPOSITIONS OF THE PHASES

Six phases were found to exist in the tantalum-oxygen system, *viz.* Ta(α), Ta_4O(β), TaO(γ), TaO_2(δ), TaO_x with $2 < x < 2.5$ (ε) and Ta_2O_5 (ζ). Several attempts were made to prepare the high temperature modification of the pentoxide reported by Lagergren and Magnéli [3]. The only result was a formation of small amounts of the ε phase, while the greater part of the tantalum pentoxide remained unaltered.

It was not possible to prepare the intermediate oxides in a pure state.

The results of the analysis of the powder photographs of the different specimens are given in Table 1.

α Phase: Ta

For pure tantalum the axial length was determined as $a = 3.311$ Å ($V = 36.30$ Å^3), and this value was found to increase up to $a = 3.341$ Å ($V = 37.29$ Å^3) for samples containing the α and β phases. It was not possible to determine the solubility of oxygen in the tantalum lattice, but the increase in volume makes a value of approximately 5 atom-% probable. This is practically equal to the solubility of oxygen in the niobium metal lattice [2].

β Phase: Ta_4O

The best samples of this bluish black, metallic oxide contained tantalum metal or tantalum pentoxide as impurity. The weight increase after oxidation to Ta_2O_5, as well as the lattice configuration, makes the formula Ta_4O for the upper composition limit very probable.

The diffraction pattern indicated an orthorhombic unit cell closely related to the body-centered α phase lattice. The unit cell dimensions were found to

Table 1. Phases obtained from different methods of preparation.

Preparation method	Phases formed
A	α, β, (ζ)
B *	γ, δ, ε, (ζ)
C *	δ, ε, (ζ)
D	α, β, (ζ)
E *	δ, ε, (ζ)

* Ternary Ta—N—O and Ta—C—O phases were also found to exist. A report of these phases will shortly appear elsewhere.

Table 2. Interatomic distances in the β phase (Ta_4O).

Atom	Surrounding atoms	Interatomic distance, Å	
Ta in 1(*a*)	8 Ta in 2(*d*) 4 Ta in 1(*a*)	2.82 3.21	2.95
Ta in 1(*b*)	8 Ta in 2(*l*) 4 Ta in 1(*b*) 4 O in 1(*h*)	3.04 3.21 2.29	3.10
Ta in 2(*l*)	4 Ta in 1(*a*) 4 Ta in 1(*b*) 4 Ta in 2(*l*) 1 O in 1(*h*)	2.82 3.04 3.21 1.96	3.02
0 in 1(*h*)	2 Ta in 2(*l*) 4 Ta in 1(*b*)	1.96 2.29	2.18

vary between $a = 7.194$ Å, $b = 3.266$ Å, $c = 3.204$ Å ($V = 75.28$ Å)^3 and $a = 7.238$ Å, $b = 3.273$ Å, $c = 3.216$ Å ($V = 76.19$ Å^3). The reflections with $h = 2n + 1$ were very weak, indicating a sublattice with the a axis half of the above value. This sublattice is body-centered, and its relation to the lattice of pure tantalum is shown by the mean axial ratio $a/2 : b : c : a_{Ta} = 1.09 : 0.99 : 0.97 : 1.00$. In spite of prolonged heat-treatment of the samples, it was not possible to obtain sharp reflections.

The most probable configuration is that corresponding to the following atomic positions in the space-group D_{2h}^1-*Pmmm* : 1Ta in 1(*a*) 0,0,0, 1Ta in 1(*b*)$\frac{1}{2}$,0,0 and 2Ta in 2(*l*)$x,\frac{1}{2},\frac{1}{2}$; $\bar{x},\frac{1}{2},\frac{1}{2}$, with x slightly different from 1/4. Good agreement between calculated and observed intensities was obtained, when $x = 0.225$. If the atomic radius of tantalum is assumed to be greater than 1.3 Å, it is not possible to place more than one oxygen atom in the metal lattice of the unit cell, namely in the position 1(*h*) $\frac{1}{2},\frac{1}{2},\frac{1}{2}$ *i. e.* at the centre of a deformed metal atom octahedron. For this reason the most oxygen-rich composition for this phase is presumed to be Ta_4O. The variation of the unit cell dimensions indicate a comparatively broad homogeneity range.

The mean interatomic distances are listed in Table 2.

An oxide analogous to the β phase in the Ta—O system occurs in the V—O system, with the approximate homogeneity range $VO_{0.15-0.25}$[6]. The structure of this phase can be regarded as a tetragonally deformed version of the body-centered vanadium lattice. The present author considers it most likely that the oxygen atom is located at (or in the vicinity of) the centre of a deformed metal atom octahedron in a superlattice containing four metal atoms — in the same way as in the Ta_4O phase. This view is supported by the fact that the upper homogeneity limit of the vanadium suboxide corresponds to the composition V_4O.

γ Phase: TaO

It was only possible to prepare this phase in the presence of fairly great amounts of higher tantalum oxides, by the oxidation with steam of tantalum carbides or nitrides. Carbon and nitrogen analyses of the samples excluded the

presence of these non-metals. The lattice of the γ phase is of the NaCl type. The cube edge was found to vary between $a = 4.422$ Å ($V = 86.45$ Å^3) and $a = 4.439$ Å ($V = 87.45$ Å^3), indicating a narrow homogeneity range.

Each Ta atom in the lattice is surrounded by 12 Ta and 6 O atoms at distances of 3.13 Å and 2.22 Å respectively.

Because of the impurities it was not possible to determine the composition of the γ phase, but it is convenient to give it the ideal formula TaO by analogy with other metallic oxides of the NaCl type, *viz.* TiO, ZrO, HfO, ThO, VO, NbO (defective lattice), UO, NpO, PuO, and AmO.

δ *Phase:* TaO_2

From Table 1 it is evident that the δ phase could be identified in several samples prepared by oxidation as well as by reduction. A fairly pure product was obtained, when TaC was oxidized with steam in the presence of ammonia at 1 100° C for two hours (ε phase occurring as impurity). The diffraction pattern of the black compound indicated the rutile structure (C4 type) [7], making the formula TaO_2 most likely. No diffraction effects indicating a superlattice formation or a lower symmetry than tetragonal were visible in the powder photographs. This is in contrast to the vanadium oxide VO_2, for which Andersson [8] reports a monoclinic structure, closely related to that of the tetragonal sublattice.

The unit cell dimensions were invariably found to be $a = 4.709$ Å, $c = 3.065$ Å, $c/a = 0.651$ ($V = 67.96$ Å^3).

The calculated density is 10.41. The observed value was 9.95.

The space-group is D_{4h}^{14}—$P4/mnm$. The tantalum ions are located in the two-fold position 2(a) 0,0,0; $\frac{1}{2},\frac{1}{2},\frac{1}{2}$, and the oxygen ions in the four-fold position 4(f) $\pm$ (x, x, 0; $\frac{1}{2} + x$, $\frac{1}{2}-x,\frac{1}{2}$). As it is not possible to determine the value of the parameter x from X-ray data, this is calculated from the formula $8x = 2 + (c/a)^2$, which gives the best space distribution. This formula gives $x = 0.303$.

Interatomic distances are listed in Table 3.

The shortest 0—0 distance is 2.62 Å, giving $r_0 2-$ = 1.31 Å. The Ta—O distances are 2.02 Å, hence $r_{Ta} 4+$ = 0.71 Å. Goldschmidt's radius value for the Ta^{5+} ion is 0.68 Å (6-coordination). If the expression $r_z = (n\,B/A\,z^2)^{1/n-1}$ given by Pauling [9] for the calculation of the radius r_z of an ion of valency z is used in this case, and the Born exponent [9] taken to be $n \sim 9$, the value $r_{Ta} 4+$ = 0.72 Å is obtained.

Table 3. Interatomic distances in the δ phase (TaO_2).

Atom	Surrounding atoms	Interatomic distance, Å
Ta	2 Ta	3.07
	8 Ta	3.67
	6 O	2.02
O	1 O	2.62
	8 O	2.86
	3 Ta	2.02

It may be of interest to note that the tantalum ions in the TaO_2 phase can be replaced by titanium and vanadium ions. The metals (Ta, Ti, V) and metal oxides (Ta_2O_5, TiO_2, V_2O_5) were carefully mixed in the proportions corresponding to the gross compositions (Ta, Ti)O_2 and (Ta, V)O_2 respectively, moulded into small brickettes and heated in thoria crucibles in a vacuum induction furnace up to about 1 800° C. The Ta/(Ti, V)ratio was varied in steps of 0.2, 0.4, 0.6, and 0.8. As indicated by the diffuse reflections in the powder photographs, it was not possible to reach equilibrium. Other oxides occurred as impurities.

The unit cell dimensions of the (Ta, Ti)O_2 samples were found to vary continuously between those of TiO_2 (rutile): $a = 4.589$ Å, $c = 2.960$ Å, $c/a = 0.645$ ($V = 62.34$ Å^3) and those of TaO_2: $a = 4.709$ Å, $c = 3.065$ Å, $c/a = 0.651$ ($V = 67.96$ Å^3). It is therefore very probable that a continuous solid solution series TiO_2—TaO_2 exists.

The solubility of VO_2 in TaO_2 was found to be comparatively small, the unit cell dimensions of the (Ta, V)O_2 phase varying between $a = 4.715$ Å, $c = 3.046$ Å, $c/a = 0.646$ ($V = 67.27$ Å^3) and those obtained for pure TaO_2.

ε Phase

The powder photograph analysis showed that the black ε-phase exists as an intermediate oxide in the TaO_2—Ta_2O_5 interval. The line distribution in the diffraction pattern indicates a complicated structure, neither related to that of TaO_2 nor to that of Ta_2O_5.

ζ Phase: Ta_2O_5

According to Zachariasen [1], Ta_2O_5 is isomorphous with α-Nb_2O_5 [5].

SUMMARY

Six phases have been found to exist in the tantalum-oxygen system.

The *α phase* (Ta) dissolves up to about 5 atom-% of oxygen in the body-centered metal lattice.

The *β phase* has a metal atom arrangement obtained by an orthorhombic deformation of the α-phase lattice. The upper phase limit corresponds to the formula Ta_4O. The metallic oxide has a bluish black colour.

The *γ phase* has the probable composition TaO, and is of the sodium chloride structure.

The black *δ phase* with the formula TaO_2 is isomorphous with rutile. It forms a complete series of solid solutions with TiO_2 (rutile).

The black *ε phase* exists as an intermediate oxide in the TaO_2—Ta_2O_5 interval. The structure seems to be complicated.

No high temperature modification of the ζ phase (Ta_2O_5) has been found to exist.

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Micro Detection of Some Elements in Organic Compounds by a Modified Emich's Method

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Bennett, Gould, Swift, and Niemann [1] have presented a system for the rapid detection of nitrogen, chlorine, bromine, iodine, arsenic, sulfur and phosphorus in a single 1 mg sample of an organic compound. The method is a development of Johns [2] modification of Emich's [3] test. In the analysis the sample is combusted in a small Pyrex tube with a mixture of calcium oxide and zinc, whereby nitrogen compounds evolve ammonia. The other elements are bound to the calcium oxide-zinc mixture. Water dissolves only the halides; hydrogen sulfide and arsine are then removed by addition of perchloric acid, and phosphate is detected in the residual perchloric acid solution.

During work on a modified Lassaigne method [4] certain substances were also tested by Emich's method [1]. Analysis by the two methods could be performed simultaneously which is found to be more advantageous than performing a double test by one method. A complete test for all the elements given in the scheme can, with use of a minimum of equipment, be performed in an hour and, due to some time consuming operations in both methods (centrifugation *etc.*), the time is not significantly increased when a Lassaigne's test is carried out simultaneously.

Analysis by the method of Bennett *et al.* is superior to earlier methods. The author has, however, noted some objections which suggest some changes in the analytical scheme. The four main points are:

1. In the combustion of some nitrogen compounds cyanide as well as ammonia is formed. The disturbing cyanide is dissolved by water but can be boiled off with sulfuric acid before the precipitation of the silver halides.

2. It is possible to detect simultaneously the presence of chlorine, bromine and iodine using an ammonia-lead(IV) oxide method [4].

3. The small sulfur content of the combustion material (of best qualities) often gives a false sulfur reaction especially when water-containing compounds are analysed.

4. The hydrogen sulfide and arsine are more distinctly separated when the evolved gases are taken up in a double layer gel tube impregnated with lead acetate and silver nitrate [4].



Fig. 1. (Scale 1 : 1). Sealed combustion tube (Corning pyrex) a) asbestos plug, b) calcium oxide — zinc mixture.

EXPERIMENTAL PART

Combustion and detection of nitrogen

A large number of sealed combustion tubes [1] (Fig. 1) can conveniently be prepared at one time and stored for future work. No change in activity has been noted after more than a year.

For the analysis both ends (only the tip of the short arm) are cut off. The sample (1—5 mg) is added. Liquids are drawn up by the packed CaO—Zn column from a long capillary and solids are just shaken down through the tube. The long arm is sealed and the combustion can with some experience be carried out in an ordinary micro burner. The evolved gas is taken up upon a wet activated litmus * paper [1], which must be observed during the heating to detect the ammonia from difficultly combusted nitrogen compounds, as these colorations are of short duration.

Detection of halogen

After the combustion the long arm is opened, water [1] is added to obtain a 3 cm high column upon the combustion mixture. The tube is centrifuged — without sealing the short arm — in a test tube (75 × 10 mm). The short arm is washed with a few drops of water and adhering water is transferred to the test tube to give a total volume of about 1/4 ml. Three drops of sulfuric acid (1 : 3) are added and the liquid is boiled for 5 minutes to remove hydrogen cyanide. One drop of 0.5 *M* $AgNO_3$ precipitates the halides ** which can be separated by an ammonia-lead(IV) oxide method [4].

Detection of sulfur and arsenic

The wet calcium oxide-zinc mixture is pushed into the bottom of a test tube (75 × 10 mm) with a stainless wire. The tube is held horizontally and 3 drops of concentrated perchloric acid are placed in the middle of the tube. The acid must not touch the combustion mixture or the opening of the tube. The tube is closed with a rubber plug containing a two-layer gel tube [4]. The under layer is impregnated with lead acetate and the upper with silver nitrate. The tube is raised to mix the combustion mixture and the acid. After a minute the tube is heated for a few seconds in a flame. The gel tube is removed after a further two minutes. Hydrogen sulfide gives a black zone in the lead acetate layer. (Sensitivity: over all 10 μg S; gel tube 1 μg.) Arsine (and phosphine, only from trivalent or elementary phosphorus) gives a black zone with a yellow top in the silver nitrate layer. (Sensitivity: over all 5 μg As; gel tube 1 μg.)

Detection of phosphorus

The residue in the test tube is treated with a few drops of water. One drop of the solution is drawn up from a corner of a molybdate paper [1]. After drying, the paper is treated with benzidine and sodium acetate solutions. A blue spot with a stronger coloured edge indicates the presence of phosphate.

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* Ordinary red litmus is sufficient when the evolved ammonia is >10 μg N, taken as H_4NCl.

** Silver sulfate sometimes precipitates, but is dissolved by a few drops of water.

DISCUSSION AND RESULTS

Detection of nitrogen

In the analysis of the compounds tabulated in the earlier work [4] it was found that some nitrogen compounds gave litmus colours difficult to distinguish from stronger blanks (5 μl water or xylene). Examples of such compounds are: diphenylamine, *p*-diazobenzenesulfonic acid, phenylbenzoyldiazomethane, triazobenzene, 2,4-dimethyl-3,5-dicarbetoxypyrrole, 2-phenylindole, and kynurenic acid.

The amount of ammonia evolved in these cases was smaller than that from azobenzene, which according to Bennett *et al.*[1] is equal to 5—10 μg N as NH_4Cl. However, in no case, did the test fail completely, when activated papers were observed during the combustion. Test samples of over 5 mg should not be used as fumes of uncombusted material make it difficult to observe the litmus papers.

Brown and Hoffpaniere [5] report that triphenyl phosphate gives a false litmus reaction with Emich's test. In the present investigation triphenyl phosphate gave no litmus colour, and a negative Lassaigne test; the tricresyl phosphate used was contaminated with nitrogen compounds which were detected by both methods.

Detection of halogen

The aqueous extracts of the combustion mixture from the nitrogen compounds ($\sim$ 5 mg) given in Table 1 have been tested for cyanide. Each solution was boiled for 5 minutes with sulfuric acid in a test tube closed with an alkaline iron (II) sulfate paper [4]. The paper was then treated with dilute sulfuric acid to convert cyanide to Prussian blue which gave a blue spot on the paper. The

Table 1. Nitrogen compounds investigated for formation of cyanide. The Prussian blue reactions have been compared with reactions from sodium cyanide solutions of known strength.

n-Propylamine	20 μg CN	Phenylhydrazine	20 μg CN
Diethylamine	20	Azobenzene	1
Tetramethylammonium iodide	50	Methylorange	30
Aniline	5	Phenylbenzoyldiazomethane	0
vic-Xylidine	5	*p*-Diazobenzenesulfonic acid	10
Mesidine	5	Triazobenzene	40
Diphenylamine	0	Pyrrole	40
Triphenylamine	1	N-Phenylpyrrole	10
Bis-(2-aminothiazole)	50	2,4-Dimethyl-3,5-dicarbethoxypyrrole	0
Acetamide	40	Indole	20
Acetanilide	20	2-Phenylindole	0
Chloropicrin	50	Imidazole	5
Nitromethane	30	Benzotriazole	0
Nitrobenzene	0	2-Methylbenzothiazole	10
Trinitrotoluene	40	Pyridine	0
iso-Amylnitrite	10	Nicotinic acid	30
Dimethylglyoxime	50	α,α'-Dipyridyl	10
Acetonitrile	50	Quinoline	1
Benzonitrile	5	Acridine	0
Phenyl-*iso*-thiocyanate	0	Uric acid	50
Ethylthiocyanate	50	Piperazine	10
Dimethylketazine	30		

spots of Prussian blue have been compared with spots obtained from sodium cyanide of known strength, giving a rough estimation of the amount of cyanide formed during the combustion. Amounts of cyanide less than 10 μg give no visible precipitate with $AgNO_3$ and are thus without practical importance. Different forms of combustion do not seem to influence the formation of cyanide.

Because of the disturbing formation of cyanide, the scheme of Bennett *et al.* for halogens has been changed. The proposed scheme is similar to the one used in the Lassaigne work. Nitric acid has also been used to acidify the solution prior to boiling off the hydrogen cyanide, but in this case iodine is also removed from the solution *.

The amount of precipitated silver halides from an Emich combustion with a given amount of halogen compound is smaller than from a Lassaigne test. The halides appear to be incompletely dissolved during the centrifugation and the combustion is incomplete. It sometimes happens that the silver precipitate is too small to be separated into its components.

Fluorine is not included in this system and attempts to extend the scheme to this element have been without success. Moreover, the chlorine in the inert, low-boiling fluorocompounds, *e. g.* freones, cannot always be detected.

Detection of sulfur

The detection of sulfur can be considered as the weakest point in this analytical method because of the occurrence of false sulfur reactions. The use of gel tubes [6] makes it possible to estimate the sulfur content roughly, thus reducing the number of false blanks. In a series of about 200 analyses there have been 26 false reactions. Of these 18 have been so weak (< 10 μg S) that it has been possible to distinguish them from real reactions. Of the remainder 4 were equivalent to 50 μg S.

It has not been possible to settle the cause of these reactions, but the small sulfur content of the glass seems to be the most likely source of sulfur. In blank tests there are more sulfur reactions when water is combusted than with xylene and there has been no distinct increase in sulfur reaction with increased amounts of zinc and calcium oxide. The calcium oxide is prepared by igniting oxalate, containing 0.001 % sulfate. Each tube therefore contains about 0.2 μg S which is below the sensitivity of the gel tube.

Detection of arsenic

The detection of arsine is simplified by the use of gel tubes. Even 1 μg As can be detected distinctly in the presence of hydrogen sulfide. With large amounts of sulfur (> 200 μg S) the whole $PbAc_2$ column is consumed and black Ag_2S is formed in the $AgNO_3$ layer. If the gel tube is removed before this occurs arsine can then be detected.

The 80-mesh zinc (Baker, lot 22 845) used in this investigation contains a little arsenic but no other disturbing elements, in contrast to "anal. pure"

* The iodine can mostly be detected with the aid of a strip of starch paper inserted in the opening of the tube.

zinc dust which generally contains nitrogen and often chlorine. The blanks often form a small yellow zone (< 1 μg As), and very occasionally a zone with a hardly visible black bottom (1 μg As). However, this causes no trouble as it is possible to estimate the amount of black arsine complex in the gel tube. The 40-mesh zinc, free from arsenic, is too coarse to be used.

On combustion of trivalent and elementary phosphorus, phosphides are formed which evolve phosphine on the addition of perchloric acid. Phosphine gives the same reaction as arsine, but is less sensitive. The blackened gel particles in the tube can then be analysed for arsenic by testing with sulfide [4].

Detection of phosphorus

The sensitivity of the phosphate test is very high (< 1 μg) and in no case was phosphorus undetected. Even when elementary or tri-valent phosphorus is combusted there is still sufficient phosphate formed to be clearly indicated.

Of the arsenic compounds tested none has given a positive phosphate test and by use of properly prepared reagents the blanks are very weak in colour. Small amounts of silicate (from the glass) do not interfere as the blue spot from the silicate-molybdate-benzidine reaction is devoid of a sharp blue edge.

SUMMARY

The system presented by Bennett *et al.* for a rapid micro detection of As, Br, Cl, I, N, P, and S in organic compounds has been checked and some changes have been proposed. It is found that:

1) Disturbing cyanide is formed on combustion of some nitrogen compounds. The cyanide can be boiled off before the precipitation of the silver halides.

2) The simultaneous presence of Cl, Br, and I can be detected using an NH_3-PbO_2-method.

3) False sulfur reactions are often obtained.

4) H_2S and AsH_3 are more distinctly separated when taken up in a double layer gel tube impregnated with $PbAc_2$ and $AgNO_3$.

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Chromatographic Separation of Anomeric Glycosides

II. New Crystalline Methylfuranosides of Galactose, Arabinose and Xylose

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In Part I of this series Augestad, Berner and Weigner [1] gave a preliminary report on the chromatographic separation of isomeric glycosides on columns of powdered cellulose. It was found that by using a suitable mixture of solvents not only ring-isomeric but even anomeric glycosides could be separated in a single operation. Previously Hough, Jones, and Wadman [2] had reported the separation of α- and β-methyl-L-rhamnopyranosides by partition chromatography on a column of "hydrocellulose". As the methylpyranosides of the common sugars are known in the pure state, the investigation was concentrated on the isolation of such methylfuranosides which have not as yet been obtained in crystalline form. In Part I the results obtained with D-fructose and D-galactose were briefly described. The β-methylfructofuranoside which was isolated as a thick syrup having a specific rotation in water of —46.9° has still not crystallized. As analysis showed the purity to be only 97 % further purification is now being attempted. In this report details are given of the isolation of the crystalline methylfuranosides of D-galactose, D- and L-arabinose, and D-xylose, of which the following are new crystalline substances:

α-Methyl-D-galactofuranoside,	m. p.	91—92°,	$[\alpha]_D^{20}$ =	+104° (in water)
α-Methyl-D-xylofuranoside	»	84°	»	+182° (» »)
β-Methyl-L-arabofuranoside	»	58°	»	+118° (» »)
β-Methyl-D-arabofuranoside	»	56—57°	»	—119° (» »)

The general procedure, details of which will be found in the experimental part, can briefly be summarized as follows. The process of glycosidation was first studied in order to find the optimal conditions for the formation of furanosides. Generally it was found that boiling a methanolic solution containing very little hydrogen chloride gave the best results. The process was followed by measuring the rotation and the amount of free sugar, and by taking paper chromatograms using the known methylpyranosides of each sugar as reference substances. The chromatographic examination at the same time enabled the solvent or solvent-mixture best suited for the subsequent

separation of the glycosides on the cellulose column to be found. Having removed the hydrogen chloride by means of silver carbonate and the methanol by evaporation a syrup was obtained, a concentrated solution of which was put directly on top of a column of powdered cellulose (Whatman, Standard Grade). The eluant was taken up in an automatic fraction-collector as described by Hough, Jones and Wadman [3]. Polarimetric readings permitted a curve to be drawn showing the main distribution of the isomeric glycosides in the fractions. In order to be able to collect those fractions which contained one glycoside only chromatographic examinations were taken on the border between subsequently eluted glycosides. From the fractions containing practically one single glycoside a syrup was obtained which in several cases crystallized spontaneously on standing for a shorter or longer time, eventually at low temperature. In repeated experiments when crystals were available for seeding crystallization took place rapidly. The glycosides were recrystallized from dry ethyl acetate.

EXPERIMENTAL

Methyl-D-galactosides

Haworth *et al.*[4,5] first prepared syrupy mixtures rich in methyl-galactofuranosides by glycosidation in methanol containing hydrochloric or sulphuric acid. Whether the reaction was carried out at room temperature or by boiling, the syrup obtained had a specific rotation of about −50°. The syrup was methylated to tetramethylmethylgalactoside the ring structure of which was proved to be that of a furanoside.

Green and Pacsu [6] prepared crystalline β-methyl-D-galactofuranoside, m. p. 63−65°, $[\alpha]_D = -108°$, by treatment of galactose-ethylmercaptal with methanol in the presence of mercuric chloride and oxide.

In our experiments a syrup rich in galactofuranosides was prepared by boling, for 6 hours, a methanolic solution containing 2 % D-galactose (C. P. quality, Pfanstiehl) and 0.013 % HCl (0.0037 *N*) when a maximum of levorotation (−44°) was reached. The solution then showed no reducing power. The hydrogen chloride was neutralized with silver carbonate. A little discoloration by silver salt was removed by heating with charcoal. On evaporating the filtered solution a completely colourless syrup was obtained. Chromatographic examinations on paper showed that a mixture of ethyl acetate, propanol-1, and water (5 : 3 : 2) gave a good separation. α-Methylgalactopyranoside prepared according to Michel and Litman [7] and β-pyranoside prepared according to Fischer [8] were used as reference substances. The paper chromatograms revealed that the syrup contained mainly furanosides, a small amount of α-pyranoside and very little β-pyranoside.

The syrup (3.5 g), diluted with a little of the eluant, was put at the top of a column (56 × 3.5 cm) of powdered cellulose and covered with a layer of cellulose. The mixture mentioned above served as mobile phase. The eluate left the column at a rate of 80 ml per hour and was collected automatically in fractions of 10 ml. After 6 hours the eluate aquired a negative rotation. The readings of the subsequent fractions were plotted against the number of the fractions as shown in Fig. 1.

Chromatographic examinations indicated where to divide the series of fractions in order to obtain pure substances. Fractions 1−12 gave 1.85 g (53.5 % of original syrup) syrup of β-furanoside, fractions 19−29 0.50 g (14.2 %) syrup of α-furanoside and fractions 34−60 0.37 g (10.8 %) syrup of α-pyranoside. The fractions containing the α-pyranoside were not worked up.

Crystalline α-methyl-D-galactofuranoside: The syrup from fractions 19−29 did not crystallize when left for a week in a vacuum desiccator. It was therefore dissolved in warm ethyl acetate and the solution placed in a refrigerator. Over night crystals (0.4 g) had developed which were recrystallized from 25 ml dry and alcohol-free ethyl acetate, m.p. 91−92°, $[\alpha]_D^{20} = +104°$ ($c = 1$ in water) and $[\alpha]_D^{20} = +101°$ ($c = 2$ in methanol). A final chromatographic examination gave only one spot. By hydrolysis at room tem-

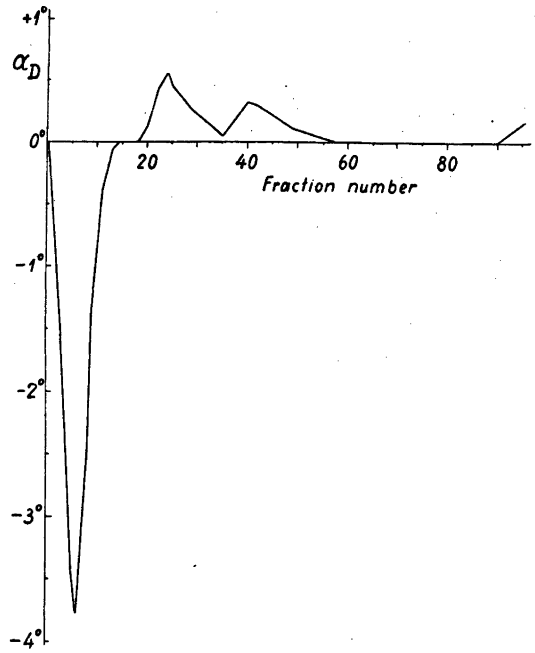

Fig. 1. Chromatographic separation of methylgalactosides: optical rotation of the fractions.

perature of a 0.5 % solution in 1.1 *N* HCl the time of half life was found to be $6^3/_4$ hours. The determination of free galactose was made by Shaffer-Hartmann's method, using the table given by Schoorl [9].

*Crystalline β-methyl-*D*-galactofuranoside:* The syrup from fractions 1—12 crystallized spontaneously when kept at −5° for a fortnight. After recrystallization from ethyl acetate the compound had, m.p. 69°, $[\alpha]_D^{20} = -112°$ ($c = 3$ in water), $[\alpha]_D^{20} = -140°$ ($c = 2.5$ in methanol).

The syrup from fractions 61—81 crystallized spontaneously overnight, and the β-methyl-D-galactopyranoside thus obtained after recrystallization had m. p. 178° and practically no optical rotation.

Methyl-D-xylosides

Haworth and Westgarth [10] by treatment of xylose at room temperature with methanol containing 1 % HCl obtained a liquid mixture of methylxylofuranosides having a specific rotation of +62°. We prepared the raw-material for the chromatographic separation by boiling, for $5^3/_4$ hours, a methanolic solution containing 2 % D-xylose (C. P. quality, Pfanstiehl) and 0.012 % HCl (0.0036 *N*). By this treatment 99 % of the sugar had reacted and the syrup isolated as described for galactose had $[\alpha]_D = +32°$.

Paper chromatograms of the syrup showed that the use of methyl ethyl ketone saturated with water as mobile phase led to a good separation of the xylosides. This solvent was therefore also used for the separation on the column. All four methyl xylosides were found to be present, the β-pyranoside, however, in minor quantity. The α- and β-methylpyranosides used for comparison were prepared according to Hudson [11] and Fischer [8] respectively. Starting with 2.3 g syrup the separation on a column of cellulose was carried out as described above. The curve for the rotation of the fractions showed that also in

this case the β-furanoside was eluted first; then followed the α-furanoside and after this the α-pyranoside. The elution was not continued until the small amount of β-pyranoside present appeared.

From fractions 1—15 the β-methyl-D-xylofuranoside (1.03 g or 45 % of original syrup) was obtained as a clear syrup having $[\alpha]_D = -82°$ in water. A paper chromatogram showed that one sugar derivative only was present, but the syrup has as yet not crystallized.*

Crystalline α-methyl-D-xylofuranoside: From fractions 21—34 a syrup (0.51 g or 22 %) was isolated which crystallized on standing overnight in a vacuum desiccator. Recrystallized from ethyl acetate it had, m. p. 84°, $[\alpha]_D^{20} = +182°$ ($c = 4$ in water). A chromatographic control gave a single spot only. The α-methylfuranoside was very easily hydrolyzable. For a 0.5 % solution in 1.06 *N* HCl the time of half life was 45 min. at room temperature.

The fractions 35—60 gave 0.39 g (17 %) syrup which crystallized in the course of a week. Recrystallized from ethyl acetate it had, m. p. 80—82°, $[\alpha]_D^{20} = +137°$ ($c = 1.2$ in water). A chromatogram showed that it was an impure α-methylpyranoside.

Methyl-L-arabinosides

Baker and Haworth [12] prepared a syrup rich in methyl-L-arabofuranosides by letting the sugar react for 17 hours at room temperature with methanol containing 2 % HCl. The syrup after having been distilled at 173—175° (0.15 mm) had $[\alpha]_D = -71.3°$ (in methanol). The rotation decreased in six months to −51.9°. This may have been due to a contamination by a small quantity of hydrogen chloride. We have found that in order to obtain stable syrups considerable attention must be paid to the removal of the hydrogen chloride.

Crystalline α-methyl-L-arabofuranoside, $[\alpha]_D = -125°$ (in water) was prepared by Green and Pacsu [6] from L-arabinoseethylmercaptal. The melting point was not determined owing to the strongly hygroscopic nature of the substance.

The α- and β-methylpyranosides of L-arabinose necessary as reference substances in our experiments were prepared according to Hudson [11].

A solution of 7 g L-arabinose (E. Merck) in 250 ml methanol containing 0.036 % HCl (0.01 *N*) was boiled for 2 h 50 min. when maximum of negative rotation ($[\alpha]_D = -48°$) was reached. The syrup was isolated as described above. Again methyl ethyl ketone saturated with water was found to be an effective mobile phase, the glycosides being eluted from the column in the order: α-furanoside, β-furanoside, β-pyranoside, and α-pyranoside. Starting with 3.3 g syrup and eluting at a rate of 200 ml per hour, the first rotation was observed after 4 hours. Fractions 1—20 gave 1.84 g (55.8 %) of levorotatory α-furanoside and fractions 37—84 0.71 g (21.5 %) of dextrorotatory β-furanoside, both as colourless syrups. Fraction 90 on chromatographic examination, besides the spot of the β-furanoside, showed a weak spot which did not coincide with those of the pyranosides and therefore obviously was due to an impurity present in the L-arabinose used. Fractions 85—110 on evaporation yielded a small quantity of crystalline β-methyl-L-arabopyranoside having m. p. 169°.

Crystalline β-methyl-L-arabofuranoside: The syrup from fractions 37—84 was dissolved in 100 parts of warm ethyl acetate and the solution placed in a refrigerator. As no crystals had appeared in four weeks one half of the solvent was distilled off. After standing a

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* *Added in proof:* The syrupy β-furanoside was later separated on a column in two nearly equal fractions having the specific rotations −85° and −87.2° respectively. The latter fraction was dissolved in 50 ml dry ethyl acetate of which 35 ml were distilled off. Placing the concentrated solution at −20° for six weeks and scratching with a glass rod the furanoside crystallized. Also the first fraction then crystallized on inoculation. After recrystallization from ethyl acetate and from ether the *β-methyl-D-xylofuranoside* had m.p. 45°, $[\alpha]_D^{21} = -89.5°$ ($c = 4.5$ in water). On hydrolyzing at room temperature with 1.09 *N* HCl the time of half life was 80 min.

further week in the refrigerator needles had been formed. Another sample of syrupy β-furanoside which had been kept for some time in a vacuum desiccator over phosphorus pentoxide crystallized overnight when inoculated with the needles. The crystals were extremely hygroscopic and necessary precautions had to be taken in handling them. After recrystallization from ethyl acetate they had, m.p. 58°, $[\alpha]_D^{20} = +118°$ ($c = 2.2$ in water). The hydrolysis of the β-furanoside in 1 *N* HCl proceeded at room temperature with a time of half life of 4 ¾ hours.

Crystalline α-methyl-L-arabofuranoside: The syrup from fractions 1—20 was kept in a desiccator for one month without crystallizing. On boiling with dry ether some of it dissolved. The remainder was dissolved in warm ethyl acetate but no crystals were formed on keeping the solution in a refrigerator for a fortnight. One half of the solvent was therefore distilled off whereupon some syrup separated which crystallized rapidly on standing in the refrigerator. The syrup recovered from the ethereal solution also crystallized on seeding. Owing to the strongly hygroscopic nature of the substance the same precautions had to be taken as in handling the β-furanoside. After recrystallization from ethyl acetate it had, m. p. 52°, $[\alpha]_D^{20} = -128°$ ($c = 4.7$ in water).

Methyl-D-arabinosides

Crystalline α-methyl-D-arabofuranoside was prepared by Montgomery and Hudson [18]. They treated the sugar with methanolic hydrogen chloride (0.2 *N*) at room temperature and stopped the reaction after 17 hours when a maximum of rotation of +45° had been observed. The crystalline α-furanoside which was described as quite hygroscopic had m. p. 65—67° (closed tube) and $[\alpha]_D = +123°$ ($c = 1.2$ in water).

In the present case a syrup rich in furanosides was prepared by boiling a solution of 6.7 g D-arabinose (C. P. quality, Pfanstiehl) in 250 ml methanol containing 0.036 % HCl (0.01 *N*) until a maximum specific rotation of +43.7° had been reached in 1 h 40 min. Analysis showed that 99.2 % of the sugar had reacted. The isolation of the syrup was carried out as described above.

The methylpyranosides used as reference substances were prepared as described by Hudson [11] for the corresponding derivatives of L-arabinose.

In the column chromatography of 3.5 g syrup methyl ethyl ketone saturated with water was used as mobile phase. The glycosides were eluted in the same sequence as in the case of L-arabinosides, the only difference being the reversed sign of rotation. Fractions 2—28 gave 1.78 g (51 %) of dextrorotatory α-furanoside and fractions 51—94 0.67 g (19.2 %) of levorotatory β-furanoside, both as syrups. From fraction 124 a small amount of crystalline β-methyl-D-arabopyranoside was obtained having m. p. 170° and a specific rotation in water of about −240°.

Crystalline β-methyl-D-arabofuranoside: 0.4 g of the syrup from fractions 51—94 was boiled with 30 ml dry ether. The undissolved part was dissolved in 30 ml ethyl acetate 25 ml of which were afterwards distilled off. The remaining mixture of syrup and solution crystallized on standing over night in a refrigerator. The hygroscopic crystals were rapidly collected and dried over phosphorus pentoxide. M. p. 56—57°, $[\alpha]_D^{20} = -119°$ ($c = 2.6$ in water). By hydrolysis at room temperature of a 0.65 % solution of the β-furanoside in 1 *N* HCl the time of half life was found to be 3 hours.

Crystalline α-methyl-D-arabofuranoside: The syrup from fractions 2—28 crystallized spontaneously when left for a couple of months in a desiccator over phosphorus pentoxide. Recrystallized from ethyl acetate, they formed needles, m. p. 52°, $[\alpha]_D^{20} = +128°$ ($c = 4$ in water); the same value was found for $c = 1.2$ in water. On recrystallization from dry ether the same kind of needles having m. p. 52° were obtained. As mentioned above Montgomery and Hudson found the m. p. 65—67°. They described their preparation as well formed transparent prisms, and it therefore seems probable that we have obtained a dimorphic form. It should be remarked that in the case of the enanthiomorphic form, the α-methyl-L-arabofuranoside, we also obtained needles with the same melting point (52°).

SUMMARY

Chromatography on a column of powdered cellulose using an appropriate solvent or solvent-mixture as mobile phase was found to give a good separation of isomeric glycosides. By this method the following new crystalline methyl-furanosides have been prepared: α-methyl-D-galactofuranoside, α-methyl-D-xylofuranoside, β-methyl-L-arabofuranoside, and β-methyl-D-arabofuranoside.

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The Influence of the Solvent on Reaction Velocity

IV.* Alkaline Hydrolysis of Ethyl Propionate and Ethyl Butyrate in Ethanol-Water Mixtures

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Comparison of results concerning the alkaline hydrolysis of ethyl acetate [1], ethyl benzoate [2], and benzyl acetate [2] in various mixed solvents reveals that the solvent effects show the same general picture irrespective of the different acid or alcoholic components in the esters. This being found, it seemed desirable, in order to get more detailed information in the matter, to study the reaction with the ethyl esters of the other simple fatty acids, the reaction rate being more sensitive to substitution of a CH_2 group in the acid than in the alcohol radical [3,4]. The present paper contributes certain data concerning ethyl propionate and ethyl butyrate, a series of mixtures of ethyl alcohol and water being used as solvent.

EXPERIMENTAL

Chemicals. The esters were prepared from the acids and ethyl alcohol [5]. The acids were commercial products of high purity. The esters, purified by repeated fractional distillation, had the following boiling points: ethyl propionate 99.4—99.7° (777 mm), ethyl butyrate 121.1—121.5° C.

The ethanol used in the kinetic experiments was commercial absolute ethanol, but purified by Walden's [6] method. The water was always freshly distilled.

Method. The alcohol-water mixtures and the solutions of ester and NaOH were prepared in the way described previously [1]. The concentration of the ester was always checked by total hydrolysis and then adjusted to the exact value. Both ester and sodium hydroxide were used in equal concentrations. For ethyl propionate 0.1 *M*, for ethyl butyrate, owing to its poorer solubility, 0.05 *M* solutions were used, so that, after mixing equal amounts (5 ml), the initial concentrations of each were 0.05 or 0.025 *M*. The fact that the experiments were carried out with different initial concentrations is not significant since the initial concentration has practically no influence upon the rate constants [7]. The reactions were carried out in vessels designed for volatile reagents and described previously [8]. The reaction was stopped by an excess of 0.02 or 0.01 *N* HCl, and the acid titrated with 0.02 or 0.01 *N* baryta solution, cresol red being used as indicator.

Since the object of the experiments was to measure the activation energies as accurately as possible, the preparation of the solvent and all other operations were carefully standardised. All the measurements were made by one of the authors (S.H.) in order to eliminate personal factors as fully as possible. Each solvent was prepared in a quantity

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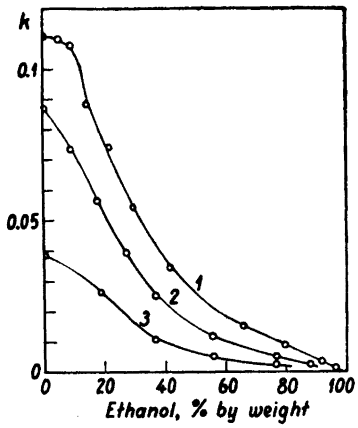


Fig. 1. Variation of the rate constant with the percentage of ethanol in the solvent at 25°. 1. Ethyl acetate, 2. Ethyl propionate, 3. Ethyl butyrate.

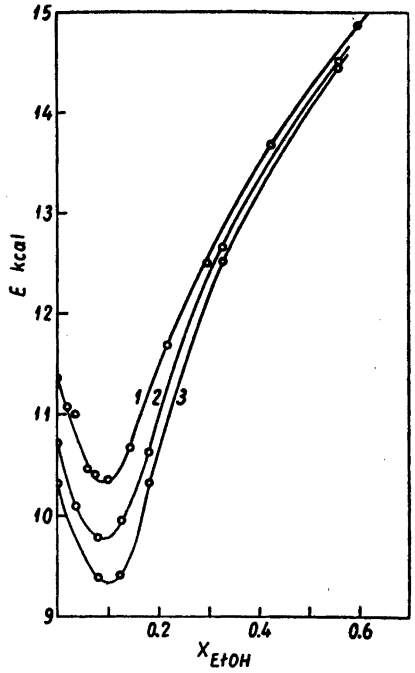


Fig. 2. Graphs to show the correlation between E and the mole fraction of ethanol in the solvent. 1. Ethyl acetate, 2. Ethyl propionate, 3. Ethyl butyrate.

sufficient for all experiments with this solvent composition. This is very important since the kinetic constants are very sensitive to the composition of the solvent. The number of observations in each run was at least seven, in most cases, however, much larger. The present results are used in direct comparison with those previously obtained for ethyl acetate in ethanol-water.

Second order kinetics were observed in all cases. The values of the rate constants obtained by the usual formula $dx/dt = k(a-x)^2$ were averaged and corrected for change of concentration due to thermal expansion of the solvent.

Table 1. Alkaline hydrolysis of ethyl propionate.

H_2O vol. %	EtOH wt. %	x_{H_2O}	10^3k liter mole^{-1} sec.$^{-1}$							E cal.	log A	ΔS^* E.U.
			0.00°	15.00°	20.00°	25.00°	35.00°	40.00°	50.00°			
100	0	1.000	15.4	46.4	66.9	87.0	133	191		10 720	6.780	−29.5
90	8.7	0.964	14.6	40.9		73.9		157		10 090	6.252	−31.9
80	17.8	0.922	12.2	32.5		56.7		122		9 770	5.912	−33.5
70	27.0	0.874	8.15	22.8		39.5		84.8		9 940	5.876	−33.6
60	36.3	0.818	4.76	13.5		25.0		57.9		10 620	6.177	−32.3
40	55.7	0.670	1.55	5.50		11.6		29.3		12 650	7.317	−27.0
20	76.5	0.440	0.538	2.27		5.29		16.3	30.2	14 500	8.339	−22.4
10	87.9	0.260	0.231	1.10		2.74		9.23	19.8	15 230	8.596	−21.2

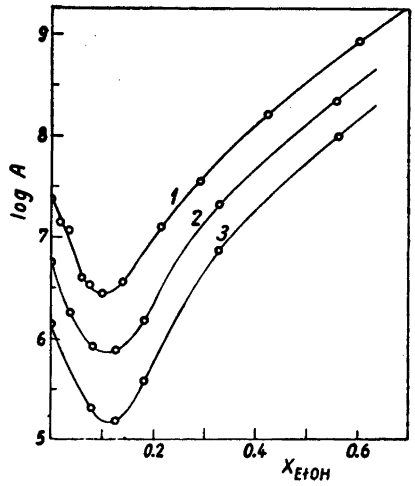


Fig. 3. Graphs to show the correlation between log A and the mole fraction of ethanol in the solvent. 1. Ethyl acetate, 2. Ethyl propionate, 3. Ethyl butyrate.

Activation energies, E, and frequency factors, A, were obtained from the variation of log k with $1/T$, the method of least squares being used in all cases. The entropy of activation, ΔS^*, was calculated from the frequency factor A by means of the equation [9].

$$A = e \frac{kT}{h} e^{\Delta S^*/R}.$$

RESULTS AND DISCUSSION

General. The values of k, E, log A, and ΔS^* are given in Tables 1 and 2. ΔS^* is calculated for 25° C. Figures 1, 2, and 3 show the plots of k, E, and log A against the alcohol content of the solvent. The values for ethyl acetate are taken from the previous investigation. We see that the curves of Figs. 2 and 3 are very similar for all three esters. The depth of the minimum in the activation energy, with regard to the value of E in pure water, is about 1 000 calories in all three cases.

The behaviour of k, E and A in alcohol-rich mixtures after the minimum is partly due to the equilibrium

$$OH^- + EtOH \rightleftharpoons EtO^- + H_2O,$$

Table 2. Alkaline hydrolysis of ethyl butyrate.

H_2O vol. %	EtOH wt. %	x_{H_2O}	10^3k liter mole^{-1} sec.$^{-1}$						E cal.	log A	ΔS^* E.U.
			0.00°	5.00°	15.00°	25.00°	40.00°	50.00°			
100	0	1.000	7.69		20.6	38.3	86.8		10 320	6.142	−32.4
80	17.8	0.922		8.68	15.7	26.6	58.3		9 390	5.316	−36.2
70	27.0	0.874		6.13	11.6	19.8	41.3		9 400	5.184	−36.8
60	36.3	0.818		2.90	5.83	10.5	23.6		10 320	5.583	−35.0
40	55.6	0.671		1.13	2.46	5.17	14.0		12 500	6.879	−29.1
20	76.6	0.438			1.08	2.55	8.17	16.5	14 440	7.984	−24.0

which lies surprisingly far to the right [10]. In the whole domain from pure water upward, on changing from one solvent mixture to another, a very important part is played by changes in the solvation of the reactants and the transition state [1,9]. Undoubtedly, the changes in the internal structure of the solvent are of importance. Many physical properties of alcohol-water mixtures, such as the partial molar volumes of the components, molar polarisation, compressibility, and viscosity, show pronounced maxima or minima in the region of 10 mole per cent of ethanol. Furthermore, many observations indicate that ethanol, when its concentration is small, is molecularly dispersed in water, greatly disturbing the structure of the water, whereas when a small amount of water is added to alcohol, single water molecules can easily enter into the chains of alcohol molecules, without any marked disturbance of the one-dimensional association and the structure of the liquid [11]. All this must be borne in mind in the interpretation of solvent effects on reaction rates.

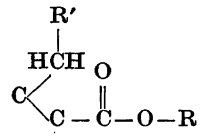
The rate constants. It has been known for a long time that the bimolecular rate constants for the alkaline hydrolysis of the esters of *n*-fatty acids decrease with increasing length of the carbon chain. There is a significant drop in the rate of hydrolysis in passing from ethyl propionate to ethyl butyrate, the change in passing from ethyl acetate to ethyl propionate being less. After butyric acid a further lengthening of the chain has only a very weak effect on the rate constants [12,13]. For the hydrolysis in pure water, the values of k at 25° C are, according to our results, 0.111, 0.087, and 0.0383 l. mole^{-1} sec.$^{-1}$ for ethyl acetate, ethyl propionate, and ethyl butyrate, respectively. The ratio of the rate constants of ethyl propionate and ethyl acetate decreases with the increasing alcohol content of the solvent, but that of ethyl butyrate and ethyl propionate is, as a first approximation, independent of the solvent composition:

Alcohol % by weight	0	17.8	27.0	36.3	55.6	76.6
k_{Pr}/k_{Ac}	0.78	0.71	0.65	0.59	0.55	0.53
k_{Bu}/k_{Pr}	0.44	0.47	0.50	0.42	0.45	0.48

The order of the activation energies and frequency factors is, as seen from Figs. 2 and 3, ethyl acetate > ethyl propionate > ethyl butyrate. Thus the differences in the rate constants are wholly accounted for by the differences in frequency factors, *i. e.* in the differences in activation entropies. These are more than sufficient to offset the effect on the reaction rates of the changes in the activation energies. This was rather surprising and contrary to the results of Evans, Gordon and Watson [13], but in better agreement with those of Smith and Levenson [14]. Evans, Gordon, and Watson thought that in the alkaline hydrolysis of aliphatic carboxylic esters the activation energy increases with the chain length of the acid component: "as a rule, E varies in accordance with the inductive effect of R", which increases in magnitude from Me to Bu. Smith and Levenson found that the energy of activation was practically constant within the precision of the measurements for this series of esters and that the changes in the velocity had to be attributed to a large extent to changes in the frequency factor. Both of these investigations were made in 85 % aqueous ethanol (by weight), where the differences in E between the three esters are very small (Fig. 2). The only data concerning the temperature dependence

of the reaction in water reported in the literature are those given by Salmi and Leimu [15], for the esters of *n*-fatty acids, but their measurements were carried out at three temperatures only and thus do not give E values accurate enough for a critical study of the problem. In the same manner as in the alkaline hydrolysis of the esters of *n*-fatty acids, k behaves in their acid hydrolysis [16] and in the acid-catalysed esterification [17]. In the alkaline hydrolysis of benzyl esters of *n*-fatty acids in 56 % aqueous acetone (by weight) the differences in the values of k are also occasioned principally by changes in A, with E remaining constant within experimental error from benzyl acetate onward [18].

As stated above, the effect of lengthening of the carbon chain in the acid part of the ester on the rate constant is certainly not explained in terms of inductive effect. The sequence of the activation energies, ethyl acetate > ethyl propionate > ethyl butyrate, is the direct opposite of that predicted by the inductive effect. The differences are especially distinct in the region from pure water to about 40 % acetone (Figs. 2 and 3). Since it appeared unlikely that the structural changes involved could have any significant effect on the carbonyl group through altered inductive or hyperconjugative effects, Smith and McReynolds [19], who thought that for each of the above sets of reactions the activation energy is practically constant, concluded that in butyric acid and higher homologs a coiled structure, in which the hydrogens on the third methylene group come close to the oxygen of the carboxyl group, is possible and is probably responsible for the rate decrease:



In the orientation assumed, approach of the hydroxyl ion (alkaline hydrolysis), water molecule (acid hydrolysis), or alcohol molecule (esterification, R = H) is sterically hindered in one direction and accordingly the frequency factor is reduced. The idea has been further developed by Newman [20]. A direct interaction between the methyl group and carbonyl oxygen was supposed also by Evans, Gordon, and Watson [13], who believed that this involves the formation of a hydrogen bond. Similar suggestions have been proposed by other investigators, too. Bennett and Mosses [21] compared the dissociation constants of propionic, butyric, and heptoic acids (1.33, 1.50, and 1.3 × 10^{-5}, respectively) and attributed the high value in the case of butyric acid to the fact that the terminal methyl, being free to move, occasionally comes into close proximity to the carbonyl group and facilitates ionisation, exerting a direct polar effect of opposite sign to its inductive effect. Dippy [22] considered that this is due to an attraction between the C—CH_3 and C = O dipoles (hydrogen-bonding), particularly in the anion. Magee, Ri, and Eyring [23], discussing the enhanced K for *n*-butyric acid, also give ring closure as the probable reason for this large value. The same view was expressed by Ayling [24] as the explanation of a number of phenomena. Independent evidence favouring this suggestion has been supplied by McReynolds and Witmeyer [25] in a study of the stabilities to race-

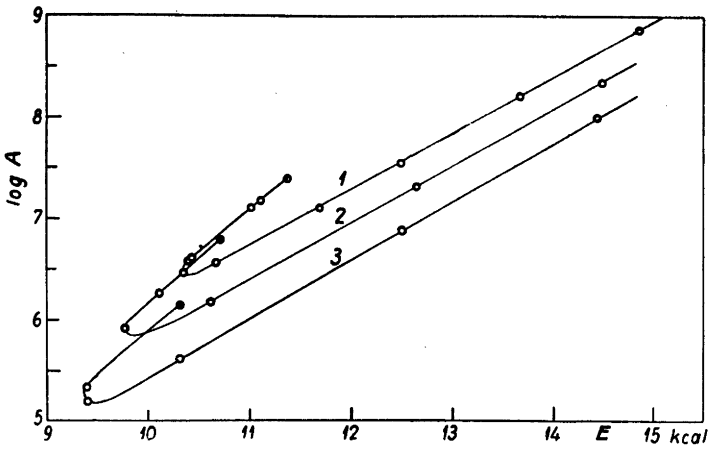


Fig. 4. Plot of log A against E. 1. Ethyl acetate, 2. Ethyl propionate, 3. Ethyl butyrate.

misation of certain salts containing aliphatic acid radicals. Anomalies were found and a hydrogen bond linking the β-carbon with the carbonyl oxygen was postulated in acid-catalysed prototropy of phenyl alkyl ketones also [26]. The *ortho*-effect in the alkaline hydrolysis of the ethyl ester of *o*-methylbenzoic acid has led to the conclusion that a direct interaction of the methyl group with the carbonyl oxygen is operative [27].

These ideas are in good agreement with our experimental results. Whatever its detailed mechanism, there is every reason to suppose that in *n*-paraffinic esters R·COOEt an additional effect opposed to the chain-transmitted (inductive) effect is operative. This additional effect tends to lower the energy of activation, and since in the alkaline hydrolysis the chief resistance to the reaction is represented by the energy required for bringing the hydroxyl ion up to the carbonyl carbon [28], it obviously tends to make the carbonyl carbon more positive. A hydrogen-bonding between the terminal methyl group of the coiled R and the oxygen atom of the carbonyl group would explain the direction of this additional effect, and there seem to be no serious objections to such a supposition [29].

The values of the activation energies show that, in contrast to the older views, the additional effect is already operative and more powerful than the inductive effect even in the case of ethyl propionate, and is obviously at maximum with ethyl butyrate, after which a further lengthening of the chain, according to all previous workers, has very little influence on the reaction rate. From molecular models it can be seen that a hydrogen bonding in propionate is geometrically not impossible, although its strength, of course, is weaker than in the case of butyrate. It is noteworthy that the difference in E is larger between ethyl acetate and ethyl propionate than between ethyl propionate and ethyl butyrate. The decrease of the frequency factor in passing from ethyl acetate to ethyl butyrate is, as concluded by Smith and Reynolds, explained in terms of increasing steric hindrance [cf.30]. In this connection it is of special

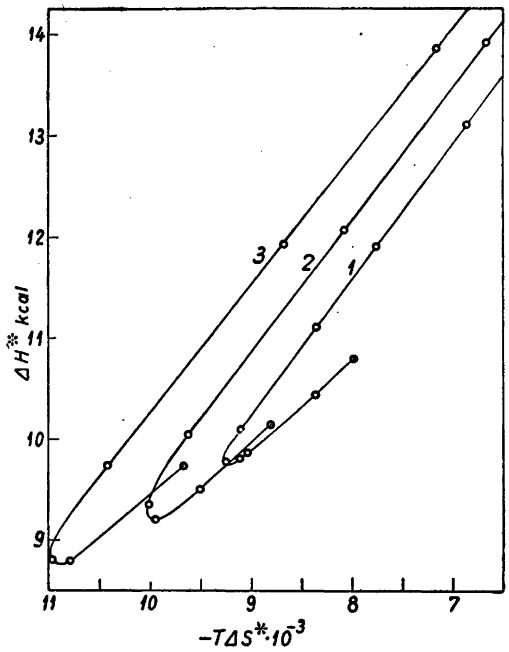


Fig. 5. Plot of ΔH^* against $-T\Delta S^*$. 1. Ethyl acetate, 2. Ethyl propionate, 3. Ethyl butyrate.

interest that behaviour of k, E, and A closely similar to that observed in the hydrolysis of carboxylic esters has been found in the alkaline and acid hydrolysis of aliphatic amides [31]. With regard to hydrolysis, there is a marked analogy between the amides and esters [32].

Figs. 2 and 3 show that the solvent effect is very similar in magnitude in all three cases. The differences between the values of log A are practically independent of the composition of the solvent. The differences between the values of E, however, become smaller as the alcohol content is increased from the mole fraction 0.1, which indicates a weak special solvent effect for each reaction. This is probably connected with differences in the changes of the solubility of the esters. With an increasing alcohol content, the solubility of the esters of the series increases the greater the length of the carbon chain, from which it directly follows that the differences between the activation energies must become smaller, since an increase in the solvation of the reactants rises the energy of activation [1], and the effect increases with the solvation. It is reasonable to assume that the transition complex, which is very polar, is, if the solvent is the some, solvated to practically the same extent in the three cases.

The relation between A and E. In the previous investigations [1,2] it was found that the plot of log A versus E is linear on either side of the minima of E and A. From Fig. 4 we see that this is also the case for the hydrolysis of

ethyl propionate and ethyl butyrate and, furthermore, that the slopes of the lines are almost identical. Between pure water and the minimum $\Delta \log A/\Delta E$ is about 0.80 (E expressed in kilocalories), in solutions containing more than 20 % by weight of ethanol $\Delta \log A/\Delta E$ is 0.56 (for ethyl acetate 0.55, ethyl propionate 0.56, ethyl butyrate 0.57). The corresponding values for the alkaline hydrolysis of ethyl benzoate in ethanol-water and in acetone-water, and benzyl acetate in acetone-water are of the same order of magnitude.[2]

The linear relationship between log A and E is equivalent to a linear relationship between the heat of activation, $\Delta H^* = E - RT$, and $T\Delta S^*$, as expressed by the equation [33,1]

$$T\Delta S^* = \alpha \Delta H^* + \beta.$$

From Fig. 5, where the plots are given, we obtain for all three esters, in the region from pure water to about 18 wt. % ethanol roughly $\alpha = 1$. In the ethanol-richer region the values for α are: ethyl acetate 0.75, ethyl propionate 0.77, ethyl butyrate 0.80.

SUMMARY

The alkaline hydrolysis of ethyl propionate and ethyl butyrate in pure water and various ethanol-water mixtures was kinetically investigated. The results are compared with previous results for ethyl acetate, and discussed.

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The Complex Formation between Iron (III) Ion and Sulfosalicylic Acid

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Phenols are characterized by, amongst other reactions, the colour which is formed with a solution of iron (III) chloride. The coloured substance formed is a complex built up by iron (III) ion and one or more phenol molecules.

Sulfosalicylic acid and iron (III) give a violet colour in a rather strongly acidic solution. When the pH is increased the colour shifts over orange to yellow.

Previous work. Wesp and Brode [1] made a review, the composition and colour of the complexes formed from 44 phenols and iron (III). Schwarzenbach and coworkers have written several papers concerning the stability of such complexes, most of them being cited in [2]. Foley and Anderson [3] investigated the system iron (III) + sulfosalicylic acid spectrophotometrically. They concluded that the colour change might depend upon a dissociation of the complex. In several buffer systems, having pH values up to 7.75, they found that the complex was built up from 1 molecule sulfosalicylic acid and 1 atom of iron (III). The apparent equilibrium constant $K = \frac{[Fe] \cdot [R]}{[FeR]}$ was found to be $1.3 \cdot 10^{-5}$ at pH 2.38 and $2.2 \cdot 10^{-5}$ at pH 5.3. [R] is here the total content of sulfosalicylic acid "without correction for ionization".

Banks and Patterson [4] in a polarographic investigation of the same complexes showed that the yellow colour obtained in neutral or alkaline solution is due to a complex built up from 1 atom iron and 3 molecules of sulfosalicylic acid. They estimated the equilibrium constant for the reaction

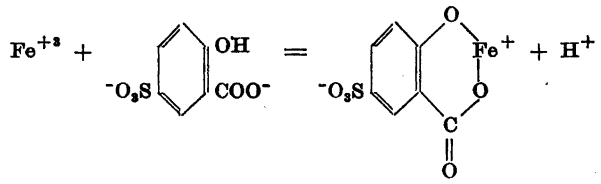
$$FeR_3^{-6} + 3\ H_2O = Fe^{+3} + 3\ RH^{-2} + 3\ OH^-$$

to be 10^{-43} C^3 (C = moles/litre). R is  . The ionic strength was 1 C and the temperature 25° C.

Kennard and Johnson [5] used the complex formation between iron (III) and sulfosalicylic acid in a photometric determination of iron. They showed that the three complexes have extinction maxima at 500, 460 and 420 mμ resp. and that the complexes are stable around pH 1.5, 5.0 and 8.2 resp.

THEORY

Sulfosalicylic acid is a tribasic acid. In water the sulfonic group is completely ionized. Thus the ions RH_2^- and RH^{-2} are present but hardly any ions, R^{-3}, since phenols are very weak acids. When 1 molecule R is bound to Fe (III), a chelate compound is formed:



This compound is a sulfonic acid which is assumed to be completely ionized.

There is a possibility that the complex $FeRH^+$ is formed. The experimental results may however be explained without including this possibility in the theory.

The higher complexes are formed in an analogous manner.

This investigation was made to estimate the numerical values of the complexity constants, defined as follows:

$$Fe^{+3} + RH^{-2} = H^+ + FeR \qquad \varkappa_1 = \frac{h \cdot [FeR]}{[RH] \cdot [Fe]}$$

$$Fe^{+3} + 2\ RH^{-2} = 2\ H^+ + FeR_2^{-3} \qquad \varkappa_2 = \frac{h^2 \cdot [FeR_2]}{[RH]^2 \cdot [Fe]}$$

and so on. Or generally written:

$$Fe^{+3} + n\ RH^{-2} = n\ H^+ + FeR_n^{3-3n}$$

$$\varkappa_n = \frac{h^n \cdot [FeR_n]}{[RH]^n \cdot [Fe]} \qquad (1)$$

where h is the hydrogen ion concentration (in mC = millimoles/litre) and [ ] the concentration of the substance indicated. The signs for the electrical charges are omitted.

To estimate these complexity constants, h and [Fe] can be measured potentiometrically. $[FeR_n]$ can be determined photometrically. Both these methods have been used here. The measurements were made at 25.0° C and, to all solutions, $NaClO_4$ was added up to a total concentration of 3 000 mC ClO_4^-. This ion strength was chosen because Hedström's [6] investigations on the hydrolysis of iron (III) and iron (II) are made at this ionic strength and thus his results could be used.

As shown by Sutton [7], ClO_4^- has a weak complex forming tendency but this tendency does not influence the results when the concentration of this ion is great and constant. It is assumed that the activity factors are constant and hence the concentrations may be used instead of activities in the equilibria.

Potentiometric determinations

The concentration of free iron (III) ion was measured in the presence of a constant amount of iron (II) with a platinum electrode. The hydrogen ion concentration was measured with a glass electrode. As a reference electrode, a silver-silver chloride electrode in 100 mC NaCl and 3 000 mC $NaClO_4$ was used. Thus the electromotive force (emf) of the following cells were measured:

$+$ Glass electrode | H^+ | Reference electrode —
$+$ Pt | Fe^{+3}, Fe^{+2} | Reference electrode —

The emf of the first cell is given by the equation

$$E_{Gl} = E_{0,Gl} + 59.16 \log h + E_j$$

where $E_{0,Gl}$ is a constant (in mV) and E_j the liquid junction potential. The glass electrode used changed its potential in the theoretically expected manner, as shown by a separate titration. E_j is approximately = —0.018 h under conditions used in this investigation, as shown by Biedermann and Sillén [8].

The emf of the second cell is given by the equation

$$E_{Pt} = E_{0,Pt} + 59.16 \log \frac{[Fe^{+3}]}{[Fe^{+2}]} + E_j$$

where $E_{0,Pt}$ is a constant. This equation can be transformed

$$E = E_{Pt} - E_j = E_{0,Pt} + 59.16 \log \frac{[Fe]_t}{[Fe^{+2}]} - 59.16 \log (b + 1)$$

where $b = \frac{[Fe]_t - [Fe^{+3}]}{[Fe^{+3}]}$ and $[Fe]_t$ is the total concentration of iron (III). If the total concentration of iron (III) and of iron (II) are each constant during an experiment, this equation can be written

$$E = E_0 - 59.16 \log (b + 1)$$

where $E_0 = E_{0,Pt} + 59.16 \log \frac{[Fe]_t}{[Fe^{+2}]}$

In order to calculate the complexity constants the following equation was used:

$$[Fe]_t = [Fe] + [FeOH] + [Fe(OH)_2] + 2[Fe_2(OH)_2] + [FeR] + [FeR_2] + [FeR_3] \quad (2)$$

The hydroxyl complexes were found by Hedström [6] and the equilibrium constants are defined by

$$\varkappa_{nm} = \frac{h^n \cdot [Fe_n(OH)_m]}{[Fe]^n}$$

By combining equations (1) and (2):

$$[Fe]_t = [Fe] + \frac{\varkappa_{11}[Fe]}{h} + \frac{\varkappa_{12}[Fe]}{h^2} + 2\frac{\varkappa_{22}[Fe]^2}{h^2} + \varkappa_1 \frac{[RH]}{h} + \varkappa_2 \frac{[RH]^2}{h^2} + \varkappa_3 \frac{[RH]^3}{h^3}$$

or by transforming

$$\frac{[Fe]_t - [Fe]}{[Fe]} = b = \varkappa' + \varkappa_1 \frac{[RH]}{h} + \varkappa_2 \frac{[RH]^2}{h^2} + \varkappa_3 \frac{[RH]^3}{h^3} \qquad (3)$$

where the small correction term $\varkappa'$ is defined by

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

The numerical values of the equilibrium constants of the hydroxyl complexes are, according to Hedström, $\varkappa_{11} = 0.90$, $\varkappa_{12} = 0.49$ and $\varkappa_{22} = 1.22$.

If the last term of equation (3) is assumed to be very small compared with the other terms, the following equation is obtained:

$$(b - \varkappa')\,\frac{h}{[RH]} = \varkappa_1 + \varkappa_2 \cdot \frac{[RH]}{h} \qquad (4)$$

The value of the left member can be calculated from experimental data. If this member is plotted against the function [RH]/h a straight line should be obtained, the intercept being $\varkappa_1$ and the slope $\varkappa_2$.

In order to calculate [RH] the dissociation constant of sulfosalicylic acid must be known:

$$K_a = \frac{[RH] \cdot h}{[RH_2]} \qquad (5)$$

Further, the following equations are valid:

$$[R]_t = [RH] + [RH_2] + [FeR] + 2\,[FeR_2] + 3\,[FeR_3] \qquad (6)$$

$$h = [H]_t - [OH]_t + [FeR] + 2\,[FeR_2] + 3\,[FeR_3] - [RH_2] + [FeOH] + 2\,[Fe(OH)_2] + 2\,[Fe_2(OH)_2] \qquad (7)$$

where $[R]_t$ is the stoichiometrical concentration of sulfosalicylic acid and $[H]_t$ and $[OH]_t$ are the stoichiometrical concentrations of hydrogen ion and hydroxyl ion resp.

From equations (6) and (7) the following equation is obtained:

$$[R]_t - h = [RH] + 2\,[RH_2] - [H]_t + [OH]_t - [FeOH] - 2\,[Fe(OH)_2] - 2\,[Fe_2(OH)_2] \qquad (8)$$

or

$$[RH] \cdot \left(1 + \frac{2h}{K_a}\right) = [R]_t + [H]_t - [OH]_t - h + [FeOH] + 2\,[Fe(OH)_2] + 2\,[Fe_2(OH)_2]$$

From this equation [RH] may be calculated.

When the values of $\varkappa_1$ and $\varkappa_2$ had been estimated as mentioned above, the value of $\frac{[RH]}{h}$ was increased, usually by decreasing h. At sufficiently low h values the complex FeR_3 was formed. Now $\varkappa_3$ could be estimated from equation (3), which could be transformed:

$$\left[(b - \varkappa')\,\frac{h}{[RH]} - \varkappa_1\right]\frac{h}{[RH]} = \varkappa_2 + \varkappa_3 \cdot \frac{[RH]}{h} \qquad (9)$$

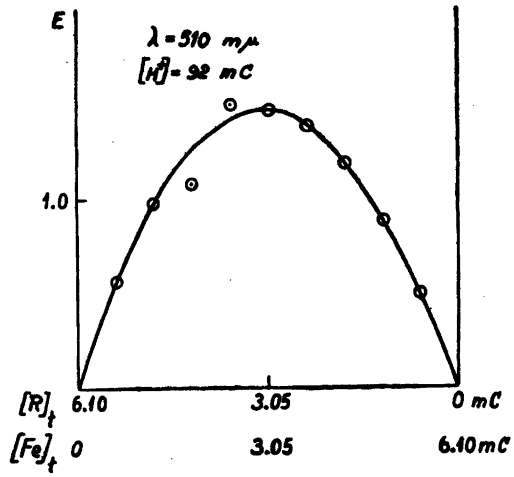


Fig. 1. Extinction (E) of various mixtures of Fe (III) *and sulfosalicylic acid.*

Here too a straight line should be formed when the left member is plotted against $\frac{[RH]}{h}$. The intercept will give the value of $\varkappa_2$ and the slope $\varkappa_3$.

Complexes with more than three R per iron atom have not been found in this investigation. This is in agreement with the supposition that chelate compounds are formed. As Fe(III) has the coordination number six, only three molecules R can be bound to one atom of Fe.

Photometric determinations

The values of the complexity constants which have been estimated potentiometrically have also been verified photometrically.

According to Job [9] the composition of a complex may be determined by the method of continuous variations. Using the same method the complexity constants may also be calculated. — In acid solution (pH about 2) sulfosalicylic acid and Fe (III) form a violet solution with extinction maximum at 510 mμ. By Job's method it was shown that this complex is built up from 1 molecule of R and 1 atom of Fe (Fig. 1). The measurements have not been used to calculate the complexity constant.

The same method could not be used to determine the composition of the orange and the yellow complexes formed at higher pH values. This is because the different complexity constants are so close to each other. Even if most of the iron (III) is in one complex form, a certain amount will be in another form (Fig. 2). The different complexes also have their extinction maxima so near each other (Fig. 3) that the extinction at one wave length will result from at least two different complexes. Another reason why Job's method could not be used is that, when the concentration of R is low, Fe (III) will be hydrolyzed or even precipitated.

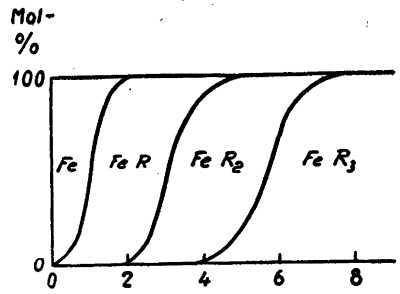


Fig. 2. Distribution of Fe (III) *(in percent) over* Fe^{+3}, FeR, FeR_2^{-3} and FeR_3^{-6}, *as a function of log* h (h *in* mC).

Another photometric method has been used instead to estimate the complexity constants. The principle is the same as that used by Heller and Schwarzenbach [10] but is modified here so that it may be also used when two complexes absorb light at the same wave length.

If only *one* complex is formed the following equations are valid:

$$\varkappa_n = \frac{h^n \cdot [FeR_n]}{[RH]^n \cdot [Fe]} \tag{1}$$

$$[Fe]_t = [Fe] + [FeR_n] = c_1 \tag{10}$$

(No appreciable amounts of hydroxyl complexes are formed)

$$[R]_t = [RH] + [RH_2] + n[FeR_n] = c_2 \tag{11}$$

$$E = \varepsilon_n \cdot [FeR_n] \tag{12}$$

$$\frac{[RH] \cdot h}{[RH_2]} = K_a \tag{5}$$

where E = the extinction of the solution in a 1 cm cell ε_n = the molar extinction.

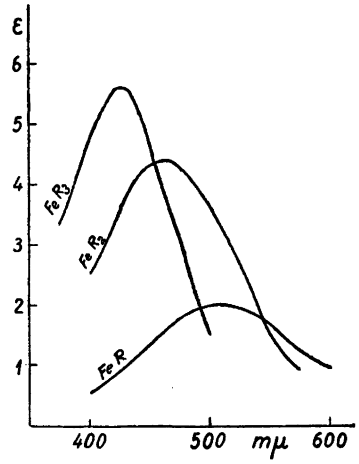


Fig. 3. Extinction curves of the three complexes FeR, FeR_2^{-3} *and* FeR_3^{-6}.

By substitution in (1) the following equation is obtained:

$$\varkappa_n = \frac{h^n \cdot \frac{E}{\varepsilon_n}}{\left(c_2 - n\frac{E}{\varepsilon_n}\right)^n \left(\frac{K_a}{h + K_a}\right)^n \left(c_1 - \frac{E}{\varepsilon_n}\right)} \tag{13}$$

Two cases may be distinguished:

1. If $c_2 \gg c_1$ the term $n\frac{E}{\varepsilon_n}$ in the denominator can be neglected and one obtains:

$$\frac{c_1}{E} = \frac{1}{\varepsilon_n} + \left[\frac{h^n}{c_2^n \left(\frac{K_a}{h + K_a}\right)^n}\right] \cdot \frac{1}{\varkappa_n \varepsilon_n} \tag{14}$$

If c_1/E is plotted against the expression in the square brackets a straight line is obtained. The intercept equals $\frac{1}{\varepsilon_n}$ and the slope is $\frac{1}{\varepsilon_n \varkappa_n}$.

2. If $\frac{1}{n}$ $c_2 = c_1 = c$ equation (13) gives:

$$\frac{c}{E} = \frac{1}{\varepsilon_n} + \left(\frac{1}{\varepsilon_n \varkappa_n}\right)^{\frac{1}{n+1}} \cdot \left[\left(\frac{h}{nE\frac{K_a}{h + K_a}}\right)^{\frac{n}{1+n}}\right] \tag{15}$$

A straight line is obtained if $\frac{c}{E}$ is plotted against the function in the square brackets. The intercept is $\frac{1}{\varepsilon_n}$ and the slope $\left(\frac{1}{\varepsilon_n \varkappa_n}\right)^{\frac{1}{1+n}}$.

On the other side, if the iron forms *two* different complexes which both contribute to the extinction at one wave length, and no other forms of iron are present, the following equations are obtained:

$$\frac{\varkappa_n}{\varkappa_{n-1}} = \frac{h \cdot [FeR_n]}{[RH] \cdot [FeR_{n-1}]} \tag{16}$$

$$E = \varepsilon_n \cdot [FeR_n] + \varepsilon_{n-1} \cdot [FeR_{n-1}] \tag{17}$$

$$[Fe]_t = [FeR_n] + [FeR_{n-1}] = c \tag{18}$$

These equations may be combined to give

$$\frac{c}{E} = \frac{1}{\varepsilon_n} + \frac{\varkappa_{n-1}}{\varepsilon_n \varkappa_n} \cdot \left[\frac{h}{[RH]} \cdot \frac{E - c\ \varepsilon_{n-1}}{E}\right] \tag{19}$$

where $\frac{c}{E}$ is a linear function of the expression in the square brackets. When $n = 1$ this equation will be identical with (14) and (15) if $\varepsilon_{n-1} = \varepsilon_0 = 0$.

By chosing suitable pH ranges from Fig. 2 it is thus possible to estimate the complexity constants $\varkappa_1$, $\varkappa_2$ and $\varkappa_3$ photometrically.

EXPERIMENTAL

Apparatus. The measurements were made with an apparatus essentially the same as that described by Forsling, Hietanen and Sillén [11]. Nitrogen was passed through the reaction mixture, the gas being first freed from oxygen using a Meyer-Ronge [12] oven and then from carbon dioxide using soda-lime. In order to avoid evaporation the gas was bubbled through a 3 000 mC solution of $NaClO_4$.

The potential of the glass electrode was measured with a valve potentiometer (type Radiometer PHM 3). The redox potential was measured with a potentiometer having a sensitivity of 0.01 mV.

The photometric measurements were made with a Beckman spectrophotometer model B.

Chemicals. A solution of iron (II) perchlorate was made by Lindstrand's [13] method from perchloric acid (A.R.) and iron (II) sulfide (A.R.) Iron (III) perchlorate was made by adding perchloric acid and hydrogen peroxide. The concentrations of the solutions were determined cerimetrically, if necessary after passage through a cadmium reductor. The concentration of ClO_4^- was determined using an ion exchange technique. The hydrogen ion concentration was determined by difference, ($[H^+]=[ClO_4^-]-3\,[Fe^{+3}]-2\,[Fe^{+2}]$).

Sodium perchlorate was made from perchloric acid (A.R.) and sodium carbonate (A.R.) added in deficit. After removing the carbonic acid using nitrogen gas, the solution was carefully neutralized with NaOH (A.R.). The concentration was determined using an ion exchange technique.

Sulfosalicylic acid was neutralized with one equivalent of sodium carbonate (A.R.) in hot solution. On cooling, the monosodium salt of sulfosalicylic acid crystallized and the salt was washed with a little water, alcohol and ether. After redissolving in water, sodium carbonate (A.R.) was added and the concentration was determined using an ion exchange technique.

Determination of the dissociation constant of sulfosalicylic acid. Before the complexity constants could be calculated, the dissociation constant, K_a, of the acid had to be determined. To a known amount of the di-sodium salt of the acid, an excess of perchloric acid was added and the solution titrated with NaOH using a quinhydrone electrode. From nine points of the titration curve, K_a was calculated. The mean value of these calculations was taken as K_a (Table 1).

Table 1. Determination of the dissociation constant, K_a, of sulfosalicylic acid.

$[R]_t$	$[H]_t$	h	K_a
19.00	14.90	3.355	2.17
18.57	12.25	2.410	2.14
18.36	10.97	2.039	2.15
18.16	9.72	1.685	2.12
17.97	8.49	1.365	2.07
17.77	7.29	1.141	2.15
17.40	4.97	0.694	2.13
17.04	2.75	0.344	2.09
16.87	1.67	0.206	2.17
			M = 2.13 ± 0.06 mC

Potentiometric experiments

The measurements were made in the following manner. A solution of Fe (II) and Fe (III) perchlorate which was acidified with a suitable amount of $HClO_4$, was "titrated" with a solution of the disodium salt of sulfosalicylic acid. At the same time an iron solution was added, so that the total concentrations of Fe (II) and Fe (III) were kept constant during the experiment. After each addition the emf was determined and it was usually found that equilibrium was attained after 15 minutes.

Table 2. Potentiometric determination of the complexity constants $\varkappa_1$ and $\varkappa_2$.

$[R]_t$	E	E_{GI}	b	h	$\frac{[RH]}{h}$	$(b-\varkappa')\frac{h}{[RH]}$
1	2	3	4	5	6	7
*	442.3					
2.900	302.7	— 73.9	227.93	1.523	0.4429	512
5.510	258.2	— 81.6	1 292.9	1.128	1.996	647
7.871	233.4	— 87.4	3 396.0	0.900	4.351	781
10.018	215.0	— 91.0	6 951.2	0.783	6.991	994
11.978	200.2	— 94.3	12 367	0.688	10.193	1 213
13.775	187.2	— 97.7	20 512	0.603	14.21	1 443
15.428	177.1	— 99.5	30 391	0.562	17.61	1 726
16.954	168.0	—101.8	43 018	0.514	21.91	1 963
18.367	160.4	—104.2	58 215	0.468	26.94	2 161
19.679	153.3	—106.1	76 744	0.435	31.84	2 410

* Too many figures are given in the tables in order not to introduce an appreciable error in the calculations.

The results of one experiment of this kind are given in some detail in Table 2. The results of the same experiment can be found in Table 3 (No. 5). In the first column of Table 2 the total concentration of sulfosalicylic acid is given. Column 2 shows the redox emf in mV. The first value (E_0) is the emf of the same solution with no sulfosalicylic acid added but to which a sufficient amount of perchloric acid was added in order to depress the hydrolysis of iron ($[H^+] = 67.5$ mC). The diffusion potential is eliminated. Column 3 gives the potential of the glass electrode. The other columns need no explanation.

Several other experiments were done in the same manner, but the conditions were varied in one respect or another. All of these experiments are collected in Table 3 and Fig. 4. In experiments 3—6 in this table the concentration of iron (III) was almost unaltered, but the concentration of iron (II) was varied from 1,5 to 14,3 mC. In spite of this variation the values of $\varkappa_1$ and $\varkappa_2$ show no trend, which shows that iron (II) does not form detectable amounts of a complex with sulfosalicylic acid under these conditions.

Table 3. Survey of experiments for the calculation of $\varkappa_1$ and $\varkappa_2$ potentiometrically.

No.	Symbol	Concentrations			$\varkappa_1$	$\varkappa_2$
		Fe (II)	Fe (III)	H^+		
1	+	0.7	3.35	0.4	420	62
2	⅄	0.7	7.53	0.3	490	65
3	×	1.5	1.65	0.3	450	55
4	⊡	3.1	1.84	0.2	550	43
5	⊙	6.5	1.75	0.3	550	63
6	◬	14.3	1.85	0.2	410	54
7	◐	0.7	0.67	22	507	—
					M = 482 ± 70	56 ± 9

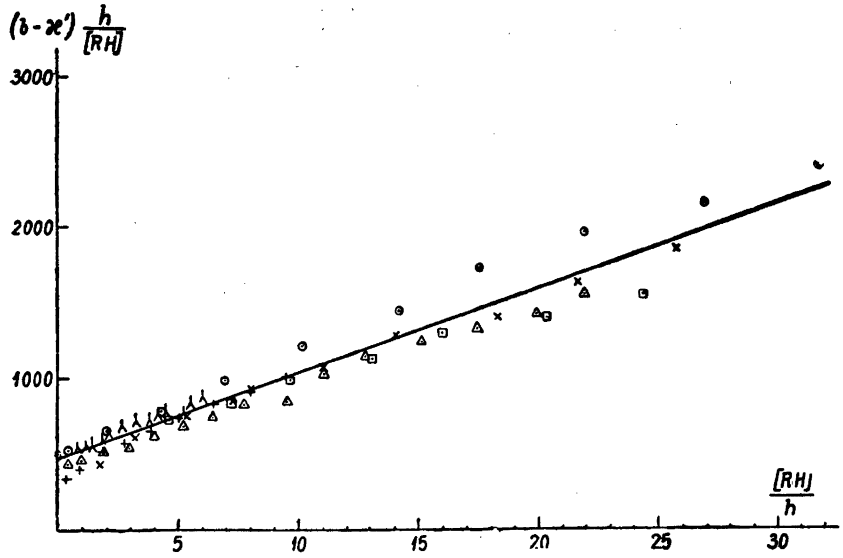


Fig. 4. Potentiometric determination of $\varkappa_1$ *and* $\varkappa_2$. *Various total concentrations of* Fe (III) *and* Fe (II). *Compare Table 3.*

In the experiments in Table 3 the concentration of iron (III) varied from 0.67 to 7.5 mC. No trend in the variation of $\varkappa$ can be seen and thus there seem to be no multinuclear complexes.

As may also be seen in Table 3 a variation in hydrogen ion concentration did not result in a variation in $\varkappa$. From this fact, it may be concluded that no appreciable amount of the complex FeRH is formed.

In the experiments performed to estimate the value of $\varkappa_3$ a technique, which differed in some respects from the preceding one, was used. Now a certain amount of sulfosalicylic acid was added to the iron solution in the beginning of the experiment. Then the hydrogen ion concentration was

Table 4. Potentiometric determination of the complexity constants $\varkappa_2$ *and* $\varkappa_3$.

$[R]_t$	$[OH]_t$	E	E_{Gl}	b	h	$\frac{[RH]}{h}$	$(A-\varkappa_1)\frac{h}{[RH]}$
1	2	3	4	5	6	7	8
		532.3					
48.62	1.792	98.0	−151.5	$2.193 \cdot 10^7$	0.0743	588.2	62.6
46.78	2.671	67.0	−169.1	$7.330 \cdot 10^7$	0.0374	1 138.2	56.2
45.08	3.487	20.0	−190.0	$4.566 \cdot 10^8$	0.0166	2 466	74.9
43.50	4.245	−55.0	−220.0	$8.459 \cdot 10^9$	0.00516	7 570	147.5
42.75	4.605	−116.0	−243.0	$9.087 \cdot 10^{10}$	0.00211	18 040	279.2

$$A = (b-\varkappa')\frac{h}{[RH]}$$

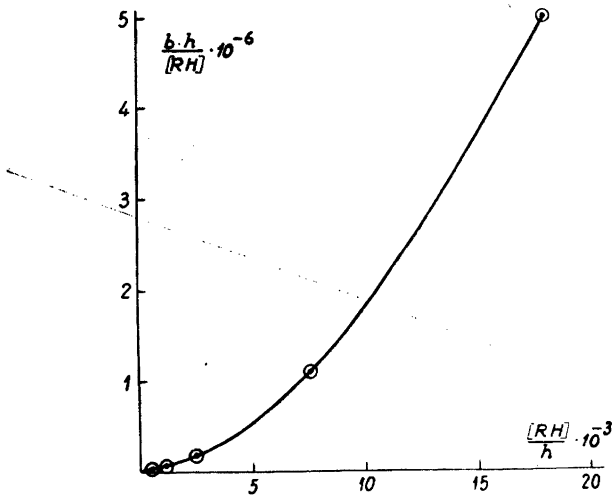


Fig. 5. Potentiometric determination of $\varkappa_2$ and $\varkappa_3$.

changed by adding NaOH. At the same time an iron solution was added so that the total concentrations of iron (II) and iron (III) were unchanged during the experiment. Such a titration is shown in Table 4. Column 1 shows the total concentration of sulfosalicylic acid which decreases a little during the titration due to the dilution of the solution. Column 2 shows the stoichiometrically calculated concentration of hydroxyl ion if no hydrolysis or complex formation occurred. Column 8 gives the left member of equation (9). Fig. 5 shows the same experiment graphically, the expression $\frac{b \cdot h}{[RH]}$ being the dependent variable. Here the curve is bent upwards, which shows that the complex FeR_3 is being formed. If the expression $\left(\frac{b \cdot h}{[RH]} - \varkappa_1\right) \frac{h}{[RH]}$ is the dependent variable the curve is a straight line, from which $\varkappa_2$ and $\varkappa_3$ may be calculated.

The results of several such experiments are collected in Table 5.

Table 5. Survey of experiments for the calculation of $\varkappa_2$ and $\varkappa_3$ potentiometrically. Total concentration of Fe (III) = *1.575, of* Fe (II) = *0.158 mC.*

$\varkappa_2$	$\varkappa_3$
48	—
42	0.013
52	—
46	0.005
50	0.014
M = 48 ± 6	0.011 ± 0.06

Table 6. Photometric determination of $\varkappa_1 \cdot [R]_t = 9.92$ mC, $[Fe]_t = 0.3024$ mC.

$[H]_t$	h	[RH]	E	$\frac{[Fe]_t}{E}$	$\frac{h}{[RH]}$
41.4	32.0	0.622	0.530	0.571	51.4
62.0	52.4	0.388	0.490	0.617	135.1
82.7	73.0	0.276	0.399	0.758	264
103.1	92.3	0.236	0.328	0.922	391
123.7	113.9	0.183	0.267	1.133	622

Photometric experiments

Using the value of $\varkappa_n$ calculated above, Fig. 2 was constructed. This figure shows in which form iron (III) is present at different "pH" values (strictly speaking, log h values). The curves are drawn on the assumption that the total concentration of sulfosalicylic acid is 10 mC and that the concentration of iron is so low that only an insignificant amount of sulfosalicylic acid is used for complex formation. From this figure the suitable "pH" values may be chosen in the photometrical determinations of the complexity constants.

In the estimation of the value of $\varkappa_1$ the "pH" range chosen was 0.94—1.50. From Fig. 2 it may be seen that in this range there is hardly any FeR_2 formed. To a certain solution of iron (III) and sulfosalicylic acid there were added varying amounts of perchloric acid. Then 3 000 mC $NaClO_4$ was added up to a certain volume. The hydrogen ion concentration and the extinction at 510 mμ were then determined. The results are shown in Table 6 and Fig. 6 curve 1. From this curve the following values are calculated: $\varepsilon_1 = 2.0$; $\varkappa_1 = 480$.

The experiment which was carried out in order to estimate the value of $\varkappa_2$ was made in the same way as the preceding one. The amounts of perchloric acid were chosen so that the "pH" values varied between the limits, 3.54 and 3.88. In this range, as may be seen in Fig. 2, only the complexes FeR and FeR_2 are present in measurable amounts. The extinctions were measured at 460 mμ. The results are shown in Table 7 and Fig. 6 curve 2, from which the values $\varepsilon_2 = 4.4$ and $\varkappa_2 = 52$ may be calculated.

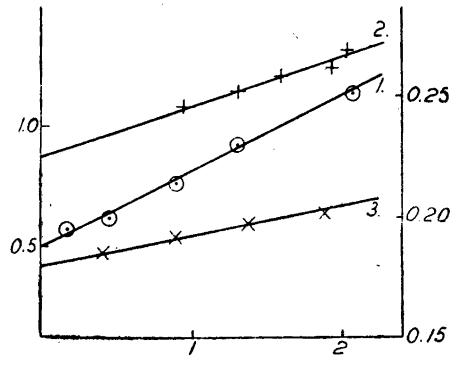


Fig. 6. Photometric determination of $\varkappa_n \cdot \frac{[Fe]_t}{E}$ *vs.* $\frac{h}{[RH]} \cdot \frac{E-[Fe]_t \cdot \varepsilon_{n-1}}{E} \cdot a$.

Curve 1: n = *1;* a = $3 \cdot 10^2$; *scale to the left*
Curve 2: n = *2;* a = 10^{-2}; *scale to the right*
Curve 3: n = *3;* a = 10^{-5}; *scale to the right.*

Table 7. Photometric determination of $\varkappa_3 \cdot [R]_t = 9.92$ mC $[Fe]_t = 0.3024$ mC.

$[H]_t$	h	[RH]	E	$\frac{[Fe]_t}{E}$	$\frac{h}{[RH]} \cdot \frac{E-[Fe]_t \cdot \varepsilon_1}{E}$
0.234	0.133	8.91	1.228	0.2462	0.00941
0.487	0.183	8.72	1.205	0.2510	0.01308
0.741	0.223	8.63	1.173	0.2578	0.01584
0.994	0.270	8.49	1.152	0.2625	0.01927
1.247	0.291	8.54	1.125	0.2688	0.02032

In the photometric determination of $\varkappa_3$, the "pH" range, 6.18—6.94, was chosen. Here most of the iron is transformed to FeR_3. No measurable amount of FeR is present. The experiment was made in an analogous manner to the foregoing ones. But, instead of adding perchloric acid, varying amounts of NaOH were added to the iron sulfosalicylic acid solution. The extinctions were measured at 425 mμ. The results are collected in Table 8 and Fig. 6 curve 3. From this curve the values $\varepsilon_3 = 5.6$ and $\varkappa_3 = 0.008$ may be calculated.

The values which were found photometrically agree fairly well with those found by potentiometrical measurements.

Table 8. Photometric determination of $\varkappa_3 \cdot [R]_t = 9.92$ mC $[Fe]_t = 0.151$ mC.

$[OH]_t$	h	[RH]	E	$\frac{[Fe]_t}{E}$	$\frac{h}{[RH]} \cdot \frac{E-[Fe]_t \cdot \varepsilon_3}{E}$
0.464	$6.56 \cdot 10^{-4}$	9.455	0.746	0.2027	$1.879 \cdot 10^{-5}$
0.489	$4.43 \cdot 10^{-4}$	9.431	0.768	0.1969	$1.370 \cdot 10^{-5}$
0.515	$2.68 \cdot 10^{-4}$	9.405	0.790	0.1914	$0.887 \cdot 10^{-5}$
0.541	$1.14 \cdot 10^{-4}$	9.379	0.820	0.1844	$0.409 \cdot 10^{-5}$

Figure 7 shows how the complex composition of an iron solution varies with the expression $\frac{[RH]}{h}$. This figure is more generally valid than Fig. 2, which only shows the conditions at a certain total concentration of sulfosalicylic acid.

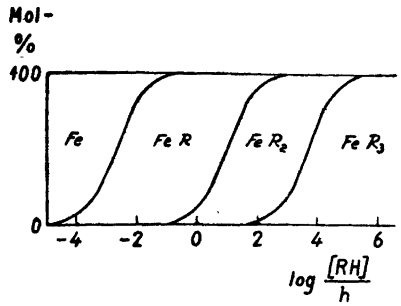


Fig. 7. Distribution of Fe (III) *(in percent) over* Fe^{+3}, FeR, FeR_2^{+} and FeR_3^{0}, *as a function of log* $\frac{[RH]}{h}$.

SUMMARY.

The complex formation between iron (III) and sulfosalicylic acid has been investigated. At 25.0° C and in a 3 000 mC solution of ClO_4^- the following constants have been determined:

1. The dissociation constant of sulfosalicylic acid (RH_3; cf. p. 266)

$$K_a = \frac{[H^+] \cdot [RH^{-2}]}{[RH_2^-]} = 2.13 \pm 0.06 \text{ mC} = 10^{-2.67 \pm 0.01} \text{ C}.$$

2. The equilibrium constants of the following reactions (both potentiometrically and photometrically):

Reaction	Equilibrium constants	
	potentiometric.	photometric.
$Fe^{+3} + RH^{-2} = H^+ + FeR$	480 ± 70	480 ± 80
$Fe^{+3} + 2\ RH^{-2} = 2\ H^+ + FeR_2^{-3}$	50 ± 15	52 ± 10
$Fe^{+3} + 3\ RH^{-2} = 3\ H^+ + FeR_3^{-6}$	0.011 ± 0.006	0.008 ± 0.002

3. The molar extinctions and the wave length of maxim. absorption:

Complex	Molar extinction $cm^{-1} \cdot mole^{-1} \cdot l$	Wave length $\lambda_{max.}$
FeR	$2 \cdot 10^3$	510 mμ
FeR_2^{-3}	$4.4 \cdot 10^3$	460 mμ
FeR_3^{-6}	$5.6 \cdot 10^3$	425 mμ

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Studies Related to Naturally Occurring Acetylene Compounds. XV. The Isolation of *trans*-Lachnophyllum Ester from *Bellis perennis* L

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In the preceding communication of this series [1] we demonstrated that acetylenic compounds are widely distributed in the tribus *Astereae* of the plant family *Compositae* and that to some extent some of the investigated genera are chemically characterised by the occurrence of specific acetylenic compounds in certain parts of the plants. With the object to collect experimental data, which perhaps once in the future might render contributions to the genesis of these genera, we have tried to investigate as many species as possible within the tribus *Astereae*. One of the few genera of *Astereae* which are represented in Scandinavia is *Bellis* as the well known daisy, *Bellis perennis* L. Wild the daisy occurs only in the coastal district of western Norway. It has, however, spread into lawns in many cultivated places. It is very scarce in the Trondheim district, but from a small collection we prepared a small sample of the essential oil of daisy, which gave a preliminary indication as to the nature of the chromophoric system. The oil from the overground parts was devoid of acetylenic chromophores. In the U.V.-spectrum of the root oil definite maxima occurred at:

λmax., oil from the root of daisy:	3 350	3 045	2 865	2 705	2 565	2 227
» *trans*-lachnophyllum ester [4]:		3 053	2 872	2 710	2 568	2 235
» *cis*- » » [5]		3 094	2 913	2 760	—	2 245

The 3 350 maximum agrees with the first maximum of the matricaria ester (II) [2,3], the following 5, as is seen from the collocation of data above, with those of *trans*-lachnophyllum ester.

trans-Lachnophyllum ester (I) is so far known only from

$$CH_3—CH_2—CH_2—C\equiv C—C\equiv C—CH=CH—COOCH_3 \quad (I)$$

$$CH_3—CH=CH—C\equiv C—C\equiv C—CH=CH—COOCH_3 \quad (II)$$

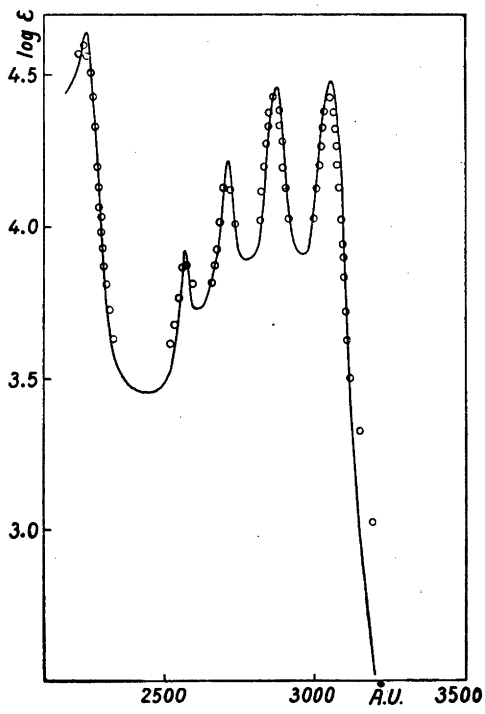

Fig. 1. Ultraviolet absorption in hexane of trans-lachnophyllum ester $CH_3-(CH_2)_2-C\equiv C-C\equiv C-CH=CH-COOCH_3$
——— *synthetic*
⊙⊙⊙⊙ *from root of Bellis perennis L.*

the synthesis, reported in the third communication of this series. The *cis*-isomer, which was discovered in *Lachnophyllum gossypinum* Bg. already in 1935 by Wiljams *et al.*[6], has been shown to be present in several essential oils from plants belonging to the *Compositae* [5,7]. We thus found it desirable to establish if the absorption maxima of the oil of the daisy root really belonged to the *trans*-isomer (I) or to quite another compound. As no further quantities of the wild daisy were available in Trondheim, we, of course, tried the much larger cultivated variants. To our disappointment it turned out that the essential oil from different cultivated varieties contained a mixture of compounds, which, to judge from the U.V.-spectra, contained about equal amounts of matricaria and lachnophyllum esters. As usual our attempts to separate this mixture by chromatography was completely unsuccessful. Thanks to energetic assistance by Miss Sonja Lystad some kilograms of the tiny root of the wild daisy were dug out from the abundant occurrences round Stavanger, in fact the procurement of the starting material was the most difficult part, and we are greatly indebted for this valuable aid. The separation of the *trans*-lachnophyllum ester from the minor amounts of matricaria ester and other compounds present was tedious, but led, as described in the experimental part, to a final fraction with the correct U.V.-spectrum of *trans*-lachnophyllum ester (*cf.* Fig. 1). The m.p. of this fraction was 16—17°, whereas 19° was given by Bruun *et al.* Of the original preparation only some mg were left, which yet showed the m.p. 15.5—16°. The amount did not allow a repurification. The

mixed m.p. was undepressed. Thus there should be no doubt that the main U.V.-maxima of the oil of the daisy root originate from the presence of the *trans*-isomer of (I).

We have not succeeded in the crystallisation of the matricaria ester from any of the chromatographic fractions enriched in that chromophore. Since 2-*cis*:8-*cis*-matricaria ester has an outstanding crystallising power, we doubt that this isomer may be present. It would be rather surprising, too, if an essential oil, which contains the *trans*-isomer of (I), should also contain the *cis* : *cis*-isomer of (II). It would be interesting to know what stereoisomer of (II) is present in daisy oils. From the unusual occurrence of *trans*-I one would expect that 2-*trans* : 8-*trans*-(II), might be found to be a naturally occurring substance. It is to be hoped that some of the other species of *Bellis* have more favourable mixtures of these acetylenic esters.

EXPERIMENTAL

The starting material consisted of the roots of some thousand plants of the wild daisy collected at Hillevåg near Stavanger and the roots of further one thousand cultivated daisies belonging to different giantvariants. When small scale extraction experiments had revealed that the yield of acetylenic compounds was the same as with steam distillation, all root material was steam distilled, total yield of essential oil 1 300 mg. In portions of about 150 mg this oil was chromatographed from petroleum ether solution on neutral alumina. The material was eluted with mixtures of petroleum ether and benzene with 10, 25, 50, 75 and 100 % of benzene. The lachnophyllum ester chromophore concentrated in the first fractions of 50 % benzene. The fractions, which according to their U.V.-spectra contained the purest chromophore, were combined and rechromatographed, with elution this time with 40 and 60 % benzene in petroleum ether. The purest fractions were combined and rechromatographed. The first washings with 40 % benzene then eluted an oil, which was practically devoid of the 3 350 chromophore in the U.V.-spectrum. This fraction (178 mg) was distilled slowly at 2×10^{-4} mm and the fraction 45—52° (airbath) was collected. This fraction solidified in the cold and could be crystallised from petroleum ether solution when cooled to −30°, m.p. 12.5—14°. These crystals (2.8 mg) were recrystallised from dilute alcohol, white needles m.p. 16.0—17.0°, mixed m.p. with synthetic (I) 16°. U.V.-spectrum *cf.* Fig. 1.

The chromatographic fractions from which the isolation of *trans*-lachnophyllum ester was successful, amounted to only some 14 % of the *Bellis* oil. In the fractions with pure benzene a chromophore was enriched with maxima close to those of lachnophyllol. The extinction coefficients were low and distillation at 0.001 mm separated these chromatographic fractions into different substances. The purest chromophore was found in the fraction b.p. 73—100°/0.0001 mm.

U.V.-maxima of this fraction:	λ max	2 830	2 678	2 527	ÅU	
	$E^{1\%}_{1\,cm}$	155	191	141		
$CH_3-(CH_2)_2-C\equiv C-C\equiv C-CH=CH-CH_2OH$ (= lachnophyllol)						
	λ max	2 825	2 665	2 520	2 395	ÅU
	$E^{1\%}_{1\,cm}$	1 320	1 480	990		

This fraction was saponified at 0° with sodium hydroxyde in acetone-water solution under pure nitrogen. Neither the unsaponifiable, nor the acid product showed selective absorption, both exhibiting only an undistinct step out below 3 100 Å. When the acid fraction had remained for some weeks in spectral hexane solution at −17°, white crystal clusters had separated, weight 1.4 mg, m.p. rather distinct at 115—117°. In order to dissolve these crystals in the original amount of spectral hexane, they had to be dissolved

in a few drops of alcohol, and this solution rapidly diluted with spectral hexane. To our surprise these crystals gave a spectrum very close to that of the lachnophyllum ester (3 045, 2 863, 2 710, 2 565). Recrystallisation brought the m.p. to 120—122.5°. As the amounts were insufficient for analysis, the *cis*- and *trans*-lachnophyllum acids were prepared for comparison by saponification of the corresponding methyl esters. *cis*-Lachnophyllum acid m.p. 72—73° undistinct, *trans*-lachnophyllum acid m.p. 123.5—124.5°. The mixed m.p. between the crystals from the essential oils of *Bellis* and the *trans*-acid was undepressed: 120.5—124.5°. By this saponification *trans*-lachnophyllum acid has obviously arisen through some displacement of an ene-diyne-chromophore into conjugation with a carboxyl group.

Similar changes in chromophores by saponification has been observed by Kavanagh *et al.*[8] with nemotin, by Anchel [9] with Drosophilin D, both acetylenic antibiotics from *Bassidiomycetes*.

The amounts available of oil of daisy prevent the elucidation of the structure of the compound which rearranges into *trans*-lachnophyllum acid. The observed rearrangement might indicate that the unusual occurrence of the *trans*-isomer of lachnophyllum ester in oil of daisy originates in a secondary displacement of the chromophoric system. The acidbase catalysed displacement of conjugated systems are known to lead to *trans*-isomers.

SUMMARY

From the essential oil of daisy there has been isolated the *trans*-isomer of lachnophyllum ester (I) whereas all hitherto known occurrences of this ester in nature comprise the *cis*-isomer. A chromatographic fraction of the daisy oil, which originally showed an ene-diyne chromophore, rearranged on saponification into an acid which turned out to be *trans*-lachnophyllum acid.

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Studies Related to Naturally Occurring Acetylene Compounds. XVI. A conjugated Tetraene — Cosmene — from the Essential Oil of *Cosmos bipinnatus*

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Recently Stavholt Baalsrud *et al.*[1] described in this journal the occurrence of a volatile polyene, b.p. ~40° at 0.3 mm Hg, present in small amounts in the essential oil of *Amellus strigosus* (Thunb.) Less. The absorption maxima in U. V. were given as:

3 091 2 959 2 822 and 2 300 (*cf.*[1] Fig. 3 p. 889)

By the spectroscopical investigations of a lot of new essential oils from plants belonging to the family of the *Compositae* cultivated at Norges Tekniske Högskole, Trondheim, in the summer 1952 this polyene was found to be present in at least the 9 following plants:

Ambrosia trifida	Root
Amellus strigosus	Leaves
Ammobium alatum grandiflorum	Leaves & root
Coreopsis Drummondi	Root
Coreopsis verticillata	Leaves & root
Cosmos bipinnatus	Flowers & leaves
Felicia amelloides	Leaves
Helianthus annus	Leaves
Pulicaria vulgaris	Leaves

In most cases the polyene is found present only in very small amounts and its presence is realised only when the substance is concentrated by chromatography or fractionation, but in *Coreopsis verticillata* and especially in *Cosmos bipinnatus* (investigated in 4 different garden varieties: *Cosmos bipinnatus* "Sensation Rosenelfe", "*praecox grandiflorus*", "*praecox nanus roseus*", "Gloria rosa") a polyene with these characteristic maxima constitutes an appreciable part of the essential oil. These oils are good starting materials for this polyene; the steam destillation however, has to be carried out in a slow stream of pure nitrogen and with exclusion of light, because the polyene, when

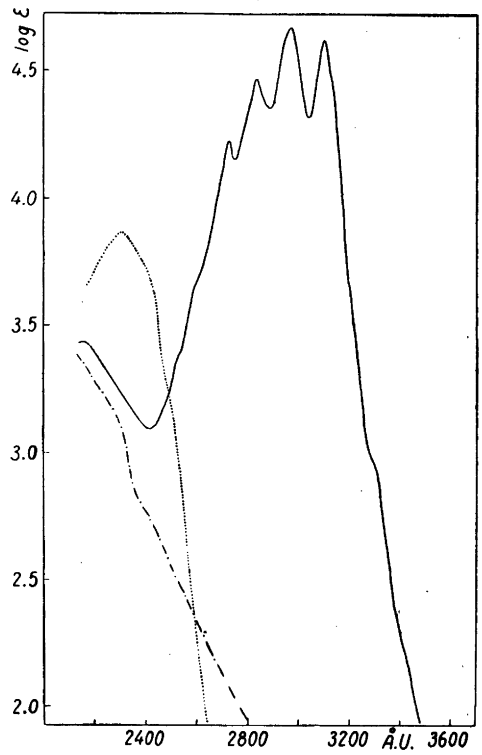

Fig. 1. Ultraviolet absorption in hexane of
——— *cosmene m.p. —1°*
····· *cosmene maleic acid anhydride mono adduct*
—·—·— *cosmene maleic acid anhydride mono adduct*

present in higher concentrations, readily polymerizes to a white solid insoluble in ordinary organic solvents.

Cosmos bipinnatus gives reasonable yields of essential oil — ~0.5 ‰ — with high extinction coefficients and has been used in the experiments to elucidate the constitution. At 0.2 mm the main portion distills at ~26° as a liquid which is colourless in absence of oxygen and light.

Stavholt Baalsrud *et al.* suggested that this polyene chromophore belonged to a hydrocarbon on the basis of the remarkable volatility and the fact that this chromophore is only slightly retarded on activated alumina in petroleum ether solution. Combustion analysis on concentrates from *Cosmos bipinnatus* confirmed this supposition. Such concentrates are utterly unstable in air and very difficult to analyse but as C + H was found > 98 % a substance with this volatility must obviously be a hydrocarbon.

The U.V. absorption maxima of this hydrocarbon — for which we propose the name Cosmene — are intermediate between octatetraene [2] and an ω,ω'-dialkyltetraene as for example parinaric acid [3].

Octatetraene	λ_{max}:	3 040	2 905	2 780	2 680
Cosmene	λ_{max}:	3 097	2 960	2 830	2 720
Parinaric acid	λ_{max}:	3 200	3 050	2 915	—

Cosmene thus might be a tetraene and it was tempting to try to obtain stable derivatives for analyses by addition of maleic anhydride.

Cosmene reacts readily with maleic anhydride at room temperature. Equimolecular amounts give a mixture of mono-adducts distilling at 90—95° at 0.0005 mm Hg as a viscous liquid. This monoadduct is obviously a mixture of isomers; the addition has to some extent taken part from the end of the tetraene system since the liquid monoadduct shows a single maximum at 2 300 ÅU, ε_{max} = 7 350, Fig. 1. A disubstituted butadiene has λ_{max} = 2 260 and ε_{max} = 21 000—22 000[4]. The diene portion of the mono-adduct thus might be about 33 %. The liquid monoadduct does not give a quite satisfactory analysis, the found values falling between the calculated values for C_9H_{12} and $C_{10}H_{14}$ + one mole maleic anhydride. A diadduct is obtained in fair yield when cosmene is distilled directly into a frozen solution of benzene-maleic anhydride which then is slowly thawed. The cosmenediadduct is a crystalline solid, m.p. 198—199 with decomposition. The U.V.-absorption curve shows only a step out below 2 400 ÅU. Analysis of the crystalline diadduct agrees well with $C_{18}H_{18}O_6$ that is $C_{10}H_{14}$ + 2 $C_4H_2O_3$.

Thus, cosmene is either a mono-ethyl- or a dimethyloctatetraene. From the infrared-spectrum we suppose that cosmene is 2 : 6-dimethyl-octatetraene. It was necessary to obtain cosmene in a state of reasonable purity before reliable conclusions could be drawn from the infrared spectrum, and it turned out to be very difficult to purify this tetraene. Our preliminary concentrates from *Cosmos bipinnatus* showed all of them the 2 300 ÅU max. in U.V., which was by far the strongest one in the *Amellus* fractions [1]. In these concentrates the hight of this 2 300-maximum was about the same as the 3 100—2 960 maxima. By repeated chromatography on alumina and distillation this 2 300 ÅU maximum was brought down about 1.0 in log $E_{1\,cm}^{1\%}$ in relation to the 3 100—2 960 maxima. These concentrates solidified at —30° and a further purification could be achieved by crystallisation from petroleum ether at —70°. The m.p. of the first crystal fraction was —7——8°. Recrystallisation brought the m.p. to —1——2° which could not be raised any further. In the U.V. spectrum of these preparations the 2 300 maximum had completely disappeared, as will be seen from Fig. 1. The best preparations had a minimum at 2 390 ÅU followed by a step out at shorter wavelengths. The extinction coefficient of the 3 100—2 960 maxima increased some 60 % by this purification. The 2 300-chromophore might belong to a related hydrocarbon with a diene-grouping.

The purification was checked by infrared spectra in the 2—15 μ region. Besides an appreciable increase in the intensity of most of the main absorption maxima, the purification led to a gradual disappearance of not less than 9 maxima of medium intensity (1 650, 1 612, 1 278, 1 105, 978, 865, 830, 783, 755 cm^{-1}).

The infrared spectrum of our purest cosmene preparation is given in Fig. 2 together with the curve given by Forrest Woods and Schwartzman for octatetraene [2] redrawn to the same scale. Both spectra are dominated by maxima characteristic of olefins. A comparison of the infrared spectrum of cosmene with those of other known olefins and polyenes makes the following conclusions justified.

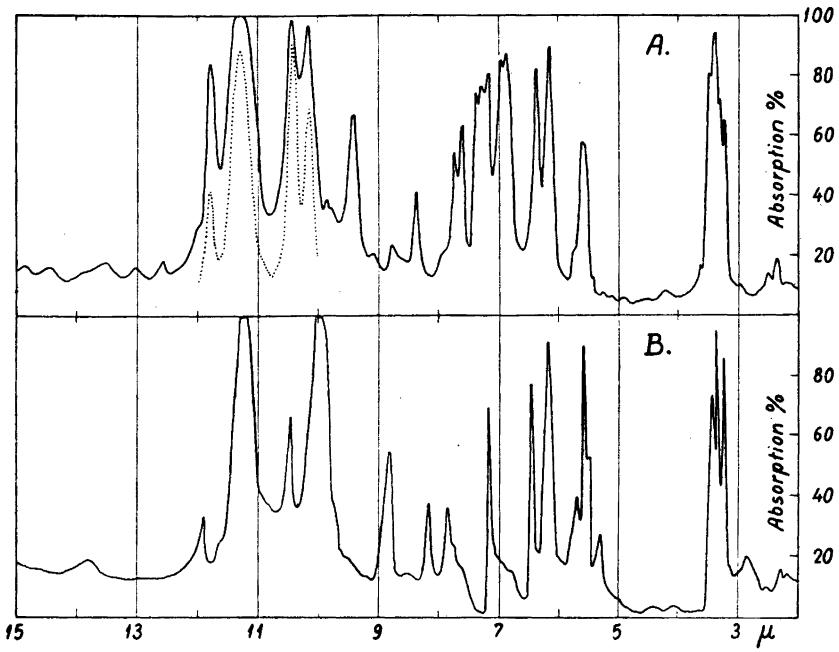

Fig. 2. Infrared absorption of
A: cosmene, liq., cell length ——— 0.025 mm ····· 0.0125 mm
B: octatetraene 1 : 3 : 5 : 7 in CS_2 soln. redrawn after [2].

1. Cosmene shows the strong 1 372 cm^{-1} band of the methyl group, which is lacking in the spectrum of octatetraene. Since there is no 770 cm^{-1} band — characteristic of an ethyl-group cosmene is obviously a dimethyl-octatetraene.

2. Cosmene shows a strong band at 958—960 cm^{-1}. A band at an identical position is present in octatetraene. This region is typical [5] of the symmetrically substituted double bond R—C = C—R′ both in the *cis* and the *trans* form.

H H

For isolated double bonds of this type the position 965—980 cm^{-1} is given; the slight displacement in octatetraene and cosmene is apparently due to conjugation. The *cis*-bond gives a strong peak in the 690—720 cm^{-1} region. Since cosmene shows only weak absorption in this region, the configuration must be *trans*.

The symmetrical *trans* double bond further gives a band of medium intensity at 1 295—1 310 cm^{-1}; cosmene has a sharp peak at 1 295 cm^{-1}.

3. The vinyl-group is characterized [5] by strong bands at 914 and 981—999, medium bands at 1 425 and 1 825 cm^{-1}. Rasmussen *et al.*[7] have shown that the 914 band of the vinyl group is displaced to 902—910 in simple dienes. In 4-methyl-1 : 3-pentadiene[8] this band lies at 894 cm^{-1} and in octatetraene [2] the position is at 898 cm^{-1}. The displacement of the 914 cm^{-1} band in dienes and polyenes cause this band to coalesce with the very strong 885—895 band of

the methylene group ($H_2C = C\langle$) if this grouping is present, *cf.* the spectra given by Sôrm, Pliva *et al.* for myrcene and natural farnesene [9,10,11].

As is seen from Fig. 2 cosmene has a rather strong 1 787 cm^{-1} band (octatetraene 1 805, 4-methyl-1 : 3-pentadiene 1 790 cm^{-1}) a strong 986 cm^{-1} band and a broad and strong band at 879—889, the two first undoubtedly belong to the vinyl group, the last to the sum of the coalescing vinyl and methylene bands.

4. The 885—895 cm^{-1} band of the methylene group is a very strong band and mostly very characteristic as it is little influenced by the rest of the molecule. The vinyl band in the 900 cm^{-1} region is regularily weaker than the 994 band, as is seen from Fig. 2. Cosmene, however, has a much stronger and broader band at 879—889 than at 986 cm^{-1}. There should be no doubt that the methylene group is present in cosmene.

Rasmussen *et al.*[7] has further demonstrated that all compounds containing the $H_2C = C$-group have bands between 1 640 and 1 668 cm^{-1}. Cosmene has two sharp peaks at 1 572 and 1 627 cm^{-1}; the corresponding peaks of octatetraene are found at 1 563 and 1 636 cm^{-1}, and thus these band might belong to the conjugated vinylgroup.

5. The tri-substituted double bond is characterized by a band of medium intensity in the 800—840 cm^{-1} region, the position of which is rather influenced by surrounding groups. Cosmene has a sharp band of medium intensity at 847 cm^{-1} which might belong to a trisubstituted double bond.

The infrared spectrum of cosmene thus rather distinctly indicates that this hydrocarbon contains all the types of double bonds present in 2 : 6-dimethyl-octa-1 : 3 : 5 : 7-tetraene (I).

$$CH_2 = \overset{\overset{CH_3}{|}}{C}-CH = CH-CH = \overset{\overset{CH_3}{|}}{C}-CH = CH_2$$

Other formulations (as 2 : 3 and 2 : 4-dimethyloctatetraene) would also satisfy the data given above. The preliminary choice of I has of course been made while a constitution as a dehydrogenated aliphatic monoterpene might be the most probable one for a branched dimethyloctatetraene. That cosmene must be branched is evident from the infrared spectrum as well as from the fact that deca-2 : 4 : 6 : 8-tetraene is a known compound, m.p. 125° [12].

The presence of ocimene (II) in the essential oil of *Tagetes glandulifera* has been demonstrated by Jones and Smith [13].

$$H_3C-\overset{\overset{CH_3}{|}}{C} = CH-CH_2-CH = \overset{\overset{CH_3}{|}}{C}-CH = CH_2 \qquad (II)$$

This occurrence has recently been confirmed by Sutherland [14], who established that the double bond position in ocimene is as in (II), in contrast to older work which preferred an isopropenyl end group. *Tagetes glandulifera* belongs to the plant family of the *Compositae*, *Tribus Heleniеae*, which tribus is rather little investigated chemically. It is interesting to note that in no fraction of the investigated essential oils originating from *Cosmos*-species or the other 8 plants mentioned above we have found spectroscopic evidence for the presence of conjugated trienes. It thus looks as if ocimene is not isomerised to allo-ocimene, but only dehydrogenated to a tetraene = cosmene.

The aliphatic sesquiterpene analogue of myrcene, natural farnesene [15], has been isolated from some plants belonging to the *Compositae* [11,1]. It should be mentioned that the essential oil of *Felicia Bergeriana (Tribus Astereae)* contains an extremely unstable substance with U.V. absorption maxima close to those of a carotenoid hexaene:

Volatile chromophore in *Felicia Bergeriana*							
$\Delta\nu \times 10^{-12}$	λ_{max} :	3 718	3 522	3 359		ÅU	(in Hexane)
		44.9		41.3			
2 : 4 : 6 : 8 : 10 : 12-Tetradecahexaene [12]	»	3 750	3 600	3 400	3 280	»	(» $CHCl_3$)
ω,ω'-Dihydrocrocetin [16]	»	3 790	3 600	3 400		»	(» alcohol)
Anhydro-vitamin-A_1 [24]	»	3 900	3 700	3 500		»	» »
iso Anhydro-vitamin A_1 [24]	»	3 700	3 500	3 300		»	» »

The hexaenic chromophore is present only in small amounts, and an isolation from natural sources would be rather futile if another much richer source of this polyene cannot be found. It would, however, be very interesting to learn if this hexaene were the sesquiterpene analogue of cosmene, that is tetradehydro-natural-farnesene. Thus with cosmene the dehydrogenated type of terpenes, so far characteristic of the tetraterpene class, is demonstrated for the first time as a natural product in the simplest of all terpenes, and the occurrence of this type in other terpenes is of general interest. It has to be remembered that it is the property of the carotenoids to be pigments and mostly rather stable solids with high crystallising power, which has made possible the isolation and elucidation of structure of this dehydrogenated class of compounds. Very unstable colourless liquid compounds occurring in the same low concentrations have very little chance to be discovered and isolated. The difficulties met with in the hydrogenated carotenoids phytofluene and phytoene are typical of the situation [17,18]. Whilst these hydrogenated carotenoids have chromophores with from 4 to 6 conjugated double bonds, it is worth while to remember that the same chromophores might belong to conjugated mono- and sesquiterpenes.

From alcoholic extracts of different *Centaurea*-species Löfgren [19,20] isolated, besides acetylenic hydrocarbons — the Centaur X hydrocarbons — some polyenes supposed to be tetraenes — the Centaur Y hydrocarbons. The U.V.-maxima of the Centaur Y-hydrocarbons are displaced some 80—98 ÅU relative to cosmene:

Centaur Y_1 and Y_2	3 195	3 045	2 915	2 800	ÅU
Cosmene	3 097	2 960	2 830	2 720	»

and so approaches the maxima of parinaric acid. We have so far not met the maxima of Centaur Y in any of our essential oils.

Centaurea also belongs to the *Compositae* family, tribus *Cynareae*. We have investigated some other members of this tribus, which seems to be very rich in acetylenic compounds and we have found some compounds, which, to judge from the frequency differences of the maxima, are polyenic. The spectral curves of the crude chromatographic fractions seem, however, to indicate aromatic compounds. The composition of these essential oils will be dealt with at a later occasion. So far the Centaur Y hydrocarbons seem to be restricted to the genus *Centaurea*.

EXPERIMENTAL

When preliminary experiments showed that the flowering and the green parts and the roots of the 4 different garden varieties of *Cosmos bipinnatus* gave essential oils with the same U.V.-spectra, the main plant material was steam distilled without separation in a 100 liter pan. When distilled without any precautions being taken, the essential oil at once polymerised into solid flakes swimming on the condensate; the main distillation was carried out with exclusion of light and in an atmosphere of pure nitrogen. All samples were stored in the cold with addition of a little hydroquinone and redistilled immediately before use. Yield of essential oil: flowers 2 ‰, leaves + stems 0.7 ‰, roots 0.4 ‰; cosmene concentration in the oil 20—40 %.

We have not succeeded in a purification of cosmene by fractional distillation alone. At 10 mm heating to the temperature necessary for distillation caused polymerisation of

most of the cosmene; at 0.3 mm the separation was unsatisfactory. The fractions boiling at about 30°/0.3 mm were combined and chromatographed on deactivated alumina [21] from petroleum ether solution and the chromatogram developed with further washings with petroleum ether. Portions of the filtrate were examined on their U.V.-absorption. Cosmene was only slightly retarded, slightly yellow zones in the chromatogram facilitate the separation of fractions. The fractions richest in the tetraene chromophore were combined, distilled and crystallised from petroleum ether at −70°. Since cosmene was very soluble in petroleum ether below zero the separation of the voluminous crystals was difficult. The best results were obtained with a diver with a glass filter which removed most of the solvent at −70° leaving a cake of solid cosmene on the filter. To remove the solvent this cosmene was redistilled, m.p. −2° − −1°. Infrared spectrum, compare Fig. 2 U.V. spectrum, compare Fig. 1.

ε_{max}	42 750	47 500	30 000	16 900	ÅU
λ_{max}	3 100	2 965	2 838	2 730	ÅU
$\Delta\nu_{max} \times 10^{-12}$	44.1	45.3	41.8		

Cosmene was too unstable for refractional measurements at ordinary temperature. The best fraction of cosmene gave immediately after redistillation, $n_D^{20} = 1.584$

cf. allo-ocimene $n_D^{25} = 1.5420$ [22]

ocimene $n_D^{25} = 1.4890$ [22]

myrcene $n_D^{21} = 1.4700$ [23]

As will be seen, the rise in conjugation from the conjugated dienes of the normal aliphatic terpenes to the trienoid and tetraenoid relatives increases the refraction index about 0.04−0.05.

Cosmene maleic anhydride mono adduct. 0.33 g of cosmene and 0.27 g of freshly distilled maleic anhydride in benzene solution was left at room temperature for 3 hours. The solvent was evaporated and the reaction mixture distilled at 0.0005 mm. Some unreacted anhydride went into the trap, and a viscous colourless oil passed over at air bath temperature 90−95°.

	Found	C 71.35, 71.28; H 7.64, 7.58
$C_{10}H_{14} + C_4H_2O_3$	calc.	» 72.37; » 6.95.
$C_9H_{12} + C_4H_2O_3$	calc.	» 71.32; » 6.47.

This liquid mixture of monoadducts could not be induced to crystallise, U.V.-spectrum in Fig. 1.

Cosmene-maleic anhydride diadduct. 0.40 g of cosmene was distilled at 0.3 mm directly into a trap containing 1.5 g of maleic anhydride in frozen benzene. The trap was slowly thawed and pure nitrogen let in only when the reaction mixture was liquid. Left under pure nitrogen for 12 hours. Worked up as above but the high vacuum distillation was interrupted at 90°. The excess maleic anhydride then was in the trap with small amounts of liquid in the receiver; the residue was solid. This residue was crystallised twice from acetone − petroleum ether. M.p. 198−199°.

$C_{10}H_{14} + 2\ C_4H_2O_3 = C_{18}H_{18}O_6$	Calc.	C 65.43; H 5.50.
	Found	» 65.53; » 5.84.

SUMMARY

In the essential oils of a dozen plants belonging to the *Compositae* there has been found a tetraene chromophore. All parts of *Cosmos bipinnatus* are rather rich in this compound which turned out to be a hydrocarbon of m.p. —1°, "Cosmene". Through a maleic anhydride diadduct the composition could be established to be $C_{10}H_{14}$; infrared studies established the structure as one of three dimethyl-octatetraenes. Of these possibilities the 2 : 6-dimethyl-octatetraene structure is tentatively chosen in analogy with normal aliphatic terpenes.

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Hyaluronic Acid

III. On Potassium Hyaluronate from the Endolymph of Sharks *

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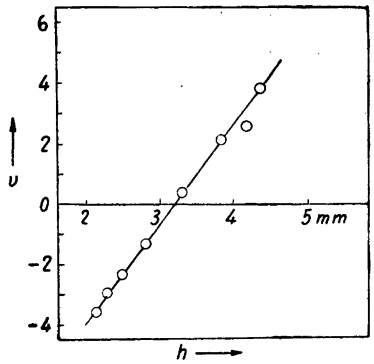
Potassium hyaluronate was isolated from the endolymph of shark ears. The nitrogen and potassium contents agreed with the theoretical values. From its osmotic pressure the molecular weight was calculated at 7.8×10^5. The substance is disaggregated by hyaluronidase.

As previously reported by Vilstrup and the author [1] it appeared likely that the endolymph of sharks contains hyaluronic acid, but the scarcity of the material prevented any attempt to its isolation. In the meantime, however, we have succeeded in collecting the endolymph from the ears of 150 sharks *(Acanthias vulgaris)*. This animal was employed as its labyrinths are especially easy of access, the skeleton is cartilageneous, and therefore technically easily manipulated. Moreover, the labyrinths of the sharks are relatively the largest known in the vertebrate system.

By a vertical section just behind the blow-holes the endolymphatic space, which contains the endolymph, is opened and the content, which is a translucent jelly, is withdrawn by gentle suction through a glass tube. By this technique 50 ml endolymph was obtained from 300 ears. The endolymph was apparently free from blood and cellular debris. The single endolymphatic clots merged into a homogeneous mass.

Immediately after withdrawing the endolymph it is cooled to —10° C, and the working up of the hyaluronic acid is started at once. During this investigation it became apparent that immediate cooling and quick working up of the starting material is very essential for the isolation of hyaluronic acid. The mucin is precipitated with about one volume per cent glacial acetic acid with vigorous stirring with a glass rod. As the endolymph is not miscible with water this precipitation must take place without the usual dilution with water. The mucin clot is washed quickly but thoroughly with water and dried in vacuum over sulphuric acid and silica gel. The dried powder is finely ground in a mortar and extracted with two or three volumes of glacial acetic acid at room temperature for three days with occasional stirring, the acid being replaced every

* Part I: *Acta Chem. Scand.* 3 (1949) 584, part II: *ibid.* 7 (1953) 603.



Fi. 1. The graph shows the relation between the height of the air column (abscissae), and the rate of movement of the upper meniscus of the air column (ordinates) in micrometer divisions in ten minutes.

twenty-four hours. By this procedure remnants of blood are removed, and the protein to which the hyaluronic acid is attached is split off. The acetic acid is now removed and the residue neutralized to pH 6.8 with a saturated potassium hydroxide solution (Glass electrode). The pulp is extracted with 4 volumes of water for three days, and the potassium hyaluronate is precipitated as a stringy mass with 4 volumes of alcohol. By adding more than the customary 1 1/2 volume of alcohol a further precipitate was formed. No flocculent precipitate was observed on standing. The preparation was purified according to Meyer [2]. The yield was 32 mg. The preparation was white and very hygroscopic; it dissolved in water in few hours, giving a clear solution.

The nitrogen content was determined by the method of Kjeldahl, modified by Blom [3]; (see Table 1). All volumetric flasks, the pipette and the burette

Table 1. The content of nitrogen and potassium, the viscosity and molecular weight of the potassium hyaluronate preparation.

% N*	% K**	Relative viscosity Conc. 1 g/l	Molecular weight $\times 10^{-5}$
3.33	9.01	24.24	7.79

* Calculated: 3.36.
** Calculated: 9.37

had been standardized. The potassium was determined as potassium sulphate; (see Table 1). The relative viscosity ($\eta_{rel} = \frac{t_H}{t_w}$, where t_H = flowtime of hyaluronate solution and t_w = flowtime of distilled water) was determined by a Kvorning and Dalgaard-Mikkelsen [4] viscosimeter; (see Table 1). Temperature 20° C (Variations about 0.01° C). The molecular weight (see Table 1) was determined by the inverted microosmometer recently described by Christiansen and the author [5]; temperature 20° C (Variations about 0.01 °C); the semipermeable testtube-shaped membrane used was a Kapcello cap. Fig. 1 shows the results of the single measurements.

As hyaluronidase is known to be highly specific towards hyaluronic acid (Chain [6], McClean [7], Robertson [8]) we tried to disaggregate our preparation

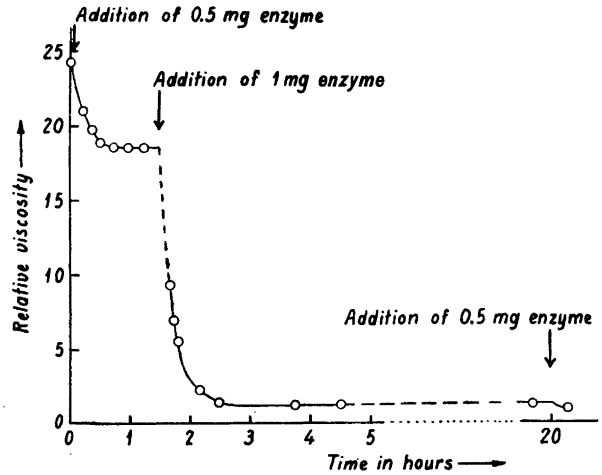


Fig. 2. Decrease in viscosity of a potassium hyaluronate solution caused by the action of testicular hyaluronidase.

by the action of a commercial (Schering) preparation of testicular hyaluronidase. When hyaluronic acid is disaggregated by hyaluronidase the viscosity of the aqueous solution is lowered.

Three millilitres of a 1 % aqueous solution of hyaluronate was incubated with 0.5 mg hyaluronidase at 20° C. The relative viscosity (measured in the viscosimeter mentioned above) which prior to the addition of enzyme was 24.24 decreased appreciably in the course of an hour. After that time the viscosity did not change, but after adding further 1 mg enzyme the viscosity decreased again and after a third addition of enzyme it equalled that of pure water. The results of the measurements are shown in Fig. 2. The diagram shows that the activity of the enzyme is rapidly inhibited. Whether this is due to destruction or adsorption of the enzyme, or the presence of inhibitory agents we do not know as yet.

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ACTA CHEMICA SCANDINAVICA 8 (1954) 295—298

*iso*Thiocyanates VII. A Convenient Synthesis of Erysoline (δ-Methylsulphonylbutyl *iso*Thiocyanate)

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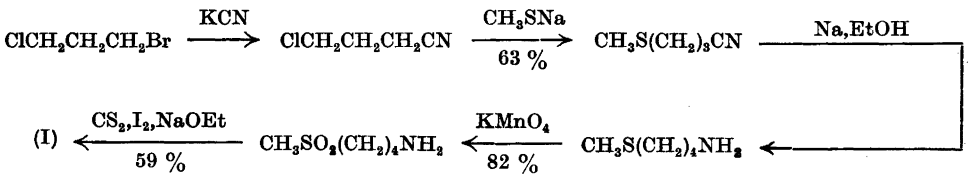
Several years ago Schneider and Kaufmann [1] isolated from seeds of the crucifer *Erysimum Perofskianum* Fisch. et M. an *iso*thiocyanate, erysoline, to which the structure (I) was assigned and synthetically confirmed.

$CH_3SO_2CH_2CH_2CH_2CH_2NCS$ (I) $\qquad$ $CH_3SO_2CH_2CH_2CH_2NCS$ (II)

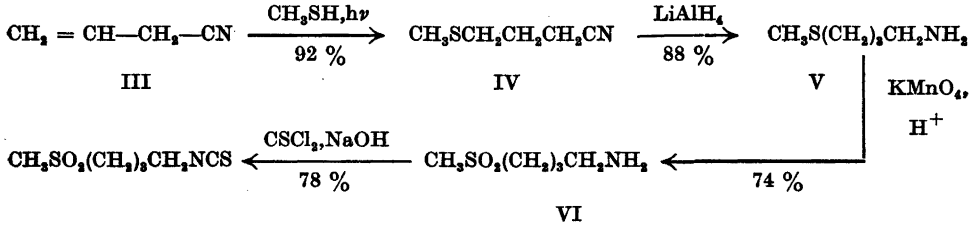
Because of the low contents of (I) in the seeds (*ca.* 0.05 %) the provision of larger quantities of erysoline from the natural source is attended with considerable difficulties. On the other hand, the synthesis described by Schneider and Kaufmann [1] implies a series of steps, which are not all convenient and satisfactory, as seen from the following scheme. The over-all yield can not be exactly estimated because some steps proceed in unstated yields but is most certainly less than 20 %.



In the course of current studies of naturally occurring *iso*thiocyanates it became necessary to have a substantial sample of erysoline at disposal. In a previous communication [2] we reported a convenient synthesis of cheiroline (II), the lower homologue of erysoline. Attempts to extend the preparative pattern to the synthesis of erysoline met with difficulties in the very first step, *viz.* the addition of methanethiol to the readily accessible allyl cyanide (III) to γ-methylthiobutyronitrile (IV). In the present case the 1,4-addition mechanism, undoubtedly operating in the addition to acrylonitrile, is precluded and no trace of (IV) was obtained under the usual reaction condi-

tions. However, the addition of mercaptans to carbon-carbon double bonds in unconjugated systems has been shown by various authors (see *e. g.* Ref.[3–6]) to be catalysed by light and peroxides, a fact which strongly suggests a free radical mechanism of the reaction. Consequently, it was not surprising to find that the addition of methanethiol to allyl cyanide under such conditions proceeded smoothly in almost quantitative yields. That the addition takes the desired course (contrary to Markownikoff's rule) appears from the identity of the further reaction products with those from the unambiguous synthetic route of Schneider and Kaufmann [1].

From γ-methylthiobutyronitrile (IV) the synthesis proceeds analogous to that of cheiroline [2] as shown in the following scheme.



The over-all yield in the above four-step reaction series is *ca.* 50 %, a considerable improvement over the *ca.* 20 % of the original synthesis. Furthermore, the present reactions can be conducted in a reasonably short time. Thus, ten grams of erysoline are readily produced in the course of three ordinary working days.

Erysoline has been characterised by its reactions with ammonia, aniline, *p*-toluidine and α-naphthylamine to the corresponding thioureas (VII, a—d).

$CH_3SO_2CH_2CH_2CH_2CH_2NHCSNH—R$

VII

a. R = H
b. R = C_6H_5
c. R = *p*-$CH_3C_6H_4$
d. R = α-$C_{10}H_7$

The physical and analytical data of these derivatives are summarized in Table 1.

Table 1.

Amine	Formula	Composition	M. p., °C	Analyses			
				Nitrogen		Sulphur	
				Calcd.	Found	Calcd.	Found
Ammonia	VII a	$C_6H_{14}O_2N_2S_2$	147 [a]	13.32	13.32	30.50	30.36
Aniline	VII b	$C_{12}H_{18}O_2N_2S_2$	105	9.78	10.04	22.39	22.55
p-Toluidine	VII c	$C_{13}H_{20}O_2N_2S_2$	108	9.33	9.41	21.34	21.32
1-Naphthylamine	VII d	$C_{16}H_{20}O_2N_2S_2$	163	8.33	8.54	19.06	19.27

[a] Ref.[1] reports 143—144°.

The ultra-violet absorption spectra of erysoline (I) and its thiourea-derivative (VII, a) were determined in ethanol solutions and found similar to those previously reported in the cheiroline series [2]. Both exhibited a broad maximum, which in the case of erysoline was located at 243 mμ (ε 910), while the thiourea curve had its peak at 242 mμ (ε 13 420) and a minimum at 223 mμ (ε 3 600).

Bacteriological tests have indicated a very pronounced effect of both cheiroline and erysoline on a rather large selection of pathogenic bacteria. Gilliver [8] formerly reported on the strong inhibitory action of cheiroline on plant pathogenic bacteria and fungi. The plant pathogenic fungus *Alternaria circinans* was included in the present studies and it was observed that both cheiroline and erysoline exhibited a 50 % growth inhibition of this organism in dilutions of 1 : 200 000 in a 2 % malt extract substrate.

EXPERIMENTAL *

γ-Methylthiobutyronitrile (IV). A mixture of 33.5 g (0.5 mole) of freshly distilled allyl cyanide [7], 24.0 g of methanethiol (0.5 mole) and 200 mg of benzoyl peroxide was placed in a quartz flask and irradiated at room temperature for 24 hours with an effective mercury-quartz lamp. The mixture was then distilled *in vacuo*, 53.0 g (92 %) of pure (IV) being collected at 101—103° and 13 mm.

Schneider and Kaufmann [1] obtained the same compound in 63 % yield from the metathetical reaction between γ-chlorobutyronitrile and sodium methylmercaptide. They report the b.p. 218° at ordinary pressure.

*δ-Methylthio-*n*-butylamine* (V). The above nitrile was reduced with lithium aluminium hydride as previously described for the lower homologue nitrile [2]. From 34.5 g of (IV) and 16.0 g of the hydride in 350 ml of anhydrous ether, a total amount of 31.6 g (88 %) of the colourless amine (V) was obtained after distillation. B. p. 77.5—78.5° at 12 mm.

The same compound, b. p. 188—190° at ordinary pressure, was formerly prepared [1] in unstated yield by reduction of the nitrile with sodium and ethanol.

*δ-Methylsulphonyl-*n*-butylamine* (VI). The oxidation of the above amine to the sulphone (VI) was conducted in acid solution, essentially as described [1]. A 74 % yield of the hydrochloride of (VI) was obtained. M. p. 162—163°. (Ref. [1] reports the m. p. 160°.)

Erysoline (I). Upon treatment of the above hydrochloride in aqueous solution with thiocarbonyl chloride and sodium hydroxide, closely following the directions from the cheiroline synthesis [2], a 78 % yield of crude erysoline with almost correct m. p., (58.5—59.5°), was obtained. An analytically pure specimen was prepared as beautiful colourless needles upon an additional recrystallization from ether, m. p. 60.0—60.5°; (Ref. [1] gives the m. p. 59—60°).

$C_6H_{11}O_2NS_2$ (193.3)	Calc.	N 7.25	S 33.16
	Found	» 7.24	» 33.16

Thiourea-derivatives (VII, a—d). Erysoline was transformed into the corresponding thiourea-derivative (VII, a) upon treatment with concentrated aqueous ammonia in ethanol solution at room temperature for 18 hours. The derivatives with the aromatic amines were prepared by heating the *iso*thiocyanate with a slight excess of the amine in ethanol to boiling for half an hour. All derivatives were recrystallized to constant melting points from 95 % ethanol. The melting points and analytical data are summarized in Table 1.

Ultra-violet absorption spectra. The spectra of (I) and (Va) were determined in ethanol solutions on a Beckman model DU quartz spectrophotometer in 1 cm cells.

* All melting points are uncorrected and determined in capillary tubes in an electrically heated block.

SUMMARY

A convenient synthesis of the *iso*thiocyanate, erysoline (I), occurring in the seeds of *Erysimum Perofskianum* Fisch. et M., is described. It proceeds from allyl cyanide in four steps with an over-all yield of *ca.* 50 %.

Erysoline has been characterized by its transformation into four thiourea-derivatives.

Microanalyses were performed in this laboratory by Mr. W. Egger. The assistance of Mr. F. Marcus in the preliminary experiments is greatly acknowledged. Thanks are due to the *Ferrosan Co.* for the performance of the bacteriological tests. The work is part of investigations supported by *Carlsbergfondet (The Carlsberg Foundation)*.

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ACTA CHEMICA SCANDINAVICA 8 (1954) 299—317

On Equilibria in Systems with Polynuclear Complex Formation. I. Methods for Deducing the Composition of the Complexes from Experimental Data. "Core + Links" Complexes

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The formation of a series of consecutive *mononuclear* complexes has been treated by a number of authors. Equations and diagrams have been given to show how the equilibria are shifted with varying composition of the solution and how the equilibrium constants involved can be calculated from experimental data of varying type and accuracy (*e.g.* N. Bjerrum [1], J. Bjerrum [2], Leden [3]).

The formation of *polynuclear* complexes has not met with the same interest. In general they have been considered as an annoying disturbance, impeding the measurement of the interesting mononuclear equilibria, and not being studied for their own sake.

Sometimes, as for instance in the chromium(III) hydrolysis studied by N. Bjerrum and Faurholt [4,5], the polynuclear complexes are formed by very slow reactions. In other systems, such as some of the copper-complex systems studied by Fronaeus [6], the polynuclear complexes seem to be formed by rapid reversible equilibria. Fronaeus eliminated them by extrapolation of his data to low concentrations, where the mononuclear complexes predominate. A similar procedure was attempted in Ahrland's paper [7] on the hydrolysis of the uranyl ion, although the main reaction products in this case are obviously polynuclear complexes.

In systems where polynuclear complexes occur, the equilibrium data have sometimes been explained in terms of reactions by which only one or two polynuclear species are formed. Such an explanation may, in a certain concentration interval, be approximately true for the equilibria between CrO_4^{2-}, $HCrO_4^-$, and $Cr_2O_7^{2-}$ in dilute acid chromate solutions (see *e.g.* Neuss and Rieman [8]), although there is certainly evidence for the formation of other polynuclear complexes, *e.g.* $Cr_3O_{10}^{2-}$. In many other cases one may doubt whether it is experimentally proved or even at all likely that Nature would select, out of all possible polynuclear complexes, exactly the one or two proposed by a certain chemist.

In later years, evidence has accumulated that in many systems an apparently unlimited series of polynuclear complexes are formed. It is believed that the first attempt to formulate such a mechanism quantitatively was the work of Granér and Sillén [9] on the hydrolysis of the ion Bi^{3+} which could be explained in terms of the formation of polynuclear complexes $Bi_{n+1}O_n^{(n+3)+}$. Later on, Brosset [10] has suggested that the aluminium ion forms complexes $Al_n(OH)_{3(n-1)}^{3+}$ in the acid pH range and $Al_n(OH)_{3n+1}^{-}$ in the alkaline range. Recently, Leden [11] has found evidence for the formation of complexes $Ag_nI_{2n+2}^{(n+2)-}$, and the corresponding complexes of thiocyanate and silver ions. As will be shown in subsequent papers, a similar mechanism can explain the equilibrium data in several other systems as well.

If polynuclear complexes are formed, the number of possible complexes, and thus of equilibrium constants, is infinitely larger than when there are only mononuclear complexes. If one finds one mechanism that can explain the experimental data, one may thus be in doubt whether the same data could not be explained equally well by a quite different mechanism.

In the present paper and a few ones following an attempt will be made to discuss the mathematical laws for the formation of polynuclear complexes, both under rather general assumptions and for a few special mechanisms. The chief aim has been to find out how much information on the mechanism one can really find from the experimental data, which by necessity are of limited accuracy and cover a limited range of concentrations.

The discussion is not limited to complexes of a metal ion and some ligand. The two complex-forming species A and B in the following treatment may be any charged or uncharged molecular species: Ag^+ and I^- or I^- and Ag^+, a metal ion and OH^- or any other ligand, a fatty acid HA and the corresponding ion A^-, H^+ and A^- or SiO_2 and OH^- etc.

In this paper we have made two fundamental assumptions: *i.e.* that we are dealing with real equilibria and that all complexes are dissolved in the solution. If equilibrium has not been attained, all arguments based on the law of mass action are of course invalid unless the slow reactions are so slow that they can be neglected.

The precipitation of a solid phase of constant composition will be found as a special case of several mechanisms discussed in the following (see part II) [12]. However, there is also the possibility that A and B form a separate phase of composition variable within a certain range. This behavior may not be expected to be usual for the hydrolysis products of metal ions or for the polynuclear silver halogenide complexes but has been proposed for the micelles in soap solutions. We hope to be able to return to this point later on in connection with work now in progress in this institution.

Equations for equilibria with a solid solution phase are not very difficult to set down. However, it is felt that one should first have real systems to test the equations in order to see which of them are really practicable with the present accuracy of measurement.

List of symbols

A	total concentration of A (2)
a	concentration of free A (1)
B	total concentration of B (2)
b	concentration of free B (1)
C	$= B =$ "total concentration of C" (32)
c	free concentration of complex C $= A_{r+t}B$ (30)
c_n	concentration of n:th complex in (21) or (31)
f	$= Sb^{-1}$, function of u (22)
k_n	equilibrium constant, defined by (21) or (31)
N	singular value for n, text around (27 to 29)
n	variable integer; number of links in a "core + links" complex
P	singular value for p, (27 to 29)
p	number of A in a complex (2)
Q	singular value for q (27 to 29)
q	number of B in a complex (2)
R	constant partial derivative in (16), obtained from spacing between $Z(\log a)_B$ curves
r	constant number of A in core (if free from B); (8 a) and Table 1
S	complexity sum (4)
s	constant number of B in core (if free from A); (8 a) and Table 1
T	constant partial derivative in (40), obtained from spacing between $\log \frac{B}{b}$ $(\log a)_B$ curves
t	constant number of A per B in link; (8 a) and Table 1
u	$= a^t b$ (10) $(= a^{-r}c$ in (30))
x	$= t \log a + \log B$ (26)
y	$= \frac{A-a}{Bt} = \frac{Z}{t}$ (25)
Z	$= \frac{A-a}{B}$ (7), average number of A bound per B
η	$= \log \frac{B}{b}$ (44)
$\varkappa_n$	equilibrium constant, defined by (9)
$\varkappa_{pq}$	equilibrium constant defined by (3)
φ	$= Sa^{-r}$, function of u (11)

General equations

We shall denote the two reactants by A and B. They may be charged but for simplicity no charges will be indicated. Let the total concentrations of the reactants, including the amounts bound in complexes, be A and B mole l^{-1}, and the free concentrations.

$$[\mathrm{A}] = a \text{ and } [\mathrm{B}] = b \qquad (1)$$

When A and B react, one or several complexes A_pB_q are formed in the solution; here p and q may be any integer. For the total concentrations A and B we have:

$$A = a + \Sigma p[A_pB_q];\ B = b + \Sigma q[A_pB_q] \tag{2}$$

where the summations are made over all possible sets (p, q) excluding (1, 0) and (0,1) which correspond to free A and B.

We shall assume that we can regard the activity factors as constant; if A or B or both are charged, the reactions must be studied in a constant ionic medium. Consequently we can use concentrations instead of activities in the law of mass action and measure ionic concentrations directly by emf methods.

For the formation of any complex A_pB_q we then have

$$pA + qB \rightleftharpoons A_pB_q;\ [A_pB_q] = \varkappa_{pq}a^pb^q, \tag{3}$$

where $\varkappa_{pq}$ is the equilibrium constant of the reaction.

It is convenient to introduce a quantity S which might be called the *complexity sum;*

$$S = \Sigma[A_pB_q] = \Sigma\varkappa_{pq}a^pb^q \tag{4}$$

From equations (1—4) we find the amount of A bound in complexes, $A—a$ mole/l:

$$A—a = \Sigma p[A_pB_q] = \Sigma p\varkappa_{pq}a^pb^q = a\left(\frac{\partial S}{\partial a}\right)_b = \left(\frac{\partial S}{\partial \ln a}\right)_b \tag{5}$$

Similarly the amount of B bound in complexes, $B—b$ mole B/l, is

$$B—b = \Sigma q\varkappa_{pq}a^pb^q = b\left(\frac{\partial S}{\partial b}\right)_a = \left(\frac{\partial S}{\partial \ln b}\right)_a \tag{6}$$

A quantity which we shall often have use for is the average number of A bound per B; we shall denote it by Z

$$Z = \frac{A—a}{B} \tag{7}$$

It follows from (7), (5), and (6)

$$Z = \frac{a\left(\frac{\partial S}{\partial a}\right)_b}{b\left[1 + \left(\frac{\partial S}{\partial b}\right)_a\right]} = \frac{\left(\frac{\partial (S+b)}{\partial \ln a}\right)_b}{\left(\frac{\partial (S+b)}{\partial \ln b}\right)_a} \tag{7 a}$$

Types of experimental data

In studies on complexity equilibria, it should always be possible to measure the total concentrations A and B by chemical analysis or to calculate them from the way the solution has been prepared. It is often convenient to let one of them, say B, be constant in each group of experiments. Then B has only a limited number of values: B_1, B_2, B_3, etc., whereas A is varied freely.

It is often possible to measure one of the free concentrations a and b, sometimes both. Since the following equations will be applicable even if A and B are interchanged, we may restrict our treatment to two cases:

Data (a, A, B). (Equations 14—38). Only a is measured but not b. The results may conveniently be plotted by giving $Z = \frac{A-a}{B}$ as a function of $\log a$, with one curve for each B value: B_1, B_2 etc. An example is the hydrolysis of the thorium ion (Hietanen [13]) where $[H^+]$, which is proportional to $[OH^-]^{-1}$, was measured by emf methods but not $[Th^{4+}]$.

Data (a, b, A, B). (Equations 39—49) Both a and b are measured. If, say, b is more accurately known than a, then $\frac{b}{B}$ and $\frac{B-b}{A}$ are the most accurately known functions but $\frac{A-a}{B}$ and $\frac{A-a}{B-b}$ may also be calculated and plotted, *e.g.* as functions of $\log a$. An example is the hydrolysis of Bi^{3+} where both $[Bi^{3+}]$ and $[H^+]$ could be measured [9].

Other types of data: Suppose that one has measured the *solubility* of a certain solid A_PB_Q (which may in addition to A and B contain solvent molecules and ions from the ionic medium) *. Then one knows that in all solutions the product a^Pb^Q is a constant, at first unknown. In general, solubility data alone are insufficient for a mathematical analysis of the equilibria in a system with polynuclear complexes, since such data are limited to a single curve in the (a, b) field. With emf methods, on the other hand, one can cover a two-dimensional area.

Two-dimensional areas in the (a, b) field are covered also by *extraction* or *ion exchange* measurements. These methods have proved valuable for studying the formation of mononuclear complexes, in which only one uncharged complex is extracted (Rydberg [14]) or only one or a few charged complexes are taken up by the ion exchanger (Fronaeus [15]). If polynuclear complexes are formed, one may expect a number of different complexes to be extracted, or sorbed in the ion exchanger. Such data may be attacked by the same general methods as given below, after introducing a few more reasonable assumptions. They shall not be discussed here, however, nor shall we discuss optical data which do not seem suitable for investigating polynuclear complexes (*cf.* Fronaeus [6], p. 89 ff) unless it is possible to devise an optical method which gives directly the number of A—B bonds.

Representation of (p, q) set

The set of all pairs of non-negative numbers (p, q) can be represented as a two-dimensional array of points in one fourth of an infinite plane **. (Fig. 1) We shall denote by black dots ● those points corresponding to complexes

* *E.g.*, if $A = I^-$, $B = Ag^+$, and the ionic medium is $NaClO_4$ in water, then $NaAgI_2(H_2O)_{3\frac{1}{2}}(s)$ would count as $A_2B(s)$.

** If one would prefer to define the reactants so that p or q may become negative (say, to consider the formation of OH^- complexes as a subtraction of H^+), the (p, q) domain would be different, but nothing would be changed in the following argument and equations.

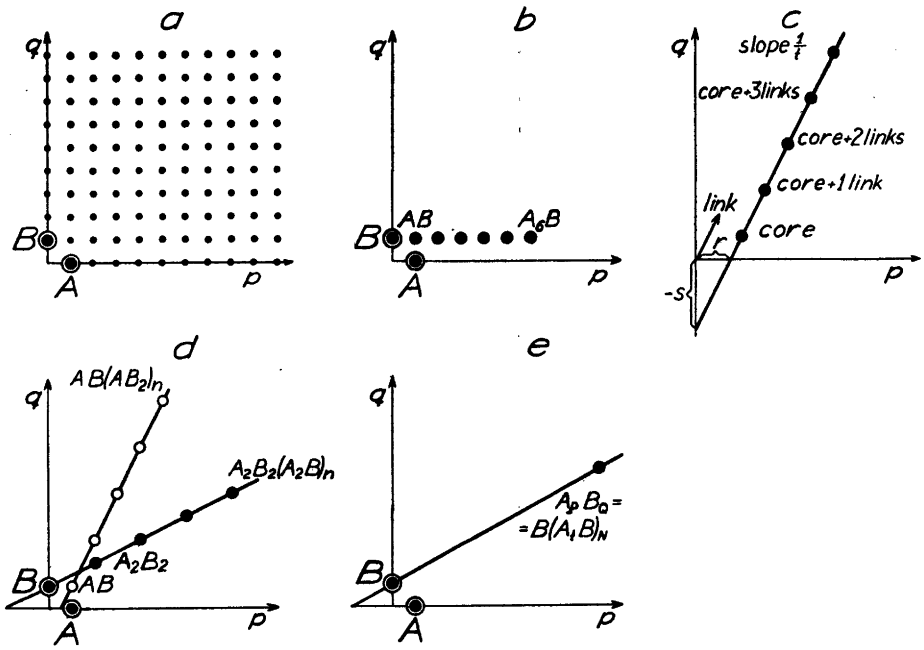

Fig. 1. Geometrical representation of the (p, q) *set. Points = pairs of integers* (p, q), *dots* ● *= pairs* (p, q) *corresponding to an existing complex* A_pB_q. *The pure components A and B are dots with circles* ⊙ *in a, b, d, and e. a) No complexes formed. b) Only mononuclear complexes* A_nB (n = 1,2 ... 6). *c) Compositions of complexes correspond to "core" +* n *"links"; the corresponding points are on a straight line with intercepts* r *and* s, *and slope 1/*t. *d) Two "core + links" mechanisms: complexes* $A_2B_2(A_2B)_n$ (r = −2, s = 1, t = 2; *dots* ●) *and complexes* $AB(AB_2)_n$ (r = ½, s = −1, t = ½; *circles* ○). *e) Only one complex formed,* $A_PB_Q = B(A_1B)_N$.

that actually occur in the solution. Fig 1 a represents a system where no complexes are formed: there are dots only for A (1,0) and for B (0,1). In Fig. 1 b there are in addition mononuclear complexes A_nB up to $n = 6$.

For any pair of reactants, it would seem unlikely that the polynuclear complexes, if they occur at all, would be spread equally over the whole area. For instance, if A and B are ions, complexes corresponding to points close to the coordinate axes are not likely to occur at a distance from the origin, since they would consist of large clusters of ions of the same sign, containing only a few opposite ions. Let us imagine that there is over each point (p, q) in the diagram, a rod, perpendicular to the paper, the height of which gives the concentration of the corresponding complex A_pB_q in a certain solution. Then it seems likely that the ends of the rods would lie on a ridge, making an angle with both coordinate axes.

For any type of data it is not so hard to devise some straight-forward mathematical method — involving, for instance, repeated division by a or b and extrapolation to 0, or repeated differentiation — that would finally give

us a complete set of equilibrium constants $\varkappa_{pq}$, one for each complex A_pB_q. Unfortunately such methods of calculation would require an accuracy for the data which is hardly ever met with in practice. So one must in practice test various simplifying assumptions.

THE "CORE + LINKS" HYPOTHESIS

Restrictions on p and q

We shall see in the following that a rather frequent type of experimental data leads to the conclusion that all complexes that exist in appreciable amounts have a composition corresponding to a certain "core" + a varying number of "links".

We shall now study the hypothesis — the "core + links" hypothesis — that no other complexes than these are formed. In the (p, q) diagram this means that all complexes that occur correspond to points on a straight line, passing through the composition of the core and with a slope depending on the composition of the link (see Fig. 1 c). In Fig. 1 d are given two specific examples: in one (black dots), the core is A_2B_2 and the link A_2B and thus the general composition of the complexes is $A_2B_2(A_2B)_n$. In the other (open circles), the core is AB and the link AB_2.

We shall characterize the line by its slope $1/t$, its intercept r with the p axis and its intercept s with the q axis (Fig. 1 c). These three quantities are related by

$$r = -ts \tag{8}$$

and the equation of the line is

$$p = tq + r = t(q-s) \tag{8 a}$$

One may also say that the complexes can be written as $A_r(A_tB)_n$ or $B_s(A_tB)_n$, where r, s and t are constants and the number n stands for any number that will make p and q integers. Table 1 shows, for a few specific examples, how

Table 1. Formulas for complexes built up of "core" + n "links". Possible ways of representing complexes

core	link	core(link)$_n$	$A_r(A_tB)_n$	$B_s(A_tB)_n$	r	s	t
A_2B_2	A_2B	$A_2B_2(A_2B)_n$	$A_{-2}(A_2B)_n$	$B(A_2B)_n$	-2	1	2
A_2B	AB	$A_2B(AB)_n$	$A(AB)_n$	$B_{-1}(AB)_n$	1	-1	1
A_3B_2	AB_2	$A_3B_2(AB_2)_n$	$A_2(A_{\frac{1}{2}}B)_n$	$B_{-4}(A_{\frac{1}{2}}B)_n$	2	-4	$\frac{1}{2}$
AB	AB_2	$AB(AB_2)_n$	$A_{\frac{1}{2}}(A_{\frac{1}{2}}B)_n$	$B_{-1}(A_{\frac{1}{2}}B)_n$	$\frac{1}{2}$	-1	$\frac{1}{2}$
B	A_tB	$B(A_tB)_n$	$A_{-t}(A_tB)_n$	$B(A_tB)_n$	$-t$	1	
A	A_tB	$A(A_tB)_n$	$A(A_tB)_n$	$B_{-1/t}(A_tB)_n$	1	$-1/t$	
—	A_tB	$(A_tB)_n$	$(A_tB)_n$	$(A_tB)_n$	0	0	

any "core + links" formula can be transformed to either one of these two forms. Of the constants, t is usually positive, whereas either r is negative, or s is negative, or both are zero. One or two of the constants or all may assume fractional values.

The "core + links" hypothesis does *not* require that *all* complexes on the line (8 a) are really formed in the solution. Perhaps only one or a few complexes really occur. What we have assumed is however that no complex A_pB_q is formed in appreciable amount which does not correspond to a point on the line (8 a):

$$p = tq + r$$

We shall follow this hypothesis and see how the experimental data can be used for finding r and t. If the experimental data lead to and are consistent with a certain line $p = tq + r$, this should indicate that the "core + links" hypothesis, even if not absolutely true, is a useful approximation.

If only one or two polynuclear complexes exist, our hypothesis is automatically valid since it is always possible to draw a straight line through one or two points (see *e.g.* Fig. 1 e).

In passing, it may be noted that this description of the "core + links" complexes as $A_r(A_tB)_n$ or $B_s(A_tB)_n$ can be applied to mononuclear complexes, AB_n ($r = 1, t = 0$), but not so well to A_nB. This little lack of symmetry has no practical importance since a system with only mononuclear complexes is easily recognized (see below) and can be well treated by the simple methods already given in the literature.

Equilibrium equations

If the "core + links" hypothesis is valid, only those equilibrium constants $\varkappa_{pq}$ that correspond to points on the line (8 a) will differ from 0. We shall denote the equilibrium constant for the formation of $A_r(A_tB)_n$ by $\varkappa_n$.

$$(r + nt)\ A + nB \rightleftharpoons A_r(A_tB)_n$$

$$[A_r(A_tB)_n] = \varkappa_n a^{r+nt} b^n = \varkappa_n a^r (a^t b)^n = \varkappa_n a^r u^n \tag{9}$$

We have introduced for convenience the variable

$$u = a^t b \tag{10}$$

Introducing (9) into (4), we find

$$S = a^r \Sigma \varkappa_n u^n = a^r \varphi(u) \tag{11}$$

Equations (11) and (5), and (7) give

$$A—a = a \left(\frac{\partial S}{\partial a}\right)_b = a^r \left[r\,\varphi(u) + tu\ \varphi'(u)\right] = BZ \tag{12}$$

In the partial derivation of φ, (10) should be remembered. Similarly we find from (11) and (6)

$$B—b = b \left(\frac{\partial S}{\partial b}\right)_a = a^r u \varphi'(u) \tag{13}$$

Equations (12), (13), and (10) will be the basis for the following discussion and for our methods for finding r and t from the data.

TREATMENT OF DATA (a,A,B)

The $Z(\log a)_B$ curves; limiting values for Z

Let us assume that we have summarized our experimental data by plotting $Z = \frac{A-a}{B}$ as a function of $\log a$. We thus have a number of curves, on each of which the total concentration B has a constant value: B_1, B_2 etc (see Fig. 2).

As a matter of fact, in practically all systems hitherto studied these curves $Z(\log a)_B$ seem either to be all parallel with a spacing ($\Delta \log a$) proportional to $\Delta \log B$ or to coincide. These two types of behavior will be dealt with separately; they both logically lead to a "core + links" mechanism. Then some equations will be given which may be used for finding r and t in other cases, assuming the "core + links" hypothesis to be valid for them too.

Sometimes the concentration of one complex $A_r(A_tB)_n$, with one special value for n, may predominate over the concentrations of all other complexes and that of free B; then the average number of A bound per B is $Z = t + \frac{r}{n}$, as can be seen directly from the formula of the complex (and can also be derived from equations 11—13).

Sometimes the *concentration of free B is negligible* within the whole range studied — an example is the formation of aluminate complexes $OH(Al(OH)_3)_n^-$ ($r = 1$, $t = 3$, n may vary) [10,16] where the concentration of free Al^{3+} is negligible in the whole range in which these complexes have been studied. Then Z has the two limiting values (compare Fig. 2 b)

$$Z = \frac{A-a}{B} \begin{cases} t + \frac{r}{n_{max}} & \text{for decreasing } \log a \\ t + \frac{r}{n_{min}} & \text{for increasing } \log a \end{cases} \Bigg\} \begin{array}{l} \text{if } r > 0 \text{ and} \\ \text{always } b \ll B \end{array} \qquad (14)$$

Equation (14) may be derived from (11—13) assuming r to be positive, as it generally seems to be in such cases. (If r were negative, n_{max} and n_{min} would change places in (14).) If n can have any value between 1 and ∞, then the limits for Z are t and $t + r$.

If, on the other hand, *free B predominates* over all complexes *at one end* of the range studied, then (compare Fig. 2 a)

$$Z = \frac{A-a}{B} \begin{cases} 0 & \text{for decreasing } \log a \ (b \approx B) \\ t + \frac{r}{n_{max}} & \text{for increasing } \log a \ (b \ll B) \end{cases} \qquad (15)$$

Here r is usually negative; if $n_{max} = \infty$, the limits are 0 and t. (If r were to be positive, the limit for large a would be $Z = t + r/n_{min}$.)

Parallel curves $Z(\log a)_B$

It is very often observed that the curves giving $Z(\log a)_B$ have the same shape, within the limits of experimental error, and are only shifted parallel with the log a axis. Moreover it is found that the spacing $\Delta \log a$ between

two curves, say for B_1 and B_2, is proportional to the logarithm of the B ratio, $\Delta \log B = \log (B_1/B_2)$. Let us denote by R the constant ratio $\Delta \log B/\Delta \log a$. We then have

$$\left(\frac{\partial \log B}{\partial \log a}\right)_Z = R = \text{constant}; \; Z(a^{-R}B) \tag{16}$$

i.e. $Z = \dfrac{A—a}{B}$ must be a function of $a^{-R}B$.

Let us start with the general equations (5), (6), and (7), permitting *a priori* any formula A_pB_q. We find

$$Z = \frac{A—a}{B} = \frac{\Sigma p\varkappa_{pq}a^pb^{q-1}}{1 + \Sigma q\varkappa_{pq}a^pb^{q-1}} \tag{17}$$

$$a^{-R}B = a^{-R}(b + \Sigma q\varkappa_{pq}a^pb^q) = a^{-R}b(1 + \Sigma q\varkappa_{pq}a^pb^{q-1}) \tag{18}$$

If Z is to be a function of $a^{-R}B$ only, then the conditions are:

1) If *b is not negligible* in comparison with B, all terms a^pb^{q-1} in the sums in (17) and (18) must be powers of $a^{-R}b$, which appears as a separate term in (18); thus $p = —R(q—1)$ or

$$p = —Rq + R \tag{19}$$

As seen by comparing (19) with (8 a) we would have "core + links" complexes with

$$R = r = —t; \; s = 1 \tag{19 a}$$

It is more convenient to write the formula of the complexes as $B(A_tB)_n$ than as $A_{-t}(A_tB)_n$. In this case the spacing gives both r and t. If only one complex A_PB_Q occurs, the $Z(\log a)_B$ curves also become parallel (eq. 29 below). This is best treated as a special case of $B(A_tB)_n$.

2) If *b is negligible* in comparison with B, the "1" disappears in the denominator of (17) and in the last term of (18). We may then multiply both sums in (17) by $a^{-R}b$ so that the sums in (17) and (18) contain the same terms $a^{p-R}b^q$. The condition for Z to be a function of $a^{-R}B$ is then that all these terms are powers of the same variable. We may always write this variable in the form a^tb, where t is a constant to be determined. We may thus from (16) deduce the equation

$$p—R = tq \tag{20}$$

By comparing (20) with (8 a) we find that we would still have "core + links" complexes; the general formula is $A_r(A_tB)_n$ where

$$r = R \tag{20 a}$$

may be found from the spacing of the curves. To find t one may use the limiting values of Z, and (14).

Consequently, if the family of curves $Z(\log a)_B$ turns out to be parallel, the chief product must be "core + links" complexes, and then the general formula can be obtained from the spacing and the limiting values. As a further step one should like to find out the details of the mechanism: whether one or several complexes are formed and the magnitude of the equilibrium constants.

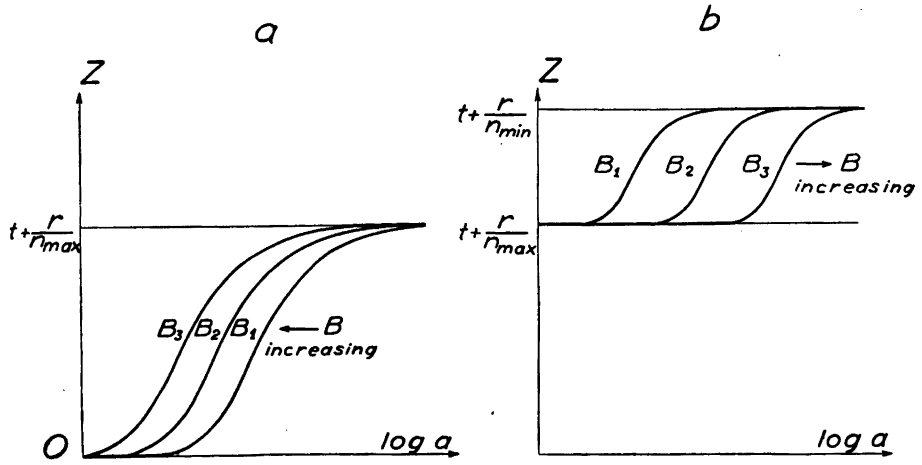

Fig. 2. Set of curves Z *(log* a *)*$_B$*, schematical. For each curve,* B *has a constant value:* B_1, B_2 *etc. The horizontal spacing between two curves at a certain* Z *is denoted by* $\triangle$ *log* a *in the text. a) Parallel curves, corresponding to complexes* $B(A_tB)_n$. *b) Parallel curves corresponding to* $A_r(A_tB)_n$ *in a range where* b *is negligible.*

A practical approach, since Z is a function of $a^{-r}B$ (16), is to plot some function y of Z versus some function x of $a^{-r}B$. Then all points should fall on a single curve $y(x)$ and our task would be confined to testing the experimental data against theoretical curves $y(x)$ calculated under various assumptions. It is advisable to eliminate the constants t and r from y and x so that the same theoretical curves y (x) can be applied to systems with different values for r and t. It is also desirable that the connection between y and x on one hand, and the measured quantities on the other, be so direct that one does not lose the feeling for the real accuracy and the possible interference of experimental errors. Suitable functions are given in (25) and (26).

1) *Complexes with B core,* $B(A_tB)_n$. If $r = -t$, we may write the general formula for the complexes as $B(A_tB)_n$. Here t is a constant whereas n may vary. We then have, denoting by k_n the equilibrium constant for the formation of the n:th complex,

$$[B(A_tB)_n] = c_n = k_n b u^n \tag{21}$$

$$u = a^t b \tag{10}$$

and from (4 to 7), (21), and (10):

$$\left.\begin{aligned} S &= \Sigma c_n = b\Sigma k_n u^n = bf(u) \\ A\text{—}a &= a\left(\frac{\partial S}{\partial a}\right)_b = ab\,\frac{\mathrm{d}f}{\mathrm{d}u}\left(\frac{\partial u}{\partial a}\right)_b = btuf' = BZ \\ B\text{—}b &= b(f + uf');\ B = b(1 + f + uf') \end{aligned}\right\} \tag{22}$$

$$Z = \frac{A\text{—}a}{B} = \frac{tuf'}{1 + f + uf'} \tag{23}$$

Here $f(u)$ is a function of u. If one compares the expressions for S in (11) and (22) and remembers (19 a) and (10), it is seen that $uf(u) = \varphi(u)$, the function in (11). It is easily verified that for Z constant, $u = a^t b$ = constant, $B = b \cdot$ constant, and thus $a^t B$ = constant, or

$$\left(\frac{\partial \log B}{\partial \log a}\right)_Z = -t \text{ in the whole range (for } r = -t) \tag{24}$$

This is equivalent to our previous equations (16) and (19 a).

A convenient way of plotting the experimental data is to give a diagram of $y(x)$ where (eq 22 and 23):

$$y = \frac{A-a}{Bt} = \frac{uf'}{1+f+uf'} \tag{25}$$

$$x = t \log a + \log B = \log u + \log (1 + f + uf') \tag{26}$$

Since each of y and x is a function of u only, y must be a function of x only and all experimental points should be on the same curve $y(x)$.

This curve can then be tested against various assumptions concerning the equilibrium constants k_n. This will be the subject of part II in this series.[12]

1 a) *Only one complex* $A_P B_Q = B(A_t B)_N$. If only one complex is formed, say $A_P B_Q = B(A_t B)_N$, we have from (4) to (7)

$$\left.\begin{aligned} S = [A_P B_Q] &= \varkappa_{PQ} a^P b^Q \\ A-a &= P\varkappa_{PQ} a^P b^Q = BZ \\ B-b &= Q\varkappa_{PQ} a^P b^Q \end{aligned}\right\} \tag{27}$$

$$Z = P\ [Q + \varkappa_{PQ}^{-1} a^{-P} b^{1-Q}]^{-1} \tag{28}$$

Thus if Z is kept constant, $a^P b^{Q-1}$ = constant, $B-b = b \cdot$ constant, $B = b \cdot$ constant, $a^P B^{Q-1}$ = constant and

$$\left(\frac{\partial \log B}{\partial \log a}\right)_Z = \frac{-P}{Q-1} = -t \tag{29}$$

The curves $Z(\log a)_B$ will be parallel in the whole range. If $Q = 1$, they will even coincide. A single complex may be treated as a special case of 1), writing the complex as $B(A_t B)_N$ with $N = Q-1$, $t = P/(Q-1)$. Then in (21), all $k_n = 0$ except k_N which has a finite value. The same functions y and x as given in (25) and (26) may be used. To find out whether one or several complexes are formed one must analyse the curve $y(x)$, as will be discussed in Part II [12].

2) *Complexes* $A_r(A_t B)_n$; *b negligible.* Let us assume that b, the concentration of free B, is negligible in the whole range so that the only species present in measurable quantities are A and the complexes $A_r(A_t B)_n$. To get equations of the same form as in 1), we shall consider the "core" of the complexes to be the smallest possible complex, $A_{r+t}B = C$. We shall use

$$\begin{aligned} &[A_{r+t}B] = [C] = c; \quad [A_{r+t}BA_tB] = [CA_rC] = c_1; \quad \ldots\ldots \\ &[A_{r+t}B(A_tB)_n] = [C(A_rC)_n] = c_n; \quad a^r c = u \end{aligned} \tag{30}$$

We can write the reactions as reactions between A and C:

$$2A_{r+t}B \rightleftharpoons A_{r+t}BA_tB + rA \text{ or } 2C \rightleftharpoons CA_{-r}C + rA$$

$$c_1 = k_1 c(a^{-r}c) = k_1 cu$$

$$(n+1)A_{r+t}B \rightleftharpoons A_{r+t}B(A_tB)_n + rnA \text{ or } (n+1)\,C \rightleftharpoons C(A_{-r}C)_n + rnA$$

$$c_n = k_n c u^n \tag{31}$$

These equations are analogous to (21). In fact, our new $u = a^{-r}c$ differs only by an unknown constant factor (the equilibrium constant of $(r+t)A + B \rightleftharpoons C$) from $a^t b$.

The total amount of C present per liter, C, is equal to B. We shall let the subscript "c" indicate the quantities obtained if the reaction mixture is considered to be made up from A and C instead of from A and B. For instance, the total amount of A, A_c, is obtained by substracting from the previous A the amount bound in C. One can verify that

$$\left.\begin{aligned} A_c &= A-(r+t)C;\ C = B \\ S_c &= \sum_1^\infty c_n = c\Sigma k_n u^n = cf(u) \\ A_c-a &= a\left(\frac{\partial S_c}{\partial a}\right)_c = -rcuf' = Z_c C \\ C-c &= c\left(\frac{\partial S_c}{\partial c}\right)_a = c(f+uf') \end{aligned}\right\} \tag{32}$$

The two quantities y and x to be plotted are (eq. 32)

$$y = \frac{A_c-a}{C\cdot(-r)} = \frac{r+t-\dfrac{A-a}{B}}{r} = \frac{uf'}{1+f+uf'} \tag{33}$$

$$x = -r\log a + \log C = -r\log a + \log B = \log u + \log(1+f+uf') \tag{34}$$

Equations (33) and (34) have the same form as (25) and (26). The same theoretical curves $y(x)$ as for the $B(A_tB)_n$ complexes can thus be used for finding the function $f(u)$ for the $C(A_{-r}C)_n$.

Coinciding curves $Z(\log a)_B$

Suppose that the curves giving $Z = \dfrac{A-a}{B}$ as a function of $\log a$ are not only parallel for various B, but also actually coincide. This means that Z is a function of a only. Since the partial derivative R in (16) becomes infinite, the formula (19) for the complexes becomes indefinite. We shall return to the general equations (5), (6), and (7), admitting a priori any complex A_pB_q

$$Z = \frac{A-a}{B} = \frac{\Sigma p\varkappa_{pq}a^p b^q}{b + \Sigma q\varkappa_{pq}a^p b^q} \tag{17 a}$$

What are the conditions that Z is to be a function of a only?

1) If *b is not negligible*, it is necessary that the sums are of first order with respect to b, so that b can be eliminated. This means that q is always = 1, so that the complexes are only *mononuclear*, A_nB.

2) If *b is negligible* in the denominator in the whole range, b can be eliminated from (17 a) provided only one value for q exists. This value need not necessarily be 1. Thus the complexes must be "*homonuclear*", either only A_nB or only A_nB_2 etc. (If p/q = constant, Z = constant. In this trivial case all Z curves coincide in a horisontal line — it is met with in a concentration range where b is negligible, and either one complex predominates, or all complexes have the formula $(A_tB)_n$.)

The methods for dealing with mononuclear complexes have been treated so fully by other authors that there is no need to go into them here. The adaption of these methods to homonuclear complexes is easy, if the need should arise.

General equations for finding r and t from (a, A, B) data

At present it may not be profitable to discuss in detail all other possible ways in which the family of curves $Z(\log a)_B$ might behave. We shall assume, as a working hypothesis, that the "core + links" hypothesis is valid. We shall then limit ourselves to some general relationships which may be helpful for finding r and t from any set of curves and also discuss how to recognize a set of curves with $r = 0$ (general formula $(A_tB)_n$). Should the need arise, the general equations given here can certainly be applied to other special cases as well.

The formulas for the *limiting values for Z*, given in (14) and (15), may also be useful for finding r and t.

Spacing for $b \ll B$. If, in a certain range of the curves, the concentration of free B, b, can be neglected in comparison with the total concentration B, we find from (12) and (13) $Z = t + r\,\dfrac{\varphi(u)}{u\varphi'(u)}$. If Z is not invariable (see below) it must be a function of u only. Consequently, if Z is kept constant, u is constant, and from (13) neglecting b: $B = a^r u\varphi'(u) = a^r \cdot$ constant, or $\log B = r \log a +$ constant. This result may be written in differential form:

$$\left(\frac{\partial \log B}{\partial \log a}\right)_Z = r \text{ for } b \ll B \tag{35}$$

and is identical with equations (16) + (20 a).

In general one may expect (35) to be approached with increasing Z. However, if the ratio p/q is the same in all complexes, then b is not negligible in comparison with B until Z becomes constant. This complication will occur either if only one complex is formed, say A_PB_Q, or if $r = 0$ and thus all complexes have general formula $(A_tB)_n$. Each of these cases is dealt with separately in this paper.

Spacing if B and one complex predominate. If we can neglect all molecular species except A, B, and one complex, say $A_PB_Q = A_r(A_tB)_N$, then we have already proved (see 29 where however we set $r = -t$):

$$\left(\frac{\partial \log B}{\partial \log a}\right)_Z = -\frac{P}{Q-1} = -\frac{r+Nt}{N-1} \qquad (29\,a)$$

This equation is, of course, useful only when b is not negligible in comparison with B; otherwise Z is constant $= P/Q$, giving a horizontal line.

Spacing for $b \approx B$. In the region where complex formation just starts, and thus Z is small, we may assume that practically only B and the lowest complex, with $n = n_{min}$, exist. Then from (29 a)

$$\left(\frac{\partial \log B}{\partial \log a}\right)_Z \to -\frac{r+n_{min}t}{n_{min}-1} \text{ for } b \approx B \qquad (36)$$

If $n_{min} = 1$, one of two cases may occur: (α) $r = -t$, which makes the expression (36) equal to $-t$. This case, which gives a series of parallel curves, has been dealt with. (β) $r + t \neq 0$, which means that the derivative in (36) is infinite and that the spacing between the curves along the log a axis approaches zero for decreasing Z.

Curves for $a^{-r}(A-a)$. If one wants to test a certain value for r and to find t, one may calculate the quantity $a^{-r}(A-a)$ and plot it as a function of log a with one curve for each B value. The following relationship may prove a useful test:

$$\left(\frac{\partial \log B}{\partial \log a}\right)_{a^{-r}(A-a)} \begin{matrix} \nearrow r \text{ for } b \ll B \\ \searrow -t \text{ for } b \approx B \end{matrix} \qquad (37)$$

As seen from (12), $a^{-r}(A-a)$ should be a function of u only. When $a^{-r}(A-a)$ is kept constant, $u = a^t b$ is constant and $B-b = a^r \cdot$ constant. For $B \gg b$, $B = a^r \cdot$ constant, which is the first relationship in (37). For $b \approx B$, on the other hand, $u \approx a^t B =$ constant, which gives the second relationship.

Complexes $(A_tB)_n$: $r = 0$. If the general formula of the complexes is $(A_tB)_n$, $r = 0$ and we have from (12) and (13)

$$\left.\begin{aligned} A-a &= tu\varphi'(u) = tBy \\ B-b &= u\varphi'(u) = By \end{aligned}\right| \qquad (38)$$

where $y = Z/t$ as in (25). We see that $b = B(1-y)$; thus b cannot be neglected in comparison with B until y becomes 1 and $Z = t$. In this special case we have no help of (35) in determining r and t.

However, if it is suspected that $(A_tB)_n$ is the general formula, one can test it, e.g., in the following way. The value for t may be obtained either from (37) or as the upper limit of Z. Then we can calculate for each point $b = B - \frac{A-a}{t}$. Now, if $t \log a + \log\left(B - \frac{A-a}{t}\right) = t \log a + \log b = \log u$ is plotted on the horizontal axis and $A-a$ (or log $(A-a)$) on the vertical, all experimental points should fall on one curve since $A-a$ should be a function of u only.

TREATMENT OF DATA (*a*,*b*,*A*,*B*)

If both a and b can be measured very accurately, and if the "core + links" hypothesis is assumed to be valid, one may devise several ways for finding r and t based on equations (11 to 13). We shall consider a rather unfavorable case: a is measured, but the difference $(A—a)$ cannot be calculated with enough accuracy to be of much help. Our main source of information will be a set of curves giving, for a series of constant B values, $\frac{b}{B}$ (or better $\log \frac{B}{b}$) as a function of $\log a$.

For low a values, both of these functions give horizontal lines, $\frac{b}{B} = 1$, and $\log \frac{B}{b} = 0$. With increasing $\log a$, $\frac{b}{B} \to 0$ and $\log \frac{B}{b} \to \infty$. At very high values for $\log a$, $b \ll B$ and the complex $A_r(A_tB)_n$ with maximum value for n, n_{max}, predominates provided r is negative, as usual; if $r > 0$, n_{min} predominates. Then we find, *e.g.*, from (27)

$$\left(\frac{\partial \log \frac{B}{b}}{\partial \log a}\right)_B = -\left(\frac{\partial \log b}{\partial \log a}\right)_B = \frac{P}{Q} = t + \frac{r}{n_{max}} \tag{39}$$

Thus if n is unbounded, the maximum slope is t.

Parallel curves $\log \frac{B}{b}$ $(\log a)_B$.

Let us assume that the curves $\log \frac{B}{b}$ $(\log a)_B$ for different B values are parallel with spacing $\Delta \log a$ proportional to $\Delta \log B$. The curves of $\frac{b}{B}$ $(\log a)$ or any other function of $\frac{B}{b}$ are then also parallel, with the same spacing. This may be expressed as

$$\left(\frac{\partial \log B}{\partial \log a}\right)_{\frac{B}{b}} = -T = \text{constant or } \frac{B}{b}\ (a^T B) \tag{40}$$

We shall start with (6), assuming *a priori* that all complexes A_pB_q may exist. We find

$$\frac{B}{b} = 1 + \Sigma q \varkappa_{pq} a^p b^{q-1};\ a^T B = a^T b + \Sigma q \varkappa_{pq} a^{p+T} b^q \tag{41}$$

The condition for $\frac{B}{b}$ to be a function of $a^T B$ only is that all terms $a^p b^{q-1}$ are powers of $a^T b$. Even if $b \ll B$, no new possibilities arise in this case. Thus

$$p = T(q—1) \text{ for all complexes} \tag{42}$$

By comparing (42) with (8 a) we find that we have "core + links" complexes with

$$r = -T,\ s = 1,\ t = T \tag{42 a}$$

The general formula for the complexes is then $B(A_tB)_n$ with $t = T$. This is the same as case 1 which we met with for parallel $Z(\log a)_B$ curves. We can apply equations (21) and (22) with $r = -t$. Using (22) we verify (the proof is similar to that of (24)):

$$\left(\frac{\partial \log B}{\partial \log a}\right)_{\frac{B}{b}} = -t \tag{43}$$

We can reduce our data to a single curve, for further discussion, by plotting

$$\eta = \log \frac{B}{b} = \log(1 + f + uf') \tag{44}$$

as a function of

$$x = t \log a + \log B = \log u + \log(1 + f + uf') \tag{26}$$

or of $\log u = t \log a + \log b$.

The analysis of such curves will be treated in part II [12].

The case with *only one complex* A_PB_Q is conveniently treated as a special case of $B(A_tB)_n$. With (27) or (43) we find

$$\left(\frac{\partial \log \frac{B}{a}}{\partial \log a}\right)_{\frac{B}{b}} = -t = -\frac{P}{Q-1} \tag{45}$$

Coinciding curves $\log \frac{B}{b}(\log a)_B$

If the curves $\log \frac{B}{b}(\log a)$ for various B coincide strictly, then $\frac{B}{b}$ is a function of a only. From (41) we find that this is possible only if always $q = 1$ which means that all complexes must be *mononuclear* in B: A_nB.

General equations for finding r and t

The *limiting slope* of the $\log \frac{B}{b}(\log a)_B$ curves, according to (39), gives $t + rn_{\max}^{-1}$ if $r < 0$; $t + rn_{\min}^{-1}$ if $r > 0$.

Spacing for $b \approx B$ and $b \ll B$. In analogy with (3 6) we find from (13) and (11) for negative r; for positive r, "min" and "max" change place:

$$\left(\frac{\partial \log B}{\partial \log a}\right)_{\frac{B}{b}} \begin{matrix} \nearrow \\ \searrow \end{matrix} \begin{matrix} -\frac{r + n_{\min}t}{n_{\min}-1} \text{ for } b \approx B \\ -\frac{r + n_{\max}t}{n_{\max}-1} \text{ for } b \ll B \end{matrix} \tag{46}$$

If $n_{min} = 1$, the lateral spacing between the curves disappears for $b \approx B$, unless $r = -t$, in which case the curves are always parallel as discussed earlier. If $n_{max} = \infty$, the limiting spacing, like the limiting slope, will give t.

Curves for $b^{-s}(B-b)$. Using a tentative value of $s = -r/t$, one may plot $b^{-s}(B-b)$ as a function of log a to give a family of curves, either with B or b constant. Since B is usually kept constant in each experimental series, the curves with B constant are obtained immediately, whereas those with b constant must be calculated by interpolation.

As one may derive from equations (13) and (8), $b^{-s}(B-b)$ should be a function of u only, if one has hit on the correct s value. One may use the equations

$$\left(\frac{\partial \log B}{\partial \log a}\right)_{b^{-s}(B-b)} \begin{cases} \nearrow -t \text{ for } b \approx B \\ \searrow -ts = r \text{ for } b \ll B \end{cases} \tag{47}$$

$$\left(\frac{\partial \log b}{\partial \log a}\right)_{b^{-s}(B-b)} = -t \text{ in the whole range} \tag{48}$$

One may then find out whether the values for t and r obtained from (47) and (48) with a tentative s are compatible with each other and with the s assumed.

Curves for $\frac{B-b}{A}$. With the aid of data (a, b, A, B) one may plot $\frac{B-b}{A}$ as a function of log b, for a series of values A_1, A_2 . . . . ., and study the limiting values and the spacing. The formulas are analogous to (14 to 36) except that B and A are interchanged and the general formula of the complex $A_r(A_tB)_n$ is replaced by $B_{r'}(AB_{t'})_{n'}$, where $r' = s = -r/t$, $t' = 1/t$, and $n' = r + nt$. The transformation of (35) and (36) gives, for example

$$\left(\frac{\partial \log A}{\partial \log b}\right)_{\frac{B-b}{A}} \begin{cases} \nearrow r' = -r/t \text{ for } a \ll A \\ \searrow \dfrac{r' + n'_{min}t'}{n'_{min}-1} = -\dfrac{n_{min}}{r + n_{min}t-1} \text{ for } a \approx A \end{cases} \tag{49}$$

If the measurements have been made for a series of constant B values, one must interpolate to get curves with constant A.

Curve for $a^{-r}(B-b)$. If one wants to test whether a certain set (r, t) is consistent with the data, one may calculate and plot $a^{-r}(B-b) = u\varphi'(u)$ versus $t \log a + \log b = \log u$. All experimental points should then be on a single curve, irrespective of B.

SUMMARY

A system is discussed in which two reactants A and B form a series of polynuclear complexes A_pB_q. The mathematical relationship between the total concentrations (A, B) and the free concentrations (a, b) of the reactants is given. A useful function called the complexity sum, S (equation 2) is introduced.

If only a, A, and B are known, one may draw curves giving $Z = \frac{A-a}{B}$ as a function of log a, with B constant for each curve. It is often observed that

these curves, with good approximation, are all parallel with spacing Δ log a proportional to Δ log B. This behavior leads to the following conclusions: 1) If b is not negligible in comparison with B, all complexes present in appreciable amounts must have the general formula $B(A_tB)_n$; here t is a constant, which is obtained from the spacing of the curves (eq 24), whereas n may vary. 2) If the curves are parallel and b negligible in the whole range, the general formula must be $A_r(A_tB)_n$, where t and r are constants, and n variable; r is obtained from the spacing.

If the $\bar{Z}(\log a)_B$ curves coincide and b is negligible, all complexes must be homonuclear, thus having the same q. If b is not negligible, they must moreover all be mononuclear in B, of general formula A_nB.

Equations are given for the case where all complexes can be written as built up from a certain "core" and a varying number of identical "links" A_tB. All existing complexes might then be written as $A_r(A_tB)_n$ or $B_s(A_tB)_n$, with $r = -ts$, but it is not necessary that all n values really occur. It is shown how the values for r and t can be found from measurements of either (a, A, B) or (a, b, A, B) in a specific system.

If the curves giving $\log \frac{B}{b}(\log a)_B$ are parallel, the formula for the complexes must be $B(A_tB)_n$ with t constant, n variable. If, moreover, they coincide, then only mononuclear complexes A_nB are possible.

This paper is limited to finding the general formula for the complexes, whilst the detailed investigation of the mechanism and the equilibrium constants *etc.* will be discussed in subsequent papers.

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On Equilibria in Systems with Polynuclear Complex Formation. II. Testing Simple Mechanisms which give "Core + Links" Complexes of Composition $B(A_tB)_n$*

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It was shown in Part I that very simple conditions are met with if all complexes that are formed by two reactants A and B can be written in the form $B(A_tB)_n$, where t is constant, and n variable. In this case, the curves giving $Z=(A-a)/B$ as a function of log a have the same shape for all values of the total concentration B and are parallel to each other.

As a matter of fact, parallel $Z(\log a)_B$ curves are often found experimentally. It was proved inversely (I, eq. 16—20) that this behavior requires a "core + links" mechanism: if b is negligible in the whole range studied, the general formula of the complexes must be $A_r(A_tB)_n$ (r and t constant, n variable) whereas if the curves are parallel in a range where b is not negligible we must have the additional condition $r=-t$ and thus the general formula $B(A_tB_n)$.

If the curves $\frac{b}{B}(\log a)_B$ and consequently $\log \frac{B}{b}(\log a)_B$ are found to be parallel, the only possible formula is $B(A_tB)_n$ (I, eq. 40—42).

It was indicated in Part I that, for further discussion of the mechanism, one may conveniently transform the coordinates so that all of the previously parallel curves coincide and the constants r and t disappear from the equations (I, eq. 25—26, 44). The experimental data are then reduced to a single curve which can be compared with a few families of theoretical curves, calculated under various simple assumptions.

In the present paper we shall only treat complexes of the general formula $B(A_tB)_n$. If the general formula of the complexes is $A_r(A_tB)_n$ and b is always negligible, one may consider the core to be $C=A_{r+t}B$ and the complexes as $C(A_{-r}C)_n$. It is then possible to use exactly the same equations and families of curves as will be derived below for $B(A_tB)_n$ (compare I, eq. 33, 34) so there is therefore no need for a separate treatment.

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* Part I: *Acta Chem. Scand.* 8 (1954) 299.

List of symbols

A total concentration of A (I, 2).
a concentration of free A (I, 1).
B total concentration of B (I, 2).
b concentration of free B (I, 1).
c_n concentration of n:th complex (1).
f $= S/b$, function of u (3).
g $= S/b$, function of v (3a).
k equilibrium constant in (17), (19), or (28).
k_n equilibrium constant defined by (1).
l_n $= k_n k^{-n}$, constant (6).
N number of links in singular complex (19).
n variable integer, number of links in "core + links" complex.
r number of A in core (if free from B).
S complexity sum (3, I 4).
t number of A per B in link.
u $= a^t b$ (2).
v $= k a^t b$ (6).
$v_{\frac{1}{2}}$ value of v for $y=\frac{1}{2}$, (33), (40), (44).
v_2 value of v that makes $B=2b$, $\eta=\log 2$ (70).
X $= x + \log 2 - x_{\frac{1}{2}}$ (10).
x $= t \log a + \log B$ (9).
$x_{\frac{1}{2}}$ value of x for $y=\frac{1}{2}$ (10).
y $= (A-a)/Bt = Z/t$ (8).
Z $= (A-a)/B$, average number of A bound per B (4).
η $= \log \frac{B}{b}$ (46).
ξ $= x +$ constant, chosen so as to make the asymptote of $\eta(\xi)$ go through the origin (51, 53); or (69, IIIc) to make all curves go through $\eta = \xi = \log 2$.

General equations

We shall assume as before that the activity factors are constant. The law of mass action gives for the formation of the complex $B(A_tB)_n$ from A and B (I, 21):

$$[B(A_tB)_n] = c_n = k_n b(a^t b)^n = k_n b u^n \tag{1}$$

Here, a and b are the free concentrations of the two reactants A and B, and the variable u is defined (I, 10) as:

$$u = a^t b \tag{2}$$

For the complexity sum S and the amounts of A and B bound in complexes (in mole/l) we find (I, 22):

$$S = \Sigma[B(A_tB)_n] = b\Sigma k_n u^n = bf(u) \tag{3}$$

$$A-a = a\left(\frac{\partial S}{\partial a}\right)_b = ab\,\frac{df}{du}\left(\frac{\partial u}{\partial a}\right)_b = btuf'(u) = BZ \tag{4}$$

$$B-b = b\left(\frac{\partial S}{\partial b}\right)_a = b[f(u) + uf'(u)] \tag{5}$$

In the following it will prove advantageous to introduce a new variable $v=ku$ and a new set of constants l_n. Here k is a constant (at first unknown) related to the equilibrium constants k_n (see eq (19) and (28)).

$$v = ku = ka^t b;\ k_n = l_n k^n \tag{6}$$

$$S = \Sigma b l_n v^n = bg(v);\ g(v) = \Sigma l_n v^n \tag{3 a}$$

Here $g(v)$ is a function of v only. From (3), (3 a), and (6) we see that

$$f(u) = g(v) = Sb^{-1};\ uf'(u) = vg'(v) \tag{7}$$

Equations (4—5) will then be transformed in the following way:

$$A—a = BZ = btvg'(v) \tag{4 a}$$

$$B—b = b[g(v) + vg'(v)];\ B = b(1 + g + vg') \tag{5 a}$$

DATA (a, A, B)

Plotting of the data

Let us remember that t, the number of A per B in a link, can be obtained from the spacing of the $Z(\log a)_B$ curves using equation (I, 24).

In any experiment, A and B, the total concentrations of the reactants, should be known from the analytical data. If, in addition, the free concentration a is measured but not b, it is convenient to calculate and plot on a diagram the two quantities y and x from (I, 25 and 26). Using these equations together with (6) and (7) we find:

$$y = \frac{A—a}{Bt} \tag{8}$$

$$y = vg'\ (1 + g + vg')^{-1} \tag{8 a}$$

$$x = t\ \log\ a + \log\ B \tag{9}$$

$$x = \log\ v—\log\ k + \log\ (1 + g + vg') \tag{9 a}$$

The expressions for x and y in (8 a) and (9 a) contain only constants and functions of the variable v, and neither a nor b separately. Then y may be plotted as a function of x only and all points should fall on a single curve which is independent of B. Thus our problem reduces to the calculation of the shape of $y(x)$ under various assumptions and a comparison of the calculated curves (from 8 a and 9 a) with the experimental one (from 8 and 9).

This comparison is made easier if we shift the *calculated* curves along the abscissa so that they all go through a single point. We shall choose as abscissa for the calculated curves:

$$X = x + \log\ 2—x_{\frac{1}{2}} \tag{10}$$

where $x_{\frac{1}{2}}$ is the value of x for $y=\frac{1}{2}$ (the "half-point"). Obviously all calculated curves $y(X)$ will then go through the point

$$X = \log\ 2,\ y = \tfrac{1}{2} \tag{11}$$

We may derive from (8 a) and (9 a)

$$x_{\frac{1}{2}} = \log 2 - \log k + \log(v^2 g')_{y=\frac{1}{2}} \quad (12)$$

which gives with (9 a) and (10)

$$X = \log v + \log (1 + g + vg') - \log (v^2 g')_{y=\frac{1}{2}} \quad (13)$$

One may prepare a transparent diagram with any calculated curve $y(X)$, place it over the diagram with the experimental curve $y(x)$, and move it parallel with the x axis. If a fit is obtained, one may use $x_{\frac{1}{2}}$ at the half-point and equation (12) to calculate the equilibrium constants.

Direct analysis

From (8a) we find $1 + g + vg' = (1 + g)/(1-y)$. We insert this into (9a), differentiate and eliminate d ln v by using d ln $v = (y^{-1}-1)$ d ln $(1 + g)$, which may be found from (8a), observing that $vg' = \mathrm{d}(1 + g)/\mathrm{d} \ln v$. The result is

$$\log (1 + g) = \int_{-\infty}^{x} y \, dx + \log(1-y) + y \log e \quad (14)$$

From (9a), inserting $1 + g + vg' = (1 + g)/(1-y)$ (8a), $\log v - \log k = u$ (6), and (14)

$$\log u = x - y \log e - \int_{-\infty}^{x} y \, dx \quad (15)$$

With data (a,A,B) one might thus use the curve $y(x)$, (14), and (15) to calculate $g(v) = f(u)$ as a function of u, and then try to find the coefficients k_n (= the equilibrium constants) in the power series (3).

From data (a,b,A,B) one finds u directly. By integrating (5) one may find

$$1 + g = 1 + f(u) = \frac{1}{u} \int_{0}^{u} \frac{B}{b} \, \mathrm{d}u \quad (16)$$

This will also give f(u) from the experimental data.

However, because of the limited accuracy of the data, these procedures seem impracticable at least with present techniques.

Choice of hypotheses

In the present paper we shall confine ourselves to studying a number of simple hypotheses, involving not more than two unknown constants to be determined with the aid of the experimental curves:

I) Our first hypothesis is that no soluble complexes are formed but that solid A_tB may *precipitate*. As usual, "A_tB(s)" may also contain solvent molecules and inert ions from the ionic medium.

II) The next simple hypothesis is that out of all conceivable complexes $B(A_tB)_n$ *only one*, $B(A_tB)_N$, is formed, where N is constant and at first unknown.

III) The other extreme hypothesis is that complexes with all positive values for n are formed by a *repeated reaction* and we shall try three different equations (28a, b, c) for the variation of the equilibrium constants k_n with n.

Hypothesis I: No soluble complex; solid A_tB may precipitate

In all the mechanisms to be considered we shall find as one limiting case those curves that would be obtained if only solid A_tB and no soluble complexes were formed (hypothesis I). We shall now derive the equations of these curves.

If some A_tB has precipitated — (B—b) mole — we have:

$$A-a = t(B-b);\ u = a^t b = k^{-1};\ v = 1 \tag{17}$$

where k^{-1} is the solubility product of A_tB (including the terms from solvent molecules and medium ions). We then find from (8), (17), (9), and (10):

$$\left.\begin{aligned} y &= \frac{A-a}{Bt} = \frac{B-b}{B} = 1-\frac{b}{B} \\ x &= \log\ a^t B = -\log\ k - \log\frac{b}{B} = -\log\ k - \log\ (1-y) \\ x_{\frac{1}{2}} &= \log\ 2 - \log\ k;\ X = x + \log\ k = -\log\ (1-y) \end{aligned}\right\} \tag{17a}$$

If the product $u = a^t b$ is less than k^{-1}, no precipitate occurs and we have $a = A$, $b = B$, $y = 0$ since there are no soluble complexes.

There will be a sharp break in the curve $y(X)$ at the point of precipitation:

$$\begin{aligned} X &= -\log(1-y) && \text{for } X > 0 \\ y &= 0 && \text{for } X < 0 \end{aligned} \tag{18}$$

The curve $y(X)$ from (18) will be seen as limiting curve in fig. 1 (hypothesis II, $N \to \infty$) and fig. 2 (hypothesis III, $k_0 \to 0$).

Hypothesis II: Only one complex formed

Let us assume that the only complex formed is $B(A_tB)_N$ and that the equilibrium constant is k^N. We then have (cf 6 and 3a):

$$S = [B(A_tB)_N] = k^N b(a^t b)^N = bv^N = bg \tag{19}$$

where

$$g(v) = v^N;\ vg'(v) = Nv^N = Ng \tag{20}$$

We may derive from (4a), (5a), and (20)

$$A-a = Ntbg;\ B-b = (N+1)bg \tag{21}$$

The equations (21) may also be obtained immediately from (19) since the concentration of the complex is bg, the number of A bound per complex is Nt and the number of B bound per complex is $(N+1)$.

We shall now see what type of $y(x)$ plot one would obtain with this mechanism. We find from (8) and (21), or (8a) and (20)

$$y = Ng\ [1 + (N+1)g]^{-1} \tag{22}$$

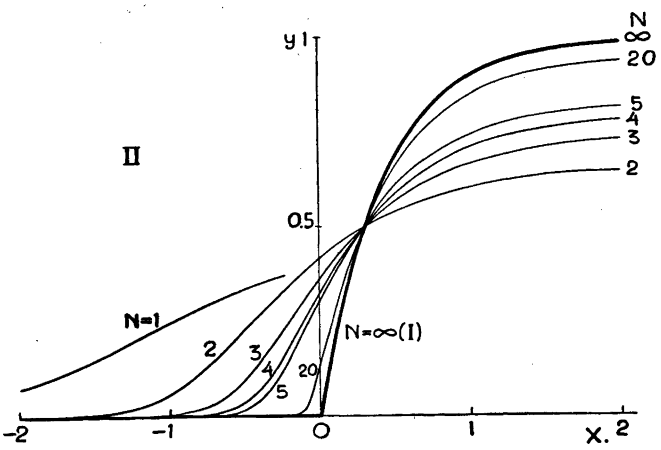

Fig. 1. Family of curves $y(X)_N$ *for hypothesis II: only one complex* $B(A_iB)_N$ *is formed.*

and from (9a) and (20)

$$x + \log k = N^{-1} \log g + \log [1 + (N + 1)g] \tag{23}$$

From (22) we find $y = \frac{1}{2}$ for $g = (N-1)^{-1}$ which gives with (23) and (10):

$$x_{\frac{1}{2}} = \log 2 - \log k + \log N - (1 + N^{-1}) \log (N-1) \tag{24}$$

$$X = x + \log 2 - x_{\frac{1}{2}} = N^{-1} \log g + \log [1 + (N + 1)g] - \log N + (1 + N^{-1}) \log (N-1) \tag{25}$$

The expressions for y and X in (22) and (25) contain only N and the single variable g. One may thus for each N draw a curve giving y as a function of X.

Fig. 1 gives a family of such curves $y(X)_N$ with various values for N: 1, 2, 3, 4, 5, 20 and ∞. For the curve with $N = 1$, the abscissa was arbitrarily chosen as $x + \log k - 1$ since this curve approaches the value $y = \frac{1}{2}$ only asymptotically (see 24). All the other curves $y(X)_N$ intersect at the half-point (11) as they should.

The limiting curve for $N \to \infty$ is $X = -\log (1-y)$ as is seen from (22) and (25) if one lets $N \to \infty$ and $g \to 0$ in such a way that Ng has finite values. (For $N \to \infty$, $Ng = 0$ we find its linear continuation, $y = 0$, $X < 0$).

Now this is equation (18) derived assuming the precipitation of solid A_iB, which may be regarded as the limiting case of the formation of complexes $B(A_iB)_n$.

If the experimental data are given in the form of a curve $y(x)$, one may test the single-complex mechanism by trying to fit the curve family $y(X)$ of fig. 1. If a fit is found, the most likely value for N is found from the shape of the curve, especially from the upper limit (see 22):

$$y \to \frac{N}{N+1} \text{ for } x \to \infty \tag{26}$$

and from the slope at the half-point:

$$\left(\frac{dy}{dX}\right)_{y=\frac{1}{2}} = \left(\frac{dy}{dx}\right)_{y=\frac{1}{2}} = \frac{(N-1)\ \ln\ 10}{2(N+3)} \tag{27}$$

From $x_{\frac{1}{2}}$, the value for x at $y = \frac{1}{2}$, one can then obtain k from equation (24).

Hypothesis III: Repeated reaction

The other extreme assumption is that all positive integral values for n are represented by complexes $B(A_tB)_n$ in the solution. We shall try the following three working hypotheses on how k_n varies with n, where k_n is the equilibrium constant for the formation of the n:th complex from A and B (1, 6):

$$\text{IIIa:}\quad k_n = k_0 k^n;\quad l_n = k_0;\quad k_{n+1}k_n^{-1} = k \tag{28a}$$

$$\text{IIIb:}\quad k_n = k_0 n k^n;\quad l_n = k_0 n;\quad k_{n+1}k_n^{-1} = k(1 + n^{-1}) \tag{28b}$$

$$\text{IIIc:}\quad k_n = \frac{k_0 k^n}{\lfloor n};\quad l_n = \frac{k_0}{\lfloor n};\quad k_{n+1}k_n^{-1} = k(n+1)^{-1} \tag{28c}$$

For all three hypotheses, the equilibrium constant for the formation of the first complex is given by $k_1 = kk_0$:

$$tA + 2B \rightleftharpoons BA_tB;\ k_1 = [BA_tB]a^{-t}b^{-2} = c_1 a^{-t}b^{-2} = kk_0 \tag{29}$$

The equilibrium constant for the addition of a new link is:

$$B(A_tB)_n + tA + B \rightleftharpoons B(A_tB)_{n+1};\ k_{n+1}k_n^{-1} = c_{n+1}c_n^{-1}a^{-t}b^{-1} \tag{30}$$

and may vary with n. With IIIa, $k_{n+1}k_n^{-1}$ has always the same value, namely k; if $k_0 = 1$, then also $k_1 = k$. With IIIb, $k_{n+1}k_n^{-1} = k(1 + n^{-1})$ tends to a constant value k, but the first few links are somewhat more easily added. With IIIc $k_{n+1}k_n^{-1} = k(n+1)^{-1}$ tends to zero with increasing n so that the formation of the higher complexes becomes increasingly difficult.

The first hypothesis is the simplest and has rendered good service for many systems. For this reason, IIIa will be treated more fully than the other two. It is also what one would expect, from statistical considerations, if the complexes are chain-like.

The two hypotheses IIIb and IIIc have been included because it seemed desirable to find out whether an equally good agreement with the experimental data could be obtained with widely different assumptions concerning the behavior of the constants, k_n. The assumptions (28b) and (28c) were not chosen on the basis of any statistical argument (which may have only a limited value in this case) but because they give an easy summation in (3) and (3a).

Hypothesis IIIa: All consecutive constants equal

For hypothesis IIIa we find, using (28a) and (3a):

$$g(v) = \sum_1^\infty l_n v^n = k_0 v(1-v)^{-1};\ vg'(v) = k_0 v(1-v)^{-2} \tag{31}$$

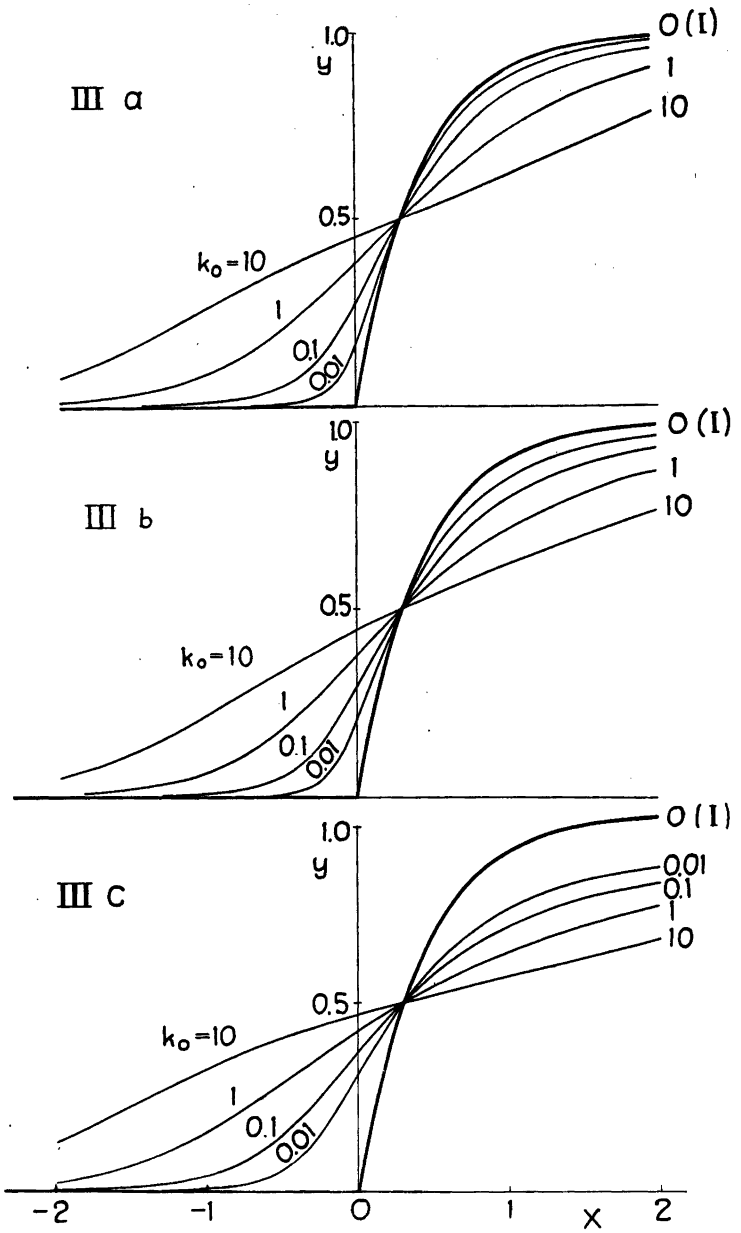

Fig. 2a—c. Families of curves $y(X)_{k_o}$ *for repeated reaction, hypotheses IIIa, IIIb, and IIIc (eq 28 a—c).*

Introducing (31) into (8a) we find

$$y = k_0 v\ [(1—v)^2 + k_0 v\ (2—v)]^{-1} \tag{32}$$

and from (32) and (12) with (31)

$$v_{\frac{1}{2}} = (1 + \sqrt{k_0})^{-1};\ x_{\frac{1}{2}} = \log\ 2—\log\ k \tag{33}$$

Finally from (10) and (33) with (9a) and (31):

$$X = x + \log\ k = \log\ v + \log\ [1 + k_0((1—v)^{-2}—1)] \tag{34}$$

With the aid of equations (32) and (34) we may construct a family of curves, giving y as a function of $X = x + \log k$ for various values of k_0. Some of these curves are given in Fig. 2a. All the curves intersect at the half-point (11) as they should.

The limiting curve for the family $y(X)_{k_0}$ is that for $k_0 = 0$, its equation being (18), $X = —\log(1—y)$. This may be demonstrated by letting $k_0 \to 0$ and $v \to 1$ in equations (32) and (34) in such a way that $k_0\ (1—v)^{-2}$ has a finite value. It may be seen intuitively that $k_0 \to 0$, k = constant means that the first complex is formed with greater and greater difficulty whereas the addition of new links is as easy as before. When v is increased towards the value 1, the formation of complexes starts suddenly and at once leads to very large complexes, which is equivalent to precipitation.

If $k_0 = 1$ all links are added with the same equilibrium constant. The equations (32) and (34) then take the simple forms:

$$y = v \tag{32a}$$

$$X = x + \log\ k = \log\ y—2\ \log\ (1—y) \tag{34a}$$

If the experimental data are seen to fit in with the family of curves in fig. 2a, one may obtain k from $x_{\frac{1}{2}}$, the x coordinate at the point $y = \frac{1}{2}$, using (33). To find k_0 one may either use the slope of the curve $y(x)$ at the half-point:

$$\left(\frac{dy}{dX}\right)_{y=\frac{1}{2}} = \left(\frac{dy}{dx}\right)_{y=\frac{1}{2}} = \tfrac{1}{2}\ \ln\ 10 \cdot (1 + 2\ \sqrt{k_0})^{-1} \tag{35}$$

as may be proved from (32) and (34). One may also, for a number of y values, measure x, calculate 10^X using (cf 10):

$$10^X = 2 \cdot 10^{(x-x_{\frac{1}{2}})} \tag{36}$$

and apply the equation

$$k_0 = y\ [1—10^X(1—y)]^2\ [10^X(1—2y)^2]^{-1} \tag{37}$$

Equation (37) may be proved as follows. From (32) and (34), $y \cdot 10^X = k_0 v^2\ (1—v)^{-2}$. If this expression is used for eliminating v from (32), (37) results after rearrangement.

Hypothesis IIIb

For hypothesis IIIb we find from (3a) and (28b)

$$g(v) = \sum_1^\infty l_n v^n = k_0 \sum_1^\infty n v^n = k_0 v(1—v)^{-2};\ v g'(v) = k_0 v(1 + v)(1—v)^{-3} \tag{38}$$

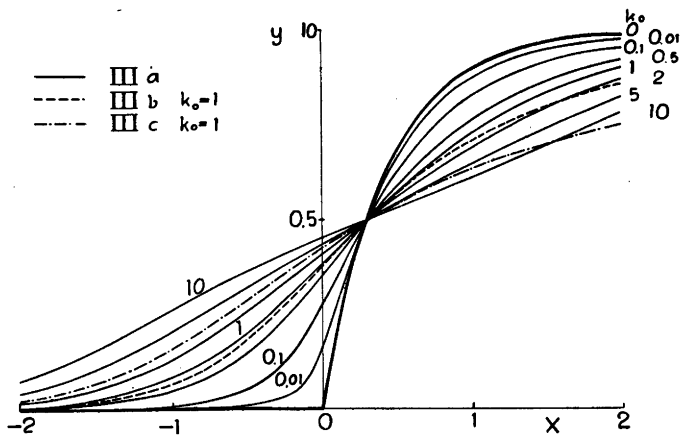

Fig. 3. Position of curves for $k_0 = 1$ *with hypotheses IIIb and IIIc in the family of curves* $y(X)_{k_0}$ *for IIIa.*

If the expressions for g and vg' from (38) are inserted into (8a) we find

$$y = k_0 v(1 + v)[(1—v)^3 + 2k_0 v]^{-1} \tag{39}$$

For $y = \frac{1}{2}$ we find from (39), and from (12) and (38)

$$2k_0 v_{\frac{1}{2}}^2 = (1—v_{\frac{1}{2}})^3;\ x_{\frac{1}{2}} + \log\ k = \log\ (1 + v_{\frac{1}{2}}) \tag{40}$$

Thus $v_{\frac{1}{2}}$ is the solution of the third-degree equation in (40) which may be solved for any k_0 value. Finally with (10) and (40), (9a) and (38):

$$X—\log\ 2 + \log\ (1 + v_{\frac{1}{2}}) = x + \log\ k = \log\ v + \log\ [1 + 2k_0 v(1—v)^{-3}] \tag{41}$$

Fig. 2b gives $y(X)_{k_0}$ as calculated from (39—41). The limiting curve for $k_0 \to 0$ is (18): $X = —\log\ (1—y)$. — Fig. 3 shows the curve for $k_0 = 1$ with hypothesis IIIb together with the curve family for IIIa.

Hypothesis IIIc

For hypothesis IIIc we find from (3a) and (28c):

$$g(v) = \sum_1^\infty l_n v^n = k_0 \sum_1^\infty \frac{v^n}{\lfloor n} = k_0(e^v—1);\ vg'(v) = k_0 v e^v \tag{42}$$

Inserting (42) into (8a) we find:

$$y = k_0 v e^v\ [1 + k_0(ve^v + e^v\ —1)]^{-1} \tag{43}$$

From (43), and from (12) and (42):

$$e^{v_{\frac{1}{2}}}(v_{\frac{1}{2}}—1) = k_0^{-1}—1;\ x_{\frac{1}{2}} = \log\ 2—\log\ k + \log\ k_0 v_{\frac{1}{2}}^2 e^{v_{\frac{1}{2}}} \tag{44}$$

From (10) and (44), (9a) and (42):

$$X + \log\ k_0 v_{\frac{1}{2}}^2 e^{v_{\frac{1}{2}}} = x + \log\ k = \log\ v + \log\ [1 + k_0(ve^v + e^v—1)] \tag{45}$$

Fig. 2c gives $y(X)_{k_0}$ as calculated from (43 to 45). Even in this case, the shape of the curve approaches that of (18) as $k_0 \to 0$. In fig. 3 the curve for $k_0 = 1$ with III c is shown together with the family $y(X)_{k_0}$ for IIIa.

For the special case $k_0 = 1$ we have $v_{\frac{1}{2}} = 1$ and the somewhat simpler expressions:

$$y = v(1 + v)^{-1} \tag{43a}$$

$$X + \log e = x + \log k = \log v + v \log e + \log (1 + v) =$$
$$= y(1-y)^{-1} \cdot \log e + \log y - 2 \log (1-y) \tag{45a}$$

DATA (a, b, A, B)

Plotting of experimental data

We shall now see how the hypotheses discussed above can be tested if one can measure both a and b, *i. e.* the free concentrations of A and B. One convenient way of plotting the experimental data is then to give

$$\eta = \log \frac{B}{b} \tag{46}$$

as a function of log a with one curve for each value for B, the total concentration of B.

It has been observed in at least two systems that, within the limits of experimental error, the curves η (log $a)_B$ are parallel and possess the same shape for all values for B, with a spacing Δ log a proportional to Δ log B. If this is exactly true, then all complexes must have, as proved in part I (eq 40—43), a "core + links" formula $B(A_tB)_n$ in which n is variable and t is a constant which can be calculated from the spacing of the curves using (I, 43).

Just as for data (a, A, B) it is convenient to transform the coordinates so that the experimental points fall on a single curve. As ordinate we shall take η; as abscissa we may choose either (cf 2)

$$\log u = t \log a + \log b \tag{47}$$

or (cf 9 and 46)

$$x = t \log a + \log B \ (= \log u + \eta) \tag{48}$$

The curve η(log u) is somewhat more compact than $\eta(x)$. With $\eta(x)$, on the other hand, the experimental uncertainties in log a and log b come out separately, one on each coordinate axis. Thus the $\eta(x)$ diagram is more suitable for discussing the experimental errors and for successive approximations.

Calculated curves

If $g(v)$ is the function (3a) that follows from a special hypothesis we want to test, we have from (5a) and (46)

$$\eta = \log (1 + g + vg') \tag{49}$$

and from (9a)

$$x + \log\ k = \log\ v + \log\ (1 + g + vg') \tag{50}$$

$$x + \log\ k = \log\ v + \eta \tag{50a}$$

On the right hand side of (50) and (49) we have functions of v only. Thus as k is constant, η should be a function of x.

If there is a maximum value for n, n_{max}, the complex with $n = n_{max}$ predominates at high values for v, and $g = l_{n_{max}} v^{n_{max}}$ in (3a). One may then prove from (50) and (49) that the maximum slope for $\eta(x)$ is $(1 + n_{max}^{-1})^{-1}$ and that $\eta(x)$ has a linear asymptote of this slope.

For comparison it is convenient to shift the calculated curves laterally so that their asymptotes always go through the origin. We shall do this by choosing the abscissa

$$\xi = x + \log\ k + n_{max}^{-1}\ [\log\ (n_{max} + 1) + \log\ l_{n_{max}}] \tag{51}$$

$$\xi = \log\ v + \log\ (1 + g + vg') + n_{max}^{-1}\ [\log\ (n_{max} + 1) + \log\ l_{n_{max}}] \tag{51a}$$

For high v values, $\eta(\xi)$ approaches the asymptote:

$$\eta = \xi\ (1 + n_{max}^{-1})^{-1} \tag{52}$$

For low v, $g \to 0$, and we have another linear asymptote

$$\eta = 0 \tag{52a}$$

If n is unbounded, we may take

$$\xi = x + \log\ k + \lim_{n \to \infty} (n^{-1} \log\ l_n) \tag{53}$$

$$\xi = \log\ v + \eta + \lim_{n \to \infty} (n^{-1} \log\ l_n) \tag{53a}$$

If the limit is finite, v has a maximum value, and for high ξ (53a) approaches asymptotically the line

$$\eta = \xi \tag{54}$$

In our three "unbounded" mechanisms, the limit expression becomes zero for IIIa and IIIb and infinite for IIIc.

The calculated curves $\eta(\xi)$ may be drawn on transparent paper and moved parallel with the abscissa axis across the experimental $\eta(x)$. If a fit is found, then (51) may be used for finding the equilibrium constants.

If the experimental data have been plotted as $\eta(\log u)$, one may test them with calculated curves giving η as a function of $(\xi—\eta)$: from (48) and (51)

$$\xi—\eta = \log\ u + \log\ k + n_{max}^{-1}\ [\log\ (n_{max} + 1) + \log\ l_{n_{max}}] \tag{55}$$

and from (49) and (51a)

$$\xi—\eta = \log\ v + n_{max}^{-1}\ [\log\ (n_{max} + 1) + \log\ l_{n_{max}}] \tag{55a}$$

Equation (55a), with the parameter v, may be used together with (49) for calculating $\eta(\xi—\eta)$ under various assumptions whereas (55) may be used for finding the equilibrium constants once a fit has been found between the experimental $\eta(\log u)$ and a calculated $\eta(\xi—\eta)$ curve.

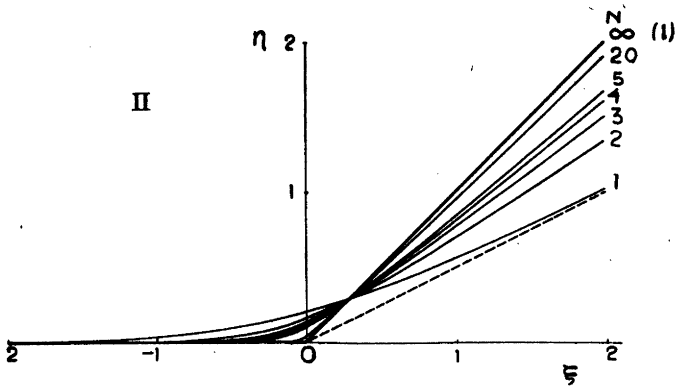

Fig. 4. Family of curves $\eta(\xi)_N$ for hypothesis II: only one complex, $B(A_iB)_N$, is formed. The dotted line is the asymptote $\eta = \frac{1}{2}\xi$ for $N = 1$.

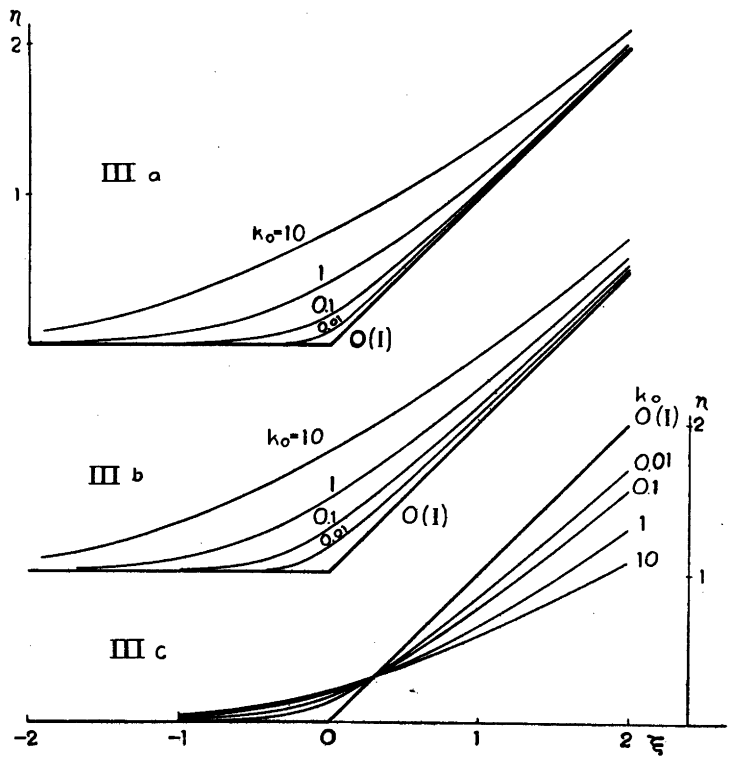

Fig. 5a—c. Families of curves $\eta(\xi)_{k_0}$ for repeated reaction, hypotheses IIIa, IIIb, and IIIc (28a—c).

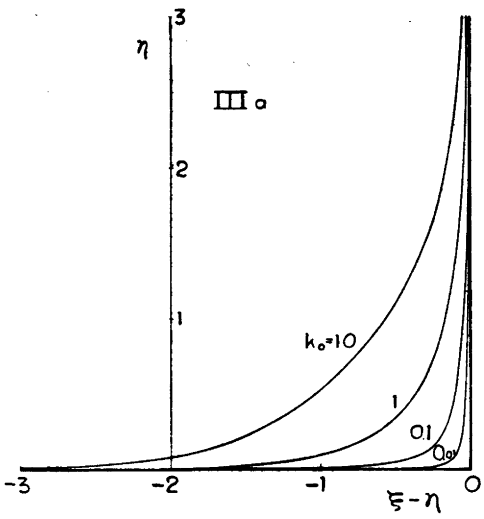

Fig. 6. Family of curves $\eta(\xi-\eta)_{k_0}$ for IIIa. Thick lines = limiting curve for $k_0 = 0$.

The asymptotes for $\eta(\xi—\eta)$ will be (cf 52a and 52)

$$\left.\begin{aligned} \eta &= 0 \\ \eta &= n_{\max}\ (\xi—\eta) \end{aligned}\right\} \tag{56}$$

If n is unbounded, the second asymptote (if it exists) will be

$$\xi—\eta = 0 \text{ (vertical line)} \tag{56a}$$

We shall now give equations for calculating the $\eta(\xi)$ and $\eta(\xi—\eta)$ curves for each of the hypotheses discussed above. Since it is generally hard to give explicit equations, we shall express η and ξ in terms of a parameter, v or g, just as we did with y and X.

Hypothesis I: No soluble complex; solid A_tB may precipitate

If some $A_tB(s)$ has precipitated, we have from (17)

$$\log u + \log k = 0$$

and with (48)

$$x = \log a^t B = \log u + \eta = \eta—\log k$$

We shall put

$$\xi = x + \log k$$

so that $\eta(\xi)$ becomes a line, $\eta = \xi$, passing through the origin.

If the product $a^t b$ is less than k^{-1}, we have no precipitate and no complex formation, and thus $b = B$ and $\eta = 0$. Each of the curves $\eta(\xi)$ and $\eta(\xi—\eta)$ will consist of two lines intersecting at the point of precipitation:

$$\eta = \xi, \quad \xi > 0, \text{ precipitate} \qquad (57)$$
$$\eta = 0, \quad \xi < 0, \text{ no precipitate}$$

$$\eta > 0, \quad \xi - \eta = 0 \text{ (vertical line), precipitate} \qquad (57a)$$
$$\eta = 0, \quad \xi - \eta < 0 \text{ (horizontal line), no precipitate}$$

The two straight lines (57) or (57a) are seen as the limiting cases of the curves $\eta(\xi)$ or $\eta(\xi-\eta)$ in fig. 4 (hypothesis II, $N \to \infty$) and fig. 5 and 6 (III, $k_0 \to 0$).

Hypothesis II: Only one complex formed, $B(A_tB)_N$

From (21), or (49) and (20), we find

$$\eta = \log \frac{B}{b} = \log\,[1 + (N+1)g] \qquad (58)$$

From (51) and (51a) since $n_{max} = N$, $l_{n_{max}} = 1$, cf (3a) and (20)

$$\xi = x + \log\, k + N^{-1} \log\,(N+1) \qquad (59)$$

$$\xi = N^{-1} \log\, g + \log\,[1 + (N+1)g] + N^{-1} \log\,(N+1) \qquad (59a)$$

Since the right-hand members in (58) and (59a) contain only the constant N and the variable g one should get a single curve for each N by plotting η as a function of either ξ or $(\xi-\eta)$. These calculated curves can then be compared with the experimental curves $\eta(x)$ or $\eta(\log u)$.

A family of curves $\eta(\xi)_N$ is given in fig. 4. All curves $\eta(\xi)$ intersect at the point

$$\eta = \xi = \log 2 \qquad (60)$$

The asymptotes of any curve are the lines (cf 52 and 52a)

$$\left.\begin{aligned} \eta &= 0 \\ \eta &= \xi\,(1 + N^{-1})^{-1} \end{aligned}\right\} \qquad (61)$$

When N tends toward infinity, the family of curves approaches a limiting curve, consisting of the two straight lines (57).

Repeated reaction, hypothesis IIIa

From (49) and (31)

$$\eta = \log\,[1 + k_0((1-v)^{-2}-1)] \qquad (62)$$

Using (28a) we find $n^{-1} \log l_n \to 0$ for $n \to \infty$, whence with (53), (53a) and (62)

$$\xi = x + \log k = \log v + \log\,[1 + k_0((1-v)^{-2}-1)] \qquad (63)$$

$$\xi - \eta = \log u + \log k = \log v \qquad (64)$$

Fig. 5 a gives $\eta(\xi)_{k_0}$ and fig. 6 $\eta(\xi-\eta)_{k_0}$ for the same k_0 values as in fig. 2c, calculated by means of equations (62 to 64). The curves in a family do not intersect. For $k_0 \to 0$, the curves tend to the limiting curves (57) and (57a),

each consisting of two straight lines, these lines being also the asymptotes for any curve.

The curves may be used for fitting the experimental data $\eta(x)$ and $\eta(\log u)$. Depending on the accuracy of the data, several methods for finding k_0 may be invented.

Repeated reaction, hypothesis IIIb

From (49) and (38)

$$\eta = \log\ [1 + 2\ k_0 v(1—v)^{-3}] \tag{65}$$

From (28 b) we find $n^{-1} \log l_n \to 0$ for $n \to \infty$, whence with (53), (53a), and (65)

$$\xi = x + \log\ k = \log\ v + \log\ [1 + 2\ k_0 v(1—v)^{-3}] \tag{66}$$

Fig. 5b gives the family $\eta(\xi)_{k_0}$. As with IIIa the limiting curve for $k_0 \to 0$ consists of the two lines (57) which are also asymptotes for each curve. The curves do not intersect.

Repeated reaction, hypothesis IIIc

From (49) and (42)

$$\eta = \log\ [1 + k_0(ve^v + e^v—1)] \tag{67}$$

As before, it follows from our definitions that

$$x + \log\ k = \log\ v + \eta \tag{50a}$$

In (53), $n^{-1} \log l_n \to —\infty$ for $n \to \infty$ (28 c), and hence (53) cannot be used. The curves $\eta(x + \log k)_{k_0}$ do not intersect. They do approach the slope + 1 but have no asymptote for increasing η. To aid the comparison with the experimental data we shall, instead of (53), make a shift in the abscissa so that all curves intersect in the point

$$\eta = \log\ 2 = \xi \tag{68}$$

This is achieved by using the abscissa (cf. 50a)

$$\xi = x + \log\ k—\log\ v_2 = \log\ v + \eta—\log\ v_2 \tag{69}$$

where v_2 is the value for v when $\eta = \log 2$. This value is the solution of

$$e^{v_2}\ (v_2 + 1) = 1 + k_0^{-1} \tag{70}$$

Fig. 5c gives the family of curves $\eta(\xi)_{k_0}$ calculated from (67) and (69). As $k_0 \to 0$ the curve $\eta(\xi)$ is seen to approach the two lines (57) like in all other cases discussed.

More complicated assumptions

It may occur that the experimental data clearly indicate, using the criteria given in Part I, that the main products are complexes of the type $B(A_tB)_n$ but that the curves $y(x)$ or $\eta(x)$ do not fit in with any of the mechanisms proposed in this paper. Then one may go to more complicated assumptions.

As a complication of hypothesis II one may assume that two or three separate complexes are formed but no others. This explanation should be resorted to only if there are strong additional arguments for these two or three complexes being preferred to others.

In a repeated reaction, one may try other formulas for the dependence of k_n of n than those given in (28). For instance, there may be a certain minimum size $n = N$ for the complex, so that $k_n = 0$ for $n < N$ and $k_n = k_0 k^n$ for $n \geq N$.

Since the available experimental data on polynuclear reactions may be fairly well explained by the simple mechanisms II or IIIa given here, it has not been thought worth while at present to give detailed calculations for such more complicated cases. The formulas are however not difficult to derive. It is felt that unless the accuracy is extremely good, one should avoid introducing more than two arbitrary constants to be determined from the experiments.

Let us suppose that the data for a certain process can be well accounted for by, say, hypothesis IIIa. Of course this fact is not a proof that (28a) is exactly followed, and that all consecutive link-addition reactions have the same equilibrium constant k. However, it would indicate that this approximation is useful for calculations and that it also gives an approximate picture of the process.

Distribution of complexes

The chief aim of this paper has been to demonstrate how the experimental data may be used for distinguishing between various possible mechanisms and for finding the equilibrium constants involved. Once these are known one may calculate the concentration of each complex in a given solution (cf 1 and 6).

$$c_n = [\mathrm{B(A_tB)_n}] = l_n b v^n \tag{71}$$

Furthermore one may calculate the fraction of all B groups present in the n:th complex (cf 71 and 5a)

$$\frac{(n+1)c_n}{B} = \frac{(n+1)l_n v^n}{1 + g + vg'} \tag{72}$$

and the average number of links per complex (cf 3a)

$$\frac{\Sigma n c_n}{\Sigma c_n} = \frac{vg'}{g} = \frac{\mathrm{d}\ \ln\ g}{\mathrm{d}\ \ln\ v} \tag{73}$$

by inserting the expression for g for the mechanism in question.

The quantities in (72) and (73) are functions of v only.

SUMMARY

The paper deals with the mathematical analysis of data for a system in which the main products of the reaction between A and B are known (by methods given in part I) to be complexes $\mathrm{B(A_tB)_n}$ with t constant and n variable.

The experimental data are suitably treated by plotting one t:th of the average number of A bound per B, $y = (A—a)/Bt$, as a function of $x =$

$t \log a + \log B$. If measurements of b are available, one may also plot $\eta = \log \frac{B}{b}$ as a function of x. In both cases, all experimental points should fall on a single curve independent of B.

These experimental curves $y(x)$ and $\eta(x)$ can then be compared with families of curves $y(X)$ and $\eta(\xi)$, calculated for various mechanisms. Here, X and ξ differ only by a constant (containing the equilibrium constants) from x, so one can move the calculated curves parallel with the horizontal axis in search for a fit.

Equations and curves are given for the following hypotheses: I) No soluble complex; solid A_tB precipitates. II) Only one complex, $B(A_tB)_N$ is formed. III) An infinite series of complexes is formed, by repeated reaction, with all positive values for n. Three equations are tried for the variation of k_n with n: IIIa: $k_n = k_0 k^n$, IIIb: $k_n = k_0 n k^n$ and IIIc: $k_n = k_0 k^n / \underline{n}$. Here k_n is the equilibrium constant for the formation of the n:th complex from A and B.

The curves for precipitation of solid A_tB (hyp. I) are found as the limiting case of a single complex (hyp. II, $N \to \infty$) and of repeated reaction (hyp. III, $k_0 \to 0$).

By transformations described in Part I, eq. 30—34, the same families of curves may also be used to treat other "core + links" mechanisms, of general formula $A_r(A_tB)_n$.

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A Convenient Method for Preparing Acetals *

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The reaction between an aldehyde and an alcohol proceeds to an equilibrium:

$$R'CHO + 2\ R''OH \rightleftharpoons R'CH(OR'')_2 + H_2O$$

the constant of which depends on the nature of the aldehyde [1,2]. The process is catalyzed by hydrogen ions [3,4], but not by hydroxyl ions, and therefore the acetalisation is of great importance in preparative chemistry as a means of protecting an aldehyde group during reactions in neutral or basic medium. For instance reduction of other groups [5,6], bromination and exchange of bromine against amino, cyano and hydroxyl groups [2] have been carried out without affecting the acetal group, which in turn is easily converted to the aldehyde group by means of aqueous acids.

Many attempts have been made to displace the equilibrium in favour of the acetal, first by using anhydrous reagents and by avoiding water in presence of acids during the isolation of the acetal. R. D. Haworth and A. Lapworth have proposed the elimination of water by distilling off the ternary azeotrope of water, alcohol and benzene [6]. L. Claisen has prepared acetals by means of orthoformic [7] and formimino [8] esters, B. Helferich and J. Hausen use orthosilicic esters [9] and W. Voss proposes the use of sulfurous esters [10].

Voss also showed that these esters do not act as alkylating agents but, because of their easy hydrolyzability, eliminate water from the equilibrium. As a matter of fact they are not able to acetalise an aldehyde except in the presence of alcohol and hydrogen ions.

The method of Haworth and Lapworth is troublesome and the others require difficultly available compounds. The author, therefore, investigated the possibility of applying esters of sulfuric and *p*-toluenesulfonic acids for the removal of water. Unlike the above mentioned esters the latter when hydrolyzed form strong acids, which catalyze the acetal formation. Consequently the use of anhydrous alcohols is not necessary; on the contrary, the small water content in commercial absolute alcohols form sufficient acid to start the reaction without adding any other catalyst. It also appeared, that neutralization of the acid could be carried out without any appreciable loss by slowly adding

* Presented before The 8th Scandinavian Chemists' Meeting in Oslo, June 1953.

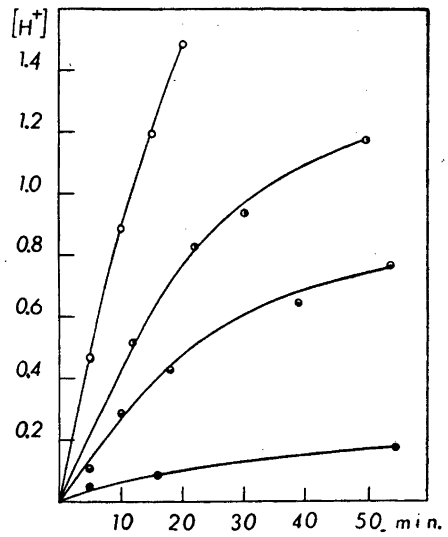

Fig. 1. Conversion-time curves.

Curve a ○ *1.42 M Me_2SO_4 + 0.95 M PhCHO in MeOH,*
» *b* ◒ *1.02 M Et_2SO_4 + 0.68 M PhCHO in EtOH,*
» *c* ● *0.87 M $MeC_6H_4SO_3Me$ + 0.87 M PhCHO in MeOH,*
» *d* ◑ *1.18 M Me_2SO_4 in MeOH.*

The temperature was in all cases the boiling point of the mixture.

the reaction mixture to a vigorously stirred mixture of sodium hydroxide and crushed ice. The yields were in the cases investigated better by this method than by the simple acetalisation with anhydrous alcohols, and of the same order of magnitude as when applying the easily hydrolysable esters mentioned above.

EXPERIMENTAL

Benzaldehyde dimethyl acetal. 10 ml benzaldehyde (0.1 mole), 80 ml methyl alcohol and 12 ml dimethyl sulfate (0.125 mole) were refluxed for half an hour and slowly added to a mixture of 150 ml 2 *N* sodium hydroxide and 100 g cracked ice under vigorous stirring. The acetal was extracted with ether (3 times 50 ml), dried over potassium carbonate and distilled *in vacuo*. Yield 13.4 g = 88 %.

By the same method the dimethyl acetals of butyraldehyde and anisaldehyde were prepared. Crotonaldehyde did not give any acetal.

In Table 1 the yields by different methods are compared.

Benzaldehyde diethyl acetal. Attempts to prepare this compound by the same method resulted in an impure product in about 55 % yield. The reason for the rather unsatis-

Table 1. Percentage yield of some acetals by different methods.

Method	H^+		Azeotrope	$HC(OEt)_3$	$CH(NH)OR$		$Si(OR)_4$		R_2SO_3		Me_2SO_4
Alkyl / Aldehyde	Me	Et	Et	Et	Me	Et	Me	Et	Me	Et	Me
Butyr-	30[11]	50[11]							80[10]	72[10]	52
Croton-						0[8]	50[9]	0[9]			0
Benz-	37[4]	56[6]	66[6]	98[7], 66[12]		84[8]	70—90[9]		81[10]	78[10]	88
Anis-	30[3]	40[6]		96[7], 88[13]	67[8]	52[8]			84[10]		65

factory result was shown to be the very slow hydrolysis of the excess diethyl sulfate by the aqueous alkali, which caused decomposition of the acetal during the subsequent distillation.

Reaction velocity. A rough estimate of the rate of reaction between benzaldehyde and methyl alcohol in the presence of dimethyl sulfate and methyl *p*-toluenesulfonate, and of benzaldehyde and ethyl alcohol with diethyl sulfate was made by titrating samples of 1 ml at appropriate intervals with methanolic sodium methoxide. The results (Fig. 1) indicate that methyl *p*-toluenesulfonate acts too slowly and that both dimethyl and diethyl sulfate react with an adequate velocity, but as mentioned above diethyl sulfate must be abandoned. Apparently the conversion exceeds 100 %, but this is due to the reaction between alcohol and dialkyl sulfate with production of ether and sulfuric acid. This was proved in the case of dimethyl sulfate by refluxing this compound with methanol and titrating samples of 1 ml (Curve d, Fig. 1). In another run the liberated methyl ether was condensed in a dry ice-acetone bath and identified by the boiling point (−23.6° / 761 mm Hg).

SUMMARY

In the acetalization of an aldehyde with methanol the yield is increased considerably by adding dimethyl sulfate.

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Separation of Conjugated and Free Bile Acids by Paper Chromatography. Bile Acids and Steroids 12

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In connection with work on bile acid metabolism it has become necessary to have a rapid method for separating bile acids directly as they appear in the bile, *i.e.* in conjugated form. Ahrens and Craig [1] have used countercurrent distribution and Norman and Bergström [2] partition chromatography for this purpose. In an investigation of bile acid metabolism where it is necessary to take bile samples at short intervals these methods, however, require too much substance and too much time. Several methods of separating bile acids using paper chromatography have recently been developed [3-6,13]. In the work described in this paper the method of Sjövall has been further investigated and developed also for the separation of conjugated bile acids.

A. SEPARATION OF FREE BILE ACIDS

Experimental

Filter paper Whatman No. 1, 4 and 3 MM can all be used. Whatman 3 MM has a greater capacity and 50—100 γ of an acid gives a well defined spot without tailing. It has earlier been used by Haslewood [6] for the separation of bile acids and their ethyl esters. Whatman 1 is mostly used when smaller amounts, 1—10 γ, are to be analysed, and when radioactive acids are run.

The paper is cut in strips 9 × 45 cm. When radioactivity is to be counted these strips are cut so that each sample runs on its own strip 1.5 cm broad, separated from the next strip by a 1 cm broad space where the paper is removed, leaving a small margin at both ends of the paper. Thus a 9 cm broad strip contains 4 such 1.5 cm strips all held together with intact paper at both ends.

For the separation of free acids the following solvents have been used.

Moving phase: *iso*propyl ether : heptane 60 : 40 v/v.

Stationary phase: 70 % acetic acid.

For the separation of desoxycholic acid and chenodesoxycholic acid isopropyl ether: heptane 20 : 80 is used as moving phase and 70 % acetic acid as stationary phase.

Equal parts of the phases are equilibrated in a separating funnel before use, at the same temperature as for the chromatography.

The cut papers are soaked in the stationary phase and dried at 100° C until they just seem dry. The acids or their sodium salts are put on the starting point 6 cm from one end of the paper. This is then hung in the chromatographic chamber overnight for a complete equilibration with both phases.

We have used both ascending and descending chromatography. The descending technique is used only for long runs, *i. e.* 16 hours and more when slow running components are to be separated. For both techniques cylindrical glass vessels 14 × 60 cm are used, their inner walls coated with filter paper wetted with moving phase. On the bottom of the vessel is placed a beaker containing stationary phase in which a strip of filter paper is hung. The stationary phase ascends on this strip and a more complete phase saturation is obtained. The vessels are closed with glass plates tightened with stopcock grease.

For the ascending chromatography the running phase is on the bottom of the vessel. During the equilibration period the chromatograms must not come in contact with the moving phase. The papers are hung with their lower edges 1 cm above the surface of the moving phase. When equilibrium has been obtained, this surface is brought up to the papers by filling more moving phase through a glass tube introduced through a hole in the cover. In this way opening of the chromatographic chamber is avoided. Another and more simple method [6] is to tilt the vessel so that the moving phase is not in contact with the paper and then, after equilibration, tilt it until the solvent reaches the paper.

When the front of the moving phase is to run off the paper, descending chromatography has to be used. The arrangements for the phase saturation are the same as for the ascending technique but the papers hang in a trough and after equilibration the trough is filled with moving phase through a small hole otherwise closed with a cork in the covering glass plate.

After equilibration for 12—16 hours the chromatograms are developed for a suitable time. The chromatography has been carried out at constant temperature (23° C).

The spots are revealed by spraying with a 10 % solution of phosphomolybdic acid in absolute alcohol and subsequent drying at 80—100° C [7]. This reagent is not specific but gives a blue colour with all the common bile acids and their conjugates with glycine and taurine. In a previous paper [4] it was stated that lithocholic acid did not give any colour. This has been found to be due to an impure phosphomolybdic acid. Cholic, desoxycholic and chenodesoxycholic acid and their conjugates can be detected after chromatography of 1—3 γ or even less, while lithocholic acid and its conjugates require 10—20 γ.

For counting of radioactivity the paper was taped on a ruler that could be moved ½ cm at a time past the GM-tube. A slit in a metal plate between the paper and the mica window allowed the counting of ½ or 1 cm of the paper each time.

Results

When pure synthetic bile acids are run the spots appear well defined with no tailing. The R_F values are, however, variable and reference acids always have to be run parallel with the mixture to be analysed. This is probably dependent upon the difficulty of keeping the concentration of acetic acid in the paper constant.

If the acids are put on the paper as sodium salts they run at the same speed as when put on as free acids, since they are set free by the acid solvents.

Sometimes it is advisable to change the moving phase. An increase of heptane in the moving phase causes a reduction of the moving rate of all the acids. It has been found for the acids with substituents at 3 α, 7 α and 12 α that this reduction for the glycine conjugates is proportionally larger than that for free cholic acid whose reduction is larger than that of dihydroxy acids. Thus if an elongated spot is suspected to contain more than one acid the heptane can be increased and a descending chromatogram developed, letting the solvent drip off the lower edge of the paper for a suitable time.

Fig. 1 a shows the separation of cholic, hyodesoxycholic, desoxycholic and lithocholic acid. With the oxygen-containing substituents in α-position at carbon atoms 3, 7 and 12 the acids separate in groups depending on the number of oxygen substituents. Desoxycholic and chenodesoxycholic acid for example

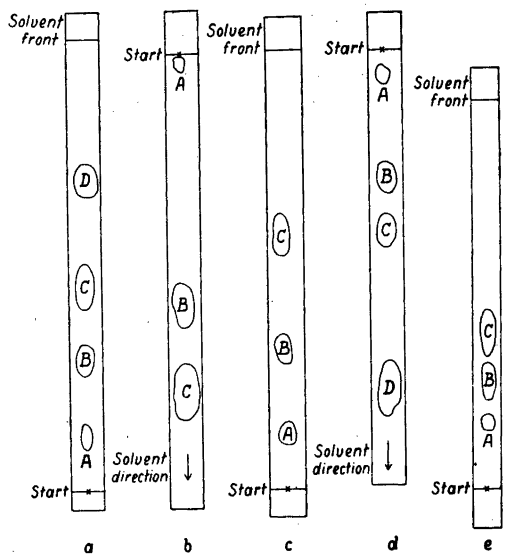

Fig. 1. Separation of different bile acids (see text).

a. Ascending, 4 hours.
Moving phase: Isopropyl ether:heptane 60 : 40 v/v.
Stationary phase: 70 % acetic acid.
Acids: Cholic (A), hyodesoxycholic (B), desoxycholic (C) and lithocholic (D).
b. Descending, 18 hours.
Moving phase: Isopropyl ether:heptane 20 : 80 v/v.
Stationary phase: As 1 a.
Acids: Impurity in the chenodesoxycholic acid (A), chenodesoxycholic (B) and desoxycholic (C).
c. Ascending, 4 hours.
Moving phase: Isopropyl ether:heptane 85 : 15 v/v.
Stationary phase: As 1 a.
Acids: Glycocholic (A), glycochenodesoxycholic (B) and glycolithocholic (C).
d. Descending, 18 hours.
Moving and stationary phase as 1 a.
Acids: Glycocholic (A), glycochenodesoxycholic (B), glycodesoxycholic (C) and cholic (D).
e. Descending, 12 hours.
Moving phase: n-Butanol saturated with 3 % acetic acid/water v/v.
Stationary phase: 70 % acetic acid.
Acids: Taurocholic (A), taurodesoxycholic (B) and taurolithocholic (C).

do not separate, neither do the ketonic acids separate from the hydroxylic acids with the same number of oxygen atoms on the skeleton. There is only a very small difference in the travelling rate. Hyodesoxycholic acid with the two hydroxyl groups at 3 α and 6 α separates clearly from the other two dihydroxy acids, desoxycholie and chenodesoxycholic acid. Acids with hydroxyl groups in β-position have not yet been tested but there are strong reasons to believe that ursodesoxycholic acid (3 α 7 β) separates from chenodesoxycholic acid (3 α 7 α) (Haslewood).

Table 1. Relationship between c/min plated on copper planchets and c/min recovered after paper chromatography.

Acid	Approx. weight γ	Copper planchets c/min	Chromatogram c/min	Recovery %
Cholic	5	390	136	34.9
Desoxycholic	3	867	324	37.4
Chenodesoxycholic	2	797	277	34.8
Lithocholic	30	948	323	34.1
Cholanic	3	432	160	37.0

In order to use the method for biological tracer work some chromatograms were run with C^{14}-labelled bile acids [8]. An estimation was made of the absorption of radioactivity in the filter paper. Table 1 shows the values obtained from five different acids. The same amount as was run on the chromatograms was plated on copper planchets and the activities compared. This was done to make it possible to correlate the curves obtained by column partition chromatography and those obtained by paper chromatography. A comparison between the activity of the spot at the starting point before the chromatogram had been developed and the activity of the spot after development showed that about 80 % was found when the chromatogram had been run. This is in rather good agreement with Rochland *et al.*[9] who recovered 85 $\pm$ 6 % when running glycine C^{14} on Whatman No. 1 paper.

The separation of desoxycholic and chenodesoxycholic acid has always been a great difficulty. The only existing preparative method is a repeated precipitation of the barium salt of chenodesoxycholic acid. As there is a small difference in moving rate with isopropyl ether : heptane as moving phase the heptane content was increased and a descending chromatogram run. Fig. 1 b shows the separation of the two acids after 18 hours descending with isopropyl ether : heptane 20 : 80 as moving phase. Heptane alone was also used as moving phase and gave after 72 hours' descending chromatography a separation of the acids. In this case, however, only 5 γ of desoxycholic acid could be run. When more substance was used the spots became elongated and with 25 γ the acid appeared as a long streak. In order to see whether this was due to a tailing that could not be detected by spraying when only small amounts of acid were used, the same amount of labelled desoxycholic acid was added to varying amounts of inactive acid. With less than 5 γ acid the activity appeared in a narrow symmetrical peak. This is shown in Fig. 2 where 3 γ labelled desoxycholic acid is separated from 2 γ labelled chenodesoxycholic acid. When, however, larger amounts of desoxycholic acid were run the activity spread over a longer area. This did not happen when chenodesoxycholic acid was used. It is possible that desoxycholic acid which easily forms choleic acids has formed a complex with acetic acid or heptane and that this is the cause of the bad capacity of the system. The addition of isopropyl ether to the moving phase largely prevents this elongation of the spots as is seen in Fig. 2 where 25 γ desoxycholic acid was run.

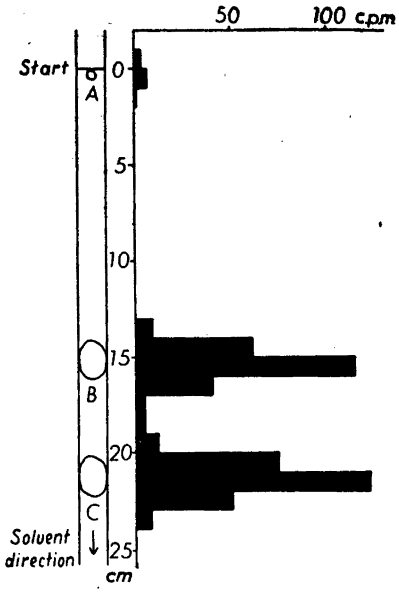

Fig. 2. Separation of labelled dihydroxy acids. The chromatogram is reproduced to the left of the diagram showing the distribution of radioactivity in the paper. A: Impurity in the chenodesoxycholic acid; B: chenodesoxycholic acid; C: desoxycholic acid.

B. SEPARATION OF CONJUGATED BILE ACIDS

1. Bile acids conjugated with glycine

Experimental. See A.

Solvent systems. Moving phase: *iso*propyl ether : heptane 80 : 20 v/v or 85 : 15. Stationary phase: 70 % acetic acid.

Spraying reagents. Phosphomolybdic acid has mostly been used. Chlorination with subsequent spraying with potassium iodide-starch [10] gives a colour with conjugated bile acids only and is useful when the question is whether a spot is a free or a conjugated bile acid. The reagent is not very sensitive and 20—50 γ of each acid has to be used for a chromatography. Probably the hydroxyl groups interfere with the reaction because glycocholanic acid gives a colour with much smaller amounts than the hydroxylated acids.

Results.

The acids used were supplied by Bergström and Norman and synthesised according to their method [11].

Fig. 1 c shows the separation of glycocholic, glycochenodesoxycholic and glycolithocholic acid. Glycodesoxycholic acid runs somewhat faster than glycochenodesoxycholic acid but the difference is not large enough for a clear separation. The glycine-conjugated desoxycholic and chenodesoxycholic acids, however, show a larger difference in moving rate than the free acids and are therefore easier to separate. This separation is seen in Fig. 1 d where the two acids are run together with glycocholic and cholic acid. The chromatogram has been developed in a descending direction for 18 hours with isopropyl ether : heptane 60 : 40 as moving phase and 70 % acetic acid as stationary phase.

The conjugation with glycine has approximately the same effect on the moving rate as one hydroxyl group in the free bile acids when isopropyl ether: heptane 80 : 20 is used as moving phase. The same has been found by Norman [12] using reversed phase partition chromatography. If, however, the heptane content is increased and descending chromatography is run for a suitable time, a clear separation is achieved of glycine-conjugated acids from free acids with one hydroxyl group more (see also under Results A). Fig. 1 d for example shows the separation of cholic acid from the glycine-conjugated dihydroxy acids.

As with the free bile acids the glycine-conjugated acids can be put on the starting line as sodium salts. The taurine conjugated acids all run slower than glycocholic acid.

2. Bile acids conjugated with taurine

Experimental.

Solvent systems. Moving phase: Equal parts of *n*-butanol and 3 % acetic acid are equilibrated in a separating funnel. The upper phase is used (butanol).

Stationary phase: 70 % acetic acid.

In this case the two phases are not equilibrated with each other because they mix easily. The equilibrium in the chromatographic chamber is therefore not a true one but it has been found that tailing of the spots is best prevented by using these phases. This is in agreement with the finding for the chromatography of other bile acids that 70 % acetic is the optimal concentration for the papers. Less acetic acid gives tailing of the spots and a higher concentration gives streaking in the direction of the solvent front.

Descending chromatography is mostly used because of the slow ascending rate of the butanol phase.

The same spraying reagents as for glycine conjugates have been used.

Results.

Fig. 1 e shows the separation of taurocholic, taurodesoxycholic and taurolithocholic acid. The acids come close together probably because of the pronounced effect of the taurine part of the molecule which makes the effect of the hydroxyl groups on the partition coefficient less prominent. The taurine conjugates of chenodesoxycholic acid and desoxycholic acid do not separate.

Even the taurine conjugated bile acids can be put on the starting line as sodium salts and still behave as acids in the chromatogram.

The glycine-conjugated acids and the free acids all run faster than taurolithocholic acid and practically with the front.

SUMMARY

A method of separating free and conjugated bile acids using paper chromatography is described.

Desoxycholic acid and chenodesoxycholic acid and their glycine conjugates have been separated.

The method has been applied to work with labelled bile acids.

Thanks are due to Professor Sune Bergström and Dr. Arne Norman for supplies of the bile acids used.

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Bifunctional Amines and Ammonium Compounds

III. Bis-β-dialkylaminoethyl Sulfides and their Quaternary Ammonium Salts

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In the second paper [1] of this series the preparation of ganglionic blocking agents chemically belonging to the class of bis-β-trialkylammoniumethyl ether salts (I) was described.

$$X^-,R_1R_2R_3N^+CH_2CH_2O\ CH_2CH_2N^+R_1'R_2'R_3',X^-$$

I

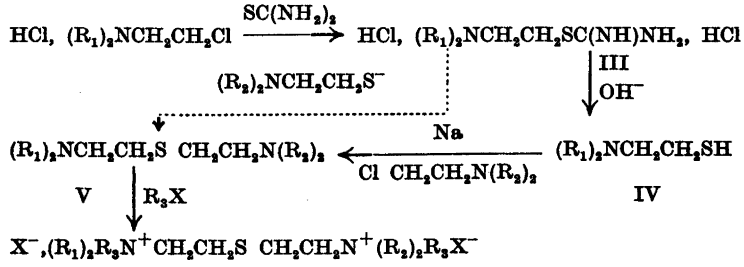
The present paper describes a number of similarly active bis-β-trialkylammoniumethyl sulfide salts (II) structurally analogous to the above mentioned ether salts.

$$X^-,R_1R_2R_3N^+CH_2CH_2S\ CH_2CH_2N^+R_1'R_2'R_3',X^-$$

II

The method of choice for the preparation of the compounds reported in this paper was the quaternization of bis-tertiary aminoethyl sulfides (V) with reactive alkylating agents dissolved in acetone containing ethanol to facilitate solvation of the resultant anion.

The precursors of the quaternary alkyl salts, the *tertiary* aminoethyl sulfides were obtained by a thioether condensation analogous to the Williamson ether synthesis. This method is advantageous in that it allows for the preparation of 'hybrid' sulfides, *e.g.* sulfides containing different N-substituents in either end of the molecule.



The mercaptans (IV) used in the thioether condensations were obtained from the β-chloroalkylamine hydrochlorides via the *iso*-thiouronium salts (III) according to Albertson and Clinton [2]. Although this procedure is the most convenient of the available methods [2-4], it has been shown in the present work that rather large amounts of the corresponding sulfide is formed simultaneously with the mercaptan, just as is the case in the direct hydrosulfide-halide condensation [3]. The formation of sulfide has been explained as a reaction between already formed mercaptan and unreacted halide [4], an explanation which is untenable for the alkaline hydrolysis of recrystallized *iso*-thiouronium salts. The simultaneous appearance of mercaptan and sulfide might however result from a nucleophilic attack of a hydroxyl ion at the carbon atom of the urea moiety in the former case and of a nucleophilic attack of mercaptide ion at the β-carbon of the aminoalkyl moiety in the latter.

The dialkylaminoethyl mercaptans, in the form of their sodium mercaptides, were condensed with β-chloroalkylamines of identical or nonidentical structure in conventional fashion, preferably in a nonpolar solvent to minimize side reactions and polymerization of the chloroamine.*

In agreement with the findings of Lawson and Reid [5] difficulties were encountered in isolating and distilling bis-β-dimethylaminoethyl sulfide. In addition, some preparations of bis-β-diethylaminoethyl sulfide showed evidence of vinyl sulfide or divinyl sulfide formation.

Attempts to prepare the mercaptans by acid hydrolysis of the thiosulfates (*cf.* Peak and Watkins [6]) were unsuccesful. The other available methods for the synthesis of bis-aminoalkyl sulfides, *viz.* direct sodium sulfide-aminoalkyl halide condensation, and mercaptan-aminoalkyl halide hydrochloride condensation to give a sulfide bis-hydrochloride according to Gilman *et al.* [3] were employed in a few cases.

Three different sulfides and six different quaternary salts having only methyl and ethyl groups as nitrogen substituents were prepared.

The preparation of the symmetric quaternary salts by another route was also investigated briefly. The reaction of bis-β-chloroethyl sulfide with tertiary amines as described by Lawson and Reid [5], who prepared the lowest homologue, bis-trimethylammoniumethyl sulfide chloride, of this series more than twenty-five years ago, proceeded much more smoothly than the corresponding reaction involving β-chloro ether. The method requires, however, the use of mustard gas, which is unpleasant to prepare and handle. The products were obtained in satisfactory yield.

A report on the pharmacological properties of the quaternary salts will appear elsewhere.

EXPERIMENTAL

β-Diethylaminoethyl-*iso*-thiouronium chloride hydrochloride (III, $R_1 = C_2H_5$)

Prepared according to Albertson and Clinton [2] from 860 g of diethylaminoethyl chloride hydrochloride (5 moles) and 400 g of tiourea (5.25 moles). No precipitation with ethyl acetate-ligroin was employed; the reaction mixture was evaporated *in vacuo* and the

* Note added in proof: Swedish Pat. 137,019 (F. Hofmann-La Roche & Co.) discloses similar sulfides prepared by condensation in ethanol-sodium ethylate.

second and third crop of crystals removed *. Yield: 1st crop 852 g, m. p. 188—192° C; 2nd crop 150 g, m. p. 185—187° C; 3rd crop 202 g, m. p. 178—185° C. Total yield 1 204 g (98 %). Crystallization from ethanol-ether raises m. p. to 196° C (Ref.[3] 195° C). Yield after recrystallization 1 165 g (94 %).

β-Dimethylaminoethyl-*iso*-thiouronium chloride hydrochloride (III, $R_1 = CH_3$)

Prepared as above from 288.2 g of dimethylaminoethyl chloride, HCl (2 moles) and 160 g of thiourea. Total yield after recrystallization 395 g (90 %). M. p. 173—175° C.

β-Diethylaminoethyl mercaptan (IV, $R_1 = C_2H_5$)

Prepared essentially as described [3] from 248 g of *iso*thiouronium salt (1 mole). Yield 71 g (53 %). B. p. 58° C at 12 mm Hg. n_D^{25} 1.4661. There was further obtained 41 g of bis-β-diethylaminoethyl sulfide (V), b. p. 147—150° C at 12—14 mm Hg. n_D^{25} 1.4736.

A solid, crystalline residue m. p. 201—203° C, is sometimes seen in the mercaptan-sulfide mixture before distillation. The residue was identified as dicyandiamide.

β-Dimethylaminoethyl mercaptan (IV, $R_1 = CH_3$)

Prepared exactly as above from 385 g (1.75 mole) of thiouronium salt. Yield 41 g (31 %). B. p. 40° C at 12 mm Hg. n_D^{25} 1.4682.

Bis-β-diethylaminoethyl sulfide (V, $R_1 = R_2 = C_2H_5$)

Prepared according to the directions given for the corresponding ether [1] from 133.2 g of α-diethylaminoethyl mercaptan (1 mole), 23 g of sodium and 172.1 g of β-diethylaminoethyl chloride, HCl. The solvent was benzene in this case. There was obtained 207 g (88 %) of the desired sulfide, slightly yellow oil, b. p. 144—150° C at 12—16 mm Hg. n_D^{25} 1.4738. (Litt.[1,2,5,6] b. p. 64.0° C at 0.4 mm Hg, b. p. 104—106° C at 0.3 mm Hg, b. p. 139—140° C at 9 mm Hg, b. p. 106—110° C at 2 mm Hg; n_D^{20} 1.4740, n_D^{20} 1.4470).

The product was also obtained by condensation of dehydrated sodium sulfide from 14 g of sodium sulfide, $9H_2O$ (0.05 mole) with β-diethylaminoethyl chloride, obtained from 17.2 g of the hydrochloride in anhydrous ethanol. Yield 4.2 g (36 %), b. p. 144—146° at 15 mm Hg.

The bis-hydrochloride was prepared by adding dry HCl gas to the amine dissolved in dry ether. The mass formed was crystallized from acetone-methanol (1 : 1), m. p. 243—245° C (decomp.) (Ref.[2,3] 252—254° C, 246—248° C). Picrate, m. p. 174—176° C.

Bis-methoiodide (As-7150) obtained from 23.2 g of sulfide (0.1 mole) and 42.6 g methyl iodide (0.3 mole) in 400 ml dry acetone and 50 ml anhydrous ethanol at room temperatur for 24 hours (violent reaction to begin with). Yield 40 g (48 %), m. p. 261° C (recrystallized twice from anhydrous methanol). $C_{14}H_{34}N_2SI_2$ (516.3) Calc. I 49.19; found 48.18.

Bis-ethobromide (As-7102) Prepared as the bis-methoiodide from 23.2 g of sulfide and 32.7 g of ethyl bromide (0.3 mole). After 3 days at room temperature, ether addition precipitates an oil which can be brought to crystallization by repeated dissolution and precipitation. Yield 10 g (22 %), m. p. 223—225° C (methanol-ether (1 : 1)). $C_{16}H_{38}N_2SBr_2$ (450.4) Calc. Br 35.5; found Br 35.5.

Bis-β-dimethylaminoethyl sulfide (V, $R_1 = R_2 = CH_3$)

Condensation of 105 g of dimethylaminoethyl mercaptan (1 mole) with chloride from 144 g of dimethylaminoethyl chloride, HCl, and 23 g of sodium gave 64.5 g (36.5 %)

* All melting and boiling points are uncorrected. The nitrogen and halogen values are macro determinations by Mrs. G. Speggers of this laboratory. Carbon and hydrogen values are micro determinations by Mr. A. Grossmann, University of Copenhagen.

of the sulfide, b. p. 103—107° C at 12—14 mm Hg. n_D^{25} 1.4751. $C_8H_{20}N_2S$ (176.3) Calc. C 54.36, H 11.2, N 15.52; found C 54.20, H 11.4, N 15.92.

Bis-hydrochloride, m. p. 273—276° C (from acetone-methanol (3 : 1)), $C_8H_{22}N_2SCl_2$ (249.3) Calc. Cl 28.44; found Cl 28.61. The bis-hydrochloride was also obtained by a condensation of mercaptan with chloride HCl as described [3], mixed m. p. with authentic sample showed no depression. Picrate m. p. 180—182° C.

Bis-methoiode (As-4366). Yield 43.5 %, m.p. 276° C (from methanol). $C_{10}H_{26}N_2SI_2$ (460.1) Calc. I 55.17; found I 53.92.

Bis-ethobromide (As-4374). Yield 50.6 %, m. p. 245—248° C (from ether-methanol (1 : 1)). $C_{12}H_{30}N_2SBr_2$ (394.2) Calc. Br 40.54; found Br 39.02.

Bis-(β-dimethyl-β′-diethyl)-aminoethyl sulfide (V, $R_1 = CH_3$; $R_2 = C_2H_5$)

Prepared as directed from 133.2 g of diethylaminoethyl mercaptan, 23 g of sodium, and 144 g of dimethylaminoethyl chloride, HCl. Yield 160 g (78.5 %), b. p. 125—127° C at 14 mm Hg. n_D^{23} 1.4688. $C_{10}H_{24}N_2S$ (204.3) Calc. C 58.75, H 11.8, N 13.72; found C 58.83, H 11.7, N 13.66.

Bis-Hydrochloride, m. p. 252—254° C (from acetone-methanol (1 : 1)). $C_{10}H_{26}N_2SCl_2$ (277.4) Calc. Cl 25.56; found Cl 25.66.

Condensation of diethylaminoethyl mercaptan and dimethylaminoethyl chloride, HCl by heating them together (cf. Gilman *et al.*[3]) gave a hydrochloride-mixture, m. p. 96° C, from which could be isolated the bis-hydrochloride of bis-diethylaminoethyl sulfide, m. p. 298° C, in small yield after 8 recrystallizations from acetone-methanol (1 : 1), but *no* bis-hydrochloride of the asymmetric sulfide. Picrate, m. p. 158—160° C.

Bis-methoiodide (As-7277). Yield 56.1 %, m. p. 271—273° C (methanol). $C_{12}H_{30}N_2SI_2$ (488.4) Calc. I 52.01; found I 50.88.

Bis-ethobromide (As-7207). Yield 43.7 %, m. p. 227° C (from acetone-ethanol (2 : 1)) $C_{14}H_{34}N_2SBr_2$ (422.4) Calc. Br 37,87; found Br 37.45.

SUMMARY

A series of bis-quaternary alkylammonium ethyl sulfide salts prepared by reaction of bis-β-dialkylaminoethyl sulfide with alkyl halide is described. The preparation of the precursors by various routes is described.

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Bifunctional Amines and Ammonium Compounds

IV.* Higher Bis-dialkylamino Ethers and Their Quaternary Ammonium Salts

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A previous paper of this series reported the preparation of bis-β-trialkylammoniumethyl ether salts (I), by a Williamson condensation and quaternization, of the bis-*tert.*-amino ether formed, with alkyl halide to give the desired salts.

$$X^-,R_1R_2R_3N^+CH_2CH_2OCH_2CH_2N^+R_1'R_2'R_3',X^-$$

This paper describes some further quaternary ammoniumalkyl ether salts, which are either homologs of I with respect to length of chain between the two charged N-atoms (II to IV), or analogs in which one or both quaternary nitrogen atoms are part of a heterocyclic nucleus (VII to XII).

$$(R_1)_2N(CH_2)_m\overset{R_3}{\underset{|}{C}}HO(CH_2)_nN(R_2)_2$$

II,	$R_1 = CH_3$,	$R_2 = C_2H_5$,	$R_3 = H$,	$m = 1$,	$n = 3$
III,	$R_1 = C_2H_5$,	$R_2 = C_2H_5$,	$R_3 = H$,	$m = 1$,	$n = 3$
IV.	$R_1 = C_2H_5$,	$R_2 = C_2H_5$,	$R_3 = H$,	$m = 2$,	$n = 3$
V,	$R_1 = CH_3$,	$R_2 = C_2H_5$,	$R_3 = CH_3$,	$m = 1$,	$n = 2$
VI,	$R_1 = CH_3$,	$R_2 = C_2H_5$,	$R_3 = CH_3$,	$m = 1$,	$n = 2$

$$ACH_2CH_2OCH_2CH_2B$$

VII,	$A = C_5H_{10}N$**,	$B = (CH_3)_2N$
VIII,	$A = C_5H_{10}N$,	$B = (C_2H_5)_2N$
IX,	$A = C_5H_{10}N$,	$B = C_5H_{10}N$
X,	$A = C_4H_8ON$***,	$B = (CH_3)_2N$
XI,	$A = C_4H_8ON$,	$B = (C_2H_5)_2N$
XII,	$A = C_4H_8ON$,	$B = C_4H_8ON$

* Part III. Acta Chem. Scand. 8 (1954) 346.
** piperidino-, *** morpholino

Twenty-two quaternary salts were obtained from these eleven amino ethers by reaction with methyl iodide and ethyl bromide in conventional fashion.

A report on the pharmacological properties of the salts will appear elsewhere.

EXPERIMENTAL *

The procedure followed for the preparation of ethers was identical to that set forth in the second paper of the series [1]. The quaternary compounds were obtained by mixing the appropriate amine with an excess of either methyl iodide or ethyl bromide, dissolved in acetone-ethanol (4 : 1). Generally the ethers with the not too bulky N-substituents reacted readily, while heating in sealed tubes was necessary to ensure complete reaction with the piperidine and morpholine substituted ethers. All yields given are after purification.

2-Dimethylaminoethyl-3-diethylaminopropyl ether (II)

From 32.8 g of 3-diethylaminopropanol (0.25 mole), 5.75 g of sodium, and 36 g of dimethylaminoethyl chloride, HCl (0.25 mole). Solvent toluene, heating period 8 hours. Yield 18 g (36 %), b. p. 104—108° C at 10—12 mm Hg. n_D^{25} 1.4373. $C_{11}H_{26}N_2O$ (202.3): Calc. C 65.33, H 12.96, N 13.85; found C 64.91, H 13.10, N 13.81.

Bis-methoiodide (As-8428). Yield 72 %, m. p. 210—212° C (from anhydrous ethanol). $C_{13}H_{32}N_2OI_2$ (486.2): Calc. I 52.21; found 51.74.

Bis-ethobromide (As-8429). Yield 48 %, m. p. 225—228° C (diss. ethanol, reprecipt. ether). $C_{15}H_{36}N_2OBr_2$ (420.3): Calc. Br 38.02; found Br 37.63.

2-Diethylaminoethyl-3-diethylaminopropyl ether (III)

From 65.6 g of 3-diethylaminopropanol (0.5 mole), 11.5 g of sodium, and 86.1 g of diethylaminoethyl chloride, HCl (0.5 mole). Solvent toluene, heating period 8 hours. Yield 54 g (47 %), b. p. 124—129° C at 15—16 mm Hg. n_D^{25} 1.4406. $C_{13}H_{30}N_2O$ (230.4): Calc. C 67.78, H 13.12, N 12.16; found C 67.47, H 13.18, N 11.92.

Bis-methoiodide (As-8302). Yield 86 %, m. p. 223° C (ethanol-water (80 : 1)). $C_{15}H_{36}N_2OI_2$ (514.3): Calc. I 48.41; found I 49.35.

Bis-ethobromide (As-8303). Yield 90 %, m. p. 225—226° C (diss. ethanol, reprecipt. ether). $C_{17}H_{40}N_2OBr_2$ (448.4): Calc. Br 35.64; found Br 35.19.

Bis-3-diethylaminopropyl ether (IV)

From 39.4 g of 3-diethylaminopropanol (0.3 mole), 6.9 g of sodium, and 55.8 g of 3-diethylaminopropyl chloride, HCl (0.3 mole). Solvent benzene, heating period 6 hours. Yield 29 g (40 %), b. p. 144—149° C at 12—14 mm Hg. n_D^{25} 1.4421. $C_{14}H_{32}N_2O$ (244.4): Calc. C 68.82, H 13.20, N 11.47; found C 68.73, H 13.11, N 11.28.

Bis-methoiodide (As-8211). Yield 57 %, m. p. 215—216° C (from anhydrous ethanol). $C_{16}H_{38}N_2OI_2$ (528.3): Calc. I 48.05; found I 47.86.

Bis-ethobromide (As-8212). Yield 43 %, m. p. 258—260° C (from anhydrous ethanol). $C_{18}H_{42}N_2OBr_2$ (462.4): Calc. Br 34.56; found Br 34.22.

2-Dimethylaminoethyl-2′-dimethylamino-*iso*-propyl ether (V)

From 44.5 g of 2-dimethylaminoethanol (0.5 mole), 12.5 g of sodium, and 93 g of 2-dimethylamino-*iso*-propyl chloride, HCl (0.5 mole). Solvent toluene, heating period 10

* All melting and boiling points are uncorrected. The nitrogen and halogen values are macro determinations by Mrs. G. Speggers of this laboratory. Carbon and hydrogen values are micro determinations by Messrs. A. Grossmann and W. Egger, University of Copenhagen.

hours. Yield 40 g (46 %), b. p. 85–88° C at 12–14 mm Hg. n_D^{25} 1.4320. $C_9H_{22}N_2O$ (174.3): Calc. C 62.03, H 12.72, N 16.08; found C 62.65, H 11.95, N 15.54.

Bis-methoiodide (As-8670). Yield 72 %, m. p. 280–282° C (from 80 % ethanol). $C_{11}H_{28}N_2OI_2$ (458.2): Calc. I 55.40; found I 55.09.

Bis-ethobromide (As-8671). Yield 92 %, m. p. 248–250° C (washed with acetone). $C_{14}H_{32}N_2OBr_2$ (392.3): Calc. Br 40.74; found Br 40.63.

2-Diethylaminoethyl-2′-dimethylamino-*iso*-propyl ether (VI)

From 58.5 g of diethylaminoethanol (0.5 mole), 12.5 g of sodium, and 93 g of 2-dimethylamino-*iso*-propyl chloride, HCl (0.5 mole + 20 % excess). Solvent toluene, heating time 8 hours. Yield 41 g (41 %), b. p. 103–106° C at 10–12 mm Hg. n_D^{25} 1.4370. $C_{11}H_{26}N_2O$ (202.3): Calc. C 65.33, H 12.96, N 13.85; found C 65.15, H 12.43, N 13.36.

Bis-methoiodide (As-8772). Yield 97 %, m. p. 244–246° C (from 80 % ethanol). $C_{13}H_{32}N_2OI_2$ (486.2): Calc. I 52.21; found I 52.15.

Bis-ethobromide (As-8773). Yield 83 %, m. p. 240–242° C (from 90 % ethanol). $C_{15}H_{36}N_2OBr_2$ (420.3): Calc. Br 38.02; found Br 38.12.

Bis-(2-dimethylamino-2′-piperidino)-ethyl ether (VII)

From 12.9 g of 2-piperidinoethanol (0.1 mole), 2.3 g of sodium, and 14.4 g of 2-dimethylaminoethyl chloride, HCl (0.1 mole). Solvent toluene, heating period 8 hours. Yield 7 g (35 %), b. p. 125–128° C at 12 mm Hg. n_D^{25} 1.4599. $C_{11}H_{24}N_2O$ (200.3): Calc. C 65.98, H 12.08, N 13.99; found C 65.67, H 11.77, N 13.63.

Bis-methoiodide (As-10793). Yield 87 %, m. p. 268–270° C (washed with acetone). $C_{13}H_{30}N_2OI_2$ (484.2): Calc. I 52.42; found I 52.59.

Bis-ethobromide (As-10794). Yield 72 %, m. p. 255–258° C (diss. ethanol, reprecipt. ether). $C_{15}H_{34}N_2OBr_2$ (418.3): Calc. Br 38.21; found Br 38.88.

Bis-(2-diethylamino-2′-piperidino)-ethyl ether (VIII)

From 27 g of 2-piperidinoethanol (0.21 mole), 4.8 g of sodium, and 35.9 g of 2-diethylaminoethyl chloride, HCl (0.21 mole). Solvent toluene, heating period 20 hours. Yield 24 g (50 %), b. p. 145–148° C at 12–14 mm Hg. n_D^{25} 1.4608. $C_{15}H_{32}N_2O$ (228.4): Calc. C 68.38, H 12.36, N 12.27; found C 68.44, H 12.50, N 11.94.

Bis-methoiodide (As-8986). Yield 92 %, m. p. 260–262° C (from 80 % ethanol). $C_{15}H_{34}N_2OI_2$ (512.3): Calc. I 49.55; found I 48.76.

Bis-ethobromide (As-8987). Yield 95 %, m. p. 226–227° C (from 96 % ethanol). $C_{17}H_{38}N_2OBr_2$ (446.4): Calc. Br 35.80; found Br 35.20.

Bis-2-piperidinoethyl ether (IX)

From 14.75 g of piperidinoethanol (0.114 mole), 2.62 g of sodium, and 21.0 g of piperidinoethyl chloride, HCl (0.114 mole). Solvent toluene, heating period 16 hours. Yield 9 g (33 %), b. p. 168–170° C at 12–14 mm Hg. n_D^{25} 1.4801. $C_{14}H_{28}N_2O$ (240.4): Calc. C 69.97, H 11.74, N 11.65; found C 69.12, H 11.92, N 11.31.

Bis-methoiodide (As-10800). Yield 86 %, m. p. 248–251° C (from 80 % ethanol). $C_{16}H_{34}N_2OI_2$ (524.3): Calc. I 48.40; found I 48.59.

Bis-ethobromide (As-10801). Yield 55 %, m. p. 205–207° C (from 95 % ethanol). $C_{18}H_{38}N_2OBr_2$ (458.4): Calc. Br 34.87; found Br 34.21.

Bis-(2-dimethylamino-2′-morpholino)-ethyl ether (X)

From 32.8 g of hydroxyethyl morpholine (0.25 mole), 5.75 g of sodium, and 36 g of dimethylaminoethyl chloride, HCl (0.25 mole). Solvent toluene, heating period 8 hours. Yield 23 g (46 %), b. p. 125–130° C at 10–12 mm Hg. n_D^{25} 1.4596. $C_{10}H_{22}N_2O_2$ (202.3): Calc. C 59.37, H 10.96, N 13.85; found C 59.46, H 10.67, N 13.47.

Bis-methoiodide (As-8026). Yield 56 %, m. p. 275—276° C (from 75 % ethanol). $C_{12}H_{28}N_2O_2I_2$ (486.2): Calc. I 52.21; found I 52.10.

Bis-ethobromide (As-8027). Yield 95 %, m. p. 256° C (from anhydrous ethanol). $C_{14}H_{32}N_2O_2Br_2$ (420.3): Calc. Br 38.02; found Br 38.30.

Bis-(2-diethylamino-2'-morpholino)-ethyl ether (XI)

From 65.6 g of hydroxyethyl morpholine (0.5 mole), 11.5 g of sodium, and 86.1 g of diethylaminoethyl chloride, HCl (0.5 mole). Solvent toluene, heating period 10 hours. Yield 72.5 g (63 %), b. p. 146—148° C at 12 mm Hg. n_D^{25} 1.4600. $C_{12}H_{26}N_2O_2$ (230.3): Calc. C 62.59, H 11.38, N 12.16; found C 62.52, H 11.14, N 11.78.

Bis-methoiodide (As-8101). Yield 96 %, m. p. 260—262° C (from 80 % ethanol). $C_{14}H_{32}N_2O_2I_2$ (514.1): Calc. I 49.36; found I 48.70.

Bis-ethobromide (As-8102). Yield 45 %, m. p. 216° C (from anhydrous ethanol). $C_{16}H_{36}N_2O_2Br_2$ (448.3): Calc. Br 35.64; found Br 34.98.

Bis-2-morpholinoethyl ether (XII)

From 19.3 g of hydroxyethyl morpholine (0.15 mole), 3.4 g of sodium, and 27.5 g of morpholinoethyl chloride, HCl (0.15 mole). Solvent toluene, heating period 8 hours. Yield 13 g (36 %), b. p. 188—192° C at 15 mm Hg. (Ref.[2] 178.0—180.5 at 7 mm), n_D^{25} 1.4772. $C_{12}H_{24}O_2N_2$ (244.3): Calc. C 59.00, H 9.90, N 11.47; found C 59.02, H 9.91, N 10.99.

Bis-methoiodide (As-7909). Yield 61 %, m. p. 252—253° C (from 75 % ethanol). $C_{14}H_{30}N_2I_2$ (528.2): Calc. I 48.05; found I 47.70.

Bis-ethobromide (As-7910). Yield 37 %, m. p. 223—225° C (diss. ethanol, reprecipt. ether). $C_{16}H_{34}O_2N_2Br_2$ (462.3): Calc. Br 34.57; found 34.33.

SUMMARY

Eleven bis-alkylaminoalkyl ethers, having ethyl-, methyl-, morpholino-, and piperidino-groups as N-substituents, and containing ethyl, *n*-propyl, and *iso*-propyl in the alkylene chain, together with the twenty-two corresponding methoiodides and ethobromides are described.

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Short Communications

The Complexity of Uranyl Fluoride

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The complex formation curve of the acetate [1] and glycolate [2] systems of the uranyl ion bends very clearly towards $\bar{n} = 3$. The third complex of these systems thus seems to be the upper limit of the complex formation, and this may be due to several reasons.

The third complex may be a coordinatively saturated one, with every ligand occupying either one of three or two of six places of coordination available around the uranyl ion. Or the complex may in fact be unsaturated, the admission of further ligands to the remaining places of coordination being impossible for space reasons. If the places of coordination around the uranyl ion are really more than three, as postulated in the last cases, then this must be evident in relation to a small ligand of strong complex forming tendency, especially if the ligand has no possibility to form chelates. The fluoride ion seems to be fit for this test. It is the smallest anion existing and gives certainly strong complexes with the uranyl ion, as seen from some rather rough investigations [3-5] previously performed.

A determination of the fluoride system will also furnish further information about the mutual strength of the halogenide systems of different cations. In this respect, the cations hitherto investigated may be divided into two distinct groups. The first of these is characterized by strong fluoride complexes whilst the complexes of the other halogenides are much weaker, with their strength decreasing in the order $Cl^- — Br^- — J^-$. Cations belonging to this group are [6-8] H^+, Fe^{3+}, In^{3+}, Zr^{4+}, very probably also [9-11,6] U^{4+}, Pu^{4+}, and Zn^{2+}. In certain cases, *e. g.* H^+ and Fe^{3+}, the heavier halogenides are even almost non-complex in spite of a strong fluoride complexity. The second group has very weak fluoride complexes while those of the other halogenides are strong, their strength increasing in the order $Cl^- — Br^- — J^-$. In this group Ag^+, Cd^{2+} and probably also Hg^{2+} are found [12,6]. A common feature of the groups is however that there is always a difference in order of magnitude between the complexity of the fluorides on one hand and that of the heavier halogenides on the other.

The determination of the free ligand concentration [A] is a convenient method for the investigation of the strongly complex fluoride system. This may be done by measuring $[H^+]$ of fluoride buffers of known composition, if the constants of the hydronium fluoride system are known for the present medium ($I = 1$ C by means of $NaClO_4$; temperature 20° C). At first these constants are therefore determined, which is performed by measuring $[H^+]'$ of fluoride buffers free from uranyl salt. All measurements of $[H^+]$ and $[H^+]'$ are performed by means of quinhydrone electrodes. The buffers are mixed by titrating known amounts of solutions of sodium fluoride, perchloric acid, sodium perchlorate and, if wanted, uranyl perchlorate direct down in the electrode vessel. The vessel is made from methacrylate plastic (plexiglas, lucite), as is also the tips of the burettes, the stems of the electrodes and all other things which may come into contact with the acid fluoride solution. This plastic is completely resistent to hydrofluoric acid, it is also transparent and easily worked. Even the "neutral" solution of sodium fluoride cannot be kept in a glass burette more than some hours before dissolving perceptible amounts of alkali out of the glass. The stock solution is therefore stored in polythene bottles.

The determination of the hydrogen fluoride system from measurements of the

concentration of the free central ion $[H^+]$ is performed according to Fronaeus [13]. Four series of the total acid concentrations C_H = 5, 10, 15 and 25 mC are carried out, with the total ligand concentration C_A increasing up to ≈335 mC in every series. It is shown that only the two mononuclear complexes HA and HA_2 exist within this range of concentration their constants

$\delta_1 = [HA]/[H^+][A]$ and $\delta_2 = [HA_2]/[H^+][A]^2$ are

$\delta_1 = 870 \pm 30$ C^{-1}, $\delta_2 = 3\,300 \pm 300$ C^{-2}

With δ_1 and δ_2 known, [A] of solutions containing uranyl may be calculated, and hence $\bar{n}$, the ligand number of UO_2^{++} (=M). It must then be presumed that HA_2 does not participate in the complex formation, but the truth of this presumption is unequivocally proved by the fact that the complex formation functions computed for different C_H, *i. e.* different $[H^+]$, are identical. If HA_2 would be perceptibly involved in the complex formation, the formation function must be dependent of $[H^+]$. Nor is this function dependent of the total uranyl concentration C_M which proves that no polynuclear complexes exist within the range of C_M measured ($\leqq$ 25 mC).

The highest [A] cannot be calculated with the stated values of δ_1 and δ_2 which are determined as means over the whole range of [A] measured. Evidently the values are modified by the change of medium associated with the exchange of perchlorate for fluoride. These high [A] are therefore computed from the difference E_A between the quinhydrone potentials of a buffer free from uranyl and a buffer of the same C_A containing uranyl, analogous to the method employed *e. g.* for the acetate system [1]. Owing to the simultaneous existence of HA and HA_2, the calculation of [A] must however in the present case be performed by a series of successive approximations.

The formation function found bends clearly towards $\bar{n} = 4$. Then the upper limit of the complex series is $UO_2F_4^{--}$, at least if [A] $\leqq$ 300 mC. The first four mononuclear complexes may thus exist. Their constants are calculated via the function $\bar{n}$ / [A], which is integrated with the lower limit $[A]_0 = 0.04$ mC, *cf.*[2] The functions X thus obtained give:

$\beta_1 = (3.9 \pm 0.3) \cdot 10^4$C^{-1} $b_1 = 39000$ C^{-1}
$\beta_2 = (8.6 \pm 0.8) \cdot 10^7$C^{-2} $b_2 = 2200$ C^{-1} $b_1/b_2 = 18$
$\beta_3 = (3.1 \pm 0.4) \cdot 10^{10}$C^{-3} $b_3 = 360$ C^{-1} $b_2/b_3 = 6$
$\beta_4 = (7.0 \pm 1.5) \cdot 10^{11}$C^{-4} $b_4 = 23$ C^{-1} $b_3/b_4 = 16$

At the moderate concentrations here used, the uranyl fluoride system is composed of solely the first four mononuclear complexes, the fourth one constituting a limiting complex. Especially the second and fourth complexes show a rather weak tendency to be formed from their predecessors, as seen from the remarcably high values of b_n/b_{n+1}. This seems to be a common feature of strong fluoride systems [14], Table 1, whereas *e. g.* the uranyl acetate system displays quite the opposite behaviour.

Evidently the uranyl ion is able to coordinate more than three ligands, *e. g.* in the case of fluoride four. The real number of places of coordination seems however rather hard to decide from the formulas of those limiting complexes which have hitherto been proved.

This work has been generously supported by *Atomkommittén (The Swedish Atomic Committee)*.

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Esterase Activity Shown by Subtilisin, a Proteolytic Enzyme from *Bacillus subtilis*

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A proteolytic enzyme preparation from *Bacillus subtilis* catalyses the transformation of ovalbumin to plakalbumin. The preparation of crystals containing this enzyme is described by Güntelberg and Ottesen [1]. Water extracts of the crude enzyme as well as the crystalline preparation, shows hydrolysing effect toward methyl esters of the lower fatty acids, as demonstrated for methyl butyrate by Güntelberg and Ottesen [2], and as will be shown, there is reason to believe that the active component is identical with the protease.

The proteolytic effect is described by Linderstrøm-Lang and Ottesen [3,4], Ottesen and Villee [5] and Ottesen and Wollenberger [6].

The investigation here deals with the esterase activity. The determinations are made by automatic titrations in a "pH-stat". Experiments were carried out in 100 ml beakers containing 50 ml of phosphate buffer (0.01 *M* sec. phosphate, 0.0005 *M* prim. phosphate and 0.1 *M* KCl) adjusted to the desired pH value with *N* NaOH. 0.0922 ml ester is used and the experiments are made at 20° C. As a measure of the activity we use the amount of ester hydrolysed in ten minutes, expressed as scale readings at the burette, containing 0.825 *N* NaOH. (100 scale readings equal 0.0653 ml). Standardizing of the proteolytic activity is made in the same way, only substituting the ester with 250 mg of casein ("Casein nach Hammarsten"), and in stead of ten minutes hydrolysis the slope of the curve at the origin is used.

To investigate whether the esterase and the protease were identical or not, the following experiments were carried out.

6 g of the crude enzyme was extracted with 60 ml 0.15 *M* $CaCl_2$ and centrifuged. The supernatant fluid (I) was made 50 % with respect to acetone and again centrifuged. The precipitate was discarded and to the new supernatant, acetone was added until the content had increased to 75 %. The precipitate was separated by centrifugation and dissolved in 17 ml of distilled water (II). An equal amount of 24 % Na_2SO_4 was added. The resulting precipitate was dissolved in a small amount of water and then dialyzed against distilled water (III). Similarly the supernatant was dialyzed (IV) in order to remove the bulk of the sulphate.

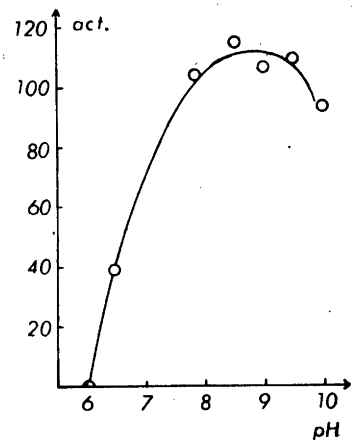

Fig. 1. Activity as function of pH.

The fractions I—IV were then standardized using casein and methyl propionate respectively as substrate. In the determination of the esterase activity of a certain fraction the amount of enzyme was always 25 times the amount used in the determination of the protease activity of the same fraction. The values of the activities of the different fractions are evidently not mutually comparable as the specific activities of the fractions are different and unknown.

		I	II	III	IV
Casein activity	(A)	138	162	20	80
ester "	(B)	113	137	15	60
F = A/B		1.22	1.18	1.33	1.33

This is taken as an evidence for the identity of the esterase and the protease, because it is estimated that the variations in F do not significantly exceed the random errors.

The esterase activity plotted against pH, using methyl propionate as sub trate and a water extract of the crude enzyme as source of enzyme was investigated (Fig. 1). The optimal pH value is approximately 8.5, and the enzyme hereby shows

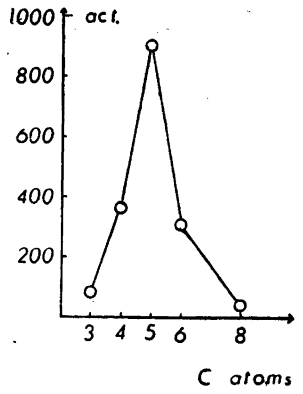

Fig. 2. Relationship between activity and chainlength (number of C-atoms) in the acid radical.

resemblance to esterases isolated from other materials as investigated by Purr [7] and others; they all find an optimum at pH 8.0–8.5.

The activity toward a few different methyl esters of the lower fatty acids was also investigated (Fig. 2).

The distict maximum found at methyl valerate is also shown by a liver esterase described by Schulte [8].

Investigations dealing with the kinetics of the ester hydrolysis are in progress in this laboratory.

The author's thanks are due to professor dr. Linderstrøm-Lang at "The Carlsberg Laboratory", who kindly has placed the crude enzyme at our disposal and to dr. E. Güntelberg for his advice and interest in the investigation.

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A Preliminary Note on the Electrophoretic Purification of Alkaline Phosphatase

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The application of electrophoresis in separation of enzymes has as yet been limited, due in particular to the laboriousness and the low yield of preparative experiments in the Tiselius apparatus. The development of a method (Carlson 1954 [1]) for electrophoresis in starch medium, where extraction of the proteins out of the medium is avoided, seemed to offer new possibilities for the use of electrophoresis in isolation of enzymes.

The electrophoresis is carried out in a vertical starch column and after the electrophoresis the separated components are forced out of the column by buffer flow and collected in fractions of suitable volume. The fractions are then available for analysis of *e. g.* protein concentration and enzyme activity.

The phosphatase preparation * used in this study was the 40 %–60 % acetone concentration fraction of a beef-kidney homogenate [2].

150 mg of this preparation was extracted with ammonium buffer of pH 9.0 and ionic strength 0.1 and the insoluble material removed by centrifugation. This extract was then electrophoretically separated in the same buffer during a period of 18 hours.

The protein concentration of the fractions was determined according to the modified biuret reaction of Goa [3]. The phosphatase activity determinations were carried out at pH 9.0 in 0.02 *M* Na-β-glycerophosphate with addition of $MgSO_4$ and phenylalanine to a final concentration of 0.01 *M* each.

The result is given in Fig. 1. As is evident, the phosphatase activity is localized to one of the slowest moving protein components holding 32 % of the protein in the preparation. Theoretically this would give a threefold enrichment of the enzyme. The

* The author is indebted to Mr. A. Bolinder, Division of Food Chemistry, Royal Institute of Technology, Stockholm, for making this preparation.

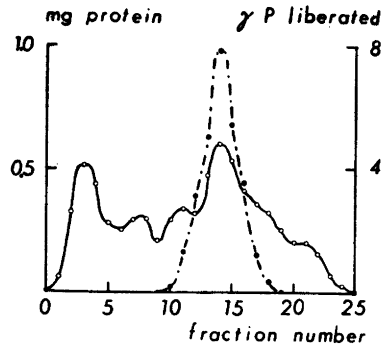

Fig. 1. Electrophoresis of the enzyme preparation. O—O *protein.* ●— —● *phosphatase activity, expressed as γ P liberated.*

yield was, however, 62 % and the enrichment in fraction number 14 was 1.85.

The possible inactivation of the enzyme during the electrophoresis due to the loss of coenzyme or to instability at the alkaline pH used, as well as the purity of the isolated enzyme, is subject to further investigations.

It has come to the author's knowledge that a similar device for electrophoresis has been developed by Drs. P. Flodin and J. Porath at the Institute of Biochemistry, Uppsala, Sweden. (*Biochim. et Biophys. Acta* **13** (1954) 175).

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Homoserine and α-Aminoadipic Acid in Green Plants

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L(+)-*Homoserine* was found in this laboratory for the first time in pea plants *(Pisum sativum)* one year ago [1]. Homoserine seemed to be lacking in the seeds but has now by using more concentrated extracts been detected in very small amounts also in non-germinating seeds. During germination [2] it is formed surprisingly quickly, attaining in 3 days about the 200 fold amount. It is later on found in all parts of the plants. According to estimations made in this laboratory homoserine is not found only in *Pisum* but seems to be a relatively common free amino acid in smaller concentrations in the plant kingdom. So far we have found it in the following plants: in the young plants of *Trifolium pratense*, in the leaves of *Acacia pentadena, Asplenium nidus, Begonia* sp., *Eucalyptus globulus, Hibiscus* and *Coleus Rehneltianus, Rhododendron* sp., *Taxus baccata* and *Peperomia rhombae*, in the leaves and the root of the apple-tree (K. Oland in this laboratory), and in the seeds of *Zea mais*. The "unknown A" which Hulme and Arthington [3] found in apples is probably also homoserine.

We have also found *α-aminoadipic acid* to be a surprisingly common amino acid in plants. By using a twodimensional run (butanolacetic acid in one direction and phenol in NH_3 atmosphere in the other direction) it is possible to separate α-aminoadipic acid from glutamic acid paper chromatographically [4]. The spot "Y" [2], found on chromatograms made from a 70 % alcohol extract of peas which had germinated 3—5 days, has appeared to be α-aminoadipic acid. This acid has moreover been found in the leaves of *Petasites officinalis, Coleus Rehneltianus, Fraxinus excelsior, Acer platanoides* (also in the yellow ones in autumn), and *Nymphaea alba*, in the seeds and seedlings of *Faba faba, Lupinus angustifolius*, and *Phaseolus vulgaris*, and in the seedlings and roots of *Trifolium pratense*. In the seed of maize Windsor [5] has found this amino acid to be a constituent of a protein.

We are deeply indepted to Professor A. Kalela, Director of the Botanical Institute, University of Helsinki, for the numerous samples of plats.

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Partition Chromatography of the Oxidised A and B Chains of Insulin

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Partition columns were introduced by Martin and Porter [1,2] for chromatographing proteins like ribonuclease and insulin. In order to obtain a sufficiently high partition coefficient in favour of the organic (stationary) phase these authors used rather high concentrations of salts in their solvent systems. When working with comparatively low molecular-weight, dialysable polypeptides, the removal of large amounts of salt from the peak fractions might conceivably cause difficulties or would at least be inconvenient. Salt-free solvent systems have been used in countercurrent distribution of naturally occurring polypeptides, particularly by Craig and his group. It must be borne in mind, however, that whereas in countercurrent distribution partition coefficients not too far from unity are preferable, values of at least 5 are required for high resolving power in partition chromatography.

In a recent paper on countercurrent distribution of insulin [3] Craig and coworkers introduced solvent systems containing di- or trichloroacetic acid (TCA) in moderate concentration. The increase in partition ratio caused by these complexing agents was very remarkable. Accordingly we considered it worth while to investigate whether solvent systems of this kind could also be used in partition chromatography of the oxidised A and B chains of insulin, which Sanger [4] separated by conventional precipitation methods.

The solvent system 2-butanol-0.01 *N* TCA (1 : 2.5) proved useful for this purpose. The columns for preparative runs (25 × 4.5 cm) were made from silane-treated Hyflo Super-Cel (150 g), which carried 75 ml of the butanol phase. Prior to chromatography the oxidised insulin was separated into crude A and B fractions by a single fractionation in ammonium acetate buffer [4]. Each fraction (120 mg) was extracted with 60 ml of aqueous phase and the extracts were chromatographed, the chromatograms being developed with aqueous phase. The elution was followed by determining the optical density at 277 mμ of the fractions collected (Fig. 1).

The A fraction emerged as a single component whereas the B fraction gave two peaks, of which the later one was the B peptide. The peak fractions were combined in each case, freed of TCA by neutralising with Dowex-2 in the bicarbonate form and lyophilised. The chromatographed A and B peptides appeared as white, amorphous solids (about 45 mg of each). In paper electrophoresis experiments (barbital buffer at pH 8.6) each peptide travelled as a single spot, thus indicating that a complete separation had been achieved.

Further details will be published in Comptes Rendus des Travaux du Laboratoire Carlsberg, Sér. chim.

The author wishes to thank Professor K. Linderstrøm-Lang for his kind interest in this work.

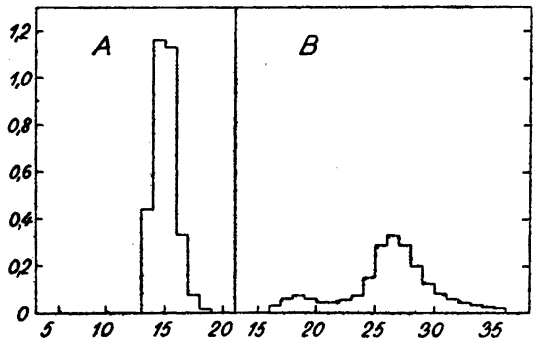

Fig. 1. Elution chromatograms of A peptide (A) and B peptide (B). Abscissa: Number of fractions (20 ml). Ordinate: Optical density of fractions at 277 mμ.

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2. Porter, R. R. *Biochem. J. (London)* **53** 320 (1953).
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4. Sanger, F. *Biochem. J. (London)* **44** 126 (1949).

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Dehydrogenation of Dihydro-Dehydro-Di-*Iso*eugenol and its Methyl Ether

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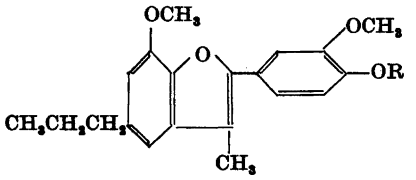
For various purposes, especially in relation to work on the U.V.-absorption spectra of lignin and lignin derivatives compound I and its methyl ether II were required.



I R = H

II R = CH_3

These substances are easily obtained by sulphur dehydrogenation of the corresponding coumarane derivatives, dihydro-dehydro-di-*iso*eugenol and its methyl ether. For spectrochemical studies compare G. Aulin-Erdtman [1].

Experimental. 3-Methyl-5-propyl-7-methoxy-2-(4-hydroxy-5-methoxy-phenyl) coumarane (I): Dihydro-dehydro-di-*iso*eugenol (12.5 g) and sublimed sulphur (1.3 g) were mixed in a 25 ml Claisen distillation flask and heated in a salt bath. The temperature was slowly raised and at 215° evolution of hydrogen sulphide began. It ceased completely after 30 minutes, by which time the temperature had reached 250°. The reaction vessel was arranged for vacuum distillation and at 220—240° (1 mm) a yellow substance (9.0 g) was collected. The distillate was dissolved in benzene, filtered through aluminium oxide and evaporated to give an almost colourless substance (5. 5 g) melting at 85—87°. Recrystallization from ligroin and from 50 % acetic acid raised the m.p. to 86.5—87.5° (needles). The substance exhibits a blueish violet fluorescence in U.V. light. (Found: C 73.6; H 6.78. Calc. for $C_{20}H_{22}O_4$ (326.4): C 73.6; H 6.80.)

Acetate: The phenol (I) was acetylated with acetic anhydride in pyridine. The reaction mixture was poured into water and the product crystallized from ethanol in needles, m.p. 124—125°. (Found: OCH_3 16.9. Calc. for $C_{20}H_{18}O_3$ $(OCH_3)_3$ (368.4): OCH_3 16.9.)

3-Methyl-5-propyl-7-methoxy-2-(4.5-dimethoxyphenyl) coumarane (II):

A. The phenol (I) was methylated with dimethyl sulphate and alkali in the usual way giving the methyl ether which crystallized from methanol in cubes, m.p. 91.5—92.0°. The substance exhibits a violet fluorescence in U.V. light. (Found: OCH_3 27.5; Calc. for $C_{18}H_{15}O$ $(OCH_3)_3$ (340.4): OCH_3 27.4.)

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1. Aulin-Erdtman, G. *Svensk Papperstidn.* **56** (1953) 91, 287.
2. Aulin-Erdtman, G. *Svensk Kem. tidskr.* **54** (1942) 169.
3. Erdtman, H. *Ann.* **503** (1933) 2830.

Received January 20, 1954.

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ÅKE SPETZ

Organisk-kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

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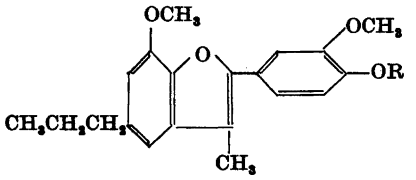

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Uracil, a Precursor of Polynucleotide Pyrimidines in the Mouse

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While it has been shown that uracil can satisfy the growth requirements of pyrimidine deficient bacteria, the experiments of Plentl and Schoenheimer [1] have demonstrated that N^{15}-uracil was not utilized by the rat for pyrimidine synthesis. Isotopic uridine [2] and uridylic acid [3], however, were incorporated into polynucleotide pyrimidines by this animal. The rat seems to be the only mammal, where a more systematic investigation of pyrimidine utilization has been carried out, and the only *free* pyrimidine — with the possible exception of thymine [3] — that could be used was orotic acid [4].

We have for some time been interested in the pyrimidine biosynthesis in the Ehrlich ascites tumor in the mouse. During this work we found that the tumor cells *in vitro* could utilize both N^{15}-orotic acid and N^{15}-uracil for the synthesis of polynucleotide pyrimidines. Further experiments showed that N^{15}-uracil was also incorporated *in vivo* into normal tissues. The results of our experiments are summarized in Table 1.

It is clear that the utilization of uracil is of the same order of magnitude in all the tissues studied as that of orotic acid. As in no case occured any incorporation of isotope into the purines, we consider it highly likely that the incorporation into pyrimidines did not depend on a more extensive degradation of the precursors. The species difference found is somewhat reminiscent of the demonstrated difference in the utilization of guanine by the rat and the mouse [5].

A detailed account of the experimental procedures will be given together with a forthcoming publication of our work with the Ehrlich ascites tumor cells.

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Received February 1, 1954.

Table 1. Incorporation of N^{15}-orotic acid (16 % excess N^{15}) and N^{15}-uracil (9 % excess N^{15}) into polynucleotide pyrimidines.

Organ	Atom % excess N^{15} calculated on basis of 100 % N^{15} in the precursor			
	Pentose nucleic acid		Desoxypentose nucleic acid	
	Uridine	Cytidylic acid	Thymine	Cytosine
Orotic acid:				
Ascites cells *in vitro*	2.18	0.36	0.16	0.07
» » *in vivo*	1.48	0.28	0.16	0.11
Mixed internal organs	0.89	0.28		
Uracil:				
Ascites cells *in vitro*	3.20	0.59	0.88	0.24
Liver *in vivo*	0.47	0.21		
Intestine *in vivo*	1.08	0.47		

In vitro: 0.1 mmoles of pyrimidine was incubated with 20 ml of ascites fluid during 3 hours. *In vivo:* 0.02 mmoles of pyrimidine / mouse was injected into ...en mice and the animals were killed after 2 hours.

On the Mucopolysaccharides of *Nucleus Pulposus*

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In 1948 Karl Meyer [1] assumed that *nucleus pulposus* must contain hyaluronic acid. Evidence for the presence of a polysaccharide was presented by Malmgren and Sylvén [2] in 1952. They isolated chondroitin sulfuric acid using the method of Einbinder and Schubert [3]. Since they obtained a fairly good yield of the polysaccharide with an almost theoretical composition they considered the bulk of the polysaccharide material in *nucleus pulposus* to consist of chondroitin sulfuric acid.

In a study of the distribution of the aminohexoses in different parts of the animal body we found that glucosamine and galactosamine occurred together and in almost equal amounts in the human *nucleus pulposus*.

Human intervertebral discs were collected from autopsy material, the lumbar and the lower thoracal discs being taken. After cleaning from surrounding tissue the *nuclei pulposi* were frozen at −20° C. When sufficient material had been collected the mass was minced in a meat chopper in the frozen state. It was then freeze-dried and further pulverized in a Turmix blendor.

Samples of this dry powder were hydrolysed with 6 *N* hydrochloric acid for 8 hours on a boiling water bath. The aminosugars were then determined colorimetrically applying the chromatographic technique of Gardell [4]. The results of this analysis together with the analysis of ester sulfate, nitrogen, ash, and moisture is given in Table 1.

Table 1.

Moisture	12.0 %
Ash	7.8 »
Nitrogen (Micro-Kjeldahl)	10.8 »
Sulfur in ester sulfates	1.2 »
Glucosamine-HCl	5.4 »
Galactosamine-HCl	5.1 »

This analysis clearly indicates the presence of a glucosamine containing polysaccharide together with chondrotinsulfuric acid. Preliminary experiments have also been made for the preparation of the polysaccharides.

Twentyfive g of the freeze-dried powder was coagulated by pouring it in small portions into 500 ml of boiling water. The material was then digested with pancreatic and intestinal glycerol extracts as described by Gardell [5]. After ten days of digestion the solution was concentrated by evaporation *in vacuo* to a syrup and precipitated with several volumes of alcohol at pH 8. The precipitate was dissolved in about 50 ml of water and the pH adjusted to 2.5 with hydrochloric acid. From this solution the crude polysaccharide was precipitated with ten volumes of alcohol. It contained 7.1 % nitrogen, 11.0 % glucosamine-HCl and 11.4 % galactosamine-HCl. The yield was 8.35 g.

This material was dissolved in about 40 ml of water, digested and precipitated as above. An almost white preparation weighing 6.18 g with a nitrogen content of 5.5 % was obtained. The digestion and precipitation procedure was repeated once more. The last precipitate was dissolved in water, neutralized, and freeze-dried. The freeze-dried preparation was extracted with 50 ml of 90 % phenol over night and centrifuged at 25 000 g. The residue was dissolved in faintly alkaline water the solution acidified to pH 2.5 and alcohol added. The polysaccharide precipitated weighed 2.69 g and contained 14 % glucosamine-HCl and 17.5 % galactosamine-HCl; the nitrogen content was 4.9 %.

Of this preparation 1.93 g was dissolved in water, freeze-dried and submitted to repeated phenol extraction. In this way 1.28 g of a white polysaccharide fraction relatively low in nitrogen and high in aminosugar and sulfur content was obtained. The analysis of this fraction is given in Table 2.

Table 2.

Moisture	12.8 %
Ash	19.9 »
Nitrogen (Micro-Kjeldahl)	2.84 »
Sulfur in ester sulfates	4.32 »
Uronic acid (Tracey [6])	13.7 »
Glucosamine-HCl	9.2 »
Galactosamine-HCl	15.6 »
Galactose	7.0 »

Galactose was determined according to Gardell [7]. The polysaccharide was hydrolysed with 1 *N* sulfuric acid 14 hours on a boiling water bath. After hydrolysis the

mixture was passed through a "Zeo-carb 225" 4.5 % crosslinked cation-exchanger in the hydrogen form, neutralized with barium hydroxide, evaporated to dryness in a desiccator and finally taken up in the solvent used in the chromatography.

Uronic acid and galactosamine are present in equimolar proportions thus there is strong evidence for the presence of chondroitin sulfuric acid. However, the sulfur content is much to high. Another interesting feature is the presence of glucosamine and galactose in equimolar proportions. This, together with the high sulfur content, strongly suggests the presence of a compound similar to the keratosulfuric acid recently described by Meyer *et al.*[8] to occur in the *cornea*. The sulfur content is sufficient to account for both these polysaccharides.

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On the Mechanism of the Heparin-induced Lipemia Clearing Reaction

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The clearing of alimentary lipemia by injection of heparin, first discovered by Hahn [1], is due to the production *in vivo* of an active substance (Anderson & Fawcett [2]) called the clearing factor. For the formation of this substance a factor from tissues and another from plasma are needed in addition to heparin (Anfinsen *et al.*[3]). By the action of clearing factor on (lipemic) serum the concentration of lipoprotein particles of the lowest density decreases, while a concomitant increase

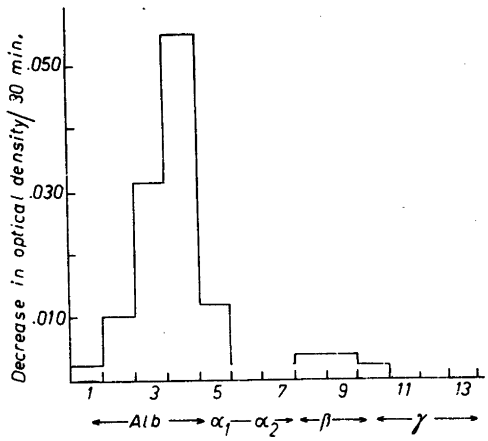

Fig. 1. Ability of electrophoretically purified serum protein fractions to clear a purified clearing factor — olive oil emulsion mixture (= electrophoretic pattern of the lipid acceptor-protein). Clearing/30 minutes/mg protein : albumin 0.010, β globulin 0.032.

occurs in the more dense low-density lipoproteins and alpha lipoproteins (Graham *et al.*[4], Anfinsen *et al.*[3], Boyle *et al.*[5], Nikkilä [6]). This means that new protein molecules enter into combination with lipids. The lipid acceptor-protein, if identical with the "coprotein" of Anfinsen *et al.*[3], is claimed to be localized in Fraction III−0 of the conventional ethanol fractionation scheme (Anfinsen *et al.*[3]). Recently, serum albumin has been reported to increase the clearing rate, probably by binding the fatty acids, which are known to be released under the action of clearing factor (Shore *et al.*[7]) and which inhibit the clearing reaction (Gordon *et al.*[8]). This paper reports briefly on some experiments carried out to throw more light on the mechanism of the clearing reaction.

As is to be expected in view of the necessity of an acceptor-protein, a tween-stabilized olive oil emulsion is not cleared upon the addition of purified (Nikkilä [9]) clearing factor. When normal serum is added to this system the clearing rate increases directly proportionally to the serum concentration up to a certain limit (20−30 % of the total volume of the mixture) but declines on the further increase of the amount of added serum, thus suggesting the presence of some slightly inhibiting substance(s) in serum. To elucidate

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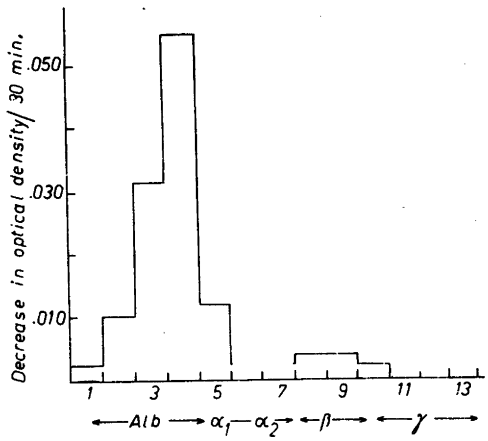

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the nature of the acceptor-protein supplied by serum, the protein fractions prepared from a normal human serum by the continuous electrophoretic technique of Grassmann and Hannig [10] were tested for their ability to initiate clearing of a purified clearing factor-olive oil emulsion mixture. Clearing took place only in samples containing albumin or beta globulin. Calculated on the basis of protein content the activities of the two fractions were in a ratio of about 3 to 1, respectively. Commercial preparations of bovine (Armour) and human albumin (Orion) possessed some acceptor-protein activity but this was clearly inferior to that of electrophoretically prepared albumin. Whole normal serum added to a system containing fat emulsion, purified clearing factor and electrophoretic albumin enhanced the clearing exactly to the extent implied by its albumin content. We believe these results make it evident that the main acceptor-protein required in the lipoprotein transformation caused by the clearing factor is serum albumin or a protein intimately related to it. No evidence is found to support the view of Gordon *et al.*[8] on the need of a second cofactor, their result being explicable, *i. a.*, by the poorer activity of chemically than of electrophoretically prepared albumin. The possibility remains, however, that the second cofactor is contained in our clearing factor preparation.

The mode of action of albumin in the clearing reaction may quite well be, as suggested by Gordon *et al.*[8], the binding of the fatty acids produced by the clearing factor. This binding, however, is probably something more essential than the mere elimination of an inhibiting factor. This is shown by the fact that *an emulsion of oleic acid* (tween-stabilized) *is instantly cleared on addition of electrophoretic albumin* but not of the other fractions. These findings provide one explanation for the clearing reaction, the primary role of the clearing factor then being to act as a lipase or a lipase activator.

Further valuable information on the clearing mechanism was accumulated from our attempts to prepare the "tissue factor" from rat pylorus, as suggested by Brown & Kauffman [11]. It appeared namely, that cell-free *saline extracts of rat duodenum* (not of stomach) *possessed high clearing activity without any addition of heparin.* Also this clearing reaction required the presence of normal serum, which, however, could be replaced by *sodium glycocholate.* The initial clearing rate as well as the net decrease of turbidity were directly proportional to concentration of the bile salt. An emulsion of oleic acid is cleared at once by glycocholate. In the clearing reaction induced by the clearing factor from postheparin serum, glycocholate not only was incapable of substituting serum (albumin) as the fatty acid acceptor but it also inhibited the reaction in the presence of serum. On the basis of these observations it seems to us highly likely that the active substance of rat duodenum is not a "tissue factor", and not even identical with the clearing factor of serum, but is a lipase. The different behaviour of the clearing activities of postheparin serum and duodenal extract against bile salt shows that the clearing factor of serum is probably not a lipase in nature but can be a lipase activator or that the heparin-induced clearing reaction does not involve fat splitting as a primary cause but only as a secondary outcome of the change in the physicochemical state of fats. Further studies are in progress and a more detailed description of our experiments will be published shortly.

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Die Reaktion zwischen Alkylenoxyden und Aminen. III *. Über eine Beziehung zwischen Reaktionsgeschwindigkeit und Dissoziationskonstante bei der Addition von Propylenoxyd an tertiäre Amine

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In den vorhergehenden Mitteilungen [1,2] dieser Serie sind hauptsächlich Messergebnisse ohne eingehendere theoretische Diskussion mitgeteilt worden. Auf Grund der Wahl der erst untersuchten Reaktionen musste Smith [1] feststellen: »Ein Zusammenhang zwischen den Dissoziationskonstanten der reagierenden Amine und ihren Additionsgeschwindigkeiten kann auch nicht entdeckt werden». Später konstatierten Eastham, deB. Darwent und Beaubien [3], dass keine Beziehungen zwischen Geschwindigkeit und Basizität noch Struktur vorzuliegen scheinen. Es ist mir jedoch gelungen, eine Beziehung von gleicher Form wie die Gleichung von Brönsted zwischen Reaktionsgeschwindigkeit und Dissoziationskonstante zu finden; später wird über strukturelle Zusammenhänge berichtet werden.

Die Geschwindigkeit von Reaktionen, die von Säuren oder Basen katalysiert werden, ist, einer allgemeinen Annahme folgend, von einem Protonenübertritt bestimmt. Die Geschwindigkeitskonstante und die entsprechende Säure-Basengleichgewichtskonstante sind durch das von Brönsted formulierte Gesetz

$$k = G \cdot \left(\frac{1}{K_B}\right)^{\beta}$$

miteinander verbunden. (K_B ist die Dissoziationskonstante; G und β sind Konstanten). Bei von Aminen basenkatalysierten Reaktionen in Wasser erhält man verschiedene Gleichungen für primäre, sekundäre und tertiäre Amine. Diese Gleichungen werden jedoch identisch, wenn man Dissoziationskonstanten der Amine in z. B. Anisol verwendet oder wenn man eine Korrektion für die Solvation des Amins in Wasser anbringt, was von Bell und Trotman-Dickenson gezeigt [4,5] worden ist.

Betreffs der vorliegenden Reaktion zeigt die Unveränderlichkeit der Konstanten bei der Addition von beispielsweise Pyridin ($K_B = 1{,}8 \cdot 10^{-9}$ bei 20 °C[6]) an Propylenoxyd, wobei eine mit den Alkalihydroxyden vergleichbare Base [7] gebildet wird, sowie auch Versuche in 0,05 *N* und 0,10 *N* Kaliumhydroxydlösung, dass keine Basenkatalyse vorliegt. Eastham, deB. Darwent und Beaubien [3] fanden keine Säurekatalyse bis pH = 4 für Aminaddition an Äthylenoxyd. Dieser Reaktionstypus wird also weder von Basen noch von Säuren katalysiert. Für die in Tabelle 1 angeführten Amine gilt doch

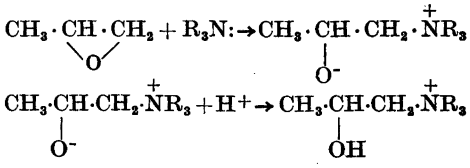
Tabelle 1. Reaktionsgeschwindigkeiten k für die Aminaddition an Propylenoxyd in Wasser bei 20° C und Dissoziationskonstanten K_B bei 25° C.

Amin	k	K_B
Trimethylamin	0,266 [2]	$5{,}45 \cdot 10^{-5}$ [9]
Methyldiäthylamin	0,0189 [8]	$2{,}2 \cdot 10^{-4}$ [10]
Triäthylamin	0,00391 [2]	$5{,}67 \cdot 10^{-4}$ [11]
Dimethyl-*n*-butylamin	0,0705	$1{,}14 \cdot 10^{-4}$

die folgende Gleichung

$$k = 4{,}8 \cdot 10^{-9} \cdot \left(\frac{1}{K_B}\right)^{1{,}8}$$

Diese Gleichung wird nicht von primären und sekundären Aminen erfüllt, was durch das oben angeführte verständlich ist. Gleichungen dieser Art sind früher hauptsächlich für säure- und basenkatalysierte Reaktionen wie für Reaktionen mit Benzolderivaten gefunden worden. Bemerkenswert ist der Exponent 1,8, weil er bei einer säure-basenkatalysierten Reaktion, deren Geschwindigkeit von einem Protonenübertritt bestimmt wird, zwischen 0 und 1 liegen muss. Die Geschwindigkeit wird bei der hier untersuchten Reaktion wahrscheinlich nicht von einem solchen Protonenübertritt bestimmt. Der Reaktionsmechanismus in Wasser dürfte in teilweiser Analogie mit der spontanen Alkoholyse von Propylenoxyd [12] folgenderweise formuliert werden können:



* Betreffs der zwei ersten Mitteilungen siehe Literaturzitate [1,2].

Tabelle 2. Addition von Dimethyl-n-butylamin an Propylenoxyd bei 20,00 °C.

	a = 0,0546				b = 0,0340			
t	124	161	204	255	314	391	504	623
p	34,1	41,1	47,5	54,3	60,5	66,9	74,2	79,4
k	0,0698	0,0703	0,0697	0,0705	0,0705	0,0703	0,0707	0,0698

k_m = 0,0702

Übrige k_D = 0,0695, 0,0719
Mittelwert sämtlicher k_D = 0,0705

Der erste Schritt soll geschwindigkeitsbestimmend sein. Dieser Mechanismus muss bedeuten, dass eine Beziehung zwischen Basizität und Geschwindigkeitskonstante für gleichartige Amine existiert, und, dass, da die reagierenden Stoffe im Endprodukt vereinigt bleiben, strukturelle Verhältnisse einen grossen Einfluss haben müssen, was alles in guter Übereinstimmung mit den experimentellen, teilweise nicht publizierten, Resultaten steht.

Experimenteller Teil. Dimethyl-n-butylamin. Das Amin wurde nach der von Hanhart und Ingold [13] modifizierten Methode von Eschweiler [14] mit geringer Änderung aus *n*-Butylaminhydrochlorid und Paraformaldehyd hergestellt. Es wurde durch Umkristallisieren des Pikrates und durch fraktionierte Destillation gereinigt. Kp_{750} = 93,3 °C, n_D^{20} = 1,3976; n_D^{25} = 1,3953; n_C^{20} = 1,3953; n_C^{25} = 1,3929; n_F^{20} = 1,4034; n_F^{25} = 1,4010; d_4^{20} = 0,7206; d_4^{25} = 0,7164; M_{gef} = 101,8; M_{ber} = 101,1.

Dissoziationskonstante des Dimethyl-n-butylamins. Die Konstanten wurden durch Leitfähigkeitsmessungen bestimmt. Für Dimethyl-*n*-butylaminhydrochlorid ergaben Doppelbestimmungen die folgenden Äquivalentleitfähigkeiten:

Λ_0 = 100 ohm^{-1} · cm^2 bei 20 °C
Λ_0 = 112 ohm^{-1} · cm^2 bei 25 °C

Folgende Dissoziationskonstanten wurden gefunden (Mittelwerte von vier bzw. zwei Bestimmungen)

$K_B = 1{,}05 \cdot 10^{-4}$ bei 20 °C
$K_B = 1{,}14 \cdot 10^{-4}$ bei 25 °C

Reaktionsgeschwindigkeit der Addition von Dimethyl-n-butylamin an Propylenoxyd, Tabelle 2. Betreffend Ausführung und Bezeichnungen siehe die vorhergehende Mitteilung [2].

Kungl. Fysiografiska Sällskapet in Lund möchte ich für Stipendien, die diese Arbeit ermöglicht haben, herzlich danken.

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Stereochemistry of Silicon

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New measurements of microwave frequencies of SiD_3Cl and SiD_3F in conjunction with earlier measurements on SiH_3Cl and SiH_3F enable the authors to calculate the silicon-chlorine distance in ordinary and deuterated silyl chlorides. The results were 2.0479 ± 0.0007 Å and 2.0486 ± 0.0007 Å respectively. The silicon-fluorine distances in ordinary and deuterated silylfluorides were found to be 1.595 ± 0.010 Å in both cases.

Silicon-hydrogen and silicon-deuterium distances and the valence angles were estimated. The changes in these quantities caused by the substitution of one hydrogen atom in silane by chlorine or fluorine are small and about the same as in the pairs CH_4, CH_3Cl and CH_4, CH_3F.

Preparation and properties of SiD_3Cl and SiD_3F are described.

In the classical paper by Kipping [1] it was shown that compounds of the type SiXYZW, where X, Y, Z, and W are mono-valent radicals, can be separated into optical isomers. This excludes a *planar* arrangement of silicon and its four ligands. Later, X-ray determination of the crystal structure of SiI_4 by Hassel and Kringstad [2] revealed that the four iodine atoms were situated in the corners of a regular tetrahedron with silicon in the centre. A pyramidal arrangement with silicon at the top was thereby eliminated. A similar result for SiH_4 was found by Tindal, Straley and Nielsen [3] by the use of infrared technique. Since the bonds of tetrahedral SiX_4 molecules almost by necessity must be directed towards the corners of a *regular* tetrahedron the interesting problem of the direction and magnitude of possible deviations from regularity in cases like SiX_3Y, SiX_2Y_2 etc. remained to be solved. In a number of cases where electron-diffraction technique could be applied [4–6] deviations were found but the results were too inaccurate to give more than a rough idea of the angular and linear irregularities so demonstrated.

By the advent of microwave technique (1945—46) conditions for further progress again became favourable. 1948, Sharbaugh [7] published measurements on $Si^{28}H_3Cl^{35}$ and $Si^{28}H_3Cl^{37}$. 1949, Townes and collaborators [8] found the microwave absorption of $Si^{30}H_3Cl^{35}$. In 1950, Sharbaugh and collaborators [9] published their results for the three isotopic species $Si^{28,29,30}H_3F$. All three

papers agreed in the statement that the silyl halides examined are symmetric tops. Such molecules have two different principal moments of inertia. The smaller moment (about the Si-halogen axis) we shall consistingly denote by I_A, the greater by I_B (adding, of course, extra symbols if we want to indicate the moment of a particular molecule). It is easy to derive that

$$(I_B - \tfrac{1}{2}I_A)m_{SiY_3X} = a^2 m_{Si} m_X + 3(a + d_Y \cos\varphi_Y)^2 m_Y m_X + 3d_Y^2 \cos^2\varphi_Y m_{Si} m_Y \quad (1)$$

where Si is a silicon isotope, Y a hydrogen isotope and X a halogen atom. m_{SiY_3X} is the mass of SiY_3X, a is the Si-X distance, d_Y the Si-Y distance. φ_Y is the supplementary angle of the X-Si-Y angle. The relation between *observed* microwave absorption frequency, ν_{obs} (cm^{-1}), and I_B is

$$\nu_{obs} = \frac{h}{4\pi^2 c I_B}(J + 1) = 2B\ (J + 1) \quad (2)$$

where J is the rotational quantum number of the *lower* of the two states involved, c the velocity of light and B the rotational constant. Experience has shown that (2) reproduces microwave spectra to a high degree of accuracy for small J-values. The highest J to be used in the following is $J = 1$. Unfortunately, microwave spectra of symmetric tops tell nothing of I_A. If, therefore, microwave absorption for, say, $Si^{29}D_3Cl^{35}$ is observed, only the magnitude of I_B (in this case denoted by $I_B^{29,D,35}$) results. This information is insufficient for a calculation of the three geometrical parameters, a, d_Y, and φ_Y. If it is assumed, however, that isotopic molecules have identical dimensions, it is seen that observation of microwave absorption for at least *three* isotopic species of the same compound enables one to carry through a calculation of a, d_Y, and φ_Y. This was what was actually done by Townes *et al.*[8] in the case of *silyl chloride*. They found $a = 2.050 \pm 0.001$ Å; $d_H = 1.50$ Å; $\varphi_H = 72°\ 2'$, but the magnitude of the two last-mentioned parameters must have been difficult to fix because of the quasi-degeneracy of the equations to solve. To avoid this complication Sharbaugh *et al.*[9] chose, in the case of *silyl fluoride*, to assume that $\varphi_H = 72°\ 6'$. They calculated $a = 1.593 \pm 0.002$ Å and $d_H = 1.50 \pm 0.03$ Å. Now, in the paper by Nielsen [3] it was shown that $d_H = 1.456$ Å in SiH_4. (Here, $\varphi_H = 70°\ 32'$). So far it looked, therefore, as if substitution of one hydrogen atom in SiH_4 by fluorine or chlorine would cause fairly large changes in the remaining Si-H bonds. Since this is definitely known *not* to be the case for the methyl halides it was decided to provide further experimental material in order to be able to calculate d_H and φ_H with somewhat higher accuracy.

EXPERIMENTAL PART

Preparation of SiD_3Cl and SiD_3F (B. B. and J. B.). 4 g Mg_2Si (from Mg and Si) was slowly added to a solution of DCl (from 60 g C_6H_5COCl and 2 g D_2O) in 22.8 g D_2O in a stream of dry hydrogen. SiD_4 and impurities (Si_2D_6 *etc.*) were condensed in a liquid-air trap. After evacuation at −190° C the temperature of the trap was raised to −125° C. The pressure rose to 380 mm Hg. In small portions the vapors were admitted to an adjacent, evacuated trap cooled in liquid air, until the vapor-pressure in the trap at 125° C had fallen to 35 mm Hg. The rest was discarded and the collected material transferred to a container, kept at −118° C from which practically the whole sample could be distilled at 470 mm Hg. In this way 100 ml portions were repeatedly prepared (1 atm., 20° C), representing a 8 % yield of SiD_4 (based on Mg_2Si).

52 ml SiD_4 (1 atm., 20° C) were brought to reaction with 38 ml DCl (1 atm., 20° C) over $AlCl_3$ (resublimed) at 100° C in a 120 ml flask. Contact between the reaction mixture and the greased stopcock was prevented by means of a drop of mercury. After three hours the reaction mixture:

$$SiD_4 + DCl \rightleftharpoons SiD_3Cl + D_2$$
$$SiD_3Cl + DCl \rightleftharpoons SiD_2Cl_2 + D_2 \text{ } etc.$$

was cooled in liquid air. The deuterium formed (27 ml at 1 atm., 20° C) was pumped off and the remaining 63 ml gases were carefully fractionated. 20 ml SiD_3Cl (vapor-pressure 45 mm Hg at −72° C) were collected. Yield: 40 % based on SiD_4. The sample so obtained was practically free of SiD_2HCl since no line corresponding to the Si-H vibration could be seen in the infrared absorption spectrum. The microwave spectrum showed that it was also free of SiD_2Cl_2. SiD_3Cl (and SiH_3Cl) can be stored for months at −78° C in a glass container without undergoing any change. At room temperature and 0.5−1 atm. it is likewise stable for shorter periods (1−2 hours). In handling the compound it is important to note that even a trace of water converts it very rapidly into $(SiD_3)_2O$ from which it is hard to separate.

SiH_3F has earlier been produced from SiH_3Cl and SbF_3 (catalyst: $SbCl_5$). In preliminary experiments it was shown that the use of $SbCl_5$ is superfluous. The following procedure was finally followed: 9.1 ml SiD_3Cl (1 atm., 20° C) was left for 12 hours in a 12 ml flask (greased stopcock protected by mercury) in contact with 250 mg resublimed SbF_3. Then the flask was cooled in liquid air and subsequently evacuated (0.2 ml D_2 was formed). The reaction mixture was fractionated at −118° C where a constant-boiling fraction (4.4 ml (1 atm., 20° C)) was obtained at 46 mm Hg. The infrared absorption spectrum showed no sign of the presence of hydrogen-containing compounds, but both microwave and infrared absorption curves showed that probably the sample was contaminated with SiD_2F_2. Since, however, the Stark patterns used in microwave spectroscopy are widely different for symmetric tops (SiD_3F) and asymmetric rotors (SiD_2F_2) the unambigous identification of a microwave line as originating from SiD_3F is not prevented.

MEASUREMENT OF MICROWAVE ABSORPTION OF SiD_3Cl AND SiD_3F. (B. B. AND J. R−A.)

Since both silicon and chlorine consist of a mixture of stable isotopes of rather high concentration (Si^{28}: 90 %; Si^{29}: 6 %; Si^{30}: 4 %; Cl^{35}: 75 %; Cl^{37}: 25 %) 'heavy' silyl chloride is a mixture of 6 isotopic species while 'heavy' silyl fluoride is composed of 3 species. Microwave absorption corresponding to four of the chlorides and all three fluorides was found (Table 1).

Table 1. Observed microwave absorption frequencies for isotopic silyl fluoride and chloride molecules.

Species	Transition	ν_{obs} MHz	$I_B \times 10^{40}$ gcm²	B MHz
$Si^{28}D_3F$	$J = 0 \rightarrow 1$	24507.0	68.461	12253.5
$Si^{29}D_3F$	»	24352.2	68.897	12176.1
$Si^{30}D_3F$	»	24204.5	69.316	12102.2
$Si^{28}D_3Cl^{35}$	$J = 1 \rightarrow 2$	23670.8	141.759	5917.7
$Si^{29}D_3Cl^{35}$	»	23402.6	143.383	5850.6
$Si^{30}D_3Cl^{35}$	»	23147.9	144.961	5787.0
$Si^{28}D_3Cl^{37}$	»	23091.4	145.316	5772.8
$Si^{28}H_3F$	$J = 0 \rightarrow 1$	28655.8	58.549	14327.9
$Si^{29}H_3F$	»	28393.4	59.090	14196.7
$Si^{30}H_3F$	»	28145.2	59.611	14072.6
$Si^{28}H_3Cl^{35}$	$J = 1 \rightarrow 2$	26695.2	125.698	6673.8
$Si^{30}H_3Cl^{35}$	»	25943.2	129.342	6485.8
$Si^{28}H_3Cl^{37}$	»	26049.6	128.813	6512.4

The observations placed below the line in the middle of Table 1 were made by the previously cited authors [7–9]. Due to the spin of the chlorine nucleus the microwave absorption of all the chlorides exhibits a hyperfine structure. Since the quadrupole coupling is very nearly the same for light and heavy silyl chloride the correction terms for quadrupole coupling found by Sharbaugh [7] for SiH_3Cl^{35} and SiH_3Cl^{37} could be taken over without appreciable error. The value $h = 6.6237.10^{-27}$ ergsec was used for Planck's constant in calculating I_B.

MOLECULAR DIMENSIONS OF SILYL CHLORIDE AND FLUORIDE.

a. Silyl chloride

It is easy to derive from (1) that

$$a_H \frac{m_{Cl^{35}}}{m_H} - 3d_H \cos\varphi_H = K^{30-28,H,35} \tag{3}$$

$$a_H \frac{m_{Si^{28}H_3}}{m_H} + 3d_H \cos\varphi_H = K^{28,H,37-35} \tag{4}$$

where $$K^{30-28,H,35} = \sqrt{\frac{(I_B^{30,H,35} - I_B^{28,H,35})\ m_{Si^{28}H_3Cl^{35}}\ m_{Si^{28}H_3Cl^{35}}}{m_H^2\ (m_{Si^{30}} - m_{Si^{28}})}}$$

and $$K^{28,H,37-35} = \sqrt{\frac{(I_B^{28,H,37} - I_B^{28,H,35})\ m_{Si^{28}H_3Cl^{37}}\ m_{Si^{28}H_3Cl^{35}}}{m_H^2\ (m_{Cl^{37}} - m_{Cl^{35}})}}$$

In the calculations to follow the atomic masses used were:

Si^{28}: 27.9866; Si^{29}: 28.9866; Si^{30}: 29.9832: Cl^{35}: 34.9786;
Cl^{37}: 36.9775; F: 19.0049; H: 1.008123; D: 2.014708,

all in the physical atomic weight scale. The corresponding value of Avogadros number used is $6.0251 \cdot 10^{23}$.

By addition of (3) and (4) a formula giving a_H as a function of measured quantities is easily derived (5):

$$a_H = \frac{m_H}{m_{Si^{28}H_3Cl^{35}}} (K^{30-28,H,35} + K^{28,H,37-35}) \tag{5}$$

By insertion it is found that

$$a_H = 2.0479 \text{ Å}$$

Microwave absorption frequencies of silyl chloride and fluoride molecules *not* containing the rare isotopes Si^{29} and Si^{30} are reliable to $\pm$ 0.2 MHz, while the rest may be assumed to be good to $\pm$ 0.3 MHz. This means an uncertainty in the calculated a_H value of 7 in the fourth decimal place so that

$$2.0472 \text{ Å} < a_H < 2.0486 \text{ Å}$$

If a similar calculation is carried through in order to find a_D, the Si-Cl distance in the 'heavy' species, we find that

$$a_D = 2.0486 \text{ Å and that } 2.0479 \text{ Å} < a_D < 2.0493 \text{ Å}$$

The values of a_H and a_D refer to the molecules in their ground state where the only internal energy left is the zero-point energy. In their ground state isotopic molecules do not possess identical structures but only very nearly so. Since the zero-point energy may be thought of as kinetic and since of two isotopic molecules the lighter has the higher zero-point energy this means that as a rule bond lengths will decrease slightly with increasing molecular weight in a series of isotopic molecules. In the calculations above it was tacitly assumed that the very small differences in zero-point energy between molecules which only differ in silicon or chlorine isotopes, have no effect on the interatomic distances. However, molecules differing in *hydrogen* isotopes have rather different zero-point energies. Here, we may expect structural differences which, due to the small masses of hydrogen and deuterium, must be expected to be most pronounced in the magnitude of the parameters d_H, d_D, φ_H, and φ_D. Indeed, the values found for a_H and a_D verify this assumption. Generally it must be expected that $d_H > d_D$ and $\varphi_H \neq \varphi_D$. In the case of the methyl halides Miller, Aamodt, Dousmanis and Townes [10] got the results of Table 2.

Table 2. Position of hydrogen and deuterium atoms in light and heavy methyl halides.

	d_H, d_D	φ_H, φ_D		d_H, d_D	φ_H, φ_D		d_H, d_D	φ_H, φ_D
CH_3Cl CD_3Cl	1.113 1.104	71° 36′ 71° 50′	CH_3Br CD_3Br	1.113 1.104	72° 21′ 72° 34′	CH_3J CD_3J	1.113 1.104	72° 33′ 72° 46′
$d_H - d_D$	0.009			0.009			0.009	
$\varphi_D - \varphi_H$		14′			13′			13′
Deviations from CH_4	0.02 0.01	1° 4′ 1° 18′		0.02 0.01	1° 49′ 2° 2′		0.02 0.01	2° 1′ 2° 14′

For the silyl chlorides and fluorides deviations from regularity of the same order of magnitude were found by Bak, Rastrup-Andersen and Bruhn [12]. Since then, Monfils[11] has studied the fine-structure of the infrared bands of silyl chloride. He found $I_A^{28,H,35} = (9.89 \pm 0.15) \times 10^{-40}$ gcm^2. Strictly spoken this result probably is a 'hybrid' between the true $I_A^{28,H,35}$ (vibrational ground level) and I_A for a vibrationally excited level (such details cannot be seen from Monfils' note) but the error committed by setting $I_A^{28,H,35} = 9.89$ 10^{-40} gcm^2 cannot be great. (In the case of OCS it has been found that the moment of inertia of a molecule, vibrationally excited by one quantum, deviated less than 0.5 % from the moment in the ground state.)

By inserting the calculated value of a_H in (3) we find that

$$d_H \cos\varphi_H = 0.476 \pm 0.002 \text{ Å} \tag{6}$$

$$I_A^{28,H,35} = 3m_H d_H^2 \sin^2\varphi_H = (9.89 \pm 0.15) \times 10^{-40} \text{ gcm}^2 \text{ gives}$$
$$d_H \sin\varphi_H = 1.405 \pm 0.012 \text{ Å} \tag{7}$$

It follows that

$$\varphi_H = 71° 15' \pm 15' \text{ and } d_H = 1.483 \text{ Å} \pm 0.010 \text{ Å}$$

In SiH_4, $\varphi_H = 70° 32'$ » $d_H = 1.456$ Å ± 0.010 Å

The deviation in φ_H and d_H, are, therefore, respectively 43′ and 0.027 Å. These numbers correspond very well with what was found for methyl chloride (Table 2). The results suggest that d_D, the Si-D distance in the heavy silyl chloride, may be one or two hundredths of an Å smaller than d_H as found for methyl chloride. Consequently we look for solutions in the region where $1.49 \text{ Å} \geqq d_D \geqq 1.46$ Å. The point of interest now is whether we shall find φ_D to be close to and a little greater than φ_H.

By inserting the value of a_D calculated above in the 'heavy' equation corresponding to (3) we find $d_D \cos\varphi_D = 0.469 \pm 0.002$. $d_D = 1.460$ Å, therefore, corresponds to $\varphi_D = 71° 11'$—$71° 21'$ while $d_D = 1.490$ Å corresponds to $\varphi_D = 71° 34'$—$71° 44'$. It is seen that φ_D and φ_H are about equal. Furthermore it is seen that if we fix d_H to 1.483 Å and if we suppose that $d_H - d_D = 0.009$ Å as in CH_3Cl we get $\varphi_D = 71° 27'$ (mean value) which happens to be 12′ greater than φ_H. For CH_3Cl and CD_3Cl this difference was found to be 14′ (Table 2).

b. Silyl fluoride

Not much could be added to the communication by Bak, Bruhn and Rastrup-Andersen [12]. Due to the lack of fluorine isotopes an equation like (5) cannot be derived giving the silicon-fluorine distance as a function of measured quantities. Since

$$a_H \frac{m_F}{m_H} - 3 d_H \cos\varphi_H = K^{30-28,H,F} \tag{8}$$

and

$$a_D \frac{m_F}{m_D} - 3 d_D \cos\varphi_D = K^{30-28,D,F} \tag{9}$$

we can derive

$$a = \frac{m_H m_D}{m_F (m_D - m_H)} \left\{ K^{30-28,H,F} - K^{30-28,D,F} + 3(d_H \cos\varphi_H - d_D \cos\varphi_D) \right\} \tag{10}$$

where the difference between a_H and a_D has been neglected which was found to be correct for silyl chloride.

By omitting the last term of this equation we find

$$a = 1.595 \pm 0.002 \text{ Å}$$

If we assume that $|d_H - d_D| \leqq 0.01$ Å and $|\varphi_H - \varphi_D| \leqq 1°$ an upper limit to the error in a, caused by this uncertainty, is 0.008 Å. Therefore,

$$a = 1.595 \pm 0.010 \text{ Å}.$$

By inserting in (8) and (9) it is found that

$$d_H \cos\varphi_H = 0.476 \pm 0.040 \text{ Å}$$

and that

$$d_D \cos\varphi_D = 0.474 \pm 0.020 \text{ Å}$$

i. e. these products are about equal to the corresponding quantities for the silyl chlorides although the uncertainty is larger due to lacking experimental data. As shown by Bak, Bruhn and Rastrup-Andersen [12] by their graphical method it can be shown that if it is assumed that also in this case $d_H - d_D \sim 0.01$ Å, φ_H and φ_D come very close to the tetrahedral value 70° 32′. But it is impossible to give $|\varphi_H - 70° 32'|$ and $|\varphi_D - \varphi_H|$ with any satisfactory degree of accuracy.

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Liquid Structure of Aqueous Nitric Acid Solutions

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The heat of vaporization E_{HNO_3} of HNO_3 from aqueous nitric acid solutions has been determined from the temperature-dependence of the partial vapor pressure of HNO_3. A formula for E_{HNO_3} similar to that for E_{HCl} derived previously is found. This formula is discussed and interpreted. The existence of the hydrate $2\ HNO_3 \cdot 3\ H_2O$ is confirmed. The structures of this and of lower hydrates are discussed. Those contain H_2O bound by a N—O bond. The existence of the ortho-acid $HNO_3 \cdot H_2O = H_3NO_4$ is discussed.

1. EMPIRICAL FORMULAE

The heat of vaporization E_{HNO_3} of HNO_3 from aqueous solutions of nitric acid has been determined from plots of $\ln P_{HNO_3}$ against $\frac{1}{T}$ at various concentrations, the partial vapor pressure P_{HNO_3} of HNO_3 being taken from the compilation by Taylor [1,2]. Only pressures P_{HNO_3} less than 100 mm Hg were included in those plots, corresponding to temperature ranges varying from 55—105° C for 20 % HNO_3 to 0—30° C for 100 % HNO_3. The results are given in Table 1 and plotted in Fig. 1 against mole fraction of HNO_3.

Table 1.

weight-% HNO_3	m	$\frac{100}{m+1}$	E_{HNO_3} kcal/mole
20	13.99	6.671	16.46
25	10.49	8.703	15.62
30	8.149	10.930	15.29
35	6.495	13.34	15.02
40	5.246	16.01	14.58
45	4.267	18.99	13.63
50	3.498	22.23	13.23
55	2.861	25.90	12.64
60	2.332	30.01	11.94
65	1.883	34.69	11.18
70	1.499	40.02	10.66
80	0.8742	53.36	10.38
90	0.3880	72.05	10.12
100	0	100.00	10.52

When m denotes the number of H_2O molecules per HNO_3 molecule we find from Fig. 1 the empirical formulae

$$E_{HNO_3} = 16.8 - \frac{16}{m+1} \text{ kcal/mole}, > m\ 1.5 \quad (1)$$

$$E_{HNO_3} = 10.4 \text{ kcal/mole}, \ m \leqq 1.5 \quad (2)$$

We will compare the formula (1) with the corresponding formula for E_{HCl} written in the form [3]

$$E_{HCl} = E_{v.d.W} + 5\ E_{Cl-H-O} - \frac{10}{m+1} E_{O-H-O}, \ m < 10 \quad (3)$$

With a suitable choice of coefficients we may write (1) in a form similar to that of (3), *viz.*

$$E_{HNO_3} = E_{v.d.W} + \left(\frac{9}{2} - 1\right) E_{O-H-O} - \left(\frac{9}{2} - 1\right) \frac{1}{m+1} E_{O-H-O} \quad (4)$$

For inserting in (4) $E_{v.d.W} = 0.47$ kcal/mole for the van der Waals energy and $E_{O-H-O} = 4.63$ kcal/mole for the energy of the O—H—O hydrogen bond [3] we have, in fact,

$$E_{HNO_3} = 16.7 - \frac{16.2}{m+1} \text{ kcal/mole} \quad (5)$$

which agrees with (1) within the accuracy of the empirical data.

A comparison of (4) and (3) suggests a common interpretation of the two formulae. In the case of HCl we have five hydrogen bonds between the Cl atom and adjacent H_2O molecules and a total number of ten H_2O molecules in the immediate vicinity of the HCl molecule [3]. Since the HNO_3 molecule can form nine hydrogen bonds with adjacent molecules we might expect the factors 5 and 10 for HCl to be replaced by 9 for HNO_3. Such a comparison indicates, however, that of the nine hydrogen bonds only one half minus one contribute to binding the HNO_3 molecule. The factor $\frac{1}{2}$ indicates that each bond is shared by two HNO_3 molecules. We will return to the discussion of (4) in section 3.

The deviations from the constant value (2) in the range 40—100 mol. % are not greater than the experimental error [1]. Further data are available for pure HNO_3 from which the heat of vaporization has been determined. From the vapor pressures of Wilson and Miles [4] and of Berl and Saenger [5] we obtain 9.30 and 9.65 kcal/mole, respectively. The vapor pressures given by Taylor are considerably lower than those given by the latter authors and by Klemenc and Rupp [6] but cover a much wider range of temperature. The calorimetrically determined heat of vaporization is 7.25 kcal/mole at 15° [7]. In view of these discrepancies we will simply take the vapor pressures of Taylor, since those may be supposed to fit better into Taylor's series of vapor pressures of the dilute acid, and, particularly, since their spread is smaller than that of the data of the other authors.

The heat of vaporization of H_2O in pure water and in aqueous nitric acid solutions is $E_{H_2O} = 10.54$ kcal/mole at 20° C. Thus, the value (2) of E_{HNO_3} in the range $m \leqq 1.5$ does not differ appreciably from E_{H_2O}.

2. DISCUSSION OF THE FORMULA (2)

There are two interesting features of (2), *viz.*

1. that $E_{HNO_3} = E_{H_2O}$ in the range $m \leqq 1.5$

b. the limit $m = 1.5$, indicating the existence of the hydrate $2\ HNO_3 \cdot 3\ H_2O$ which we will now discuss.

Three hydrates have been reported previously in the range $m \leqq 1.5$ with $m = \frac{1}{2}$, $m = 1$, and $m = 1\frac{1}{2}$. Of those the monohydrate is very well known; it has been isolated and studied in the solid as well as in the liquid state.

The hydrate $2\ HNO_3 \cdot 3\ H_2O$ has been found by a number of investigators. Kolb [8] found a maximum in the contraction of the solution at this concentration. Veley and Manley [9] concluded on this hydrate from their curves of electrical conductivity and refraction index. Creighton and Githens [10] pointed out that the vapor pressure has a minimum at this composition. Erdmann claimed to have isolated this hydrate by freezing (—39°) [11] and also studied its molecular weight in the vapor [12]. Küster and Kremann [13] ridiculed the results of Erdmann in a paper, the arrogance of which is, fortunately, rare in scientific literature, and claimed that this hydrate is a mixture of the wellknown mono- and tri-hydrates. In view of this criticism Gmelin [14] and Mellor [15] quote both views and consider the existence of this hydrate as still undecided. More recent views on the subject are unknown to the author.

The basic error in the argument of Küster and Kremann is the postulate that a hydrate can not exist in aqueous solution unless it can be isolated from the solution. Thus, the present author has shown in a series of papers [16] that the hydrates $HNO_3 \cdot 6\ H_2O$ and $HNO_3 \cdot 15\ H_2O$ exist in the solution although they can not be isolated by freezing. Similarly, the hydrate $2\ HNO_3 \cdot H_2O$ found by Erdmann [11] and equally ridiculed by Küster and Kremann has been shown to exist in aqueous solution by Chédin [17].

The curve in Fig. 1 seems to establish the existence of the hydrate $2HNO_3 \cdot 3H_2O$. The fact that no other hydrate shows up in that curve does not prove, of course, that no other hydrates exist.

The existence of hydrates with $m = \frac{1}{2}$ and $m = 1\frac{1}{2}$ indicates that those hydrates and, may be, the other hydrates as well, form aggregates of an even number, *e. g.* pairs, of HNO_3 molecules or consist of hydrates of the anhydride, $N_2O_5 \cdot m\ H_2O$.

In water and in aqueous solutions, the molecules are held together in a structure by hydrogen bonds. The energy of the O—H—O bond is independent of to what kind of molecule the O atoms belong. Thus, O—H—O bonds between H_2O molecules, H_2O and H_2SO_4 molecules, H_2O and HNO_3 molecules, *etc.* all have the same energy. This follows from (4) and from the corresponding formulae for E_{H_2O} in HNO_3 and H_2SO_4 solutions [3] as well as from the vapor pressure [3]. Hence, an O—H—O bond between two HNO_3 molecules has the energy $E_{O-H-O} = 4.6$ kcal/mole, and we must assume that there are two H atoms per evaporating molecule forming bonds with the liquid structure. This means that the HNO_3 molecules either form pairs or dimers, *e. g.* $(HNO_3)_2 = N_2O_5 \cdot H_2O$, or that the H_2O molecules in the hydrates are instru-

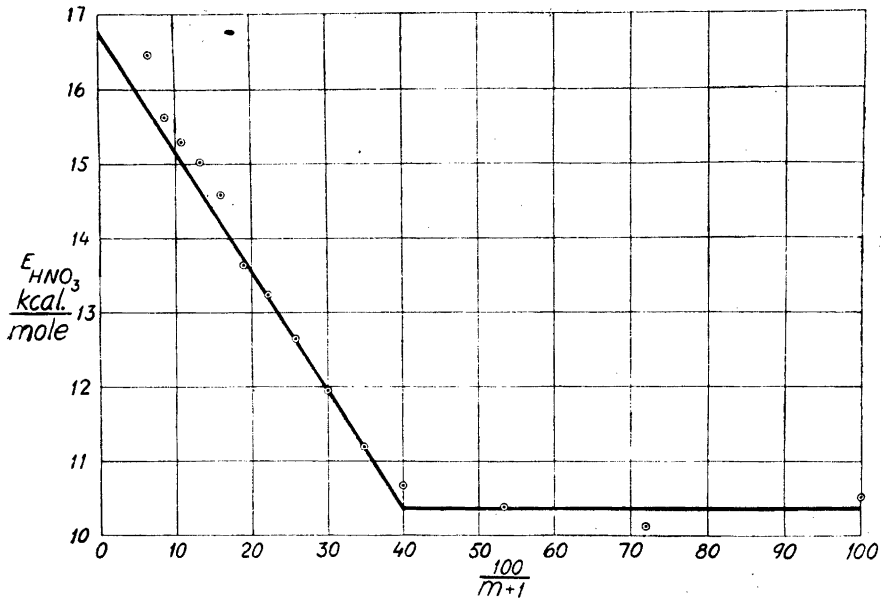


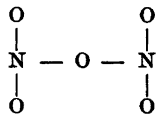
Fig. 1.

mental in binding the HNO_3 molecules to the structure, *viz.* by supplying the lacking number of H atoms for the formation of the bonds. By writing the dimer in the form $N_2O_5 \cdot H_2O$ we see that there is little difference between those two alternatives.

In order to discuss the structures of and the bonds in the various hydrates we will start out by considering the structural possibilities for the reaction

$$N_2O_5 + H_2O \rightarrow (HNO_3)_2 = 2\ HNO_3 \qquad (6)$$

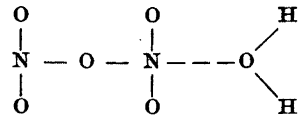
Concerning the dimer $(HNO_3)_2$, the "nitronium nitrate", reference is made to Gmelin [14]. Further reference may be made to Jones and Thorn [18] and to Chédin and coworkers who studied association of HNO_3 molecules [19,20], and the complex ions $(HNO_3)NO_3^-$ [21] and $(HNO_3)_2NO_3^-$ [22]. The structure of gaseous N_2O_5 is



(7)

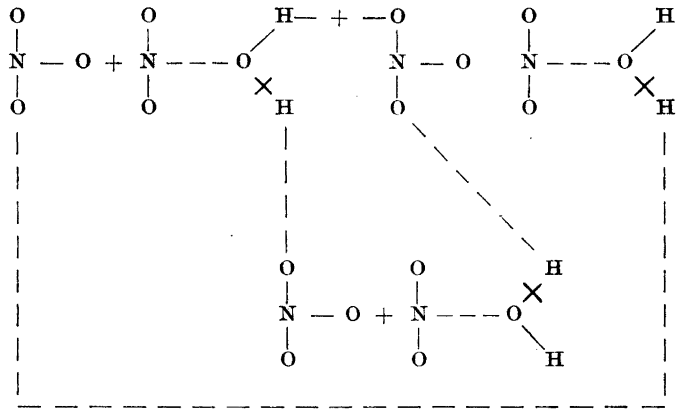
The reaction (6) may be effected by splitting N_2O_5 into NO_2 and NO_3 and by adding OH to NO_2 and H to NO_3. In both cases, there must be three N—O bonds per HNO_3 molecule. We will therefore assume that the hydrate $N_2O_5 \cdot H_2O$ is the first reaction product in (6) and that the H_2O molecule is

bound to the N_2O_5 molecule by a N—O bond. The structure of $N_2O_5 \cdot H_2O$ is then



(8)

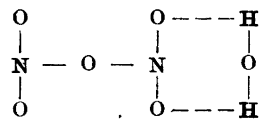
If a number of such hydrate molecules are bound together with hydrogen bonds as shown schematically by the formula



(9)

and the bonds are broken as indicated by the perpendicular lines, HNO_3 molecules will form which may be held together by hydrogen bonds or which may separate after evaporation. Since each $N_2O_5 \cdot H_2O$ molecule according to (8) has two H atoms for forming hydrogen bonds with the liquid structure, the heat of vaporization of one $N_2O_5 \cdot H_2O$ molecule or of the $(HNO_3)_2$ dimer is equal to E_{H_2O}.

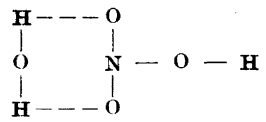
If, instead of (8), we assume the structure



(10)

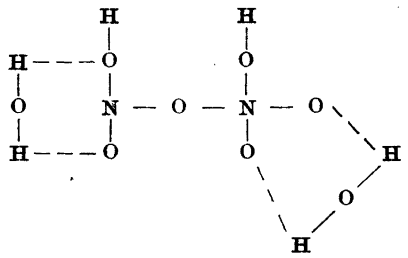
i. e. that the H_2O molecule is bound in the hydrate $N_2O_5 \cdot H_2O$ by hydrogen bonds, the reaction (6) is not structurally possible.

We will now apply the same arguments to the hydrate $HNO_3 \cdot H_2O$. The structure of this hydrate has been the subject of much controversy [14]. According to Chédin and Fénéant [19,2] its structure should be of the same type as (10), *i. e.*



(11)

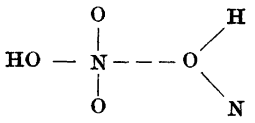
This hydrate contains only one H atom for the formation of a hydrogen bond with the liquid structure, whereas two are required. We must therefore assume the formation of a dimer $(HNO_3 \cdot H_2O)_2 = 2HNO_3 \cdot 2H_2O$ with two H atoms available for the formation of hydrogen bonds with the liquid structure. But then there is no H atom available for internal binding within the dimer and we are led to the conclusion that a N—O bond has to be formed for holding the dimer together.



(12)

The same reason as were just given against (10) are valid against (11) and (12) as will be shown presently.

It has seemed natural to compare the hydrate $HNO_3 \cdot H_2O$ to $HPO_3 \cdot H_2O = H_3PO_4$ and to $SO_3 \cdot H_2O = H_2SO_4$. Adopting this view we will assume that the H_2O molecule is bound in $HNO_3 \cdot H_2O$ by a N—O-bond and write $HNO_3 \cdot H_2O$ in the form



(13)

i. e. the same structure as that of H_3PO_4 [24]. Salts of the acid H_3NO_4 are known, *viz.* Na_3NO_4, $Na_3NO_4 \cdot H_2O$, $Na_2HNO_4 \cdot H_2O$, and $K_3NO_4 \cdot H_2O$ [14]. The existence of such salts indicates that the N—O bond may strengthen into a valence bond. The structure (13) contains three H atoms which may form bonds with the liquid structure. However, if those molecules combine in pairs held together by two hydrogen bonds there will be a total of four, *i. e.* two per $HNO_3 \cdot H_2O$ molecule, H atoms available for the formation of external hydrogen bonds. Thus, if the mono-hydrate $HNO_3 \cdot H_2O$ evaporates in the dimer form $(HNO_3 \cdot H_2O)_2 = 2HNO_3 \cdot 2H_2O$, we may expect the heat of vaporization per $HNO_3 \cdot H_2O$ molecule to be equal to that of an H_2O molecule. Obviously, the alkali salts quoted can not be formed from (12) but very well from (13).

When further H_2O is added to the mono-hydrate dimer, *i. e.* to pairs of the structure (13), we arrive at the hydrate $2HNO_3 \cdot 3H_2O$ with four external and, consequently, four internal hydrogen bonds. The third H_2O molecule is then bound to the $(HNO_3 \cdot H_2O)_2$ molecule by hydrogen bonds and not by a N—O bond.

3. DISCUSSION OF THE FORMULA (4)

In the structure of water, each O atom is surrounded by four nearest neighbors forming a tetrahedron with one O atom at each corner and one O atom in the centre. We may assume that the same co-ordination number applies to the O atoms of the HNO_3 molecule [25]. Hence, each O atom of the HNO_3 molecule will have one N atom and three O atoms, belonging to other HNO_3 molecules or to H_2O molecules, as nearest neighbors. The total number of nearest neighbors per HNO_3 molecule is then 9. Thus, when $m > 9$ the HNO_3 molecule may have its full number of H_2O neighbors without sharing any of those with any other HNO_3 molecule. However, as has been shown by the author in connection with the dissociation of the HNO_3 molecule in aqueous solution [25,26], this occurs at low temperatures only. At higher temperatures, above 21° C, the actual number required is $m = 15$ owing to the formation of a hexa-hydrate with whose H_2O molecules the $(m\text{-}6)$ further added H_2O molecules may bind.

However, the coordination number 9 may still be valid for $m < 9$, because some of the H_2O molecules may be shared by several HNO_3 molecules or replaced by O atoms of neighbor HNO_3 molecules. This is indicated by the formula (4) to be the fact for $m > 1.5$. As discussed in section 2, for $m \leqq 1.5$, the structure is held together by H_2O molecules bound with N—O bonds to the HNO_3 molecules and to the liquid structure by hydrogen bonds, the hydrates forming dimers of two HNO_3 molecules. If this state is preserved at higher values of m, two external hydrogen bonds per HNO_3 molecule will be lost, because one will be engaged in forming the dimer and one is replaced by an internal N—O bond. Hence, the total number of external bonds will not be 9 but $9—2 = 7$. Since those are shared by the two HNO_3 molecules in the dimer, the number of bonds per HNO_3 molecule will be $\frac{7}{2}$ as indicated by the empirical formula (4).

When treating the partial vapor pressure of water above nitric acid solutions [3] we found the limiting formula

$$p = \frac{m-1}{m+1} \tag{14}$$

instead of Raoult's law

$$p = \frac{m}{m+1} \tag{15}$$

The formula (14) shows that one of the m H_2O molecules does not contribute to the vapor pressure, *i. e.* it is bound in a hydrate. Although this conclusion was actually drawn, no satisfactory explanation could be given, because we assumed at that time as well as previously [25] that the mono-hydrate has the structure (11) which is not possible at values of m greater than 1. It should be pointed out in this connection that the N—O bond according to (13) does not affect the derivation of the degree of dissociation [25], since we are only concerned with hydrogen bonds in that derivation.

In concluding this section it should be pointed out that the validity of (4) has been established only in the range $14 > m > 1.5$. At lower concentrations we would expect the HNO_3 pairs to separate and the factor $\frac{1}{2}$ in (4) to disappear and, thus, E_{HNO_3} to increase above the value given by (4). Unfortunately, data are lacking in the range $m > 14$.

4. CONCLUSIONS

A. From the following evidence:

1. the existence of the hydrates $N_2O_5 \cdot H_2O$, $HNO_3 \cdot \frac{1}{2} H_2O$, and $HNO_3 \cdot 1\frac{1}{2} H_2O$;
2. the existence of the "nitronium nitrate" $(HNO_3)_2$ and the complex ion $(HNO_3)NO^-_3$;
3. the factor $\frac{1}{2}$ in (4);
4. the formula (2);

we conclude upon the existence of dimers or structural units containing two HNO_3 molecules.

B. From the following evidence:

1. the existence of salts of the type Na_3NO_4;
2. the term —1 in the formula (4);
3. the formula (14);
4. the structural possibilities for reactions;
5. a comparison with $HPO_3 \cdot H_2O = H_3PO_4$, $SO_3 \cdot H_2O = H_2SO_4$,

we conclude upon the existence of a N—O bond in the mono-hydrate which may strengthen into a valence bond with formation of the ortho-acid, $HNO_3 \cdot H_2O = H_3NO_4$.

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Hydrolysis of α-Bromo-*t*-Butylmalonic Acid

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In connection with an investigation of the kinetics of the hydrolysis of some organic bromides, a short account of which has already been given [1], the hydrolysis of α-bromo-*t*-butylmalonic acid in alkaline solution was also studied. The hydrolyses were usually followed by titration of the alkali consumed in the reaction. With the above mentioned acid this method, however, gave unsatisfactory results. The titration showed an initial comparatively rapid consumption of alkali, which soon became much slower and no useful kinetics could be obtained from these results. Titration of the liberated bromide ions gave however good first order kinetics. The initial rapid decrease in alkalinity corresponded approximately in speed to the liberation of bromide ions, whereas the later, slower part of the consumption of alkali proceeded long after all the organically bound bromine had been converted into bromide ion. However the total consumption of alkali in the hydrolysis was one equivalent. It is thus evident that there must be formed some bromine-free intermediate, which slowly reacts further with consumption of alkali.

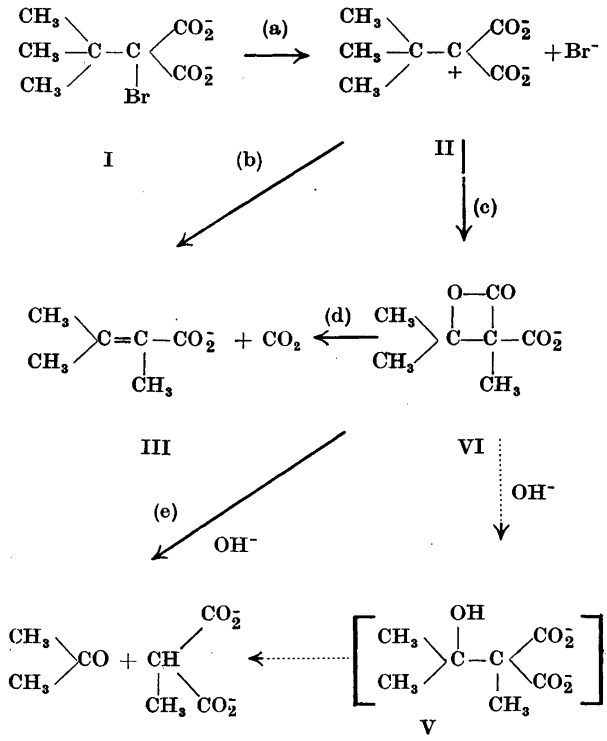
In order to get some idea of the nature of this slower reaction a calculation of the rate constant was made taking as zero time a time when the first rapid reaction is completed, as judged by the complete conversion of bound bromine into bromide ion. Although a rather good first order constant could be obtained from each run, a comparison of the rate constants from runs with increasing concentration of alkali showed a definite increase in the rate. It is thus evident that this second reaction is at least partly bimolecular, although no agreement between second order rate constants from different runs could be obtained. As will be shown later carbon dioxide is liberated in the reaction, which makes the determination of the hydroxyl ion concentration by titration, using phenolphtalein as indicator, unreliable, and this is supposed to be the cause to these differences.

A search for the products of hydrolysis gave then further information about this rather complex reaction. As reaction products were found trimethylacrylic acid, methylmalonic acid and acetone. It was further found that the proportion of these products varied with the concentration of alkali, as is seen from Table 1.

Table 1.

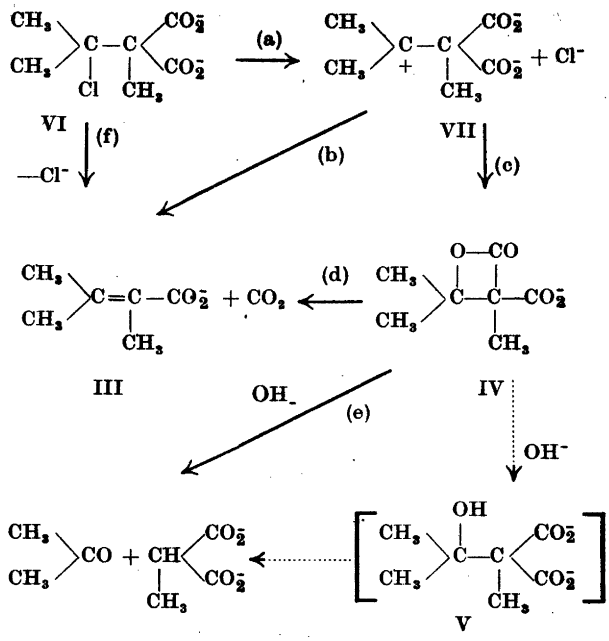
Molar proportion alkali : acid	[OH^-]	Acetone mole %	Methyl-malonic acid mole %	Trimethyl-acrylic acid mole %
3 : 1	0.1 *N*	11	11	76
4 : 1	0.1 *N*	28	27	68
4 : 1	0.2 *N*	33	32	62
4 : 1	0.3 *N*	43	43	54
4 : 1	0.5 *N*	49	40	53
6 : 1	0.2 *N*	41	35	55
8 : 1	0.2 *N*	43	41	60

The yield of acetone and methylmalonic acid thus increases not only with an increase in the molar proportion total alkali: free acid, but also with an increase in the concentration of free alkali, whereas the yield of trimethylacrylic acid correspondingly decreases. When the hydrolysis was carried out with the free acid without any addition of alkali no acetone was formed and the only isolable acid was trimethylacrylic acid. The sum of acetone or methylmalonic acid and trimethylacrylic acid accounts for about 90—100 % of the reaction. On the basis of the above mentioned facts the following mechanism is proposed for the hydrolysis of *α*-bromo-*t*-butylmalonic acid in alkaline solution.



The reaction (a) is the first order reaction measured by the release of bromide ion. The formed carbonium ion (II) then reacts in two different ways, (b) and (c), both reactions being faster than (a) but of about equal speed. The reaction (b) accounts for the initial faster consumption of alkali because the carbon dioxide formed acts as an acid towards phenolphtalein, that was used as indicator in the titration. The reaction (c) gives a β-lactone (IV) which then reacts according to (d) and (e), which reactions correspond to the later slower consumption of alkali. The formation of β-lactones in the hydrolysis of β-halogensubstituted acids has been noted before and Johansson [2] succeeded in isolating such a lactone from the hydrolysis of β-bromobutyric acid. That (III) cannot be formed exclusively by path (b), but must be formed partly also by path (d) follows from the greater amount of acetone and methylmalonic acid formed when the hydroxyl ion concentration is increased. If only (b) were prevailing no such change should be expected, since the proportion of trimethylacrylic acid to methylmalonic acid and acetone would depend solely on the relative rates of (b) and (c), which must be assumed to be independent of the concentration of alkali. On the other hand the bimolecular reaction (e) certainly increases its speed with increasing hydroxyl ion concentration, whereas (d) can be assumed to be unimolecular and thus independent of the hydroxyl ion concentration, which leads to the observed increase in yield of the products from (e). Although decarboxylation of β-lactones is well known, it has been stated [3,4] that this does not occur in the presence of water. In this case we are, however, forced to assume that loss of carbon dioxide can occur also in the presence of water. Whether the formation of acetone and methylmalonic acid from lactone (IV) goes directly or whether the corresponding β-hydroxyacid (V) is an intermediate can not be said with certainty. Usually alkylsubstituted β-hydroxy acids [5], even β-hydroxymalonic acids [6,7] are stable towards alkali, whereas β-hydroxy acids with aryl and aralkyl groups sometimes are split in the same way as that pictured above [5,8].

In order to find some further support for the mechanism described above the hydrolysis of methyl-1-chloro*iso*propylmalonic acid (VI) was studied. This acid, which has not been described before, was prepared by chlorination of methyl*iso*propylmalonic acid with sulphurylchloride in the presence of catalytic amounts of benzoyl peroxide [9]. Although the structure of the chlorination product obtained has not been determined by any independent means, analysis and the products of hydrolysis clearly show that it must be the desired acid (VI), Hydrolysis of this acid gave the same three products as were obtained in the hydrolysis of α-bromo-t-butylmalonic acid *viz.* acetone, methylmalonic acid and trimethylacrylic acid in 21, 18 and 62 % yield, respectively. The yield of acetone and methylmalonic acid is lower and that of trimethylacrylic acid higher than from α-bromo-t-butylmalonic acid under comparable conditions. This reaction can be assumed to take the following course:



The higher yield of trimethylacrylic acid can be explained as the result of a direct decarboxylative elimination [10] (f). Although there is no kinetic evidence for the unimolecular nature of reaction (a), this can be inferred from analogy, (VI) being a tertiary halogenide. There is further no direct evidence for the formation of the β-lactone (IV) but as the rearrangement of (II) into (IV) evidently must go through (VII) its inclusion in the scheme seems inevitable. This scheme is thus very closely related to the earlier one, the main difference being the lack of any rearrangement in the latter case.

This rearrangement is of course a typical Wagner-rearrangement, but the interesting point here is that in the series $(CH_3)_3CCH_2Br$, $(CH_3)_3CCHBrCO_2^-$ and $(CH_3)_3CCBr(CO_2^-)_2$, the first [11] and the third undergo hydrolysis with rearrangement, whereas the second gives a high yield of trimethyllactic acid, thus hydrolysing without rearrangement. The mechanism is in all three cases definitely S_N1 [11] and it appears thus probable that there must be two different factors which decide whether a rearrangement of the primarily formed cation takes place or not.

EXPERIMENTAL

α-Bromo-t-butylmalonic acid. *t*-Butylmalonic acid was prepared according to Wideqvist [12]. Its conversion into *α*-bromo-*t*-butylmalonic acid was carried out according to Abderhalden and Rossner [13]. After recrystallisation from ether-light petroleum it had m.p. 188°. (Lit. 183° [13], 180–183° [14]) (Found: Br 32.5; $C_7H_{11}O_4Br$ requires Br 33.4).

In order to put its structure beyond any doubts it was converted, by heating to 200°, into *α*-bromo-*t*-butylacetic acid, m.p. 72°, undepressed when mixed with an authentic sample [15]. It was further reduced with zinc and acetic acid to *t*-butylmalonic acid of m.p. 152° (alone or in mixture test).

Hydrolysis of α-bromo-t-butylmalonic acid

a) *Kinetics.* The solutions in water were made up at 0° and samples of 5 ml were sealed into bulbs. These were placed in a termostat heated to the appropriate temperature and shaken for one minute. One bulb was taken out and broken, either under 100 ml of ice cold water and titrated with hydrochloric acid using phenolphtalein as indicator, or under 100 ml 6 *N* nitric acid, a known excess of silver nitrate solution added and the excess titrated with ammonium rhodanide. The time when this bulb was taken out was taken as zero time, unless stated otherwise. The other bulbs were then treated in the same manner after appropriate time intervals.

Table 2. Temp. 59.8°. $(a-x) = [(CH_3)_3CCBr(CO_2^-)_2]$ and $(b-x) = [OH^-]$ expressed in ml 0.0264 N HCl per 5 ml sample.

Time (min)	0	3	6	9	12	16	20	30
$(a-x)$	10.50	5.83	4.23	3.33	2.54	1.74	1.32	0.79
$(b-x)$	19.82	15.05	13.45	12.55	11.76	10.96	10.54	10.01
$k_1 \cdot 10^3$ (sec^{-1})		3.27	2.53	2.13	1.97	1.71	1.15	0.75
$k_2 \cdot 10^2$ (sec^{-1} $gmol^{-1}$ l)		4.50	2.97	2.63	2.56	2.58	2.47	2.17

Table 3. Temp. 48.2°. $[(CH_3)_3CCBr(CO_2^-)_2]$ expressed in ml 0.0244 N NH_4CNS per 5 ml sample.

Time (min)	0	2	4	5	6	8	10
$[(CH_3)_3CCBr(CO_2^-)_2]$	9.63	5.45	3.24	2.50	2.13	1.09	0.53
$k_1 \cdot 10^3$ (sec^{-1})		4.73	4.54	4.42	4.19	4.54	4.83
					Mean 4.59		

By extrapolation from the figures of Table 3 a time of 13 min. can be derived at as the time all the $(CH_3)_3CCBr(CO_2^-)_2$ has disappeared. This time is in the following three runs taken as zero time.

Table 4. Temp 48.2°. $(a-x)$ and $(b-x)$ expressed in ml 0.0268 N HCl per 5 ml sample. Initial $[NaOH] = 0.075$ N.

Time (min)	0	7	15	25	35	45
$(a-x)$	5.11	4.19	3.68	2.90	2.48	2.04
$(b-x)$	8.61	7.69	7.18	6.40	5.98	5.54
$k_1 \cdot 10^4$ (sec^{-1})		4.73	3.64	3.78	3.44	3.40
						Mean 3.80
$k_2 \cdot 10^3$ ($sec^{-1}gmol^{-1}$ l)		10.85	8.69	9.60	9.11	9.43
						Mean 9.54

Table 5. The same, but initial $[NaOH] = 0.11$ N.

Time (min)	0	6	12	18	24	30	38	46	54
$(a-x)$	5.01	4.17	3.35	2.78	2.43	2.01	1.60	1.39	1.09
$(b-x)$	15.55	14.71	13.89	13.32	12.97	12.55	12.14	11.93	11.63
$k_1 \cdot 10^4$ (sec^{-1})		5.10	5.59	5.45	5.02	5.07	5.01	4.65	4.70
							Mean 5.07		
$k_2 \cdot 10^3$ ($sec^{-1}gmol^{-1}$ l)		6.30	7.13	7.12	6.67	6.87	6.91	6.52	6.76
							Mean 6.79		

Table 6. The same, but initial [NaOH] = 0.19 N.

Time (min)	0	7	15	25	35	45	55
$(a-x)$	4.41	2.77	2.25	1.37	0.96	0.73	0.36
$(b-x)$	29.56	27.92	27.40	26.52	26.11	25.88	25.51
$k_1 \cdot 10^4$ (sec^{-1})		(11.07)	7.31	7.91	7.26	6.66	7.60
						Mean	7.35
$k_2 \cdot 10^3$ ($sec^{-1}gmol^{-1}$ l)		(7.21)	4.92	5.25	4.95	4.58	5.30
						Mean	5.00

b) *Hydrolysis products.* The experiments, the results of which are given in Table 1, were made in the following way. An appropriate amount of the acid was dissolved in a potassium hydroxide solution of known concentration. This was heated in a flask provided with a reflux condenser at 50° for three hours. The content of the flask was after cooling neutralised and distilled until no more acetone came over. In the distillate the acetone was determined by titration with iodine [16]. The acetone was further identified as its 2,4-dinitrophenylhydrazone, m.p. 120°. Mixed with authentic acetone 2,4-dinitrophenylhydrazone of m.p. 121—123° it had m.p. 120—122°.

The solution from which the acetone had been removed was, when necessary, further evaporated till a volume of about 40 ml. This was then acidified, whereby trimethylacrylic acid precipitated. One extraction of the mother liquor with ether gave a further amount. After recrystallisation from dilute alcohol it had m.p. 69—70°. No depression when mixed with an authentic sample. (Found: C 63.25; H 8.8; Eq.wt. 115.1; $C_6H_{10}O_2$ requires C 63.1; H 8.8; Eq.wt. 114.1).

The solution remaining after the separation of trimethylacrylic acid was carefully evaporated to dryness and the residue was extracted with ether. Evaporation of the ether gave methylmalonic acid. After recrystallisation from light petroleum it had m.p. 130—132°, undepressed when mixed with an authentic sample. (Found: C 40.8; H 5.2; Eq. wt. 59.3; $C_4H_6O_4$ requires C 40.7; H 5.1; Eq.wt. 59.0).

Methyl-1-chloroisopropylmalonic acid. Methyl*iso*propylmalonic acid (3.0 g) and sulphuryl chloride (2.55 g) were dissolved in benzene (20 ml). Benzoyl peroxide (23 mg) was added as catalyst, and the mixture heated on a steam bath for 9 hours. The benzene was then evaporated, first on a steam bath and finally in vacuum. The residue (3.5 g) was recrystallised from ether-light petroleum or ether-benzene giving a substance of m.p. 170°. The yield of pure product was very poor, only 0.3 g. (Found: C 43.5; 5.8; $C_7H_{11}O_4Cl$ requires C 43.2; H 5.7).

The hydrolysis of this acid was carried out in the same way as described for α-bromo-*t*-butylmalonic acid, using a molar proportion of alkali to acid of 6.5 : 1, the concentration of the free alkali being 0.6 *N*. The products of hydrolysis were isolated and identified as before.

α-Bromo-t-butylacetic acid. This acid was prepared according to Homeyer, Whitmore and Wallingford [14]. It had m.p. 72°. (Lit. 72—73° [14]). 2.5 g was dissolved in 0.1 *N* sodium hydroxide solution (250 ml) and heated on a steam bath for 4 hours. The solution was neutralised and evaporated to a small volume (No acetone could be detected in the distillate). It was then acidified with hydrochloric acid and evaporated to dryness in a vacuum desiccator over sodium hydroxide. The residue was extracted with ether and the ether evaporated leaving 1.4 g (82 %) of β,β,β-trimethyllactic acid. After recrystallisation from chloroform it had m.p. 87—88°. (Lit. 87—88° [17], 90—91° [18], 86.5—87.5° [19].) (Found: C 54.5; H 9.2; Eq.wt. 132.4; $C_5H_{12}O_3$ requires C 54.5; H 9.15; Eq.wt. 132.2.) The isomeric, also possible hydrolysis product, α,β-dimethyl-β-hydroxybutyric acid is an oil [20,21].

SUMMARY

Hydrolysis of α-bromo-*t*-butylmalonic acid in alkaline solution gives trimethylacrylic acid, methylmalonic acid and acetone. The mechanism of this reaction is discussed.

A part of this investigation has been carried out at the University College, London. One of the present authors (J. G.) wants to express his gratitude to the *British Council* for a Grant-in-Aid, which made this visit possible. He wishes also to thank Professor C. K. Ingold for the hospitality of the Laboratory, and for his help, given in discussions during the stay in London and in correspondence later on.

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Note on the Preparation of γ-Pyridone

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König and Greiner's method for the preparation of γ-pyridone from pyridine was thoroughly checked. It is superior to the method by Robinson and Thornley both with regard to time spent and amount of material used. A procedure giving analytically pure γ-pyridone in a 20 per cent yield from pyridine is described. Unsuccessful attempts to prepare the intermediate N-(4-pyridyl)-pyridinium chloride hydrochloride in a state of high purity are reported.

In connection with the preparation of 4-deutero-pyridine (I) some 4-substituted pyridine derivative had to be produced. Since (I) may be prepared from 4-chloro-pyridine which can be made from γ-pyridone (II) [1] a laboratory method for the preparation of (II) was looked for.

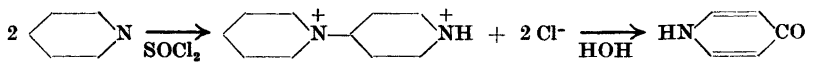
EXPERIMENTAL PART

1°. Robinson and Thornley's method [2] (compare King and Ware [3])

Chelidonic acid was satisfactorily prepared according to "Organic Synthesis"[4]. Its conversion to chelidamic acid[2] was likewise successful. 60 g chelidamic acid heated to 260° C gave 30 g white, but impure γ-pyridone (m.p. 122—5° C). The purification of γ-pyridone so produced has not been described. By conversion to hydrate, extraction of the hydrate with hot chloroform a.s.o. as described under 3°, 10 g γ-pyridone of highest quality (m.p. 150—1° C) and an additional quantity of 5—6 g impure compound (m.p. 140—43° C) was obtained. Total yield: 50 % based on chelidamic acid. King and Ware[3] reported a 95 % yield but gave no criterion of the purity. If this series of preparations is started from 430 g diethyl oxalate and acetone, 20—30 g γ-pyridone can be prepared in 5 working-days.

2°. König and Greiner's method [5]

The route to γ-pyridone found by König and Greiner is as follows:



(III) (II)

First, it was attempted to prepare *pure* N-(4-pyridyl)-pyridinium chloride hydrochloride (III) in satisfactory yield. All the attempts were fruitless. The amount of crystalline material collected according to König and Greiner definitely increased without change in quality by letting pyridine react at room temperature with thionyl chloride for three days instead of boiling the mixture for a shorter period. Well-dried pyridine was applied in all experiments but aside from this the use of high-grade pyridine and thionyl chloride had almost no effect on the yield. In a typical example, 165 g pyridine gave 145 g impure (III), dried *in vacuo* over conc. sulfuric acid. The chlorine equivalent weight of the light-brown product was 123—5 (theory: 114.6). It decomposed on heating. When 144 g of this crude product was dissolved in hydrochloric acid and treated for one hour on a steam bath with active carbon, 108 g of almost white, crystalline material (IV) was obtained after evaporation of the water and addition of alcohol as described by König and Greiner. By this procedure the chlorine equivalent weight was improved to 116—8 but still no real melting point was found contrary to the findings of König and Greiner. The crystals, heated in a stream of nitrogen, began to decompose at 170—80° forming a liquid and a white solid which were both deposited on the cold parts of the flask, and leaving a coloured solid on the hot bottom (the heating was carried out by immersing the flask in an oil-bath). Not even a trace of HCl was hereby liberated.

We were never able to obtain the two modifications of (III), mentioned by König and Greiner, neither were we able to prepare crystals with a satisfactory content of C, N, and H as reported by König and Greiner. (Found for (IV): C 49.4; N 12.3; H 4.8. Calc. for $C_{10}H_{10}N_2Cl_2$ (229.2): C 52.4; N 12.2; H 4.4). The ultraviolet absorption spectrum had two pronounced peaks at 2 330 and 2 640 Å. At these wave-lengths an aqueous solution of (IV), containing 0.01003 g per litre, had the optical density 0.327 and 0.288, respectively. (Cell-length 10 mm).

In a series of attempts to purify (IV) the chlorine equivalent weight, the C, N, and H contents and the ultraviolet absorption spectrum were constantly checked but no method giving rise to obvious improvements was found (recrystallization from methanol; ethanol; a mixture of ethanol and conc. hydrochloric acid; precipitation from methanolic solutions by ethyl ether; repeated treatments with hydrochloric acid and active carbon). Therefore, it is irrational to try to purify (IV) by any of these methods. In connection with the preparation of pyridone König and Greiner themselves recommend to omit isolation of purified (III) but their paper leaves the impression that this is a matter of convenience and not a matter of rational method.

König and Greiner report that an aqueous solution of crude (III) can be hydrolyzed to yield 70 g crude γ-pyridone. The result of their subsequent purification processes was not reported. After several experiments the procedure given under 3° was adopted for laboratory preparation of γ-pyridone.

3°. Laboratory preparation of γ-pyridone from pyridine

300 ml thionyl chloride (technical grade) was added drop by drop at room temperature to 170 ml pyridine (dry) placed in a 1 l three-necked flask with separatory funnel (for $SOCl_2$), thermometer, and an exit calcium chloride tube. The addition of the acid chloride was regulated so that the temperature was kept below 50° C. After 72 hours at room temperature during which the reaction mixture slowly turned brown, the excess thionyl chloride was evaporated *in vacuo*, the reaction flask being immersed in a water bath which was gradually heated from 50—100° in the course of 1—2 hours. The solid contents of the flask was afterwards cooled to 0° C and a freshly prepared mixture of 170 ml abs. alcohol and 17 ml conc. hydrochloric acid (0°) was added. The crystals were carefully crushed and the whole mixture left at 0° C for 30 minutes. Then the crystals were separated by suction on a sintered glass-filter and afterwards washed three times with 50 ml of the above-mentioned mixture of alcohol and conc. hydrochloric acid. The last traces of alcohol were removed by washing with dry ether and subsequent suction. The yield was 145 g crude (III), dried *in vacuo* over conc. sulfuric acid.

The whole quantity was dissolved in 250 ml water and the solution filtered through paper on a Büchner-funnel. The filtrate was heated on a steam-bath for 1 hour with 20 g active carbon and afterwards filtered through filter-paper on a Büchner-funnel. The

almost colourless solution was now boiled for 72 hours in a round-bottomed flask with reflux condenser. After neutralization of the acid formed (by means of approximately 85 g anhydrous sodium carbonate) *all* the water was removed by destillation *in vacuo*. To help remove the water completely and to loosen the crystalline mixture from the flask 500 ml dry chloroform was added and afterwards again removed by destillation *in vacuo*. It was now generally easy to get the solid material out of the flask. In an extraction apparatus γ-pyridone was now separated from inorganic material by boiling dry chloroform. The extraction lasted 24 hours. After cooling of the resulting, brown chloroform solution (300 ml) crude γ-pyridone was precipitated by addition of an excess of ethyl ether. After drying *in vacuo* over conc. sulfuric acid the product so obtained weighed 30 g (m. p. 133—40° C).

Purification of this quantity was accomplished by dissolving in 300 ml dry chloroform, filtering, and boiling the filtrate placed in a round-bottomed flask together with 90 % of the water necessary for the formation of the hydrate (5 g). On cooling, the hydrate crystallizes out *very* readily (which is not the case with γ-pyridone itself). In the chloroform the coloured impurities concentrate. The chloroform solution is removed by decantation, fresh chloroform is added to the hydrate and the mixture is again boiled, shaken and cooled, after which the chloroform is decanted. By sufficient repetition of this procedure (3—4 times further) the colourless hydrate of γ-pyridone is obtained. This is very easily reconverted to γ-pyridone by heating in a flask, immersed in boiling water and constantly being evacuated by the water aspirator. The almost colourless γ-pyridone so obtained weighs 20 g, representing a 20 % yield based on pyridine. Depending on the extent to which the hydrate has been purified the melting point lies at 147—151° C. (Analysis found: C 63.16; N 14.71; H 5.50. Calc. for C_5H_5NO: C 63.15; N 14.73; H 5.30.)

The saturated solution of γ-pyridone in chloroform contains 4 g per 100 ml solution at 20° C, 12—13 g at 61° C. In spite of this fact we have been unable to use recrystallization from chloroform as a purification procedure because of the pronounced tendency of γ-pyridone to form super-saturated solutions.

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On the Quantitative Determination of Solids by Means of Infrared Spectroscopy. Determination of Benzylpenicillin in Crystalline Sodium Salts

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M. Stimson and M. T. O'Donnel [1] in 1952 published an article on the preparation of discs of potassium bromide able to transmit infrared radiation by the compression at high pressure of finely ground potassium bromide. According to this method it should be possible to record the infrared spectrum of a substance in the solid state by mixing the substance with potassium bromide. Similar and more comprehensive experiments have been made independently by the above authors by U. Schiedt and H. Rheinwein [2].

Both the above mentioned articles deal only with qualitative evaluations of the spectra, but Schiedt and Rheinwein mention that the spectra may also be used for quantitative determinations. As the number of solvents which are suitable for absorption measurements in the infrared range is extremely limited [3], it is evident that every improvement of the experimental technique in connection with the recording of spectra of solids must be considered an important advance. The methods hitherto used, according to which the absorption measured is either that of a suspension of the substance in liquid paraffin or that of a thin film of the substance on a sodium chloride window, are not readily reproducible and consequently more suitable for qualitative determination.

The new method seemed to involve many advantages and was therefore subjected to a close study. Both the authors quoted employ a compression device which allows for the evacuation of the potassium bromide powder before and during the pressing. By means of such a device Schiedt and Rheinwein are able to prepare discs with a transmittancy of 90 % in the wavelength range 7—15 μ. For the purpose of the present work it was, unfortunately, not possible to work under similar conditions, and a simpler method was tried. A pressing device, as illustrated in Fig. 1, was made from steel and hardened to 60° Rockwell "C" hardness. The die was surrounded by a ring of mild steel to prevent accidents in case the die should burst. A hydraulic press was used to establish the high pressure, and after the pressing the resulting potassium bro-

mide disc was fitted in a suitable holder in the spectrograph for the purpose of the measurements. The spectrograph used was a Beckman I R. II spectrograph with sodium chloride prism and recorder. This simple procedure has also been tried by Schiedt and Rheinwein, who consider it to be unsatisfactory because the discs have a cloudy appearance, presumably on account of air inclusions.

In spite of the cloudy appearance the transmittancy of such discs is not less than 70—80 % within the range 7—15 μ.

As the intensity losses are due to reflection and scattering, it should be expected that the measured transmittancy would be dependent on the orientation of the disc in the spectrograph, an assumption which has been confirmed by experiments. Experiments proved, however, that the transmittancy for a certain orientation of the disc is constant within the range 7—15 μ. From this it will be seen that it will be impossible to prepare blank discs for quantitative measurements, while it will be possible to use the base-line technique proposed by Wright [4], thus eliminating the necessity of a blank value.

By means of the above described simple pressing technique it has proved possible to verify most of Schiedt and Rheinwein's results. Out of several alkali halogenides potassium bromide thus appeared to be the most suitable. By way of example it may be mentioned that the transmittancies of two discs of sodium chloride and potassium bromide — prepared under identical conditions — were 30—40 % and 65—75 %, respectively. It was also found that fine grinding and effective drying are factors which increase the transmittancy of the discs. For the subsequent experiments the substance used was micronized potassium bromide a.g., Merck, which after the micronizing was dried for 24 hours at 110° C.

The aim of the first experiments was to investigate the influence of the pressure used for preparing the discs on the transmittancy. Discs of 0.5 g with a diameter of 16 mm were prepared at different pressures, ranging from 25 tons to 45 tons. The period through which the pressure acted was in all cases 2 minutes. On examination it proved impossible to measure any significant differences in the transmittancy of the discs.

The aim of the following experiments was to investigate whether the duration of the compression had any influence on the transmittancy. Discs were therefore prepared at a pressure of 25 tons and with the same weight and diameter as mentioned above, but the period of compression varied from 15 seconds to 10 minutes. A period of 1 minute proved to be sufficient to produce maximum transmittancy, for which reason a pressure of 25 tons and a period of compression of 1 minute were subsequently considered to produce optimum conditions.

The discs resulting from this procedure have — as mentioned above — a transmittancy of 70—80 % within the range 7—15 μ.

DETERMINATION OF BENZYLPENICILLIN

The determination of benzylpenicillin in crystalline sodium salts is an example of the application of this method for a quantitative purpose. Two modifications of a method for the determination of benzylpenicillin in crystalline salts by means of infrared spectroscopy have been described in the litera-

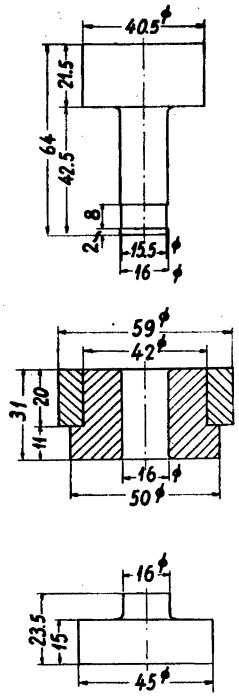

Fig. 1. Pressing device.

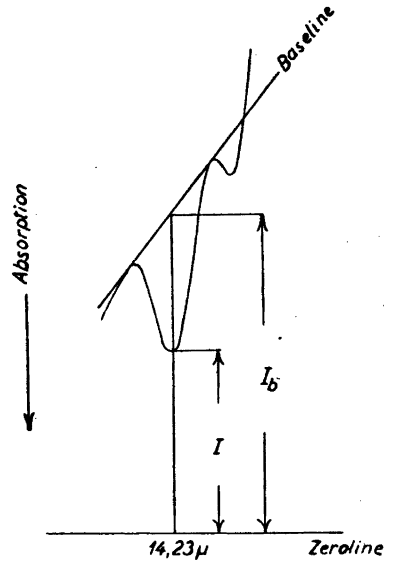

Fig. 2. Determination of base-line extinction
Base-line extinction $= \log I_b/I$.

ture by Barnes and coworkers [5] and E. A. Garlock jr. and D. C. Grove [6], respectively.

Both modifications are based on measurements of suspensions of penicillin in liquid paraffin, and both utilize the absorption band with maximum at 14.23 μ which is characteristic of benzylpenicillin. The difference between the procedures lies in the different ways in which the thickness of the layer is eliminated. Barnes *et al.* apply the internal standard technique using D,L-alanine as internal standard. This procedure, however, requires that a constant ratio always be maintained between the amount of substance weighed out for the analysis and the amount of D,L-alanine used. Garlock and Grove use a cell which ensures constant thickness of the layer. None of the methods are quite satisfactory, and the author's own experiments show that analyses according to both of them must be made with the greatest care and by an experienced analyst to give reliable results.

When applying the new technique, the thickness of the layer may be neglected as it is possible to weigh the finished disc.

Lambert-Beer's law applies in the following form:

$$\log \frac{I_0}{I} = E \,.\, c \,.\, l \qquad \text{(I)}$$

where I_0 is the incident energy, I the energy which has passed through the disc, while E is a constant, l the thickness of the layer and c the concentration.

If F denotes the area of the disc and a is an expression of the quantity of substance uniformly distributed throughout the disc, the following equation will apply:

$$c = \frac{a}{F \cdot l} \qquad \text{(II)}$$

Substituting this in (I), we obtain

$$\log \frac{I_0}{I} = \frac{E \cdot a}{\mathrm{F}} = K \cdot a$$

As the press used is always the same, K is a constant.

If a mixture of sodium benzylpenicillin and potassium bromide of known composition is prepared, it should be possible to obtain a straight line relationship between the weight of the discs prepared from this mixture and the extinction, that is, a standard curve.

It has already been mentioned that it is impossible to prepare a blank disc, for which reason the extinction must be replaced by the base-line extinction. The meaning of this term appears in Fig. 2 which shows the infrared spectrum of sodium benzylpenicillin in the range about 14.2 μ as produced by the recorder of the spectrograph.

For the present experiments a crystalline sodium salt was used which fulfilled the requirements of the Danish Pharmacopoea as to identity and purity [7]. The content of benzylpenicillin was found to be 98 % according to the N-ethylpiperidine precipitation method. 249 mg of this salt was ground carefully in a mortar and mixed with 50.00 g micronized potassium bromide, a. g. Merck. Samples of 0.2—1.0 g of this mixture were now subjected to a pressure of 25 tons for 1 minute. The absorption of the resulting discs was measured at once in the range 13.8—14.8 μ; slit and amplification being chosen once and for all so as to cause the recorder to give full deflection at 13.8 μ. After the recording of the spectrum, the disc was weighed on an analytical balance to obtain an expression of the quantity of substance involved.

Fig. 3. illustrates the results of a series of measurements. The base-line extinction is in this case plotted against the weight of the disc. It can be seen from this graph that the relationship is only linear up to about 0.6 g of mixture, after this value the curve flattens out considerably. The reason is that the absorption exhibited by the heaviest discs is too great to allow for a reasonably accurate determination of the base-line extinction. Experiments now showed that by using a mixture containing only half the amount of sodium benzylpenicillin (0.25 %) it was possible to obtain the same standard curve when applying discs of twice the original weight.

The following method was used for a series of analyses: 0.0500 g of substance was finely ground and mixed thoroughly with 10.00 g of micronized potassium bromide, a. g. Merck. When applying a micro balance, it is possible to use quantities only one tenth of those mentioned. From this mixture discs with a weight of 0.3—0.5 g were prepared as described above. The spectrum was recorded under standard conditions, and the base-line extinction at 14.23 μ

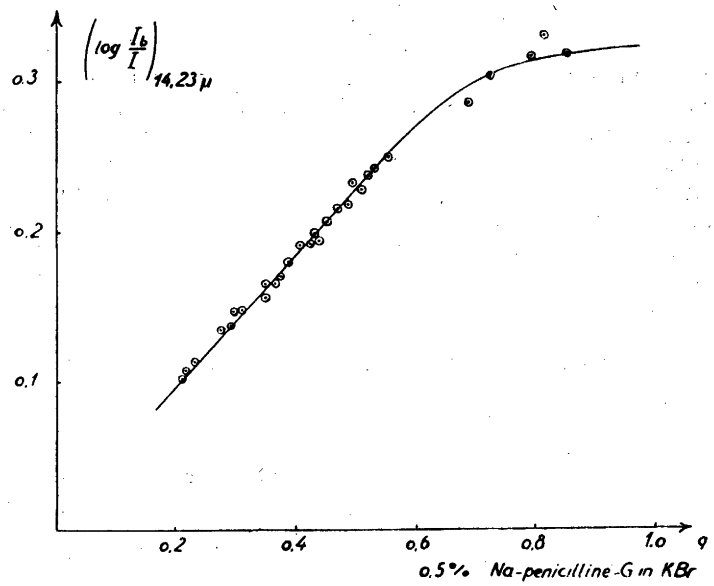

Fig. 3. Standard curve for the determination of benzylpenicillin.

was determined. When the weight of the disc has been determined, the necessary data are available for the calculation by means of the standard curve of the content of benzylpenicillin. A series of determinations made on the same salt sample (Table 1) illustrates the reproducibility of the method.

Table 1. Repeated determinations on the same salt sample.

84.3 %	benzylpenicillin
87.6 %	
83.4 %	
87.2 %	
88.7 %	
Mean value 86.2 %	

Four salt samples of different origin were analysed, according to the precipitation method described in the Danish Pharmacopoea and also according to the method described in the present work. A comparison of the results is given in Table 2.

Table 2.

Salt sample no.	N. E. P.	I. R.	Diff.
1	95.7 % G	96.2 % G	−0.5 %
2	90.3 % —	93.1 % —	−2.8 %
3	90.1 % —	89.0 % —	+1.1 %
4	87.6 % —	86.2 % —	+1.4 %

COMMENTS

Barnes *et al.*[5] have given a thorough discussion of the sources of error which may be the result of absorption bands near 14.2 μ due to interfering substances. It is consequently necessary to examine the whole spectrum qualitatively in order to exclude the possibility of interference from impurities.

The grinding of the sample to be analyzed has been performed in a mortar before the admixture of potassium bromide. Since the absorption is to some extent dependent on the particle size [8], it is essential to perform the grinding carefully so as to obtain uniform particle size. Schiedt and Rheinwein suggest solution of the sample in alcohol followed by admixture of a known quantity of potassium bromide and evaporation in vacuum of the alcohol. This method may perhaps more readily produce a uniform mixture of the two substances, but it is doubtful whether the particle size obtained is uniform from time to time. As sodium penicillin is rather labile, this method has not been taken into consideration for the purpose of the present work.

In comparison with the usual N-ethylpiperidine method the method suggested in the above is distinguished by being rapid and just as reliable, the analytical error being about 3 %. In comparison with previously used methods based on infrared spectroscopy the method is more readily reproducible, because the elimination of the thickness of the layer does not require special measures.

SUMMARY

Compression at high pressure of finely ground potassium bromide results in discs with 70—80 % transmittancy in the wavelength range 7—15 μ. The addition of substances to the bromide enables recordings of the infrared spectra of such substances in the solid state. The spectra recorded may be utilized for quantitative determinations when applying the base-line technique proposed by Wright. On this basis a method for the determination of benzylpenicillin in crystalline sodium salts has been developed and described in the present work, the measurements being referred to the specific maximum at 14.23 μ which is characteristic of benzylpenicillin.

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Protein Studies of Endolymph and Perilymph of the Inner Ear

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The amount of total protein in the endo- and perilymph of sharks has been estimated at 1.8 and 1.2 % respectively. It is found that the endolymph contains α, β and γ globulins, but no albumin, and that the perilymph contains both α, β and γ globulins and albumin.

A study of the literature shows that our knowledge concerning the chemical composition of the liquids of the inner ear (the labyrinth) is very small and rather fragmentary. As a part of a general investigation on these fluids we report here the determination of total protein and the ratios of the various protein fractions. The reason why sharks (*Acanthias vulgaris*) are employed is mentioned in a recent paper by one of the authors [1].

DETERMINATION OF PROTEIN

The amount of total protein was determined by precipitation partly with 1 volume of 20 % trichloroacetic acid, partly with 1 volume of absolute alcohol at —10° C just after withdrawal of the fluids. The trichloroacetic acid was removed from the precipitates by extraction with ether. All protein precipitates were dried to constant weight *in vacuo* over silica gel and phosphorus pentoxide. The precipitate obtained by addition of alcohol was soluble in water. The results are in Table 1.

Table 1. The protein content of endolymph and perilymph and the nitrogen content of these proteins.

Fluid	Precipitating agent	% Protein	% N in the precipitates	
Endolymph	CCl_3COOH	1.76	15.27 *	15.30 *
	C_2H_5OH	1.71	15.02 *	
Perilymph	CCl_3COOH	1.18	13.98 *	13.35 **
	C_2H_5OH	1.11	13.27 **	

* Determined by C. E. J. according to Kjeldahl modified by Blom [3].
** Determined at the University Institute of Chemistry, Copenhagen.

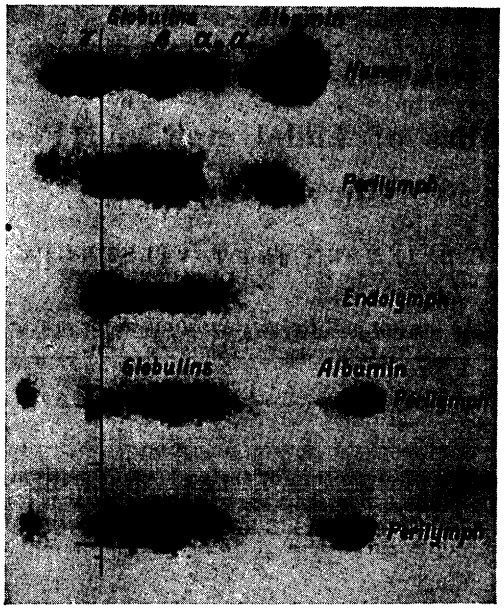

Fig. 1. The electrophorogrammes of endolymph and perilymph. The vertical line indicates the spots where the applications of the various samples have taken place. The separation of albumin from the globulins is evident in all perilymph electrophorogrammes. Only in lowest diagram (perilymph) a fair separation of the α, β and γ globulins is observed.

In the use of these and other reagents precipitating proteins it must be borne in mind that certain of the reagents are better precipitating agents in some instances than in others depending upon the particular type of protein that is to be precipitated.

In order to verify the protein nature of the precipitates the nitrogen content was determined. The results are in Table 1. The precipitates also give the ordinary reactions for protein *e. g.* the biuret test, the test for sulphur and Millons test.

PAPER ELECTROPHORESIS OF THE ENDOLYMPH AND THE PERILYMPH

On account of the scantiness of the fluids we had to make use of a micro-method to separate the proteins in the endolymph and the perilymph. We chose the procedure of paper electrophoresis which is a typical micro-method, requiring only very small quantities for analysis (5—50 μl).

Immediately prior to the electrophoresis the endolymph was liquified by a trace of hyaluronidase, as described in a recent paper by the present authors [2]. Thus the sample for analysis acquired a consistency suitable for the procedure of electrophoresis. The perilymph was used in its natural state. A sample of 25 μl is applicated as a spot on a 15 × 35 cm buffered filter paper (Whatman

No. 3). The buffer is a barbituric buffer pH 8.6: 0.05 *M* sodium diethylbarbiturate (10.3 g per litre) 0.01 *M* diethylbarbituric acid (1.84 g per litre); ionic strength 0.05. A potential gradient of 3 V/cm is applied across the paper for 24 hours in a closed chamber.

The strip is dried, and the various protein fractions stained by immersing the strip for 5 minutes in 1 % bromophenol blue in saturated alcoholic mercuric chloride solution. The mercuric chloride fixes the proteins in the paper strip. For further fixation the strip is then placed in a 1 % solution of mercuric chloride for 15 minutes. After washing for 10 minutes in methanol, 10 minutes in ethanol and again in methanol for 3 minutes, the strip is dried. Fig. 1 shows the electrophorogrammes with the electrophorogram of human serum as reference. At our selected pH (8.6) all the protein fractions are negatively charged, and so it could be expected that all the protein particles would migrate in the same direction (towards the positive pole). The apparently anomalous behaviour of the γ globulin in moving towards the negative pole is caused by a displacement of the entire protein pattern towards the negative pole as a result of an electroendosmotic flow in this direction.

The intensity of the staining together with the area covered by the dye may be used for exact estimation of the relative ratios of protein components in the sample examined, provided care is taken of the fact that albumin binds relatively more dye than do the globulins. However, if the extinctions for the globulin-bound amounts of dye are multiplied by a factor 1.6 we have a relatively good measure of the ratio of albumin/globulin.

This ratio was found to be zero for the endolymph and roughly 21/79 for the perilymph (for further details see Köiw [3]) The average figure for normal human serum is 70/30 (we found 75/25).

We wish to thank V. Kornerup, M. D., the Finsen Institute, Copenhagen, for valuable collaboration in performing the electrophoresis. Our thank is due to Professor J. A. Christiansen for his interest in this investigation.

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ACTA CHEMICA SCANDINAVICA 8 (1954) 402—406

On the Theory of Non-Isothermal Systems

HANS HOLTAN JR.*

Recently the thermodynamics of irreversible processes [1] has been applied to thermocells [2-4]. For the homogeneous potential difference the following expression was obtained

$$F \operatorname{grad} \varphi = -\sum_{K=1}^{n} (t_K/z_K) (\operatorname{grad} \mu_K)_{P,T} - \sum_{K=1}^{n} (t_K/z_K) Q_K^* (\operatorname{grad} T)/T \quad (1)$$

where F is Faraday's number, φ the electric potential, t_k and z_k the transference number and charge of component k respectively; μ_k is the chemical potential of component k expressed in mole units, and Q^* is the molar heat of transfer of component k, *i. e.* the heat transported with one mole of component k at uniform temperature, and T is the absolute temperature.

An expression quite analogous with (1) may easily be derived by means of the *principle of thermal interaction* which originally was introduced by the author [5] as an alternative to the Eastman-Wagner [6,7] theory. In the language of Brönsted's energetics [8,9] the principle states that a "loss of work" connected to the movement of matter may be compensated partly or completely by the transport of free entropy in the opposite direction.

Consider a transport of one mole of component k from a region of a conductor of temperature T, pressure P, electric potential φ and chemical potential μ to a region of temperature $T + \mathrm{d}T$, pressure $P + \mathrm{d}P$, electric potential $\varphi + \mathrm{d}\varphi$ and chemical potential $\mu_k + \mathrm{d}\mu_k$. Such a transport is accompanied by the "loss of work" (loss of potential energy):

$$\sum \partial A = -S_k \mathrm{d}T - z_k F \mathrm{d}\varphi + V_k \mathrm{d}P - \mathrm{d}\mu_k + S_k''' \mathrm{d}T \quad (2)$$

where ΣA is the total "loss of work", S_k and V_k are the transported entropy and volume respectively and S_k''' the compensated entropy of component k. The term $S_k'''\mathrm{d}T$ takes care of the interaction between the flow of matter and the flow of heat which takes place at all times in a system where temperature gradients are present.

In the stationary state

$$\sum \partial A = 0. \quad (3)$$

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As we have

$$\mathrm{d}\,\mu_K = (\mathrm{d}\mu_K)_{P,T} + V_K\,\mathrm{d}\,P - S_K\,\mathrm{d}\,T \tag{4}$$

we obtain from (2) the following equation in the stationary state when multiplying both sides of the equation with t_k and summing from $k = 1$ to $k = n$:

$$F\,\mathrm{d}\,\varphi = -\sum_{K=1}^{n} \frac{t_K}{z_K}\,(\mathrm{d}\,\mu_K)_{P,T} + \sum_{K=1}^{n} \frac{t_K}{z_K}\,S_K'''\,\mathrm{d}\,T \tag{5}$$

We see that (5) is quite analogous with (1) and comparison shows that

$$S_K''' = -\,Q_K^*/T \tag{6}$$

As the expressions for the heterogeneous potential differences contain no interaction terms, the expression for the total thermopotential difference will also be quite analogous according to both methods. As shown by the author [2] this expression reads in cases where there are no concentration gradients:

$$F\,\Delta\varphi_t = -\sum_{K=1}^{n} (t_K/z_K)\,Q_K^*\,\Delta\,T/T - (Q_{el}^*/T)\,\Delta\,T - \Delta\,S\,\Delta\,T \tag{7}$$

where $\Delta\,\varphi_t$ is the resultant potential difference, Q_{el}^* and S_{el} the molar heat of transfer and the molar entropy of the electrons in the metallic part of the cell respectively, and $\Delta\,S$ the change of entropy accompanying the heterogeneous electrode reaction when one Faraday of electricity passes the interface.

The same expression is of course obtained by means of the principle of thermal interaction except for the fact that Q_{el}^* should be replaced by $-S_{el}'''\,T$, and Q_k^* by $-S_k'''\,T$ according to (6).

As shown by the author [2] it is of advantage to split up the expression (7) in the following way

$$F\,\Delta\,\varphi_t = -\sum_{K=1}^{n} (t_K/z_K)\,(Q_K^* + T\,S_K)/T - (Q_{el}^* + T\,S_{el})/T - \Delta\,S + \sum_{K=1}^{n} (t_K/z_K)S_K + S_{el} \tag{8}$$

The first part (the two first terms on the right hand side) contains heats of transfer, which are kinetic quantities, while the second part (the three last terms on the right-hand side) consists of well known quantities.

A number of calculations of the second part have been performed by the author [2]. It is remarkable that for thermocells with solid electrolyte the calculated values agree with the experimental ones [10–12], which means that the first part of the thermocell expression is negligible in these cases.

Considering (8) we see that the first term on the right hand side disappears if

$$Q_K^* = -\,T\,S_K \tag{9}$$

or if, *cf.* (6)

$$S_k''' = S_k \tag{10}$$

Therefore, if the compensated entropy of component k is equal to the total entropy of component k ("full compensation"), the first term vanishes.

As the second term is small in any case (the thermopotentials in metallic thermocouples are of a smaller order of magnitude than in electrolytic cells), the first part will practically vanish when (9) or (10) are fulfilled, and the expression for the thermopotential becomes purely thermostatic. This thermostatic expression for the potential in thermocells with solid electrolyte has formerly been derived by the author [5] by means of the principle of thermal interaction by introducing (10) from the beginning. This procedure is not quite logical and it had been better to derive the general expression for the thermopotential difference first, as has been done here. From the fact that calculations of the thermostatic part of the potential difference for thermocells with solid electrolyte show agreement with experimental results, one should be careful not to conclude that the compensation is complete also in other cases, or in other words that stationary states in general can be described by means of thermostatic variables alone. This fact has been stressed by Mazur [13].

The compensated entropy is in fact a kinetic quantity and it is a good approximation to replace it by the thermostatic entropy only in special cases, and the introduction of the compensated entropy in relation (2) is in reality analogous with the way of expressing the interaction by means of phenomenological relations as done in the general theory of irreversible processes [1]. The basis of this theory is formed by the Onsager relations, which permit a coupling of phenomena occurring in quite different situations. For instance the heat of transfer appearing in the expressions for the electrical potential differences in *non-isothermal* systems is given as the heat transported with the unit mass at *uniform temperature, i. e.* in a quite different situation.

The derivation of the homogeneous thermopotential difference is extremely simple when the principle of thermal interaction is applied. One arrives directly at expression (5) without the application of Onsager's relations of reciprocity. However, if we want to derive for instance relation (6) or the second Thomson relation we must of course apply the Onsager relations as is done in the general theory of irreversible processes. It is, however, not always necessary to compare with different situations, and the kinetic quantity, the compensated entropy may as well be calculated directly from kinetic theories as the related quantity, the heat of transfer, given in a different physical situation. The principle of thermal interaction is lacking in generality as it of course only applies to phenomena in non-isothermal systems, but it is probably possible to generalize the principle to include a wider range of phenomena.

It is of interest to consider the value of the compensated entropy in some extreme cases.

Consider first a uncharged gaseous system contained in two containers separated by a tube. The two containers are supposed to have the same volume, but the temperatures differ by $\mathrm{d}T$ and the pressures by $\mathrm{d}P$, respectively.

Since no net volume is transported equation (2) reads in this case for a transport of one mole of substance between the two containers [14]

$$\sum \delta A = -S_K \,\mathrm{d}T - \mathrm{d}\mu_K + S_K''' \,\mathrm{d}T \qquad (11)$$

We then get in the stationary state (*cf.* (3))

$$\mathrm{d}P/\mathrm{d}T = S_K'''/V_K \tag{12}$$

If the separation tube is very narrow (of dimensions comparable with the mean free path of the molecules of the gas) and the pressure low, we find by comparison with the Knudsen equation that

$$S_K''' = R/2 \tag{13}$$

We see that the energy of compensation $S_k''' T$ equals $RT/2$, which means that only one degree of translational motion is compensated for; a quite reasonable result for a substance where there are no forces acting between the particles. As shown by de Groot [1] the heat of transfer for this case can easily be derived from the kinetic gas theory.

If there were forces acting between the particles of the gas passing between the two containers, one expects a larger value for the compensated entropy.

Consider then another extreme case, *viz.* thermocells with solid salts. We have seen that the compensation is complete in this case. Thus, in this case where the particles are bound to their places by rather strong forces, practically all their entropy is compensated for.

For intermediate cases we will expect values of S_k''' between $R/2$ and S_k, and therefore, for instance for thermocells with electrolytic solutions, the kinetic part of the thermopotential should not be of negligible magnitude, and the expression for the Soret effect in solutions should also be expected to contain a kinetic term, *i. e.* it can not be expressed in terms of thermostatic variables alone. A calculation of the compensated entropy or the heat of transfer in these cases must be based on a kinetic treatment taking the structure, the forces and the degrees of freedom into account.

The splitting up of the general thermocell equation as performed by the author [2] in applying the general theory of irreversible processes to thermocells is rather artificial and the result found that the kinetic part of the expression is of negligible magnitude when the electrolyte is a solid salt is surprising from this point of view. The result is more logical from the point of view of the principle of thermal interaction, and the author was actually lead to this splitting up of the thermopotential difference expression from his previous knowledge of the principle of thermal interaction.

Krogh-Moe [15] has recently indicated that the principle of thermal interaction is embodied in the principle of least dissipation of energy [16].

SUMMARY

The expression for the thermopotential difference in non-isothermal cells (thermocells) can be derived simply and logically by means of the principle of thermal interaction, and the results obtained have been shown to be consistent with those obtained by applying the general (but for this purpose more complicated) theory of irreversible processes.

A discussion of the two quantities, heat of transfer and compensated entropy has been put forward. The latter quantity is in reality introduced in a way analogous to the introduction of phenomenological relations in the thermodynamical theory of irreversible processes, and this fact explains why the two methods lead to consistent results.

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Die Verschiedenheit der durch Amylasen spaltbaren Bindungen der Kartoffelstärke

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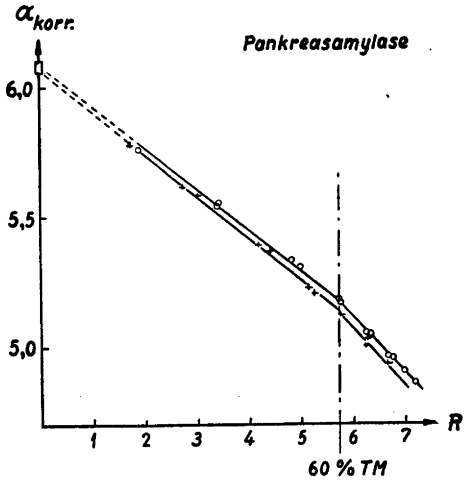
Der Abbau von Kartoffelstärkekleister durch Pankreas-, Subtilis- und Gersten-Amylasen wird durch gleichzeitige Messungen des Drehungs- und Reduktionsvermögens verfolgt. Die Änderung der Drehung per Mol gespaltener Bindungen, $[Sp]_D$, ist verschieden für die verschiedenen Amylasen und abhängig vom Abbaugrad. Dieses Resultat lässt sich nur dadurch erklären, dass Stärke verschiedene durch Amylasen spaltbare Bindungen enthält.

In der vorigen Arbeit [1] haben wir uns die Frage gestellt: »Welche Drehungsänderungen sind mit der Spaltung einer Glucosidbindung in der Stärke oder in Stärkeabbauprodukten verbunden, und zwar von Gleichgewichtszustand zu Gleichgewichtszustand gemessen.» Die Änderung der Drehung per Mol gespaltener Bindungen haben wir mit $[Sp]_D$ bezeichnet. Versuche über die Säurehydrolyse von Kartoffelstärkekleister ergaben, dass $[Sp]_D$ während der Hydrolyse nicht konstant ist. Von Punkt zu Punkt berechnet ist $[Sp]_D$ anfangs ca. $-160°$ und fällt während des Abbaus, um bei einem Abbau von ca. 60 % seinen minimalen Wert ca. $-280°$ zu erreichen.

In der vorliegenden Arbeit bringen wir Resultate von Untersuchungen über den Abbau von Kartoffelstärkekleister durch Amylasen. Der Stärkeabbau wurde bei 40° durchgeführt, und zwar bei pH 6—7 in Versuchen mit Pankreasamylase, bei pH 5,2 in Versuchen mit Subtilisamylase und Gerstenamylase. Aus dem Ansatz wurden in passenden Intervallen Proben entnommen, in denen die Enzymwirkung entweder durch Kochen oder durch Ansäuern auf pH 1—2 sistiert wurde. In den Proben wurden Reduktions- und Drehungsvermögen bestimmt. In einigen Serien wurde das Reduktionsvermögen mit einer Kupfermethode, in anderen Serien mit einer Jodmethode bestimmt. Das Drehungsvermögen ist erst nach einem Abbau von ca. 10 % TM bestimmbar, da die Stärkelösung vorher nicht klar genug ist. Die Resultate sind graphisch dargestellt. Das Reduktionsvermögen, R, in mmol reduzierender Zucker per 100,0 g Lösung berechnet, ist Abscisse; das Drehungsvermögen, α_{korr}, auf 1 dm-Rohr umgerechnet und mit der Dichte korrigiert, ist Ordinate.

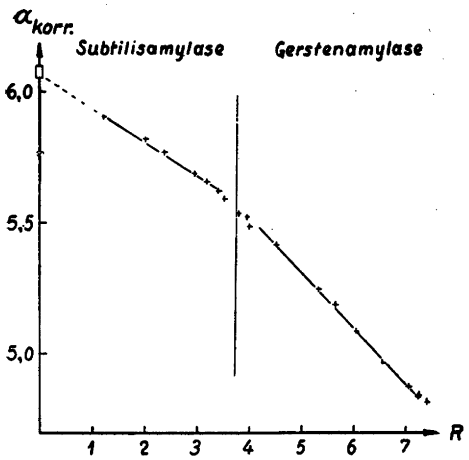


Figur 1. Abbau von Kartoffelstärke. Kleister 3,00 %, pH = 6—7, t = 40°.
R: mmol reduzierender Zucker per 100,0 g Lösung,
+ Kupfermethode, ○ Jodmethode.
α_{korr}: Drehung, korrigiert für Dichte, 1 dm-Rohr, D-Linie.
$[Sp]_D$: Kupfermethode, bis 60 % TM: −164°; nach 60 % TM: −216°. Jodmethode, bis 60 % TM: −159°; nach 60 % TM: −215°.

Die Resultate der Versuche mit Pankreasamylase sind in Figur 1 dargestellt. Die beiden Methoden zur Bestimmung des Reduktionsvermögens geben Kurven, die etwas gegen einander verschoben sind. Es ist uns nicht möglich gewesen einen plausiblen Grund dafür zu finden. Bis zu einem Abbaugrad von ca. 60 % TM, d.h. bis zu einer Spaltung von ca. 30 % aller Bindungen der Stärke, liegen die Punkte auf einer Geraden. Nach 60 % TM liegen die Punkte ebenfalls auf einer Geraden, die aber eine andere Neigung hat. Die Neigung der Geraden ist $[Sp]_D \cdot 10^{-3}$; sie wird nach der Methode der kleinsten Quadrate berechnet.

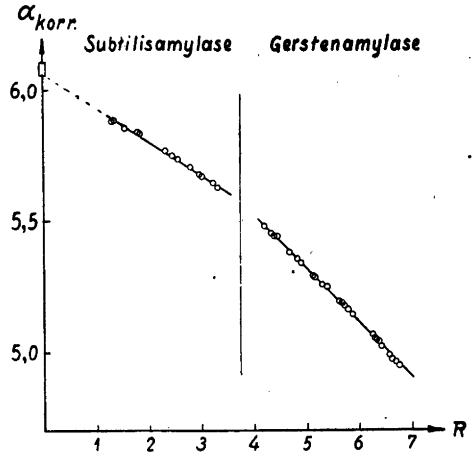
In Figur 2 und 3 sind die Resultate der Versuche mit Subtilisamylase und Gerstenamylase dargestellt. Im Gegensatz zu den Versuchen mit Pankreasamylase geben die beiden Methoden zur Bestimmung des Reduktionsvermögens Kurven, die sich decken, weshalb wir es für nötig gefunden haben, die Resultate getrennt wiederzugeben. Die linken Hälften der Figuren zeigen den Abbau durch Subtilisamylase, die rechten Hälften einen fortgesetzten Abbau durch Gerstenamylase, nachdem die Subtilisamylase den schnellen Teil ihres Angriffes durchgeführt hat und durch Kochen sistiert worden ist. Für jedes Enzym liegen die Punkte auf Geraden, deren Neigung und damit $[Sp]_D$ verschieden ist.

In Tabelle 1 haben wir die $[Sp]_D$-Werte zusammengestellt. Innerhalb der Fehlergrenzen sind die mittels der Kupfermethode und die mittels der Jodmethode gefundenen Werte paarweise gleich. Der Stärkeabbau durch Pankreasamylase gibt zwei $[Sp]_D$-Werte. Die Änderung tritt abrupt bei ca. 60 % TM ein und zwar gleichzeitig mit der Überschreitung des achroischen Punktes und mit der früher gefundenen grossen Geschwindigkeitsabnahme [2] des Stärkeabbaus. Der Abbau von Stärkekleister durch Subtilisamylase wird, wie früher gezeigt [3], bei einem Abbaugrad von 35—40 % TM gehemmt; die Abbauprodukte werden durch Gerstenamylase schnell gespalten. Der $[Sp]_D$-Wert für den Abbau von Stärkekleister durch Subtilisamylase ist verschieden von dem



Figur 2. Abbau von Kartoffelstärke. Kleister 3,00 %, pH = 5,2, t = 40°.

R: mmol reduzierender Zucker per 100,0 g Lösung, Kupfermethode.
α_{korr}: *Drehung, korrigiert für Dichte, 1 dm-Rohr, D-Linie.*
$[Sp]_D$: *Subtilisamylase: −129°. Gerstenamylase: −205°.*



Figur 3. Abbau von Kartoffelstärke. Kleister 3,00 %, pH = 5,2, t = 40°.

R: mmol reduzierender Zucker per 100,0 g Lösung, Jodmethode.
α_{korr}: *Drehung, korrigiert für Dichte, 1 dm-Rohr, D-Linie.*
$[Sp]_D$: *Subtilisamylase: −129°. Gerstenamylase: −204°.*

$[Sp]_D$-Wert für den weiteren Abbau der hinterlassenen Spaltprodukte durch Gerstenamylase.

Beim enzymatischen Abbau von Kartoffelstärkekleister erhält man also verschiedene [Sp]/-Werte, die innerhalb gewisser Abbaugrenzen konstant sind. Nach unserer Ansicht kann dieses Resultat nur dadurch erklärt werden, dass die Stärke verschiedene durch Amylasen spaltbare Bindungen enthält.

Nach der heute üblichen Auffassung enthält Stärke nur zwei Bindungstypen, nämlich 1α4-Bindungen und 1α6-Bindungen. Die 1α6-Bindungen sind gegen Amylasen resistent; Isomaltose, 6-(α-Glucosido)- Glucose, wurde ja nach intensiver Behandlung von Stärke mit Amylasen isoliert [4,5]. Hiernach können Amylasen nur 1α4-Bindungen spalten. Unter dieser Voraussetzung werden

Tabelle 1.

Amylase	Abbau % TM	Kupfermethode $[Sp]_D$	Jodmethode $[Sp]_D$
Pankreas-	10—60	−164°	−159°
Pankreas-	60—75	−216°	−215°
Subtilis-	10—35	−129°	−129°
Gersten- nach Subtilis-	45—70	−205°	−204°

wir gezwungen anzunehmen, dass die Bezeichnung 1α4-Bindung nicht genügt, um einen Bindungstypus zu karakterisieren.

Die Anwesenheit von 1α5-Bindungen in der Kartoffelstärke halten wir immer noch für möglich; die Anschauung von alternierenden 1α4- und 1α5-Bindungen in einem Teil der Stärke [1] haben wir jedoch verlassen.

METHODIK

Stärke. Kartoffelstärke. Superior Qualität.

Pankreasamylase. Aus frischem Schweinepankreas wird nach üblichen Methoden ein Dauerpräparat hergestellt.

Subtilisamylase. Eine 1-%ige Lösung eines Trockenpräparates von Subtilisamylase wird mit Phosphatpuffer auf pH 7,0 eingestellt und 15 Minuten auf 70° erwärmt.

Gerstenamylase. Ein Gerstenauszug wird durch Ansäuern (pH 3,8, 0°, 16 Stunden) von jeder Spur von α-Amylase befreit. Durch Fällung mit Tannin wird ein Dauerpräparat hergestellt.

Bestimmung des Drehungsvermögens. Polarisationsapparat von Schmidt und Haensch, Monochromator, D-Linie, 4 dm-Rohr, 19°–21°.

Bestimmung des Reduktionsvermögens. Kupfermethode: Kaliumkupferkarbonatmethode nach Blom–Rosted [6]. Jodmethode: Methode nach Willstätter–Schudel in der von uns beschriebenen Ausführung [1].

Stärkekleister. 15,00 g trockene, aschenfreie Stärke werden in einem, mit zugehörigem Propeller tarierten, 500 ml Weithals-Stehkolben abgewogen und durch Rühren mit einem schnell laufenden Motor in 400 ml Wasser aufgeschlämmt. Ohne das Rühren zu unterbrechen, wird der Kolben in ein siedendes Wasserbad getaucht und nach 15 Minuten in ein Wasserbad von *ca.* 40° überführt. Nach weiteren 15 Minuten wird das Rühren unterbrochen.

Abbau durch Pankreasamylase. a) (Oktober 1951). Der Kleister wird mit 10,0 ml 1 *M* KH_2PO_4, 10,0 ml 1 *M* Na_2HPO_4, 10,0 ml 1 *M* NaCl und 1,0 bis 10,0 ml einer 0,5-%igen Lösung von Pankreasamylase versetzt; der Inhalt des Kolbens wird auf 500,0 g mit Wasser aufgewogen und eine Minute gerührt. Der Kolben wird verschlossen in ein Wasserbad von 40° getaucht. Zu verschiedenen Zeitpunkten (15 Minuten bis 4 Stunden) werden Proben von ca. 100 g entnommen, die in verschlossenen Kolben 10–15 Minuten in einem siedenden Wasserbad erhitzt werden, um die Wirkung der Amylase zu sistieren. Nach Abkühlen werden die Lösungen durch 9 cm Filter, worauf ca. 75 mg Kieselgur angebracht sind, filtriert. In den letzten 25 ml der Filtrate werden Drehungsvermögen, Dichte und Reduktionsvermögen nach der Kupfermethode bestimmt.

b) (Juni 1952). Der Kleister wird mit 2,0 bis 30,0 ml einer 2-%igen Lösung von Pankreasamylase versetzt und eine Minute gerührt. Der Kolben wird verschlossen in ein Wasserbad von 40° getaucht. Zu verschiedenen Zeitpunkten (20 bis 160 Minuten) werden Proben von ca. 90 g entnommen und in tarierten Kolben, ca. 2 g 4 *N* HCl enthaltend, gegossen. Dadurch wird pH auf ca. 1 verschoben, um die Wirkung der Pankreasamylase zu sistieren. Durch Wiegen vor und nach den einzelnen Manipulationen wird genauer Kontakt mit der ursprünglichen Stärkemenge bewahrt. Nach Abkühlen und Filtrieren, wie unter a) beschrieben, werden in den letzten 25 ml der Filtrate Drehungsvermögen, Dichte und Reduktionsvermögen nach der Jodmethode bestimmt.

Abbau durch Subtilisamylase. Der Abbau wird in derselben Weise wie unter Pankreasamylase beschrieben durchgeführt, nur werden in allen Versuchen als Puffer 15,0 ml 1,2 *M* CH_3COONa und 5,0 ml 1,2 *M* CH_3COOH benutzt. Die Enzymmengen betragen 4,0 bis 15,0 ml einer 1-%igen Lösung von Subtilisamylase, und der Abbau dauert höchstens 200 Minuten. (a) Januar 1951, b) Mai 1952.)

Abbau der von Subtilisamylase hinterlassenen Spaltprodukte durch Gerstenamylase. Der Kleister wird mit Acetatpuffer und Subtilisamylase und evt. einigen Tropfen Toluol versetzt und eine Minute gerührt. Der Kolben wird verschlossen in ein Wasserbad von 40° getaucht. Nach passender Zeit (3 bis 18 Stunden) wird die Amylasewirkung durch Kochen sistiert. Während des ganzen Versuches wird durch Wiegen vor und nach den einzelnen Manipulationen genauer Kontakt mit der ursprünglichen Stärkemenge bewahrt.

a) (Februar 1951). Eine Probe wird entnommen und der Rest des Kolbeninhalts mit Gerstenamylase (3 bis 20 mg) versetzt. Der Abbau wird wie unter Pankreasamylase a) beschrieben durchgeführt. Gesamtdauer bis zu 4 Stunden.

b) (April 1953). Die gesamte Portion des durch Subtilisamylase abgebauten Kleisters wird durch ein 15 cm Filter, worauf ca. 200 mg Kieselgur angebracht sind, filtriert; die ersten 25 ml des Filtrates werden verworfen. Eine Probe wird entnommen, der Rest der klaren Lösung wird mit Gerstenamylase (8 bis 16 mg) versetzt und der Kolben in ein Wasserbad von 40° getaucht. Zu verschiedenen Zeitpunkten (10 bis 160 Minuten) werden Proben entnommen, worin die Wirkung der Amylase durch Ansäuern sistiert wird. Ohne Filtrieren werden die Lösungen wie unter Pankreasamylase b) beschrieben analysiert.

Blindwert. Zwecks Bestimmung der Blindwerte, sowohl des Drehungs- als des Reduktionsvermögens, werden in allen Fällen vollständige Parallelversuche ohne Stärke durchgeführt.

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Kinetics of the Exchange of Cadmium between Amalgam and Cadmium Salt Solutions at Equilibrium Potential. II. The Influence of Complex Formation

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In a previous paper by the present author [1] it was mentioned that the cadmium exchange reaction could be divided into different partial processes, *e. g.*, the electron exchange including the loosening and coordinating of ligands, and the transport of cadmium in both phases up to the interface. For the analogous zinc exchange Randles and Somerton [2] have shown by their impedance measurements that the rate of the electron exchange is influenced by halogenide ions, and for that reason it is to be expected that these ions influence the electron exchange for cadmium also, especially as the halogenide complexes of cadmium are much stronger than those of zinc (*cf.* Leden [3], Sillén and Liljeqvist [4]).

On the other hand it was proved in the previous investigation by the use of a radioactive isotope that in cadmium perchlorate solutions under the concentration conditions chosen the over-all exchange reaction at equilibrium potential difference between amalgam and solution was controlled by the diffusion of cadmium ions through the Nernst diffusion layer of the solution. Thus, as long as the same rate law is valid, the effect of complex formation upon the whole exchange reaction is determined by the difference in diffusibility of various complexes, and it is the aim of the present investigation to study this effect for some ligands that give complexes of medium strength with the cadmium ion.

THEORETICAL

In the previous investigation it was proved that the rate h of the cadmium exchange is proportional to the total cadmium concentration C of the perchlorate solutions and independent of the concentration q in the amalgam, that is:

$$h = k_0 \cdot C \qquad (1)$$

where k_0 is called the rate constant of the process. An equation of the same form was derived on the basis of a diffusion-controlled exchange, and then the relation: $k_0 = \text{constant} \cdot \frac{D}{\delta}$ was obtained. Here D is the diffusion coefficient of the cadmium ion and δ the thickness of the Nernst diffusion layer of the solution. In our measurements k_0 has the dimension $cm^2 \cdot g^{-1}$, and the proportionality factor, simply called "constant", is equal to $\frac{s_0}{m_0} \lim_{x \to 0} \frac{dt}{dx}$ where s_0 and m_0 are the surface area and weight of an amalgam droplet and t the time of contact for the passage of the droplet through a solution layer of the thickness x. For the measurements below it will be shown that $\delta^{-1} \cdot \lim_{x \to 0} \frac{dt}{dx}$ no doubt has a constant value in the different measurement series. Then, if the the same rate law still holds, variations in the value of the rate constant obtained for different solutions can be referred to variations in the diffusion coefficient.

Now, if the cadmium solution contains a ligand A giving a system of mononuclear complexes of the type MA_j (j = 1,2,...N), each of these complexes takes part in the exchange process with an individual rate constant k_j and diffusion coefficient D_j. Furthermore, if we denote by α_j the fraction of the total cadmium concentration that is present as the complex MA_j, the expression for the rate of exchange becomes:

$$h = C \cdot \sum_{j=0}^{N} \alpha_j k_j \tag{2}$$

Then for the average rate constant k we have:

$$k = \sum_{j=0}^{N} \alpha_j k_j \tag{3}$$

and for the ligand number (Fronæus [5]) in the complex solution:

$$n = \sum_{j=1}^{N} j \alpha_j \tag{4}$$

When the solution contains some radioactive cadmium, Cd*, the average constant k is, of course, also valid for the (Cd*, Cd) and (Cd, Cd*) processes (*cf.* Fronaeus [1]). Thus the differential equation, derived in the previous paper [1], for the radioactive exchange takes the form

$$\frac{d\bar{q}_a}{dx} = k \frac{C_a q_i - C_i q_a}{q_a + q_i} \tag{5}$$

for a complex solution. The subscripts a and i refer to radioactive and inactive cadmium respectively, and $\bar{q}_a$ is an average concentration in the amalgam droplet, while q_a is the one close to the interface at the end of the contact time. From eq. (5) it is evident that $\frac{d}{dx}\left(\frac{\bar{q}_a}{C_a}\right)$ for $x = 0$ (*i. e.*, $q_a = 0$) gives

the value of k. The determination can be performed graphically from a plot of $\bar{q}_a/C_a$ against x. If the values of the quantities a_j are known for varying ligand concentrations and the corresponding k-values are obtained from the experiments, it is possible to calculate according to eq.(3) the rate constant k_j for the different complexes.

Such a calculation is, of course, quite formal, if the k_j-values obtained are not specific to the complexes in question but depend upon various factors. For instance, it cannot be excluded *a priori* that close to the amalgam the diffusion coefficient of a charged complex could be influenced by the magnitude of the potential difference between amalgam and solution. In the previous paper [1] it was proved that k_0 in perchlorate solutions was a constant and thus independent of the potential variations accompanying the changes of C. For a rather strong complex system, on the other hand, we have the possibility of keeping the concentration of the saturated complex MA_N constant but still being able to vary the potential by changing the excess of ligand. Then we can see whether k_N is dependent on the amalgam potential or not, and at the same time it should be evident whether a partial exchange of perchlorate ions of the neutral salt medium for the ligand could affect appreciably the k_N-values.

EXPERIMENTAL

Chemicals used. Stock solutions of sodium bromide, sodium iodide, and ammonium perchlorate of analytical grade were prepared and their concentrations were checked by the use of a cation exchanger in the hydrogen form. From carbon dioxide-free ammonia and ammonium perchlorate a buffer was obtained. The other chemicals used were prepared as before [1].

The radioactive exchange measurements were carried out with the apparatus of the previous investigation [1] and under the same experimental conditions. Thus the rate constants obtained in this investigation can be compared with the k_0-value determined before. For a description of the calculation of the average concentration $\bar{q}_a$ from the experimental data the reader

Table 1. Determination of the ligand number $\bar{n}$ for some values of the bromide, iodide, and ammonia concentrations. The concentration of cadmium perchlorate is 5.00 mC.

C_{Br^-} mC	E mV	$\bar{n}$	C_{I^-} mC	E mV	$\bar{n}$	C_{NH_3} mC	E mV	$\bar{n}$
100	23.3	1.1	100	52.6	3.5	100	100.5	3.4
200	35.0	1.6	200	86.1	3.9	200	135.2	3.8
300	44.6	2.2	300	107.5	4.0	300	155.2	3.9
400	52.6	2.4	400	121.5	4.1	400	169.8	4.0
500	60.3	2.7	500	133.7	3.9			
600	66.7	3.0	600	142.2	4.2			
700	72.7	3.1	700	150.5	4.2			
800	77.0	3.1	800	155.5	3.8			
900	81.7	3.2	900	161.5	3.8			

Table 2. Measurements of the exchange of radioactive cadmium between amalgam and solution in the presence of different complex forming ligands.

Conc. of ligand mC	q_i % by weight	C_i mC	$C_a \cdot 10^{-1}$ cpm	x cm	C'_a cpm	m g	$\frac{\bar{q}_a}{C_a} \cdot 10^2$ $cm^3 \cdot g^{-1}$
	1.00	2.23	1 140	3.0	32.0	1.002	4.2
	1.00	2.23	1 140	4.9	58.5	1.107	7.0
	1.00	2.23	1 140	8.1	95.0	1.080	11.6
	1.00	2.23	1 140	12.1	174	1.280	17.9
$C_{Br^-} = 900$							
	1.00	13.4	5 870	3.1	203	1.249	4.2
	1.00	13.4	5 870	6.4	406	1.255	8.3
	1.00	13.4	5 870	10.0	743	1.570	12.0
	1.00	13.4	5 870	12.5	377	0.715	13.5
	1.00	2.23	1 000	2.8	27.0	1.152	3.5
$C_{Br^-} = 450$	1.00	2.23	1 000	5.7	42.0	0.908	6.9
	1.00	2.23	1 000	9.4	63.0	0.815	11.6
	1.00	2.23	1 000	11.8	129	1.366	14.2
	1.00	2.23	1 150	3.1	28.5	0.850	4.4
$C_{I^-} = 900$	1.00	2.23	1 150	5.0	54.0	1 142	6.2
	1.00	2.23	1 150	8.5	100	1.180	11.1
	1.00	2.23	1 150	11.2	122	1.093	14.5
	1.00	2.23	1 080	2.5	33.0	1.224	3.7
$C_{I^-} = 450$	1.00	2.23	1 080	5.3	35.5	0.740	6.7
	1.00	2.23	1 080	9.0	120	1.459	11.4
	1.00	2.23	1 080	11.6	134	1.252	14.9
	2.00	2.23	672	3.8	24.5	1.253	4.4
$C_{NH_3} = 300$	2.00	2.23	672	5.3	42.0	1.423	6.6
	2.00	2.23	672	8.1	44.0	1.040	9.5
	2.00	2.23	672	13.0	127	1.738	16.3

is referred to the earlier paper. The ligands selected were the bromide and iodide ions and ammonia. The complex solutions had the compositions:

$$\begin{cases} (C_i + C_a) & \text{mC } Cd(ClO_4)_2 \\ 20 & \text{mC } HClO_4 \\ C_A & \text{mC NaBr or NaI} \\ (900 - C_A) & \text{mC } NaClO_4 \end{cases} \qquad \begin{cases} (C_i + C_a) \text{ mC } Cd(ClO_4)_2 \\ 300 \text{ mC } NH_3 \\ 900 \text{ mC } NH_4ClO_4 \end{cases}$$

The complexity has been investigated by Leden [3] but at another ionic strength, and for that reason an approximate determination of the ligand number $\bar{n}$ was performed potentiometrically. These measurements are reported in Table 1. $\bar{E}$ denotes the potential difference between the amalgam in a solution without the ligand and in the complex solution at the same cadmium concentration. For the calculation of $\bar{n}$ the Bodländer equation (*cf.* Fronæus [5]) was used.

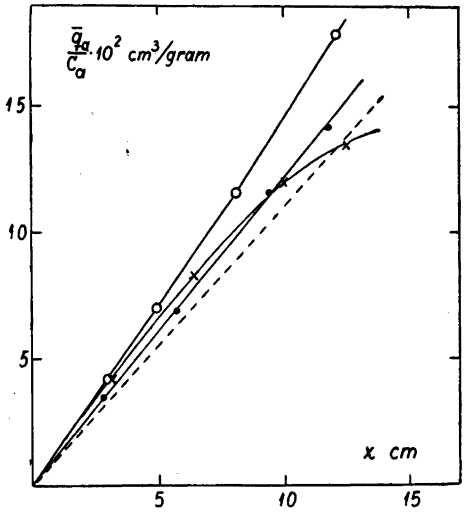

Fig. 1. $\frac{\bar{q}_a}{C_a}$ *as a function of x for* $q_i = 1.00$ *per cent and with different complex bromide solutions. 1.* $C_i = 2.23$ *mC,* $C_{Br^-} = 900$ *mC (○); 2.* $C_i = 13.4$ *mC,* $C_{Br^-} = 900$ *mC (×); 3.* $C_i = 2.23$ *mC,* $C_{Br^-} = 450$ *mC (●). The slope of the dashed line indicates the value of* k_0.

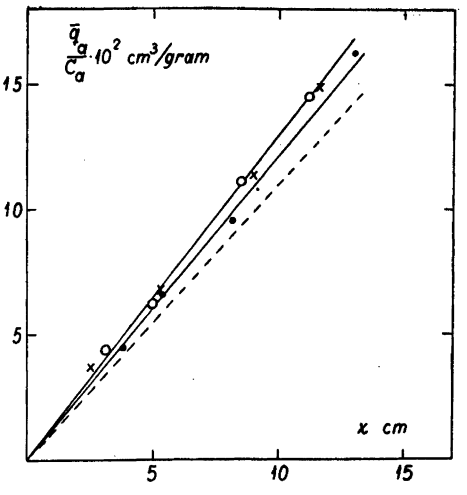

Fig. 2. $\frac{\bar{q}_a}{C_a}$ *as a function of x for different complex iodide and ammonia solutions. 1.* $q_i = 1.00$ *per cent,* $C_i = 2.23$ *mC,* $C_{I^-} = 900$ *mC (○) or 450 mC (×); 2.* $q_i = 2.00$ *per cent,* $C_i = 2.23$ *mC,* $C_{NH_3} = 300$ *mC (●). The slope of the dashed line indicates the value of* k_0.

The values obtained in the exchange measurements are collected in Table 2. The first two series are carried out at the same bromide concentration (900 mC) but at different values of $C_i \approx C_i + C_a$. In Fig. 1 it is seen that the curves 1 and 2 have the same slope $k = 1.42 \cdot 10^{-2}$ cm² · g⁻¹ for $x = 0$, and this shows that the rate constant is independent of the potential change of about 23 mV accompanying the variation of C_i. At this bromide concentration $\bar{n}$ has the value 3.2, and then we can put approximately $\alpha_3 \approx 1$ and obtain $k_3 = 1.4 \cdot 10^{-2}$ cm² · g⁻¹. For $C_{Br^-} = 450$ mC the corresponding curve 3 in Fig. 1 gives a somewhat lower value $k = 1.23 \cdot 10^{-2}$ cm² · g⁻¹. Since in this case $\bar{n} = 2.5$, we put $\alpha_2 \approx \alpha_3 \approx 0.5$, and from eq. (3) we get: $k_2 + k_3 = 2.45 \cdot 10^{-2}$ cm² · g⁻¹ and $k_2 = 1.1 \cdot 10^{-2}$ cm² · g⁻¹, which is about the same as the k_0-value determined in the previous paper.

The measurements on the iodide system were carried out at $C_{I^-} = 900$ mC and 450 mC. In both cases we had $\bar{n} = 4$, $\alpha_4 = 1$ and $C_i = 2.23$ mC. Thus the concentration of the exchanging saturated complex CdI_4^{2-} was constant, but the potential difference E changed about 35 mV. In Fig. 2 it is seen that the same curve 1 was obtained for the two iodide concentrations. From this result we conclude that either the expression $\delta^{-1} \lim_{x \to 0} \frac{dt}{dx}$ was constant and thus D specific for the complex and not appreciably dependent upon E and the change of the salt medium, or else both $\delta^{-1} \lim_{x \to 0} \frac{dt}{dx}$ and D depend upon the factors

mentioned, but the variations compensate each other, so that k remains constant. The first-mentioned alternative seems most probable and it will be shown in the following section that it must be valid, and then we have $k_4 = 1.30 \cdot 10^{-2}$ cm$^2 \cdot$ g^{-1}. As the conditions must be similar in bromide solutions, it is likely that the rate constants k_2 and k_3 obtained above also have a physical meaning.

Finally a measurement series with ammonia as the ligand was performed. In this case the salt medium was 900 mC ammonium perchlorate and C_{NH_3} was 300 mC. From Table 1 we get $\bar{n} = 4$ and $\alpha_4 = 1$. Curve 2 in Fig. 2 gives us $k_4 = 1.20 \cdot 10^{-2}$ cm$^2 \cdot$ g^{-1}.

DETERMINATION OF ABSOLUTE VALUES OF THE RATE CONSTANTS

As mentioned before the values of the reaction rate constants, hitherto determined in this paper and in the previous one, are relative, that is, they include the factor $\frac{s_0}{m_0} \lim_{x \to 0} \frac{dt}{dx}$, which depends upon the apparatus used. Though the aim of the present investigation has been attained very well by the determination of these relative values, it is for several reasons of great interest to know also the absolute values of the rate constants, and this necessitates a determination of the factor mentioned. In the following the performance of that part of the investigation will be described, and in connection with this the reader is referred to Fig. 1 of the previous paper [1], where the apparatus used is drawn.

When leaving the mouthpiece of the funnel A the liquid amalgam at first formed a thin, well-defined squirt which then divided into a row of small droplets. While the amalgam squirted out it was photographed against a luminous background, after which the radius r of the squirt was measured on the photographic plate by means of a microscope with an ocular scale. The graduation on the burette of the apparatus was photographed on the same plate and gave us a calibration of the ocular scale.

In addition, the weight w of the amalgam that was collected per unit time was determined by separate measurements. Then, if ϱ is the density of the amalgam and v_0 the velocity of the squirt, the following is valid:

$$\varrho \cdot \pi \cdot r^2 \cdot v_0 = w \tag{6}$$

and from this relation the value of v_0 was computed.

After this a determination of the average radius of the amalgam droplets formed was undertaken. For this purpose a lower part of the burette, where the squirt had divided into the row of droplets which were still present in the gas phase, was photographed in the same way. Then a time of exposure as short as 10^{-4} sec. (electronic light-flash) was chosen, so that a clear marking of the fast droplets was obtained on the plate. It was, however, impossible to measure directly on the plate the droplet radius, but this had to be determined indirectly. The velocity v of a droplet at a point at a known distance from the end of the coherent squirt was computed from the law of the free fall. The number of droplets per unit length was put equal to the average number n_1 per unit length within an interval, symmetrically surrounding the point. Then the average radius R was obtained from the relation:

$$\varrho \cdot \frac{4\pi}{3} \cdot R^3 \cdot n_1 \cdot v = w \tag{7}$$

Finally the part of the burette that contained the solution was photographed during the passage of the droplets, and the average number n_1 of droplets per unit length in this phase was measured. Because of the turbulence in the solution during the experiments it was necessary to use a great number of plates to obtain a reliable value of n_1. Furthermore, owing to the retardation of the droplets in the solution n_1 was a function of the layer thickness, and the measurements had to be repeated for different x-values. Then the time t of contact between amalgam and solution could be calculated from the relation:

$$\varrho \cdot \frac{4\pi}{3} \cdot R^3 \cdot n_1 \cdot x = w \cdot t \qquad (8)$$

With the apparatus used R had the value $(3.5 \pm 0.1) \cdot 10^{-2}$ cm and ϱ was approximately $= 13.6\ \mathrm{g \cdot cm^{-3}}$. In a series of measurements, when the salt medium had the composition: 20 mC $HClO_4$ + 900 mC $NaClO_4$ the following values of t / x were obtained:

x cm	t / x sec · cm^{-1}
5.0	0.016 ± 0.002
7.0	0.017 ± 0.003
10.0	0.024 ± 0.003

The value $0.016 \pm 0.002\ \mathrm{sec \cdot cm^{-1}}$ can be accepted as $\lim \frac{dt}{dx}$ for $x \to 0$, and then we get:

$$\frac{s_0}{m_0} \lim_{x \to 0} \frac{dt}{dx} = 0.10 \pm 0.01\ \mathrm{sec \cdot cm \cdot g^{-1}}$$

In order to decide whether this value is applicable also to the complex solutions measurements of the density and relative determinations of the viscosity of the different liquids were performed at a constant temperature. It was found that when all sodium perchlorate was exchanged for sodium bromide or iodide the variations in density were within 4 % and the variations in viscosity within 2 %. Only with the solution containing the ammonia buffer did the value of the viscosity differ as much as 7 % from that of the perchlorate solution. Thus if the experimental random errors in the photographic measurements are taken into consideration, the use of the same values of t / x for all the solutions is justified. This was checked experimentally on the bromide solutions. Then for the absolute values of the rate constants the following is valid: $K = 10 \cdot k$ cm · sec^{-1}. Especially for the perchlorate solutions we have $K_0 = 0.11$ cm · sec^{-1}, which is a considerable specific exchange rate. The absolute values of the other rate constants are collected in Table 3.

Table 3. Absolute values of the rate constants obtained for the different complexes.

Ligand	K_3 cm · sec^{-1}	K_3 cm · sec^{-1}	K_4 cm · sec^{-1}
Br^-	0.11	0.14	
I^-			0.13
NH_3			0.12

As the variations in viscosity and density were so slight, the thickness δ of the diffusion layer must be nearly constant in the different solutions. For a calculation of δ the relation: $K = D / \delta$ can be applied to the perchlorate solutions. An accurate value of D for the cadmium ion in the salt medium used is not known, but if we presuppose that it is of the order of magnitude 10^{-5} $cm^2 \cdot sec^{-1}$, we find $\delta \approx 10^{-4}$ cm at a linear velocity of about 60 $cm \cdot sec^{-1}$ of the amalgam droplets.

Of course the diffusion layer will not maintain a spherical shape around the droplet, and thus the calculated value of δ will be the average layer depth.

DISCUSSION

The results of this investigation make it clear that the rate of the cadmium exchange is only slightly affected by the formation of complexes of moderate strength, and this is what could be expected for a diffusion-controlled process, where the rate constant is proportional to the diffusion coefficient. When the water ligands are displaced by bromide or iodide ions or by ammonia, the diffusion coefficient D increases somewhat. As the magnitude of the potential difference between amalgam and solution and small changes of the salt medium have no influence on the D-value of a certain complex, the differences between the diffusion coefficients of the complexes investigated probably must be ascribed to differences in the sizes of the ions or molecules.

SUMMARY

The influence of complex formation on the exchange of cadmium between amalgam and solution is studied by a radioassay procedure developed in a previous work [1]. The bromide and iodide ions and ammonia are selected as ligands, and the concentrations of the different complexes in the solutions are known.

The rate constant is not very greatly influenced by the formation of these complexes of moderate strength, as the exchange is still diffusion-controlled. For the different complexes the rate constants are proportional to the corresponding diffusion coefficients.

It is shown that the differences in the values obtained of the rate constants are probably ascribable entirely to different size of the complexes.

My sincere thanks are due to Professor S. Bodforss for his kind interest in the investigation and to Fil.mag. C. O. Östman and Mr. L. Å. Appelqvist for their valuable assistance in the measurements.

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Über synthetische Versuche in der Reihe des Patulins und verwandter Verbindungen. II *. Zur Synthese der Di- und Tetrahydro-desoxy-patulinsäure

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Die *Dihydro-desoxy-patulinsäure* (V), von verschiedenen Forschern aus dem Antibiotikum Patulin und von Woodward und Singh über das *Desoxy-patulin*(IX) durch Hydrierung als Öl erhalten [1], wurde von uns vor einiger Zeit über die bis dahin unbekannte *4-Oxy-tetrahydropyranyl-(3)-essigsäure*(IV) auf dem durch die Formeln I → V bezeichneten Reaktionswege dargestellt und — da auch wir zunächst ein Öl erhielten — durch bekannte Derivate identifiziert [2].

Da die Verbindung V allgemein als *Dihydro-desoxy-patulinsäure* und deren *Lacton*(VII) als *Dihydro-desoxy-patulin* bezeichnet wird, werden wir künftig konsequent die *4-Oxy-tetrahydropyranyl-(3)-essigsäure*(IV) »*Tetrahydro-desoxy-patulinsäure*» und deren *Lacton*(VI), das bereits das fertige Skelett des Patulins enthält, »*Tetrahydro-desoxy-patulin*» nennen.

Während unsere Untersuchung damals im Zeichen konstitutiver Fragestellungen in Verbindung mit der Formaldehyd-Olefin-Reaktion stand und wir daher an Einzelheiten vorbeigehen mussten, haben wir in der vorliegenden Arbeit das Fehlende zu ergänzen versucht. Hierzu gehörte die Reindarstellung und die genaue Ausbeuteermittlung der Reaktions-Zwischenprodukte. Hauptgegenstand der Untersuchung war jedoch die Aufklärung der Vorgänge, die sich bei der Oxydation der *Tetrahydro-desoxy-patulinsäure*(IV) bzw. des *Tetrahydro-desoxy-patulins* (VI) abspielen. Nachdem sich die *Tetrahydro-desoxy-patulinsäure*(IV) bzw. deren *Lacton*(VI) durch Chromsäure-Oxydation in das *Dihydro-desoxy-patulin*(VII) bzw. die *Dihydro-desoxy-patulinsäure*(V) überführen liess [2], schien es nicht ausgeschlossen, auf dem gleichen Wege zu höheren, patulinähnlicheren Oxydations- bzw. Dehydrierungsprodukten, ja u. U. zum *Patulin*(X) selbst, zu gelangen. Nichtzutreffendenfalls sollte jedenfalls die Ursache des Versagens ermittelt werden.

* I. Mitteilung: Olsen, S. *Acta Chem. Scand.* **5** (1951) 1326.

I. DIE REAKTIONS-ZWISCHENSTUFEN

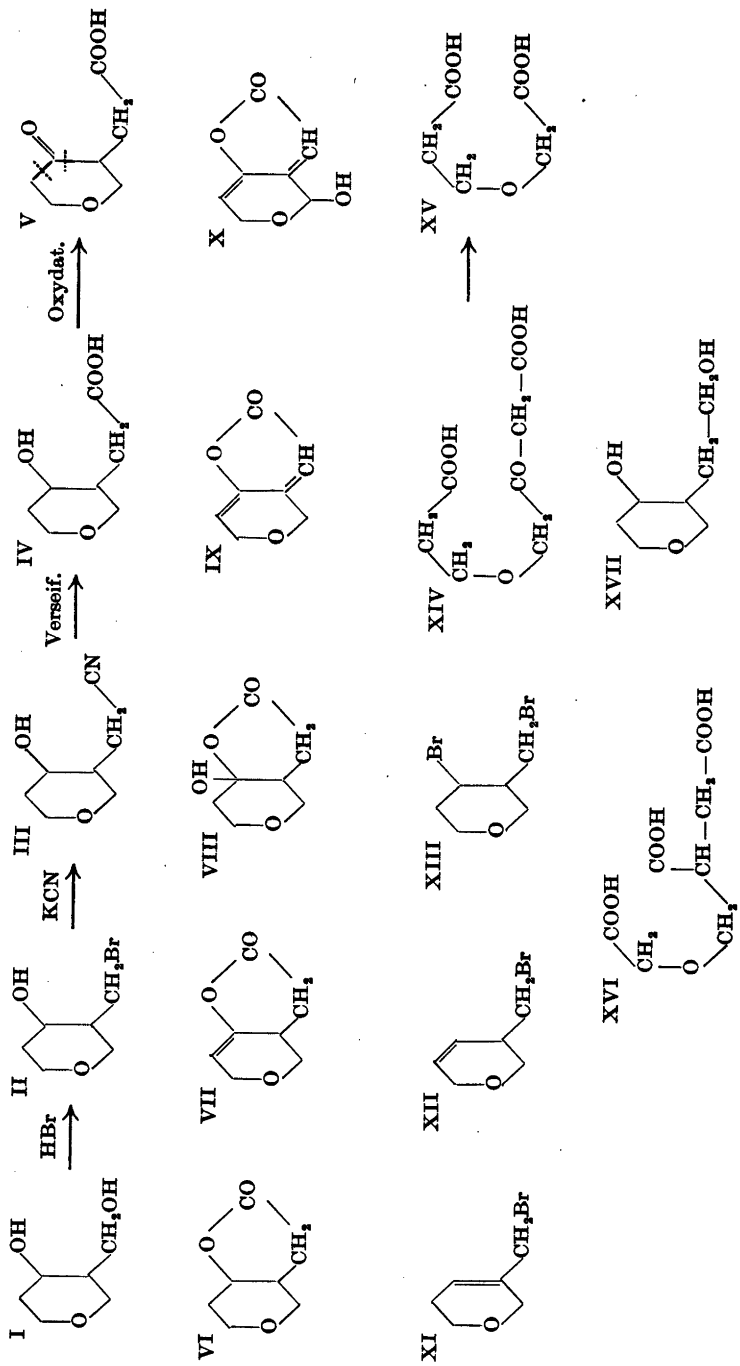
Beim Einleiten von trockenem Bromwasserstoff in *3-Oxymethyl-4-oxy-tetrahydro-pyran*(I) erhält man neben 77 % *3-Brommethyl-4-oxy-tetrahydro-pyran*(II) zwei niedriger siedende, bisher unbekannte Verbindungen vom $Sdp._{13}$85—87° und $Sdp._{10,5}$ 118°, denen die Formeln eines *3-Brommethyl-Δ^3-dihydropyrans*(XI) bzw. *3-Brommethyl-Δ^4-dihydropyrans*(XII)* und eines *3-Brommethyl-4-bromtetrahydropyrans*(XIII) zukommen. Das *3-Brommethyl-4-oxy-tetrahydropyran*(II) geht beim Behandeln mit Kaliumcyanid in 73 % Ausbeute in das *4-Oxy-tetrahydropyranyl-(3)-acetonitril*(III) vom $Sdp._{10}$ 166° über, das bei der *sauren* Verseifung rasch und in 72 % Ausbeute das *Tetrahydro-desoxy-patulin*(VI) liefert. Der früher angegebene $Sdp._{0,4}$ 137—142° ist also bedeutend zu hoch und wurde wahrscheinlich durch die Anwesenheit der freien *Säure*(IV) bedingt. Die Verbindung wurde in das aus der vorigen Untersuchung bekannte *Hydrazid*, das bisher unbekannte *Amid* vom Schmp. 122—123° und mittels verdünnter Säure in die freie *Tetrahydro-desoxy-patulinsäure* übergeführt.

II. DIE CHROMSÄURE-OXYDATION DES TETRAHYDRO-DESOXY-PATULINS

Um über den Angriff des Oxydationsmittels (CrO_3—H_2SO_4) Klarheit zu gewinnen, wurde das *Tetrahydro-desoxy-patulin*(VI) unter verschiedenen Bedingungen und mit ansteigenden Mengen Oxydationsmittel, entsprechend 1—4 Grammatomen Sauerstoff, behandelt. Der Theorie nach sollte zur Darstellung der *Dihydro-desoxy-patulinsäure*(V) 1 Grammatom Sauerstoff per Grammolekül Lacton erforderlich sein. In diesem Falle erhält man aber viel Ausgangsmaterial zurück. Andererseits haben wir bei Anwendung von 4 Grammatomen Sauerstoff per Grammolekül Lacton — nachdem das Oxydationsmittel völlig umgesetzt war — aus dem Oxydationsgemisch auch *Dihydro-desoxy-patulinsäure*(V), ja sogar unveränderte *Tetrahydro-desoxy-patulinsäure*(IV) isolieren können! Hieraus geht hervor, dass man bezüglich der Menge des Oxydationsmittels — je nach dem beabsichtigten Zweck — rein empirisch zu Werke gehen muss. Bei Verwendung von 1—4 Grammatomen Sauerstoff per Mol *Tetrahydro-desoxy-patulin* ist es uns gelungen, *Dihydro-desoxy-patulinsäure*(V), die bisher nur als Öl bekannt war, zum ersten Male in *reiner kristallisierter* Form herzustellen. Es ist eine farblose Verbindung vom Schmp. 85—86°, die in saurer Lösung mit ihrem Lacton, dem *Dihydro-desoxy-patulin*(VII), möglicherweise auch dem *Lactol*(VIII) im Gleichgewicht zu stehen scheint. Die bei verschiedenen Oxydationsversuchen erzielte Maximalausbeute betrug 25 %. Bei keinem unserer Versuche haben wir eine höher oxydierte, patulinähnlichere Substanz als die *Dihydro-desoxy-patulinsäure* isolieren können. Die Ursache hierfür ergibt sich aus dem 3. Oxydationsversuch (4 Grammatome Sauerstoff) des experimentellen Teiles. Aus diesem Versuch geht nämlich hervor, dass die zuerst entstehende *Dihydro-desoxy-patulinsäure* gegenüber dem Oxydationsmittel empfindlich ist. Sie wird durch dieses teilweise an den in der

* Für die Formulierung XI bzw. XII sprechen ausser den physikalischen Konstanten einige Reaktionen, über die in Kürze berichtet werden wird.

Formeln.



Formel V punktiert gezeichneten Stellen oxydativ aufgespalten, einerseits unter intermediärer Bildung der *γ-(β-Carboxy-äthoxy)-acetessigsäure*(XIV), die sofort zur *β-[Carboxy-methoxy]-propionsäure*(XV) weiter oxydiert wird, andererseits unter Bildung der beständigeren, bisher nicht bekannten *β-Carboxy-γ-[carboxy-methoxy]-buttersäure*(XVI) vom Schmp. 143—145°. — Man könnte die Bildung der *β-[Carboxy-methoxy]-propionsäure* natürlich auch durch oxydativen Abbau der *β-Carboxy-γ-[carboxy-methoxy]-buttersäure*(XVI) erklären. Dagegen spricht jedoch, dass wir bei der Oxydation des *Tetrahydro-desoxy-patulins* mit überschüssiger Salpetersäure nur diese Säure(XVI), die übrigens in Äther und Benzol schwer, in Wasser und Aceton dagegen leicht löslich ist, aber nicht die *β-[Carboxy-methoxy]-propionsäure*(XV) erhielten. — Versuche, das *Tetrahydro-desoxy-patulin* katalytisch mit elementarem Sauerstoff, mit Hydrogenperoxyd oder Bleitetraacetat zu oxydieren, führten zu keinem Ergebnis. — Erwähnt sei, dass sich das *Tetrahydro-desoxy-patulin* mit Lithium-Aluminium-Hydrid glatt und in guter Ausbeute zu dem bisher unbekannten *4-Oxy-tetrahydropyranyl-(3)-äthylalkohol*(XVII) vom $Sdp._{12}$ 181—183° reduzieren liess.

EXPERIMENTELLER TEIL

Umsetzung von 3-Oxymethyl-4-oxy-tetrahydropyran mit Bromwasserstoff

In 322 g *3-Oxymethyl-4-oxy-tetrahydropyran* (I), dargestellt nach Olsen [2], Sdp.$_7$ 152—155°, leitete man unter gleichzeitigem Erwärmen auf dem Wasserbade trockenen Bromwasserstoff bis zu einer Gewichtszunahme von 296 g (entsprechend ca. 1,5 Mol) ein. Bei der Destillation des dunkelfarbigen Reaktionsgemisches erhielt man neben niedriger und höher siedenden Anteilen als Hauptfraktion 316 g eines gelben Öles vom $Sdp._9$ 130—133°, das sich beim Stehen allmählich dunkel färbt: *3-Brommethyl-4-oxy-tetrahydropyran*(II), Ausbeute 66 %. Zur Analyse wurde die Substanz mehrmals destilliert: Farbloses Öl vom $Sdp._{10}$ 136°, $n_D^{22} = 1{,}5178$, $d_4^{22} = 1{,}5504$; MR = 38,12 (ber. 38,66).

$C_6H_{11}O_2Br$ (195,1) Ber. C 36,95 H 5,68 Br 40,97
Gef. » 36,83 » 5,57 » 41,43

Aus den niedriger siedenden Anteilen, deren Menge je nach den Versuchsbedingungen wechseln kann, wurden durch Destillation zwei weitere Fraktionen erhalten:

1) $Sdp._{13}$ 85—87°, farbloses, dünnflüssiges, die Augen stark angreifendes Öl, das sich beim Stehen verfärbt, J.Z. 130,5 (ber. 143,3); $n_D^{22} = 1{,}5274$; $d_4^{22} = 1{,}477$; MR = 36,87 (ber. 36,66):
3-Brommethyl-Δ³-dihydropyran (XI) bzw.
3-Brommethyl-Δ⁴-dihydropyran (XII)

2) $Sdp._{10.5}$ 118°, farbloses, dünnflüssiges Öl, das sich beim Stehen nicht verfärbt, J.Z. 0 (ber. 0);
$n_D^{22} = 1{,}5416$; $d_4^{22} = 1{,}8058$; MR = 44,93 (ber. 44,90).
3-Brommethyl-4-brom-tetrahydropyran(XIII)
$C_6H_{10}OBr_2$ (258,0) Ber. C 27,94 H 3,91 Br 61,94
Gef. » 28,63 » 3,99 » 61,23

4-Oxy-tetrahydropyranyl-(3)-acetonitril (III)

In eine Lösung von 300 g *3-Brommethyl-4-oxy-tetrahydropyran* (II), $Sdp._9$ 130—133°, in 440 ml abs. Alkohol trug man 200 g feinpulverisiertes Kaliumcyanid ein. Das Gemisch wurde 60 Stunden unter Rückflusskühlung erhitzt und nach dem Erkalten vom Salz befreit. Bei der Destillation erhielt man 158 g eines schwach gelben Öles vom Sdp., 163—166°. Ausbeute 73 %. Die Substanz wurde zur Analyse erneut destilliert: Farbloses Öl, $Sdp._{10}$ 166°; $n_D^{19} = 1{,}4818$; $d_4^{16} = 1{,}1476$; MR = 35,03 (ber. 35,33)

$C_7H_{11}O_2N$ (141,2) Ber. C 59,57 H 7,85 N 9,92
Gef. » 59,20 » 7,68 » 9,78

Tetrahydro-desoxy-patulin (VI)

155 g *4-Oxy-tetrahydropyranyl-(3)-acetonitril* (III), Sdp.$_9$ 163—166°, wurden nach Zusatz von 180 ml konz. Salzsäure auf dem Wasserbade vorsichtig erwärmt. Bei etwa 50° erfolgte eine heftige Reaktion unter Abscheidung von Ammoniumchlorid. Im Anschluss erwärmte man 2 Stunden auf dem Wasserbade. Nach dem Abkühlen im Kühlschrank wurde das Ammoniumchlorid abgesaugt, das Filtrat mit 40 prozentiger Kalilauge stark alkalisch gemacht und so lange erhitzt, bis alles Ammoniak verschwunden war. Nach dem Ansäuern des Reaktionsgemisches (kongosauer) wurde unter vermindertem Druck bis zu einem dicken Brei eingedampft, der erschöpfend mit Aceton digeriert wurde. Bei der Destillation der vereinigten Acetonauszüge erhielt man 112 g eines farblosen Öles vom Sdp.$_8$ 133—134°; $n_D^{23} = 1{,}4814$; $d_4^{23} = 1{,}2179$; MR = 33,24 (ber. 33,32); V.Z. 400 (ber. 395). Ausbeute 72 %.

Tetrahydro-desoxy-patulinsäure (IV)

0,89 g *Tetrahydro-desoxy-patulin* (VI) wurden mit verdünnter Schwefelsäure 2 Stunden auf dem Wasserbade erwärmt. Die orangefarbene Lösung ätherte man mehrfach aus und dampfte die vereinigten Ätherauszüge auf 5 ml ein. Beim Erkalten kristallisierte eine farblose, etwas ölige Substanz, die abgesaugt und aus Essigester umkristallisiert wurde (0,16 g). Farblose Nadeln. Schmp. 108—110°.

Hydrazid: 0.5 g *Tetrahydro-desoxy-patulin* (VI), gelöst in 3 ml Methanol, wurde mit 0,24 g Hydrazin-hydrat (60 %) 1 Stunde gekocht. Farblose Nadeln aus Alkohol. Schmp. 163—165°.

Amid: Entsteht beim Sättigen einer Lösung des *Tetrahydro-desoxy-patulins* in abs. Alkohol mit Ammoniak in der Kälte. Aus Äthylacetat. Schmp. 122—123°.

$C_7H_{13}O_3N$ (159,2)	Ber.	C 52,82	H 8,23	N 8,80
	Gef.	» 52,88	» 8,20	» 8,83

Oxydation des Tetrahydro-desoxy-patulins mit Chromsäure

1) *1,5 Grammatome Sauerstoff.* 4,6 g *Tetrahydro-desoxy-patulin* (VI), gelöst in 20 ml Wasser, wurden tropfenweise mit einer Lösung von 3,2 g Chromsäureanhydrid und 3,1 ml konz. Schwefelsäure in 25 ml Wasser versetzt und nach 15 Minuten Stehen bei 50° auf dem siedenden Wasserbade 30 Minuten erhitzt. Die blaugrüne Reaktionsmischung schüttelte man mehrfach mit Äther aus und trocknete die vereinigten Ätherauszüge über Natriumsulfat. Nach Verdampfen des Äthers hinterblieben 3,6 g braunes Öl, das nach Methylierung mit Diazomethan mit Hydrazin-hydrat (60 %) umgesetzt wurde und dabei 0,22 g des bekannten *Pyridazinon-Derivates der Dihydro-desoxy-patulinsäure* vom Schmp. 158—160° ergab. Zur Analyse wurde die Substanz im Ölpumpenvakuum bei ca. 120° sublimiert.

$C_7H_{10}O_2N_2$ (154,2)	Ber.	C 54,53	H 6,54	N 18,17
	Gef.	» 54,47	» 6,55	» 18,19

Sie wurde durch Behandeln mit Brom in Eisessig in das bekannte *Oxypyridazin-Derivat* übergeführt, das nach Sublimation im Ölpumpenvakuum bei ca. 130° in farblosen Kristallen vom Schmp. 228—231° erhalten wurde.

$C_7H_8O_2N_2$ (152,2)	Ber.	C 55,24	H 5,30	N 18,41
	Gef.	» 55,29	» 5,20	» 18,81

2) *2 Grammatome Sauerstoff. Kristallisierte Dihydro-desoxy-patulin-säure (V).* 2,74 g *Tetrahydro-desoxy-patulin* (VI), gelöst in 10 ml Wasser, wurden tropfenweise mit einer Lösung von 2,58 g Chromsäureanhydrid und 2,5 ml konz. Schwefelsäure in 15 ml Wasser unter Eiskühlung versetzt, wonach das Reaktionsgemisch 3 Stunden in Eis und 5 Tage bei Zimmertemperatur sich selbst überlassen blieb. Die Aufarbeitung, wie vorstehend beschrieben, führte zu 1,83 g eines schwach gelben Öles, das nach dem Lösen in Essigester nach längerer Zeit teilweise kristallisierte. Die Kristalle wurden abgesaugt und fraktioniert aus Äther umgelöst. Man erhielt 0,19 g *Tetrahydro-desoxy-patulinsäure* (IV) vom

Schmp. 108—110° und 0,29 g *Dihydro-desoxy-patulinsäure* (V) vom Schmp. 85—86°. Bei der Titration verbrauchten 22,85 mg der Säure V 1,462 ml *N*/10 NaOH, was einem Äquivalentgewicht von 156,3 entspricht.

$C_7H_{10}O_4$ (158,2) Ber. C 53,15 H 6,37
Gef. » 53,17 » 6,25

Die Säure V liess sich in das *2,4-Dinitro-phenylhydrazon* vom Schmp. 193—196°, in das *Methylester-2,4-dinitro-phenylhydrazon* vom Schmp. 149—151° und das *Pyridazinon-Derivat* vom Schmp. 158—160° überführen.

3) *4 Grammatome Sauerstoff.* 9,9 g *Tetrahydro-desoxy-patulin* (VI), gelöst in 40 ml Wasser, wurden, wie vorstehend beschrieben, in der Kälte mit einer Lösung von 18,7 g Chromsäureanhydrid und 17 ml konz. Schwefelsäure in 50 ml Wasser oxydiert und, nachdem die Flüssigkeit eine blau-grüne Farbe angenommen hatte, aufgearbeitet. Man erhielt nach dem Verdampfen des Äthers 8,1 g schwach gelbes Öl, das nach dem Impfen mit *Dihydro-desoxy-patulinsäure* teilweise kristallisierte. Die Kristalle (*A*) wurden vom Öl (*B*) (3,9 g) getrennt und mit Äther ausgekocht, wobei 0,42 g farblose Substanz (*C*) ungelöst blieb. Ätherlösung *(D)*. Die *Substanz C* wurde aus Aceton-Benzol umkristallisiert. Farblose Kristalle. Schmp. 143—145°. Bei der Titration verbrauchten 17,69 mg Substanz 2,590 ml *N*/10 Natronlauge, was einem Äquivalentgewicht von 68,31 (ber. 68,71) entspricht.

β-Carboxy-γ-[carboxy-methoxy]-buttersäure (XVI)

$C_7H_{10}O_7$ (206,2) Ber. C 40,78 H 4,89
Gef. » 40,96 » 5,10

Bei der Aufarbeitung der *Ätherlösung D* erhielt man 2 g
Dihydro-desoxy-patulinsäure (V) vom Schmp. 85—86°, 0,3 g
Tetrahydro-desoxy-patulinsäure (IV) vom Schmp. 108—110° und 0,44 g
β-[Carboxy-methoxy]-propionsäure (XV) vom Schmp. und Mischschmp. 93—95°

$C_5H_8O_5$ (148,1) Ber. C 40,55 H 5,44
Gef. » 40,33 » 5,47

Nach längerem Stehen war das *Öl B* fast völlig kristallisiert. Bei der Aufarbeitung, wie vorstehend beschrieben, erhielt man weitere 1,3 g *Tetrahydro-desoxy-patulinsäure* (IV), 0,8 g *Dihydro-desoxy-patulinsäure* (V) und geringe Mengen *β-Carboxy-γ-[carboxy-methoxy]-buttersäure* (XVI) und *β-[Carboxy-methoxy]-propionsäure* (XV).

β-Carboxy-γ-[carboxy-methoxy]-buttersäure (XVI)

Bei der Oxydation von 1 g *Tetrahydro-desoxy-patulin* (VI) mit 4 ml Salpetersäure (1 : 1) und anschliessendem Eindampfen des Reaktionsgemisches hinterblieb ein dickflüssiges Öl, das beim Anreiben mit Aceton teilweise kristallisierte. Man gewann sc 0,13 g farblose Substanz, die, aus Aceton-Benzol umkristallisiert, bei 143—145° schmolz und sich bei der Mischprobe als *β-Carboxy-γ-[carboxy-methoxy]-buttersäure* erwies.

4-Oxy-tetrahydropyranyl-(3)-äthylalkohol (XVII)

(Experimentell bearbeitet von Ernst Finsnes)

Unter Benutzung der von Nystrom und Brown [3] gegebenen Vorschrift wurde eine Lösung von 10 g *Tetrahydro-desoxy-patulin* (VI) in 40 ml abs. Äther unter mechanischem Rühren in eine Suspension von 3,8 g Lithium-Aluminium-Hydrid so schnell eingetropft, dass sich das Reaktionsgemisch eben im Sieden hielt. Dieses wurde in üblicher Weise aufgearbeitet. Bei der Destillation erhielt man 6,5 g farbloses Öl vom $Sdp._{12}$ 181—183°.
Bisphenylurethan aus Benzol Schmp. 127—130°.

$C_{21}H_{24}O_5N_2$ (384.4) Ber. C 65.81 H 6.32 N 7.12
Gef. » 65.61 » 6.29 » 7.29

ZUSAMMENFASSUNG

Die vor einiger Zeit beschriebene Synthese der *Tetrahydro-desoxy-patulinsäure* wurde mit sehr guter Ausbeute reproduziert. Deren Chromsäure-Oxydation zur *Dihydro-desoxy-patulinsäure* wurde ebenfalls reproduziert und dabei diese Säure erstmalig *kristallisiert* erhalten. Die *Dihydro-desoxy-patulinsäure* erleidet bei der Oxydation teilweise eine Ringsprengung unter Bildung der bisher unbekannten *β-Carboxy-γ-[carboxy-methoxy]-buttersäure* und der *β-[Carboxy-methoxy]-propionsäure*. Die Darstellung einer Reihe anderer, bisher unbekannter Verbindungen wird beschrieben.

Norges Almenvitenskapelige Forskningsråd und dem *Fridtjof Nansen-Fond* dankt man für finanzielle Unterstützung.

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S-Benzylthiuronium Derivatives

Determination by Titration with Perchloric Acid, and some Comments on the Melting Points

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Titration in non-aqueous solution can be widely used in the identification of organic compounds. We have shown, in a previous paper [1], that xanthogenates can be titrated with perchloric acid in glacial acetic acid solution, and it has now been proved that S-benzylthiuronium derivatives of carboxylic acids can be determined in the same way.

In the determination of the equivalent weight of an acid, which may be found in the form of ester, salt, amide, nitrile or other compound, the S-benzylthiuronium derivative is easily prepared, and the nitrogen content of the derivative is determined by the Kjeldahl method as proposed by Veibel [2]. This procedure is extensively used for carboxylic acids, which are easily soluble in water and therefore difficult to isolate in the pure state from aqueous medium.

In our method the S-benzylthiuronium salt is dissolved in glacial acetic acid and titrated directly with perchloric acid in the same solvent. The titration may be carried out in the course of a few minutes, and the end-point determined either visually or potentiometrically. The reaction is represented as follows:

$$HClO_4 + CH_3COOH \longrightarrow CH_3COOH_2^+ + ClO_4^-$$

$$C_6H_5CH_2-S-C(=NH_2^+)-NH_2 \, , \, {}^-OOCR + CH_3COOH_2^+ \longrightarrow C_6H_5CH_2-S-C(=NH_2^+)-NH_2 + RCOOH + CH_3COOH$$

The S-benzylthiuronium salts have also been used in isolating carboxylic acids from natural products and from reaction mixtures, since they are easily and quickly prepared.

Fig. 1. shows the potentiometric titration curves for two derivatives. In the visual titration crystal violet is used as indicator, and the colour changes of

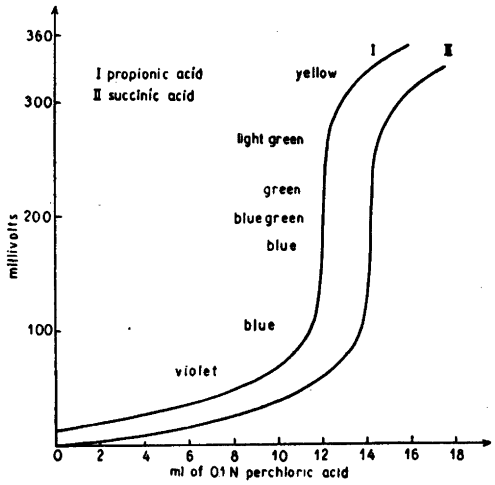


Fig. 1. Potentiometric titration curves of S-benzylthiuronium propionate and S-benzylthiuronium succinate, and colour changes of the indicator (crystal violet) during the titration.

crystal violet during the titration are given in the same figure. The greatest potential break appears at the colour change from blue to blue-green.

Some of the S-benzylthiuronium salts contain water of crystallization, which can interfere in the titration, since a water content in the medium gives fading end-points. However, it has been found that this slight increase of the water content in the solvent has no influence, and Fig. 1 shows that the titration curves for the propionic acid salt (anhydrous) and the succinic acid salt (2 molecules of water) are almost identical.

The water of crystallization in the S-benzylthiuronium salts may be determined by the Karl Fischer method. The modification proposed by Johansson [3] is used.

EXPERIMENTAL

Preparation of the S-benzylthiuronium salts. The derivatives are prepared according to Vogel [4] p. 359; this procedure is a modification of that given by Veibel and Lillelund [5], Veibel and Ottung [6] and by Donleavy [7].

Titration of the S-benzylthiuronium salts of carboxylic acids. Reagents used: Glacial acetic acid, not less than 99.7 % CH_3COOH. 0.1 *N* perchloric acid in glacial acetic acid. Crystal violet indicator, 0.5 g crystal violet dissolved in 100 ml glacial acetic acid. The 0.1 *N* perchloric acid is prepared and standardized according to Markunas and Riddick [8], except that we have adjusted the water content by the Karl Fischer method to between 0.02 and 0.05 %.

Procedure: 0.100—0.300 g of the derivative is dissolved in 15—20 ml glacial acetic acid. 2 drops of crystal violet indicator are added and the solution is titrated with 0.1 *N* perchloric acid to a blue-green colour. The subsequent colour of the solution will be green, and only about 0.02 ml 0.1 *N* perchloric acid is required to produce the colour change from blue-green to green. Sometimes the S-benzylthiuronium perchlorate is precipitated but this does not interfere with the titration. The results of the visual titration are given in Table 1 and indicate that the method is accurate.

The potentiometric titration is carried out using a calomel electrode, fibre type, as reference and a glass electrode connected to a potentiometer (Radiometer, pH-meter 3).

When calculating the equivalent weight by means of the S-benzylthiuronium salt, it should be remembered that some salts contain water of crystallization.

Table 1. Analysis of S-benzylthiuronium derivatives of carboxylic acids by titration with perchloric acid.

Acid	Amount g	ml $HClO_4$ 0.1000 *N*	Equivalent weight		Deviation %
			calc.	found	
Formic	0.2647	12.37	212.3	214.0	0.8
Acetic	0.2286	9.30	244.3 [a]	245.8	0.6
Propionic	0.2039	8.50	240.3	239.9	0.2
n-Butyric	0.0924	3.62	254.3	255.2	0.4
*Iso*butyric	0.2397	9.43	254.3	254.2	—
n-Valeric	0.3466	12.90	268.4	268.7	0.1
*Iso*valeric	0.2537	9.48	268.4	267.6	0.3
n-Caproic	0.3131	11.07	282.4	282.8	0.1
n-Caprylic	0.4537	14.60	310.5	310.8	0.1
Monochloroacetic	0.2225	8.57	260.7	259.6	0.4
Trichloroacetic	0.3907	11.80	329.6	331.1	0.5
Oxalic	0.2422	11.40	211.3	212.5	0.6
Succinic	0.2825	11.56	243.3 [b]	244.4	0.5
Adipic monosalt	0.4230	13.44	312.4	314.7	0.7
Adipic disalt	0.2856	11.91	239.3	239.8	0.2
Sebacic	0.5075	18.91	267.4	268.4	0.4

[a] The derivative contains one molecule of water of crystallization.
[b] The derivative contains two molecules of water of crystallization.

S-*p*-chloro-[9], S-*p*-bromo[10]- and S-*p*-nitrobenzylthiuronium salts may also be used in the identification of carboxylic acids, and we have found that these derivatives also can be titrated by the method described.

S-benzylthiuronium salts of sulfonic acids. The sulfonic acids are also characterized through their S-benzylthiuronium salts [5,7,11], and we have prepared the derivative of benzene-, *p*-toluene- and β-naphthalenesulfonic acid, but none of these can be titrated with perchloric acid. This must be due to the fact that the sulfonic acids are highly ionized in glacial acetic acid, as pointed out by Hantzsch and Langbein [12]. Trichloroacetic acid, which has approximately the same acid strength in aqueous solution as benzenesulfonic acid, gives a salt which is readily titrated.

Titration of other types of thiuronium compounds. In the identification of alkylhalides the S-alkylthiuronium picrates are used and it has been found, that they may be accurately titrated in glacial acetic acid solution, and in this way the equivalent weight of unknown halides can be determined indirectly. The titration is carried out as described for the S-benzylthiuronium salts of carboxylic acids. As proposed by Veibel [12], the picrate itself can be used as an indicator when titrating visually, due to the colour change of the solution from yellow to colourless, when all the picrate ions are converted to picric acid.

COMMENTS ON THE PREPARATION OF THE DERIVATIVES

It is well known that it is important not to allow the reaction mixture to become appreciably alkaline, since the free S-benzylthiuronium base then decomposes rapidly yielding benzyl mercaptan. In order to prevent this decomposition the carboxylic acid should only be partially neutralized [5,6]. This is accomplished by adding alkali to the colour change of *methyl red*. Donleavy [7], however, states that the neutral sodium or potassium salt of the organic acid should be used, *e. g.* neutralization using *phenolphthalein* and then addition of hydrochloric acid until the colour of the indicator disappears.

We have tried both procedures, and our experiments show that these methods are equally well applicable, except in the preparation of the adipic acid derivative.

Adipic acid can give derivatives with either one mole or two moles of S-benzylthiuronium chloride. On using methyl red in the neutralization a derivative consisting of a mixture of the monosalt and the disalt was precipitated. A pure disalt was produced when using phenolphthalein as the indicator, while the pure monosalt could be prepared by addition of S-benzylthiuronium chloride to an exactly half-neutralized adipic acid.

COMMENTS ON THE MELTING POINTS OF THE DERIVATIVES

The derivatives of carboxylic acids. The melting points of the S-benzylthiuronium salts recorded in the literature show large deviations. In order to explain these discrepancies we have carried out experiments on some of the factors which can influence the determination. Melting points from our experiments and from the literature are listed in Table 2. It is seen that there are great differences between the melting points found by Veibel *et al.*[5,6] and by Donleavy [7]. Melting points for the S-*p*-chloro- and the S-*p*-bromobenzylthiuronium salts are also given in the table in order to demonstrate the small dispersion in the melting points for all types of S-benzylthiuronium salts.

Our melting points were determined by the capillary tube method. An electrically-heated paraffin bath with mechanical stirrer [22] was used, so that the rate of heating could be accurately regulated. Only the temperature at which the compound melts completely is registered.

First we investigated the position of the melting point when the temperature of the heating-bath was previously raised to within 15° C of the melting point, before introducing the substance, and secondly the melting point was determined when the capillary tube was inserted in the heating-bath at room temperature. In both cases the same melting point was registered, when the rate of heating was constant.

Table 2. Melting points of some S-benzylthiuronium derivatives.

Acid	S-benzylthiuronium salt				S-*p*-chloro-benzyl-[9]	S-*p*-bromo-benzyl-[10]
	Own results [a]	Veibel *et al.* [5,6]	Donleavy [7]	Other authors		
Formic	152° C	150—151°C	146° C	150—151 °C[14]	148°C	148°C
Acetic	136	135—136	134	143—144[14],136—138[15] 135—136[16,17],146—147[18]	140	149
Propionic	153	151—152	148	—	143	146
n-Butyric	150	—	146	—	139	142
*Iso*butyric	150	—	143	—	—	—
n-Valeric	156	—	—	—	142	146
*Iso*valeric	159	—	153	—	148	138
n-Caproic	157	—	—	154—155[18]	143	146
n-Caprylic	157	—	—	—	—	147
Monochloro-acetic	160	159—160	—	156[16]	158	154
Trichloro-acetic	149	148—149	—	150[16]	148	146
Oxalic	198	195—196; 203	193	199—200[14],191—192[16]	194	194
Succinic	154	154—155	149	153[19],145—146[20]	167	167
Adipic monosalt	164	—	—	—	—	—
Adipic disalt	163	—	—	159 [b]	—	—
Sebacic	155	—	—	—	—	—

a) All our m.p. are corrected and determined by the capillary tube method; rate of heating: 4° C per minute.

b) The melting point of the adipic acid derivative is given by Smith and Jones [21] and as it is prepared according to Donleavy [7] one must expect it to be the disalt.

Next we altered the rate of heating for the last 15° C below the melting point, and this proved to be of great significance. Rates of heating of 1° C per minute and 4° C per minute were used. We observed that there was a difference of up to 9° C between the melting points found for the same sample, when heating at 1° C/min and at 4° C/min. The results are shown in Table 3. For all the examples given there was a deviation of several degrees when the rate of heating was varied.

The discordant values for the melting points given by Veibel *et al.*[5,6] and Donleavy [7] can be explained from what has been mentioned above. On com-

Table 3. Melting points of some S-benzylthiuronium derivatives. Dependence on the rate of heating.

Acid	Rate of heating *		Deviation
	1° C/min	4° C/min.	
Formic	147° C	152° C	5° C
Acetic	131	136	5
Propionic	146	153	7
n-Butyric	144	150	6
*Iso*butyric	143	150	7
n-Valeric	149	156	7
*Iso*valeric	151	159	8
n-Caproic	150	157	7
n-Caprylic	150	157	7
Monochloroacetic	151	160	9
Trichloroacetic	142	149	7
Oxalic	193	199	6
Succinic	147	154	7
Adipic monosalt	157	164	7
Adipic disalt	154	163	9
Sebacic	148	155	7
Benzenesulfonic	149	149	0
p-Toluenesulfonic	183	183	0
β-Naphthalene-sulfonic	192	192	0

* In determining the melting points given in the table, the temperature of the paraffin bath was raised to within 15° C of the melting point before introducing the capillary tube.

paring the results in Table 2 and our results in Table 3 it appears that Donleavy probably used a slower rate of heating than Veibel *et al.* The melting points found by us (rate of heating: 4° C per minute) are in excellent accordance with those given by Veibel *et al.*

The explanation of the fact that the melting points are highly dependent on the rate of heating must be that these salts undergo destruction around the melting point. This is accompanied by an evolution of gas by most of the salts. Probably this destruction commences below the true melting point, when the temperature of the bath is raised too slowly. Thus the derivative

becomes contaminated with its own destruction products and the melting point will naturally be depressed.

It has not been emphasized previously that the melting points are *highly* dependent on the rate of heating. Vogel [4 p. 360] has pointed out that there is several degrees difference in the melting point of a S-benzylthiuronium salt determined on the hot stage and in a paraffin bath, and Kass *et al.*[23] have mentioned that the S-benzylthiuronium derivatives of octadecenoic acids cannot be used due to similarity of melting points and dependence of the melting point on rate of heating.

An attempt was made to use the Kofler "Heizbank" but this apparatus cannot be employed for these derivatives.

The conclusion must be that it is essential to use an apparatus which affords an accurate regulation of the rate of heating, and for the reasons stated above we prefer a rate of heating of 4° C per minute. Furthermore, we wish to stress that it is important to analyse the derivatives, since the melting points cannot always be used in the identification due to similarity.

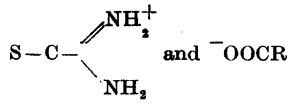
The derivatives of sulfonic acids. The melting points of three sulfonic acids salts are recorded in Table 3, and it is seen that the melting points for this type of S-benzylthiuronium salts do not show any dependence on the rate of heating. The melting point is sharp and these derivatives are valuable for the characterization of sulfonic acids. It has also been found that the Kofler "Heizbank" may be used in the determination.

However, if the sulfonic acid contains other functional groups, *e. g.* sulfanilic acid, it has been found that there is a dependence on the rate of heating.

The derivatives of adipic acid. There is only a very small difference in the melting points of the disalt and the monosalt prepared from adipic acid (Table 2) and furthermore, it has been found that a mixture of the two salts showed no depression of the melting point. According to this it is impossible to deduce the composition of a adipic acid derivative on the basis of the melting point.

In order to confirm that the mixed derivative which was prepared from adipic acid using methyl red as indicator in the neutralization really was a mixture of the monosalt and the disalt, such as the titration with perchloric acid had shown, an X-ray analysis was carried out. This confirmed that it was a mechanical mixture of the two salts.

The similarity of the melting points of the two salts of adipic acid and of their mixture must be explained on the basis of the fact that the melting points of the substituted and the unsubstituted S-benzylthiuronium salts of the carboxylic acids given in Table 2 cover only a small range of temperature. This suggests, as pointed out by Walker [24] that "the forces which bind crystals of this type together are comparable in strength throughout the series of carboxylic acids and that the radicals attached to the two groups



respectively make little or no contribution to the stability of the crystal."

On basis on this explanation one would expect that other derivatives of this type will show melting points which are not well dispersed. We have prepared the S-*p*-nitrobenzylthiuronium derivatives of propionic, *n*-butyric, *iso*butyric, *n*-valeric and *iso*valeric acid and the melting points were 139° C, 137° C, 137° C, 144° C and 137° C respectively (rate of heating: 4° C/min.).

The S-*p*-nitrobenzylthiuronium chloride and the derivatives were prepared according to Rupe and Zweidler [25]. Our derivatives were analyzed by titration with perchloric acid and the observed results agreed with the theoretical values within 0.5 %. These derivatives have no advantage over the other S-benzylthiuronium salts, and furthermore we have observed that they are sensitive to light.

Although the melting points of S-benzylthiuronium salts show a poor dispersion and are strongly dependent on the rate of heating, they have the advantage that they are very easily prepared and, furthermore, it has now been shown that the equivalent weight is readily determined by titration. Therefore, one must expect that they still will be used in the identification of carboxylic acids.

SUMMARY

It is shown that the S-benzylthiuronium salts of carboxylic acids can be accurately titrated with perchloric acid in glacial acetic acid solution. The titration can be carried out either potentiometrically or visually using crystal violet as an indicator.

The method described is recommended for the determination of the equivalent weight of carboxylic acids which are difficult to isolate as pure compounds.

Some of the derivatives contain water of crystallization which is determined according to Karl Fischer.

The derivatives of sulfonic acids are practically neutral in acetic acid solution and cannot be titrated by the method described.

S-alkylthiuronium picrates prepared from aliphatic halides are also titrated with perchloric acid. The equivalent weight of the halide can thus be determined indirectly.

Melting points of nineteen S-benzylthiuronium derivatives, some of which have not previously been recorded in the literature, are given.

A survey of the literature shows that there are disagreements concerning the melting points given for the S-benzylthiuronium salts of carboxylic acids. It is shown that the reason for this is that the melting points are extremely dependent on the rate of heating.

The S-*p*-nitrobenzylthiuronium salts of some aliphatic carboxylic acids have been prepared, but these derivatives appear to have no advantage over other S-benzylthiuronium salts.

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Diheterolevulosan III and IV

The Structure of Diheterolevulosan IV *

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The anhydride mixture resulting from the treatment of D-fructose with concentrated hydrochloric acid [1] has been shown to contain two compounds, diheterolevulosan I and II, the structures of which have been proved to be di-D-fructopyranosido 1,2′:2,1′-dianhydride and a D-fructopyranose-D-fructofuranose 1,2′:2,1′-dianhydride repectively [2–6]. Later, during the course of the present investigation, Wolfrom and coworkers have reported the isolation of a third substance of the same type [7], which they designated diheterolevulosan III, shown by periodate oxidation to be either a di-D-fructopyranose 1,2′-2,3′-dianhydride or an anomer of diheterolevulosan II. According to the same authors, application of the Hudson isorotation rules to the rotatory data of the two compounds supports the latter view.

In our work on the isolation and structure of diheterolevulosan I and II [6], we used paper chromatographic methods, but in some cases there were discrepancies between the chromatographic and the optical rotatory data, and the investigation was therefore continued using other methods.

The anhydride mixture prepared as before was divided by crystallisation into separate fractions, shown in the diagram, Fig. 1, followed by further fractionation on a carbon-celite column using the gradient elution technique (linear gradient) with ethanol as eluant. The elution was followed polarimetrically and by paper chromatography. A typical run is shown in Fig. 2. The components were usually isolated as their acetates which were fairly easy to purify by crystallisation. Diheterolevulosan I and II and the two new compounds diheterolevulosan III and IV were obtained in this manner from the two mother liquors. In addition some amorphous material probably containing the fructose anhydrides described by Wolfrom *et al.* together with some compounds of reducing character were obtained. The crystalline fractions 11, 12 and 131 only yielded diheterolevulosan I, II and IV. As the resolution of diheterolevulosan I, II and IV was not complete on the columns used,

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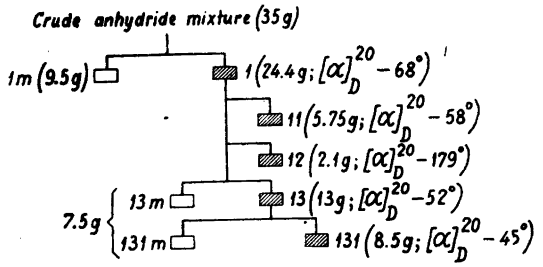

Fig. 1. Recrystallisation chart. Fractionation of the crude anhydride mixture from hydrochloric acid treated D*-fructose.*

a quantitative estimation of the relative amounts of these compounds must be omitted. Nevertheless the relatively large amount of diheterolevulosan IV obtained by us compared with the small amount of syrup unaccounted for in Wolfrom's experiments and which may contain the same substance is noteworthy. A possible explanation is that a higher reaction temperature was used in our experiments than in Wolfrom's. On the other hand the yield of diheterolevulosan III is of the same order in both experiments.

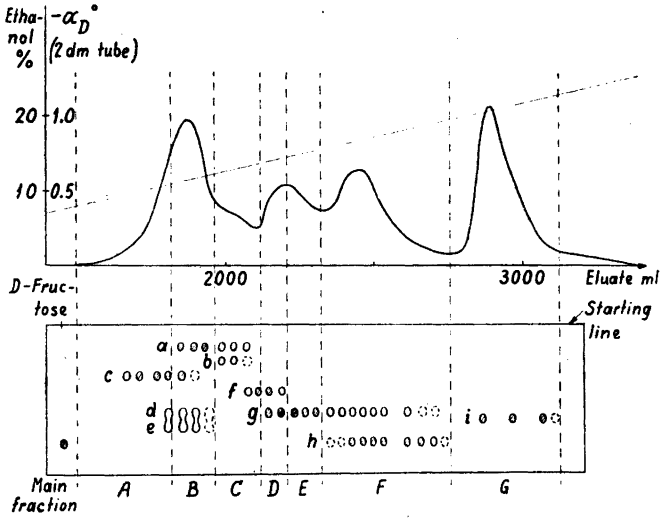

Fig. 2. Chromatographic separation on a carbon column of fructose anhydride mixture from hydrochloric acid treated D*-fructose, using the gradient elution technique. Optical rotation curve and paper chromatogram of the fractions. Spot on paper chromatogram: a—e unknown substance; f diheterolevulosan I; g diheterolevulosan II and IV; h unknown substance; i diheterolevulosan III.*

Though X-ray crystallographic data have not been determined for our preparation of diheterolevulosan III its identity with the substance described by Wolfrom *et al.* is evident from the following facts:

	Wolfrom *et al.*	Present investigation
Diheterolevulosan III	M.p. 255—258° (no dec.)	M.p. 240—242° (dec.)
	$[\alpha]_D^{26}$ −179° (water, c = 3.6)	$[\alpha]_D^{20}$ −183° (water, c = 2)
Acetate	M.p. 135.5—136.5°	M.p. 130—131°
	$[\alpha]_D^{25}$ −169° (chloroform, c = 2)	$[\alpha]_D^{20}$ −159° (chloroform, c = 2)

On periodic acid oxidation both preparations yield one mole of formic acid with the consumption of three moles of oxidant.

When oxidized with periodic acid, diheterolevulosan IV (m.p. 279—281°, $[\alpha]_D^{20}$ −309° in water) consumes four moles of oxidant with the formation of two moles of formic acid. The acetate (m.p. 268—269°, $[\alpha]_D^{20}$ −199° in chloroform) shows a remarkable stability towards heat and is easily purified by sublimation at 12 mm pressure.

On the paper chromatogram, using the butanol-ethanol-water solvent, diheterolevulosan II and IV travel with approximately the same rate and only a little slower than diheterolevulosan III. Diheterolevulosan IV is only with difficulty made visible with resorcinol-hydrochloric acid spraying reagent probably owing to its resistance to hydrolysis.

The structure of diheterolevulosan IV has been shown by the periodic acid oxidation and by its complete methylation and subsequent hydrolysis and identification of the fructose trimethyl ether formed following the general procedure described in a preceding paper [6] to be that of a di-D-fructopyranose 1,2′:2,1′-dianhydride. It therefore obviously is an anomer of diheterolevulosan I ($[\alpha]_D^{20}$ −44° in water).

Applying the Hudson isorotation rules (*cf.* Wolfrom *et al.*[7]) to this pair, assuming diheterolevulosan I to be the α,α'- and diheterolevulosan IV the β,β'- form:

$[M]_D^{20}$	of diheterolevulosan I,	(-44×324)	$= -14\,200$
$[M]_D^{20}$	» » IV,	(-309×324)	$= -100\,100$
	Subtracting	$4A$	$= 85\,900$
		A	$= 21\,500$

This value is in good agreement with that calculable for the two methyl D-fructopyranosides:

$[M]_D^{20}$	of α-D-form	$(+46 \times 194)$	$= 8\,900$
$[M]_D^{20}$	» β-D-form	(-172×194)	$= -33\,400$
	Subtracting	$2A$	$= 42\,300$
		A	$= 21\,200$

Thus the anomeric structure of diheterolevulosan I and IV is established with some certainty.

EXPERIMENTAL

Preparation and fractionation of the anhydrides. D-Fructose (75 g) was dissolved in concentrated hydrochloric acid (300 ml) and allowed to stand 72 hours at 0°. After removal of the hydrochloric acid and fermentation of unchanged fructose as described earlier the resultant syrup (35 g) was dissolved in a small volume of hot methanol and allowed to stand at room temperature for three weeks yielding crystalline material (fraction 1, 24.4 g; $[\alpha]_D^{20}$ −68° in water) and an amorphous mother liquor product (fraction lm, 9.5 g).

A few crystals of diheterolevulosan I were added to the solution of fraction I in hot water (17 ml) and methanol (30 ml). After standing for ten days at 0° the product formed semispherical aggregates which were collected (fraction 11, 5.75 g; $[\alpha]_{20}^{D}$ −58° in water). Paper chromatogram indicated the presence chiefly of diheterolevulosan I together with smaller amounts of one or several of the other diheterolevulosans. During the filtration a micro-crystalline precipitate started to form in the mother liquor which was then allowed to stand in the cold for a further few days yielding the crystalline fraction 12 (2.1 g; $[\alpha]_D^{20}$ −179° in water). Two rather faint spots corresponding to diheterolevulosan I and II, the former predominating, were obtained on the paper chromatogram. After removal of the solvents from the mother liquor, the resultant syrup was dissolved in methanol (25 ml) yielding the crystalline fraction 13 (13 g; $[\alpha]_D^{20}$ −52° in water) and an amorphous mother liquor product (fraction 13 m). On the paper chromatogram fraction 13 gave a strong spot on the same level as diheterolevulosan II and a weaker due to diheterolevulosan I. It was recrystallised from methanol-ethanol-water producing crystals (fraction 131, 8.5 g) and a mother liquor (fraction 131 m).

Fraction 1 m (9.5 g) was dissolved in water (130 ml) and adsorbed on the top of a column (35 × 4.5 cm) of equal parts (by weight) of "carbo animalis pro analysi" (J. P. Riedel and E. de Haen) and Celite. The carbohydrates were then eluted with aqueous ethanol (4 000 ml), the concentration of which was increased linearly from 0 to 30 %. The eluate was divided by means of an automatic fraction collector into fractions (*ca* 25 ml each), which were then investigated with respect to their optical rotation (2 dm tube) and by means of paper chromatography (Fig. 2). They were divided into main fractions (A−G) as indicated in the figure. After evaporation *in vacuo* and dissolution in a small volume of methanol, fractions A and B remained in solution while fractions C−G gave crystals which were collected and characterized by paper chromatography and optical rotation measurements.

Fractions A and B amounted to 400 and 800 mg respectively. Fraction C (430 mg) yielded diheterolevulosan I (220 mg; $[\alpha]_D^{20}$ −43° in water), fraction D (850 mg) diheterolevulosan II (330 mg; $[\alpha]_D^{20}$ −39°, contaminated with diheterolevulosan I), fraction E (900 mg) diheterolevulosan II (560 mg; $[\alpha]_D^{20}$ −36°), fraction F (1.1 g) diheterolevulosan II and IV (460 mg; $[\alpha]_D^{20}$ −108°, single spot on paper chromatograms) and fraction G (560 mg) diheterolevulosan III (380 mg, $[\alpha]_D^{20}$ −168°).

Upon evaporation under reduced pressure of the eluate from the column preceding fraction A, an oil (1.0 g) was obtained which on paper chromatography appeared to contain mainly glycerol together with very small amounts of fructose. The eluate succeeding fraction G gave on evaporation a syrup (440 mg) of complex composition whilst washings from the column (2 000 ml 50 % ethanol) when concentrated under reduced pressure (40°) gave a residue (600 mg) which suddenly underwent spontaneous decomposition.

When acetylated in acetic anhydride-pyridine solution the crystalline material from fraction F yielded impure diheterolevulosan IV hexaacetate (160 mg from ethanol, m.p. 260−265°). A pure product was obtained by recrystallisation from ethanol and by sublimation under water pump pressure, m.p. 268−269° (under sublimation), $[\alpha]_D^{20}$ −199° (chloroform, $c = 2$). (Analysis: Found C 50.2; H 5.67. Calc. for $C_{24}H_{32}O_{16}$: C 50.0; H 5.60.)

The crystalline material from fraction G was acetylated in a similar manner yielding diheterolevulosan III hexaacetate (520 mg) from ethanol in thin plates, m.p. 129−131°. Despite repeated recrystallisations from ethanol the melting point could not be raised

above 130.5—131° (*cf.* Wolfrom *et al.*, m.p. 135.5—136.5°). $[\alpha]_D^{20}$ —159° (chloroform, c = 2). (Analysis: Found C 50.1; H 5.63. Calc. for $C_{24}H_{32}O_{16}$: C 50.0; H 5.60.)

The relatively large amount of material from fraction 1 m which remained amorphous even after separation on the carbon column is explained by the fact that the mother liquors remaining show a very complicated composition on paper chromatograms. The three dominating amorphous components were found in fractions A, B and F. They have not been investigated further.

Fraction 13 m + 131 m (7.5 g) was separated in the same manner as 1 m, the optical rotation curve obtained being very similar to that shown in Fig. 2. However, relatively more material was obtained in the crystalline state. Diheterolevulosan III hexaacetate (240 mg, m.p. 128—129°) and diheterolevulosan IV hexaacetate (600 mg, m.p. 263—267°) were obtained as well as diheterolevulosan I and II (*ca* 5 g together).

Fraction 11 (5.75 g) was acetylated without further separation, yielding diheterolevulosan I hexaacetate (6.5 g, m.p. 165—168°) and diheterolevulosan IV hexaacetate (170 mg, m.p. 267—268°).

Fraction 12 (2.1 g) was added to a carbon column (23 × 3.3 cm) and eluted with aqueous ethanol (2 000 ml) the concentration of which was raised linearly from 0 to 40 %. In the optical rotation curve there was only one peak corresponding to a partly separated mixture of diheterolevulosan I, II and IV. Unfortunately, owing to an accident with the fraction collector, the last parts of the eluate where diheterolevulosan III should eventually be found were lost. The eluate was evaporated to give two fractions the first of which contained mainly diheterolevulosan I and II and the second (1.09 g, $[\alpha]_D^{20}$ —250°) diheterolevulosan II and IV. The latter, when acetylated, gave diheterolevulosan IV hexaacetate (1.2 g, m.p. 265—267°).

Fraction 131 (8.5 g; $[\alpha]_D^{20}$ —45°) was separated on a carbon column in the same way as 1 m, the optical rotation curve of the eluate showing two peaks close together corresponding to diheterolevulosan I and II and diheterolevulosan IV respectively. Diheterolevulosan III if it had been present should have given rise to a third peak well separated from the two obtained. The first part of the eluate gave a mixture of diheterolevulosan I and II (5.8 g, $[\alpha]_D^{20}$ —39°) and the last a mixture (1.75 g) which upon acetylation yielded diheterolevulosan IV hexaacetate (130 mg, m.p. 260—264°).

Diheterolevulosan III. Diheterolevulosan III hexaacetate (200 mg) in absolute ethanol (5 ml) containing sodium ethoxide (100 mg) was allowed to stand at room temperature overnight. After dilution with water the reaction mixture was passed through the Amberlite resin, IR 120, and then evaporated under reduced pressure. The residue was dissolved in ethanol and yielded diheterolevulosan III (95 mg) which on recrystallisation from aqueous ethanol had m.p. 240—242° (dec.), $[\alpha]_D^{20}$ —183° (water, c = 1.8). (Analysis: Found C 44.4; H 6.14. Calc. for $C_{12}H_{20}O_{10}$: C 44.4; H 6.22. Periodic acid consumption (10 mg of substance in 0.1 *C* H_5IO_6 (2 ml), 24 hours at room temperature) 3.12 moles. Formic acid liberated when a sample (10 mg) dissolved in 0.25 *C* sodium metaperiodate (1 ml) and water (5 ml) was allowed to stand at room temperature overnight: 0.9 moles.)

Diheterolevulosan IV. Deacetylation of the acetate (200 mg), as described above, yielded diheterolevulosan IV (97 mg), which on recrystallisation from aqueous ethanol formed heavy prisms, m.p. 279—281° (dec.), $[\alpha]_D^{20}$ —309° (water, c = 2). (Analysis: Found C 44.5; H 6.12. Calc. for $C_{12}H_{20}O_{10}$: C 44.4; H 6.22. Periodic acid consumption: 4.12 moles. Formic acid liberated on sodium metaperiodate oxidation: 1.7 moles.)

Diheterolevulosan IV hexamethyl ether. Dioxane (5 ml) was added to diheterolevulosan IV hexaacetate (290 mg = 0.5 mmol) and powdered sodium hydroxide (600 mg = 15 mmol) in a flask with an effective mechanical stirrer. Methyl sulphate (0.6 ml = 6 mmol) was added slowly (0.5 hour) at 60—65° under vigorous stirring and the temperature was then raised to 70° for 2 hours. The mixture was diluted with dioxane (5 ml) and allowed to stand at room temperature overnight. It was filtered, the filter cake being carefully washed with dry benzene, and the filtrate evaporated to dryness. The residue (dried over silica gel) was dissolved in liquid ammonia (*ca* 5 ml) and potassium metal added till a stable blue colour was obtained. After evaporation of the ammonia the residue was dissolved in dry methyl iodide (1 ml) and left overnight. The excess of methyl iodide was removed, the reaction product extracted with chloroform and the solution passed through

a short column of alumina. On the addition of light petrol to the strongly concentrated chloroform solution diheterolevulosan IV hexamethyl ether crystallised in hard aggregates (156 mg = 77 %, m.p. 119—122°). Distillation ($b.p._{0.1}$ *ca* 170° bath temperature) and recrystallisation from chloroform-light petrol yielded prisms, m.p. 122—122.5°, $[\alpha]_D^{20}$ −243° (chloroform, $c = 2$), properties unchanged after two recrystallisations from isopropyl ether. (Analysis: Found OCH_3 44.6. Calc. for $C_{12}H_{14}O_4(OCH_3)_6$: 45.6.)

Hydrolysis of diheterolevulosan IV hexamethyl ether. A preliminary experiment showed that the hydrolysis of the methyl ether reached *ca* 30 % completion when run under the same conditions as used for hydrolysis of diheterolevulosan I hexamethyl ether (1.5 *N* H_2SO_4, 80° and 18.5 hours). Paper chromatograms indicated the formation of fructose 3,4,5-trimethyl ether as the only hydrolysis product. It was found that the methyl ether of diheterolevulosan IV could be separated from the fructose trimethyl ether formed by partition between water and chloroform analogous to the corresponding methyl ethers of diheterolevulosan I and II.

A solution of diheterolevulosan IV hexamethyl ether (110 mg) in 1.5 *N* sulphuric acid (1.1 ml) was kept at 80° for 15.5 hours. It was then extracted with chloroform (4 × 0.5 ml) and the combined extracts were washed with water (1.5 ml) which in its turn was extracted with chloroform (2 × 0.5 ml). All chloroform phases were then combined and evaporated, and the residue crystallised from isopropyl ether, yielding unchanged hexamethyl ether (60 mg). The mother liquor was evaporated to dryness, the residue dissolved in water (0.4 ml) and this solution extracted with chloroform (3 × 0.15 ml) to recover the last traces of unchanged hexamethyl ether. The two aqueous solutions were combined with the hydrolysate, forming the fructose trimethyl ether fraction, whilst the remaining chloroform phase after evaporation was combined with the recovered hexamethyl ether (60 mg) and the combined material thus obtained was subjected, after the addition of fresh methyl ether (30 mg), to repeated hydrolysis following the scheme given above.

After the second hydrolysis the chloroform soluble material recovered weighed 75 mg (largely crystalline). Starting from this lot the hydrolysis was repeated twice, yielding in the end 30 mg of unchanged hexamethyl ether. The amount of material consumed was then 140 − 30 = 110 mg.

The combined extracted hydrolysates were neutralised with barium carbonate, filtered, evaporated and the residue extracted with chloroform. The chloroform solution was filtered and evaporated yielding crude fructose trimethyl ether as a pale yellow oil (122 mg). For further purification this was dissolved in a benzene-ethanol (520 : 85) solvent saturated with water (2.5 ml), added to the top of a hydrocellulose column (20 × 2 cm), and eluted with the same solvent. The eluate was collected in 3 ml fractions and the fructose methyl ether located by putting a few drops of each fraction on a filter) paper and then spraying with aniline hydrogen phthalate reagent. Evaporation of the fractions (36—42) containing the bulk of the fructose methyl ether yielded an oil (93 mg which after distillation ($b.p._{0.8}$ 130° bath temperature) amounted to 88 mg (73 % of theory) and had $[\alpha]_D^{20}$ −134° (water, $c = 2$), which compares favourably with $[\alpha]_D^{20}$ −128° (water, $c = 2$) observed for an authentic sample of D-fructose 3,4,5-trimethyl ether.

D-*Arabonamide 2,3,4-trimethyl ether from* D-*fructose 3,4,5-trimethyl ether.* To a solution of fructose trimethyl ether (66 mg) in acetic acid (1 ml) and water (0.5 ml) the stoichiometric amount (135 mg) of lead tetraacetate was added. After standing at room temperature for two hours, the solution was diluted with water (1 ml) and acetic acid (0.5 ml), freed from lead dioxide by centrifuging, and the supernatant liquid saturated with hydrogen sulphide and centrifuged. Evaporation of the clear solution gave the crude lactone derivative, which was distilled at water pump pressure (bath temperature 150—160°) and then dissolved in liquid ammonia. After the ammonia had evaporated, the crystalline residue was dissolved in acetone and the solution after filtration evaporated to 0.5 ml. When isopropyl ether (0.5 ml) was added, D-arabonamide 2,3,4-trimethyl ether separated as needles (40 mg). From the mother liquor a further quantity (10 mg) of substance was obtained making a total yield of 80 % of theory. M.p. 102.5—103°, unchanged after recrystallisation from the same solvents. When mixed with an authentic specimen m.p. 102.5—103°) the melting point was again unchanged.

The overall yield of arabonamide derivative calculated upon diheterolevulosan IV hexamethyl ether consumed is 58 % of theory, proving that diheterolevulosan IV is built up from 1,2-linked D-fructopyranose units only.

SUMMARY

The anhydride mixture resulting from treatment of D-fructose with concentrated hydrochloric acid has been separated on carbon columns using the gradient elution technique. Two new compounds, diheterolevulosan III and IV were isolated, the former evidently being identical with diheterolevulosan III reported by Wolfrom and coworkers. Diheterolevulosan IV has been proved to be di-D-fructopyranose 1,2′-2,1′-dianhydride. Thus diheterolevulosan I and IV are anomers, and from their optical rotation it can be concluded that the former is the α,α'- and the latter the β,β'-form.

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Studies on Aspartase

IV. On the Effect of pH on Aspartase

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The catalytic power of aspartase seems to be highly sensitive to changes of pH. A sharp pH optimum has been found to be situated in the neutral pH-region [1]. From this optimum the acid branch of the pH curve falls steeply [1]. Nothing is known of the character of the alkaline branch of the activity curve.

The present investigation has been undertaken in order to elucidate the influence of pH on the activity and stability of aspartase and thus to find out something of the factors which determine the characteristic features of the pH-activity curve. The aspartase has been obtained from propionic acid bacteria.

EXPERIMENTAL

Enzyme preparation: Propionibacterium peterssonii (strain of this laboratory) was the starting material. The bacteria were cultivated as earlier and prepared as a dry powder [2]. From the dry powder a crude enzyme solution was obtained by *n*-butanol treatment, using a method described earlier [2].

Activity measurements: The initial velocity of the reaction served as measure of the enzymatic activity. It was obtained graphically from a complete experimental curve and expressed in μg NH_3—N liberated or bound within 1 hour at 37° C and in the buffer designated. The activity is expressed as a percentage of the maximum of the initial velocity obtained.

The test solution was exactly the same as used earlier [2] with L-aspartic acid except that enzyme solutions were used instead of dry bacteria. When the enzyme solution was buffered no phosphate buffer was used.

When fumaric acid and ammonia were used as substrates in the synthesis of L-aspartic acid the test solution was the same as in the deamination experiments but instead of 2 ml 0.1 *M* L-aspartic acid, 2 ml 0.1 *M* fumaric acid (pH 7.3) and ammonium chloride were employed.

Ammonia was determined in exactly the same way as described earlier [2].

The hydrogen ion concentration was determined with a glass electrode (Beckman).

The buffer solutions in the experiments were prepared by mixing of the following solutions: sodium acetate (0.1 *M*)-acetic acid (0.1 *M*), sodium dihydrogen (0.33 *M*)-sodium monohydrogen phosphate (0.33 *M*), tris(hydroxymethyl)aminomethane [3] (0.2 *M*)-hydrochloric acid [3] (0.1 *M*) and sodium acetate (1/7 *M*) + the sodium salt of veronal (1/7 *M*)-hydrochloric acid [4] (0.1 *M*).

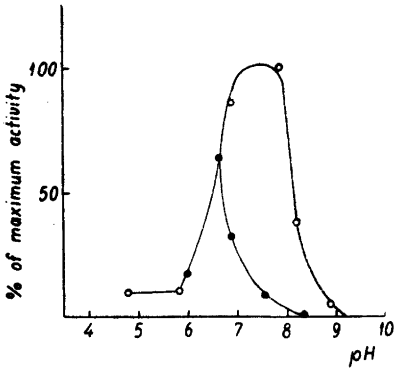

Fig. 1. Influence of pH on aspartase activity. Initial reaction velocities (expressed as percentages of the maximum activity) measured at 37° C in acetate and tris (hydroxymethyl)-aminomethane buffers (○) and in phosphate buffer (●) with L-*aspartic acid as substrate.*

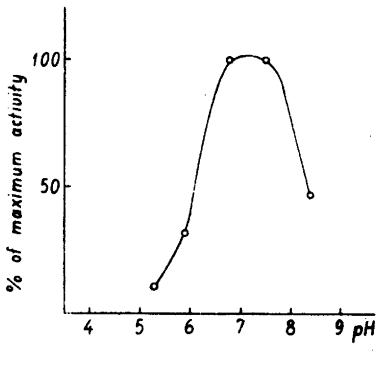

Fig. 2. Influence of pH on aspartase activity. Initial reaction velocities measured at 37° C in veronal-acetate buffer with fumaric acid and ammonia as substrates.

The pH-activity curve. In the deaminating experiments acetate buffer was used in the test solution from pH 4.5 to 5.7 in a total concentration of 0.03 *M* in relation to acetate. Phosphate buffer was used from pH 6 to 8.5 in a total concentration of 0.066 *M* in relation to the PO_4-group. Sodium chloride was added to this buffer to obtain a constant ionic strength equal to 0.2. The tris(hydroxymethyl)aminomethane buffer was used in a final concentration of 0.03 *M* in relation to tris(hydroxymethyl)aminomethane.

The synthesis experiments were performed in the buffer of Michaelis [4] at a final concentration of 0.01 *M* in relation to sodium acetate and the sodium salt of veronal. The pH was measured at the beginning and the end of the experiment and the average was used for the activity curve.

The pH-stability curve: 4 ml enzyme solution was used in all the experiments. The pH was adjusted with 0.1 *M* acetic acid, 0.1 *M* sodium hydroxide and tris(hydroxymethyl)aminomethane buffer with a final concentration of 0.033 *M*. The enzyme solution was then incubated at 37° C for 24 hours. During this time pH was controlled and, if it changed, adjusted to its original value. The pH was measured at the beginning and the end of the incubation and the average was used for the stability curve. After incubation pH was adjusted to 7.2 and the activity of the preparation was measured at this pH as described above.

RESULTS

The correlation between pH and activity of aspartase is illustrated in Fig. 1 with L-aspartic acid and in Fig. 2 with fumaric acid and ammonia as substrates. No greater difference can be observed between these two curves although a lesser shift of the acid branch of the synthesis curve towards the acid side may be noticed. Fig. 1 shows the strong inhibitory effect of phosphate, which causes an apparent shift of the pH-optimum towards the acid side. Since the total concentration of phosphate (PO_4) and ionic strength were kept constant in all pH values the only change with rising pH was a shift of dihydrogen phosphate to monohydrogen phosphate. The increasing inhibitory effect with the rise in

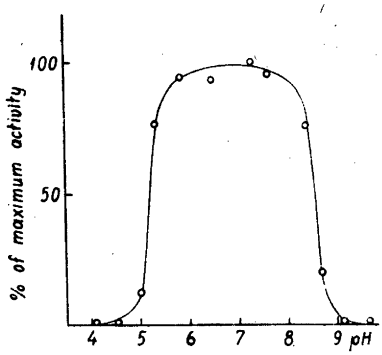

Fig. 3. Influence of pH on the stability of aspartase at 37° C.

the concentration of the monohydrogen ion of phosphate is strongly indicative of the existence of two valencies available for combination with the inhibitor.

The same concentration of phosphate as used here affects the activity of fumarase too [5]. But contrary to aspartase, the pH-optimum of fumarase is shifted towards the alkaline side by phosphate and a considerable activation is observed over most of the pH-range studied [5].

In an earlier paper no inhibitory effect of phosphate was observed [6]. Then, however, the concentration of monohydrogen phosphate was low, since the experiments were performed at pH 7.

The effect of pH on enzymatic activity is considered to be a summation of at least three factors: the stability of the enzyme, the ionization of the groups involved in the formation of the enzyme-substrate compound, and the ionization of the substrate.

In order to discover the extent to which the effect limiting the activity of aspartase at acid and alkaline pH values is due to enzyme destruction, the pH stability of aspartase was studied (Fig. 3). Aspartase seems to be stable between pH 6 and 8 but on the acid side of 5.5 and the alkaline side of 8.5 aspartase is irreversibly changed to an inactive protein. Comparing the pH-activity curves with the pH-stability curve reveals that the alkaline branch of the pH activity curve depends on instability of the enzyme at these pH values. The acid branches exhibit no such similarities as those of the alkaline branches.

DISCUSSION

Since the enzymes are proteins it seems rather obvious that their activity must depend on the electrical charge or on the state of dissociation of the enzyme. The variation of the activity with pH has therefore been interpreted in terms of acceptance and donation of protons by the active enzyme. This concept was further expanded to include ionization of the substrate.

Aspartase was found to exercise its catalytic power within the pH-range 6 to 9. Accordingly, only the zwitter-ion of L-aspartic acid forms the active substrate. (The pK values of aspartic acid are as follows: pK_1 = 1.88, pK_2 = 3.75 and pK_3 = 9.60) [7]. Hence it is quite evident that the ionization of the

substrate did not influence the shape of the pH-activity curve to any greater extent although a decrease of active substrate took place at the highest alkaline values (*e.g.* 10 % at pH 8.6). Since initial velocity is proportional to the product of the active substrate concentration and the active enzyme concentration, the pH-activity curve in Fig. 1 represents the concentration of active enzyme only.

An evaluation of the dissociation constants of the active groups of the enzyme may be possible from data in the pH-activity curve, which may give indications as to the possible structure of the functional groups in the active surface.

The acid branch of the activity curve shows a pK of 6.2—6.5 (the pH value corresponding to half-maximum activity). As the pH rises increasing quantities of the enzyme are changed into an active form till its concentration again begins to decrease. The alkaline branch of the pH activity curve indicates a pK value of 8.2—8.5. The alkaline branches of the pH activity and pH stability curves seem to be nearly identical. It may at least be assumed that the same groups are responsible for the decrease in activity in both cases. No similar consonance is observable in the acid branches.

The acid group (s) in aspartase with pK = 6.2—6.5 permits the exclusion of hydroxyl groups (the pK of the phenolic group in tyrosine is equal to 10.07) [7], sulphydryl groups (the pK of this group is 8.18[8] or 10.28[7], *cf.* below), the guanidine group (the pK of the guanidine group in arginine is equal to 12.48) [7]. The dissociation constants of most amino groups differ considerably from the value of the acid group in aspartase, although in special peptidic combinations such as tyrosylarginine (with pK equal to 7.39) [7] and phenylarginine (with pK equal to 7.57) [7] the constants are not so far from that of the acid group. Nor is it likely that carboxyl groups of aspartic acid and glutamic acid are responsible for the dissociation constant above. The free second carboxyl group of aspartic and glutamic acid have the pK value 3.65 and 4.25, respectively [7]. In special peptides these values are slightly higher (*e.g.* in aspartylglycine the pK is equal to 4.53 and in lycylglutamic acid to 4.47) [7].

Ring nitrogen, however, as in imidazole derivatives seems to have dissociation constants which concur well with the value of the acid group in aspartase. The pK value of imidazole nitrogen in histidine is equal to 6.00 [7] (in histidylhistidine the pK values of the imidazole groups are 5.60 and 6.80) [7].

Free histidine in acid solution exists in the form of imidazolium ion, in which there is resonance of the double bond [9]:



The imidazolium ion is capable of dissociating a proton (with a p$K \approx 6$), giving rise to the following resonating structure in alkaline solutions [9]:



A B

Since B is less stable than A it is almost completely suppressed in the imidazole.

The importance of the acid group in the enzyme is explained by the suggestion that aspartase is a metal protein [6], a suggestion strongly confirmed in this study by the observations concerning the inhibitory effect of phosphate. If the metal is bound by ionic bonds to any group in the enzyme it replaces a proton. From the discussion above it can be concluded that the metal atom must be bound to imidazole groups, *i.e.* to the negatively-charged N-atom in the imidazole nucleus (formula B above). After coupling with the positive metal the unstable form (B) is stabilized by induction from the metal.

Histidine has been found to bind many divalent metals very readily, surpassed only by cysteine in this respect, with the surprising exception of Mn and Mg[10]. These data, however, are not valid for the combination of metal with histidine residues in a protein since the carboxyl and amino groups present in the histidine are not available when the histidine forms part of a peptide chain.

The dissociating basic group of aspartase with a pK value equal to 8.2—8.5, responsible for the alkaline branch of the activity curve, rules out carboxylic guanidium and phenolic ions. The pK value of this group seems to be in the sulphydryl or amino group range. There seems, however, to be some doubt about the pK values of the sulphydryl group in cysteine. The treatise of Schmidt [8] favours pK 8.18, whereas Cohn and Edsall [7] give pK 10.28. The most recent investigation shows up the difficulties of attributing the pK values to either of the thiol groups or the amino groups respectively [11].

In the case of aspartase it is known from inactivation by specific reagents that free sulphydryl groups are essential to the enzymatic action. The alkaline group with a pK value 8.2—8.5 is therefore assumed to indicate the dissociation of a sulphydryl group. The undissociated form of the group seems to be essential to the activity of the enzyme.

SUMMARY

1. Aspartase activity exhibits a pH optimum at 7.5 with a steep decrease towards the acid and alkaline sides.

2. Phosphate buffer exercises an inhibitory effect on the catalytic action of aspartase.

3. Aspartase is stable between pH 6 and 8. On the acid side of pH 5.5 and the alkaline side of 8.5 the enzyme is irreversibly changed to an inactive protein.

4. The results are discussed and the dependence of aspartase activity on pH is interpreted in terms of acid and basic groups in the active centre. On

the basis of dissociation constants it is suggested that histidine residues and undissociated sulphydryl groups are functional groups in the active aspartase. The histidine residues are assumed to link the active metal to the enzyme.

I am indebted to Professor A. I. Virtanen for his stimulating interest in this work and for valuable discussions.

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The Preparation of Apurinic Acids and A New Method for the Quantitative Determination of the Purine Contents of Deoxyribonucleic Acids

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It has been demonstrated [1,2] that the purine deoxyribonucleosides on treatment with an acidic type of resin, such as Amberlite IR-120 (H) in the hydrogen form, are hydrolysed to the purines and the free sugars. This finding suggested that the contact of an aqueous solution of deoxyribonucleic acid (DNA) with such a resin would result in the liberation of the purines with the simultaneous formation of the so called apurinic acid [3]. When liberated, the purines would be retained by the acidic resin and the aqueous solution should contain only apurinic acid.

The present paper describes the preparation of the apurinic acids from the DNA's isolated from the following sources: calf thymus gland, soft herring and cod roe, wheat germ and *Mycobacterium phlei* by a method which involves treatment of their aqueous solutions with Amberlite IR-120 (H). In addition a quantitative micro-method for the determination of the purines of DNA based on the removal of purines by the resin is described, and applied to the above mentioned DNA's.

EXPERIMENTAL

Preparation of resin. Amberlite IR-120 (H) (50 g, 40—60 mesh) was treated with hydrochloric acid (1 l, 4 *N*) in a column, washed free of acid with water, washed with alcohol (200 ml), ether (200 ml) and finally dried at 60° C until free from ether. This product was used in all the subsequent experiments.

Deoxyribonucleic acids. The DNA's from calf thymus gland, soft herring roe, wheat germ and *M. phlei* employed in the present investigations were those described in a previous publication [4] (DNA from calf thymus gland preparation T_1, and DNA from hering roe was H_1). The DNA used from soft cod roe has been described in a recent paper [1].

Nitrogen and phosphorus analyses. The methods described in a following paper were used [2].

Standard purines. Adenine (supplied by B.D.H, Ltd.) was purified by recrystallisation once from hot water. Guanine (supplied by B.D.H, Ltd.) was recrystallised according to the procedure of Horbaczewski [5]. The recrystallised products were dried over

phosphorus pentoxide in vacuum (20 mm Hg) at 110° C. The molecular extinction coefficients in hydrochloric acid (4 *N*) of the standard adenine and guanine were determined in the Beckmann spectrophotometer at four wavelengths. These were:

	270 mμ	260 mμ	250 mμ	245 mμ
Adenine	9 700	11 150	9 350	7 600
Guanine	5 450	8 300	10 200	10 200

Preparation of apurinic acid from calf thymus DNA

An aqueous solution (40 ml) of DNA from calf thymus gland (1 mg / ml approx., freeze-dried material) and Amberlite IR-120 (H) (5.7 g) were mixed in a flask fitted with a quick fit stopper and shaken gently at room temperature. The initial ultra violet absorption of the DNA solution was determined by diluting an aliquot (0.3 ml) with phosphate buffer (to 5 ml with 0.1 *M* buffer, pH 7.0) and examining the solution at 260 mμ in the Beckmann spectrophotometer. At intervals (*i. e.* every day) the ultra violet absorption of the solution being treated with the resin was determined in the same way. After fourteen days the resin was filtered off, washed with water (10 ml), the solution neutralised with dilute alkali to pH 7.0 and evaporated to dryness in the frozen state. 25 mg of a white product was obtained. It was analysed for nitrogen and phosphorus and the atomic N/P ratio calculated.

General method for the determination of the purines of DNA

DNA (approx. 7 mg, freeze-dried material) was dissolved in water (7 ml) and part of the solution (5 ml) was transferred to a small flask (volume 10 ml) containing Amberlite IR-120 (H) (0.72 g). The flask was closed with a slightly greased quick fit stopper. It was then shaken gently at room temperature for twelve days. The total phosphorus content of the solution (5 ml) was calculated by determining the content of an aliquot (0.4 ml) of the remaining of the original DNA solution. In addition the ultra-violet absorption spectrum was determined by diluting an aliquot (0.3 ml) with buffer (to 5 ml with 0.1 *M* phosphate buffer, pH 7.0) and examining the diluted solution in the Beckmann spectrophotometer in the range 230—290 mμ. When twelve days had elapsed the solution was filtered from the resin using a small glass column (diameter 1.3 cm, length 9.5 cm) fitted with a glass disc which served as a filter. The column was connected with a filtering tube and the water pump. The filtrate containing the apurinic acid was analysed for phosphorus by withdrawing aliquots (0.4 ml) and the ultra-violet absorption spectrum determined as above (this latter spectrum and that of the original DNA are not required for the determination of the purines). The remaining of the filtrate was neutralized with sodium hydroxide and the apurinic acid isolated after evaporation in the frozen state in vacuum.

The resin was transferred quantitatively from the flask to the column with some water (20 ml) and washed (with 20 ml of water). To remove the purines, the resin was eluted with hydrochloric acid (4 *N*, 100 ml) at a rate of approximate 1 ml/min. The eluate was collected in a volumetric flask (100 ml). Examination of subsequent eluate at 260 mμ indicated that the volume used for elution was sufficient to remove the purines quantitatively. The ultra-violet absorption of the eluate was determined in 1 cm cells at several wavelengths using 4 *N* HCl as a blank. The amounts of adenine and guanine present in the eluate could be calculated by using the following formulae:

$$\text{Adenine (in } \mu\text{g)} = \frac{E\lambda_1 \cdot G\lambda_2 - E\lambda_2 \cdot G\lambda_1}{A\lambda_1 \cdot G\lambda_2 - A\lambda_2 \cdot G\lambda_1} \cdot A \cdot 100 \qquad \text{(a)}$$

$$\text{Guanine (in } \mu\text{g)} = \frac{E\lambda_1 - \frac{\text{Adenine} \cdot A\lambda_1}{100 \cdot A}}{G\lambda_1} \qquad \text{(b)}$$

Formulae (a) and (b) were obtained by solution of the following two equations:

$$\text{Adenine} \cdot \frac{A\lambda_1}{A} + \text{Guanine} \cdot \frac{G\lambda_1}{G} = E\lambda_1 \cdot 100$$

$$\text{Adenine} \cdot \frac{A\lambda_2}{A} + \text{Guanine} \cdot \frac{G\lambda_2}{G} = E\lambda_2 \cdot 100$$

In the two formulae, $E\lambda_1$ and $E\lambda_2$ designate the optical densities at the two wavelengths λ_1 and λ_2 ($\lambda_1 > \lambda_2$). Two sets of wavelengths were used for the calculations: 270 and 250 mμ, as well as 260 and 245 mμ · $G\lambda_1$, $G\lambda_2$, $A\lambda_1$ and $A\lambda_2$ are the optical densities of the standard adenine and guanine solutions (in 4 N HCl) in 1 cm cells at the two wavelengths, and A and G the concentrations (in μg/ml) of the standard solutions. All concentrations of purines determined in this investigation are the mean values of the figures obtained by calculations at the two sets of wavelengths mentioned above. The formulae (a) and (b) are similar to those used by Laland *et al.*[4] for the determination of amounts of adenine and cytosine in a mixture, and to those described by Chargaff *et al.*[6] for the determination of mixture of uridylic acid and cytidylic acid and now recently for adenine and guanine [3].

Chromatographic analyses of the apurinic acids

The apurinic acid (2 mg) was heated with perchloric acid [7] (0.4 ml, 35 %) on a boiling water bath for 90 minutes. An aliquot of the hydrolysate (0.05 ml, containing approx. 0.035 mg of phosphorus) was put on a paper chromatogram, developed with the *iso*propanol-hydrochloric acid mixture (65 % aqueous *iso*propanol (v/v), 2 N with respect to hydrochloric acid) of Smith and Wyatt [8] and the chromatogram photographed according to the technique of Markham and Smith [9]. In all the apurinic acids examined, the chromatograms revealed the presence of the pyrimidines but only traces of the purines were detectable.

CONTROL EXPERIMENTS

Estimations of prepared mixtures of adenine and guanine

Guanine (454 μg) in dilute hydrochloric acid solution (0.1 N, 0.5 ml) and adenine (440 μg) also in hydrochloric acid solution (0.1 N, 0.25 ml) were mixed with water (5 ml) and Amberlite IR-120 (H) (0.72 g) in a small flask. The flask was kept at room temperature for two hours with occasional shaking. The resin was filtered off quantitatively in the manner described in the section of the purine determination of the DNA's. Examination of the filtrate in the spectrophotometer at 260 mμ indicated that negligible amounts of purines were left in the filtrate. The purines absorbed by the resin were eluted with hydrochloric acid (100 ml, 4 N). The ultra-violet absorptions of the eluate at 270 and 250 mμ, as well as that at 260 and 245 mμ were used to calculate the amounts of adenine and guanine by application of the formulae previously given in this paper.

Resin blank

To determine the ultra-violet absorption of the eluate of the resin used in the determination of the purines, the following experiment was carried out. Amberlite IR-120(H) (0.72 g) was shaken in the usual manner with water (5 ml) for twelve days at room temperature. The resin was then filtered off and eluted as described previously. The eluate was examined against hydrochloric acid (4 N) at 270, 260, 250 and 245 mμ and exhibited negligible absorption (approx. 0.01 in optical density).

Liberation of purines due to the acidity of a DNA solution when mixed with IR-120 (H)

DNA solution (5 ml, approx. 1 mg/ml) was mixed with Amberlite IR-120(H) (0.72 g) and left at room temperature with occasional shaking, and the resin filtered off after one hour. A few drops of chloroform were added to the filtrate to prevent growth. The solution was kept in a stoppered flask at room temperature for fourteen days, and then neutralized with dilute alkali. Phosphorus analysis were carried out on aliquots (0.5 ml). The presence of any free purines in the solution was determined by quantitative paper chromatography (0.25 ml of the solution was put on the chromatogram). Three determinations were carried out. The chromatograms were developed with a mixture of *n*-butanol and aqueous ammonia (0.6 *N*) (6 : 1, v/v) [10]. The ultra violet prints of the chromatograms indicated the presence of two minor spots having R_F values identical with that of adenine and guanine, respectively. The spots were cut out and eluted with hydrochloric acid (3 ml, 0.1 *N*) and the amounts of purines determined quantitatively by ultra-violet spectroscopy.

Examination of the eluate of the resin containing the purines

Part of the hydrochloric acid eluate (40 ml) of the resin containing the purines from calf thymus DNA (approx. 5 mg) (see the section for the determination of the purines), was added ethanol (200 ml) and concentrated in vacuum. When concentrated to a small volume (approx. 10 ml) further addition of ethanol was made and concentration again carried out. This was repeated three times after which time the hydrochloric acid was practically removed. The final volume was 1 ml and aliquots were examined by paper chromatography using the *iso*propanol-hydrochloric acid mixture [8]. The ultra-violet print of the chromatograms demonstrated the presence of only adenine and guanine. Analysis of the concentrate (0.5 ml) revealed the absence of phosphorus.

RESULTS AND DISCUSSION

Apurinic acids. On treatment of an aqueous solution of the sodium salt of DNA from calf thymus with Amberlite IR-120 (H) in the hydrogen form at room temperature, a rapid increase in the ultra violet absorption at 260 mμ takes place: Fig. 1. This can be explained in the following way. When treating the DNA solution with the resin, a partly exchange of the sodium ions and the hydrogen ions of the resin occurs which results in a fall in pH to 2.7—2.9. It is well known fact that such a change in the pH of a native DNA solution is followed by an increase in the ultra-violet absorption [11].

It appears from Fig. 1 that on continued treament of the DNA solution with the resin, a slow decrease in the ultra-violet absorption takes place, reaching a steady state after twelve days. The decreased ultra-violet absorption has been shown to be due to the complete liberation of the purines with the subsequent absorption of the latter by the resin. This was demonstrated by paper chromatography of the hydrolysate of the material remaining in the solution, and of the hydrochloric acid eluate of the resin. It is reasonable to assume that before any molecule is hydrolysed by a resin, it must at least be located on its surface. The macromolecules of DNA will be attracted to the sulphonic acid groups of the resin because of the amino groups of the former. The DNA molecules are far too large to enter the small pores of the resin and therefore only the surface can accommodate the molecules to be hydrolysed.

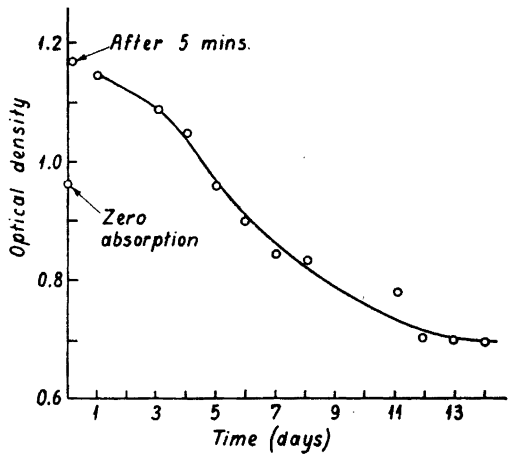

Fig. 1. Changes in optical density at 260 mμ of a solution of calf thymus DNA on treatment with Amberlite IR-120 (H).

On the other hand, smaller molecules such as the deoxyribonucleosides which have been shown to be hydrolysed very rapidly by the resin are accommodated by the whole resin particle. Thus the rate of liberation of the purines from DNA would be expected to be very much slower than for instance in the case of the purine nucleosides. This is borne out by the time (12 days) required for the complete removal of the purines in the DNA, compared with the almost instantaneous hydrolysis of the purine deoxyribosides [1,2].

The acid pH (2.7—2.9) of the solution of DNA being treated with the acidic resin could, in part, be responsible for the liberation of the purines. Experiments demonstrated that in the case of DNA from calf thymus, 13.4 and 17.9 % of the total adenine and guanine respectively were liberated because of the acidity of the solution. Thus under the present experimental conditions the resin was responsible for the liberation of more than 80 % of the purines.

In the method of Chargaff *et al.*[3] for the preparation of apurinic acid, a solution of DNA with pH 1.6 at 37° C is dialysed in a cellophane bag against a buffered solution of the same pH. The method presented in this paper would seem to have some advantages over Chargaff's method. The reaction proceeds at a higher pH and at a lower temperature than that used by Chargaff *et al.*[3], and consequently less degradation is likely to occur. When isolating the apurinic acid it is only necessary to filter off the resin from the solution, and the product can be obtained in pure state upon lyophilization of the filtrate. Paper chromatography and phosphorus analyses of the eluate of the resin demonstrated that the former removed purines only from the DNA solution. The phosphorus concentration of the DNA solution had increased slightly (2 %) after the treatment with the resin, because of the absorption of some water by the anhydrous resin. The yield of apurinic acid by the present method is necessarily 100 %. The apurinic acid from DNA from calf thymus had an atomic nitrogen over phosphorus ratio of 1.39, which

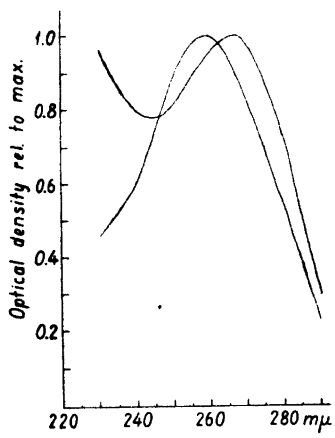

Fig. 2.

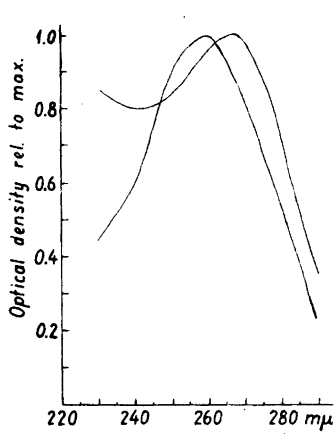

Fig. 3.

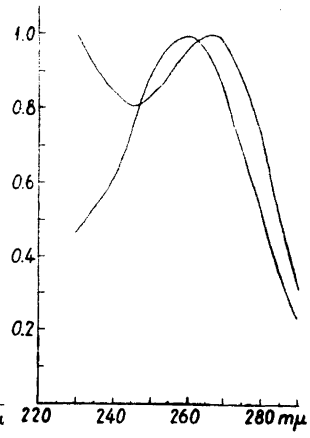

Fig. 4.

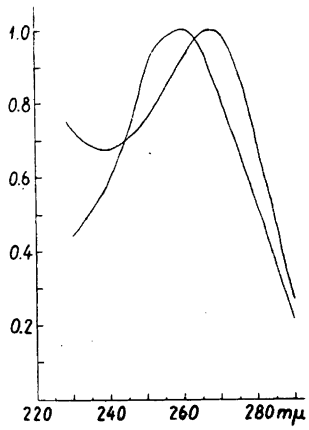

Fig. 5.

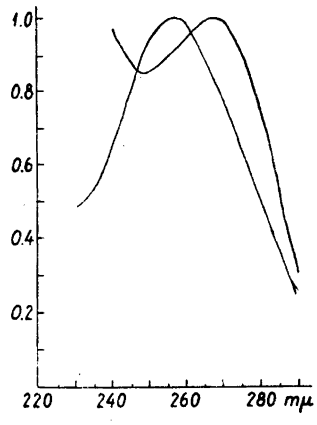

Fig. 6.

Figs. 2, 3, 4, 5 and 6 represent the ultra-violet absorption spectra at pH 7.0 in phosphate buffer of apurinic acids and the parent DNA's isolated respectively from the following sources: calf thymus, herring roe, cod roe, wheat germ and M. p h l e i. In every figure the ultra-violet absorption spectrum with the maximum of absorption at the lowest wavelength is that of the DNA and the other spectrum is that of the corresponding apurinic acid.

is close to that reported by Chargaff *et al.*[3] (1.40 and in some cases 1.20.) It reduced Fehling solution upon heating and reacted on a piece of filter paper when heated with aniline hydrogen phthalate.

Apurinic acids prepared by the resin method from the other DNA's examined in this investigation, were shown to be devoid of purines. The ultra violet absorption of all the apurinic acids and their corresponding DNA's are shown in the Figs. 2, 3, 4, 5 and 6. It is seen from these Figs. that in all the apurinic acids the ultra-violet absorption maximum is shifted towards

higher wavelength *i. e.* 268 and 269 mμ, when compared with parent DNA. Chargaff *et al.*[3] report similar result in the case of apurinic acid from calf thymus DNA.

Table 1 demonstrates that the decrease in the value of $\varepsilon\ (P)_{260}$ (atomic extinction coefficient with regard to phosphorus [12]) when a DNA is converted to the corresponding apurinic acid is in the range of 1 800—2 200 in the six cases examined, and consequently does not differ a great deal in DNA's from such different sources as *e. g.* calf thymus and *M.phlei.*

Table 1. Molecular extinction coefficients with regard to phosphorus at pH *7.0 for DNA's and the corresponding apurinic acids.*

Source of DNA	$\varepsilon\ (P)_{260}$ for DNA	$\varepsilon\ (P)_{260}$ for apurinic acid
Calf thymus	6 300	4 350
Herring roe	6 500	4 300
Cod roe	6 900	4 700
Wheat germ	6 600	4 750
M. phlei	6 250	4 450

The freeze-dried apurinic acids from calf thymus DNA darkens on storage in a dark bottle; this has also been noticed for the apurinic acids prepared from herring roe, cod roe, wheat germ and *M.phlei* DNA's. Whether this is due to a reaction of the free aldehyde groups of the apurinic acid with traces of proteins present in the original DNA or to intermolecular reactions in the apurinic acid, can not be decided upon at the moment.

Determination of purines. The complete removal of the purines from a DNA by Amberlite IR-120(H) led to the development of a micro-method for the determination of the former. In order to check the method, known amounts of adenine and guanine were determined under experimental conditions similar to those used in the determination of the purines of a DNA. The results are presented in Table 2.

Table 2. Determination of known amounts of a mixture of adenine and guanine.

	Adenine (μg)	Guanine (μg)
Theory	440	454
Found when calc. at 270 and 250 mμ	440	453
Found when calc. at 260 and 250 mμ	440	450

The determination of the purines of a DNA as carried out in this investigation requires only 6—7 mg of material. However, if necessary, 3—3.5 mg of DNA can be used by reducing the amount of resin and the volume of the eluate to the half.

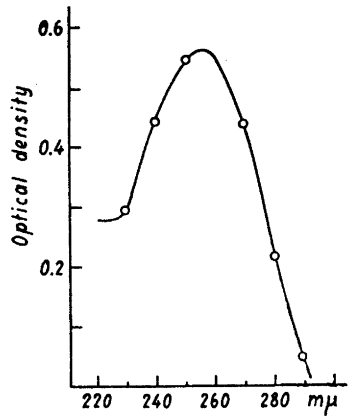

Fig. 7. The drawn curve is the ultra-violet absorption spectrum of the resin eluate containing the purines from calf thymus DNA. The open circles are the theoretical values for the ultra violet absorption.

The deviation in separate analyses of the determination of the purines in DNA is demonstrated in Table 3. Each set of figures are the mean values of the figures obtained by calculation at the two sets of wavelengths mentioned in the experimental section.

Table 3. Separate analyses of the purine content of the DNA's from calf thymus and wheat germ.

Analysis No.	Source of of DNA	Moles of purines per 4 atoms of phosphorus	
		Adenine	Guanine
1	Calf thymus	0.97	0.73
2	Calf thymus	1.05	0.795
1	Wheat germ	0.98	0.78
2	Wheat germ	0.975	0.795

The figures for the two wheat-germ analyses agree well, but the agreement is not quite so good in the case of calf thymus DNA. However, the ratio adenine/guanine is very nearly the same in the two analyses (the ratio is 1.33 and 1.32 respectively). Variations in separate analyses as here presented are largely due to variations in the phosphorus determination and less to the determinations of the purines.

The ultra-violet absorption spectrum of a resin eluate agrees with the theoretical values. In Fig. 7 the drawn curve shows the ultra-violet absorption spectrum of the resin eluate containing the purines from calf thymus DNA. The open circles represent the theoretical values for the ultra-violet absorption at several wavelengths. These values have been calculated from the ultra violet absorption of the standard purine solutions, and from the results of calculations of the amounts of adenine and guanine in the eluate. These were calculated by means of the ultra-violet absorption of the eluate at 260 and 245 mμ.

Table 4 summarises the results of the purine analyses of a number of DNA's by the method described in this paper. In brackets are quoted the figures reported in an earlier paper [7], and obtained by quantitative paper chromatography of hydrolysates of the same specimens. (No figures reported for cod roe DNA.)

Table 4. The purine contents of DNA's.

Source of DNA	Moles of purines per 4 atoms of phosphorus	
	Adenine	Guanine
Calf thymus	1.01 (0.99)	0.76 (0.77)
Herring roe	0.95 (1.0)	0.73 (0.71)
Cod roe	0.94	0.76
Wheat germ	0.98 (0.90)	0.79 (0.67)
M.phlei	0.50 (0.57)	1.14 (1.0)

The figures reported for the DNA's from wheat germ and calf thymus are the mean of the figures given in Table 3.

The determination of purines in a DNA by paper chromatography usually involves the use of acid hydrolysis (formic acid or perchloric acid) at high temperature, prior to the chromatographic separation of the purines and pyrimidines. This rather drastic treatment is likely to destroy some of the purines. In this respect the present method is probably preferable since the purine determination is carried out at room temperature, and consequently less destruction of the purines should occur. Admittedly the total time required for the purine analysis according to the present method is long, but it should be borne in mind that the actual time required is not more than that of quantitative paper chromatography. By raising the temperature at which the present purine analysis is carried out to *e. g.* 37° C a reduction in the total time could be achieved. No attempt, however, has yet been made to carry out the analysis at this temperature.

Finally it is natural to consider the possible extension of the methods discussed in this paper to ribosenucleic acids (RNA). Undoubtedly, the lability of the purines in RNA suggests that these methods could be applied to this group of nucleic acids.

SUMMARY

1. A new method for the preparation of apurinic acid from DNA is described and applied to number of different DNA's. The method involves the use of a sulphonic acid type of resin.

2. A micro-method for the determination of the purines in DNA requiring 6—7 mg material is reported and applied to several DNA's. The results are compared with those obtained by quantitative paper chromatography.

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The Crystal Structure of Barium Tetrathionate Dihydrate

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This article reports the first crystal structure analysis of a salt of tetrathionic acid. A preliminary account has been published [1]. The only other salt of the polythionic acids, the structure of which has been determined by X-ray methods, is potassium trithionate [2].

Barium tetrathionate dihydrate, $BaS_4O_6 \cdot 2H_2O$, was first isolated by Fordos and Gélis in 1842 [3]. For the present work, the salt was prepared by the original method, *viz.*, by interaction of iodine with an aqueous suspension of barium thiosulphate.

X-RAY DATA

The salt, crystallized from water by addition of ethanol, appears as prisms elongated in the direction of the a axis and bounded by $\{011\}$ and $\{001\}$. Twinning on (001) was occasionally observed.

From oscillation and Weissenberg photographs, taken with Cu$K\alpha$ radiation, $\lambda = 1.54$ Å, the following unit cell dimensions were derived [4]: $a = 5.17$ Å, $b = 9.46$ Å, $c = 19.07$ Å, $\beta = 96°$. With four molecules of $BaS_4O_6 \cdot 2H_2O$ per unit cell, the calculated density is 2.84 g/cm^3, as compared with the measured value [5] of 2.78 g/cm^3.

The only systematic absences occur in the $h0l$ reflections when l is odd, and in the $0k0$ reflections when k is odd. The space group is therefore $C_{2h}{}^5$—$P2_1/c$, from which follows that the asymmetric unit is one molecule.

The intensities of the $0kl$ and $h0l$ reflections were estimated visually from zero layer Weissenberg photographs about the a and b axes. 161 of the 237 $0kl$ reflections and 106 of the 130 $h0l$ reflections attainable with Cu$K\alpha$ radiation were recorded on the films with measurable intensity. The crystal specimens used had cross-sections of about 0.05 × 0.05 mm, and no correction was applied for absorption. The intensities were corrected for the Lorentz and polarization factors, using the method of Goldschmidt and Pitt [6]. The values were later placed on an approximately absolute scale by comparison of observed and calculated structure factors, a temperature factor exp—($B \sin^2\Theta/\lambda^2$) with $B = 1.4$ Å^2 for the $0kl$ reflections and $B = 1.6$ Å^2 for the $h0l$ reflections being applied to the calculated structure factors. The latter were based upon

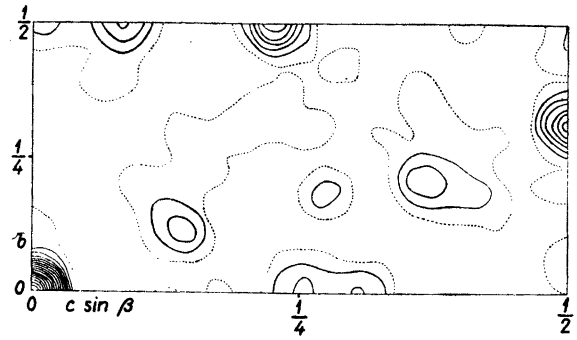

Fig. 1. Patterson projection along the a *axis. Contours at arbitrary but equal intervals.*

the Hartree atomic scattering curves for oxygen and sulphur, and on the Thomas-Fermi curve for xenon in the case of the barium ion. The same curve was used for all four sulphur atoms, and no account was taken of the hydrogen atoms of the water molecules.

The Patterson and Fourier summations were made at intervals of 6° along all three axes, using Beevers-Lipson strips. Peaks were located by Booth's method [7].

ANALYSIS OF STRUCTURE

Use was made of the heavy atom technique, the position of the barium ion being found from Patterson projections along the a and b axes.

The asymmetric parts of these projections are reproduced in Figs. 1 and 2. The barium-barium vectors are easily recognized as the highest peaks in the maps beside the origin peaks. The following barium coordinates were derived from the Patterson projections: $x = 0.250$, $y = 0.600$, $z = 0.364$. Only the y coordinate differs, by 0.007, from the final value.

Fourier projections along the a and b axes were subsequently carried out, starting with signs of the reflections calculated from the barium contributions alone. The positions of the sulphur atoms, and partly the oxygen atoms, could be recognized in the first maps. The structure was refined by the Fourier method of successive approximations. The final electron density maps, $\varrho(yz)$ and $\varrho(xz)$, are shown in Figs. 3 and 4, and explanatory diagrams are reproduced in Figs. 5 and 6.

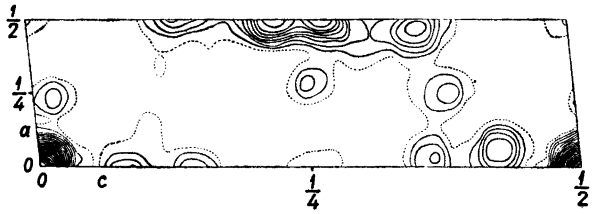

Fig. 2. Patterson projection along the b *axis. Contours as in Fig. 1.*

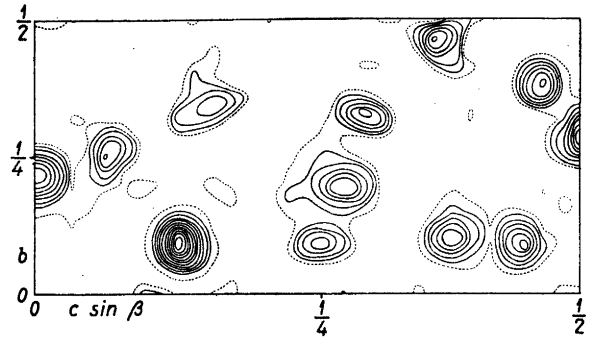

Fig. 3. Projection of $BaS_4O_6 \cdot 2H_2O$ along the a *axis,* ϱ(yz). *A quarter of a unit cell (one asymmetric unit) is shown. The 4-electron line is dotted. Contour intervals: 10 e.Å^{-2} for the barium ion, 4 e.Å^{-2} for sulphur atoms, and 2 e.Å^{-2} for oxygen atoms and water molecules.*

In $\varrho(yz)$ all atoms are resolved except the oxygen atoms O_2 and O_5, which each overlap with one of the terminal sulphur atoms. In $\varrho(xz)$, O_1 overlaps with S_1 and O_2 lies near a screw axis and thus overlaps with another O_2 atom. The latter atoms were placed exactly on the screw axis although, from the shape of the peak in $\varrho(xz)$, this does not appear to be quite correct. The coordinates, xO_1, yO_2 and yO_5, which could not be derived from any resolved peak in $\varrho(yz)$ or $\varrho(xz)$, were chosen so as to make the arrangement around the sulphur atoms conform with that of the other oxygen atoms. The overlapping with oxygen atoms introduces an uncertainty in the y coordinate of S_4 and the three S_1 coordinates, although attempts were made to estimate and correct for the displacing effect of the oxygen atoms.

The following z coordinates, from $\varrho(yz)$ and $\varrho(xz)$, respectively, differed in the two projections: S_1, 0.285 and 0.292; S_3, 0.446 and 0.444; O_2, 0.244 and 0.250; O_3, 0.253 and 0.261; O_5, 0.507 and 0.515; $(H_2O)_2$, 0.333 and 0.347. The discrepancies are due partly to overlapping from opposite directions.

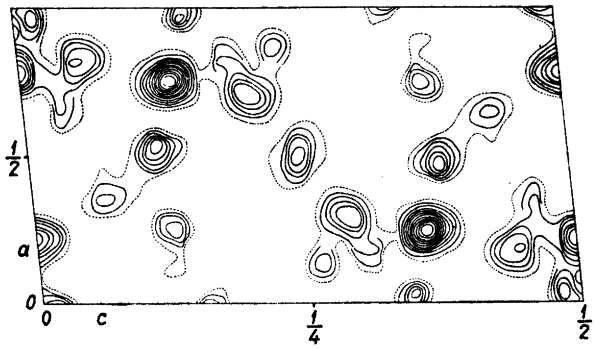

Fig. 4. Projection of $BaS_4O_6 \cdot 2H_2O$ along the b *axis,* ϱ(xz). *Half a unit cell (two asymmetric units) is shown. The 6-electron line is dotted. Contour intervals as in Fig. 3.*

The atomic coordinates finally chosen are collected in Table 1. The coordinates, x, y and z, are in fractions of cell edges, and refer to the monoclinic crystal axes, while x', y' and z', in Å, denote orthogonal coordinates referred to the a', b and c axes, where a' is chosen normal to c in the ac plane.

Table 1. Atomic coordinates. Origin at a centre of symmetry.

	x	y	z	x' (Å)	y' (Å)	z' (Å)
Ba	0.250	0.593	0.364	1.29	5.61	6.81
S_1	0.298	0.190	0.288	1.53	1.80	5.33
S_2	0.473	0.101	0.382	2.43	0.96	7.03
S_3	0.183	0.090	0.445	0.94	0.85	8.39
S_4	0.217	0.283	0.502	1.11	2.68	9.46
O_1	0.200	0.331	0.299	1.03	3.13	5.59
O_2	0.500	0.220	0.250	2.57	2.08	4.50
O_3	0.112	0.098	0.257	0.57	0.93	4.84
O_4	0.350	0.381	0.465	1.80	3.60	8.68
O_5	− 0.036	0.328	0.515	− 0.19	3.10	9.84
O_6	0.362	0.251	0.569	1.87	2.37	10.66
$(H_2O)_1$	0.750	0.463	0.370	3.86	4.38	6.65
$(H_2O)_2$	0.027	0.850	0.340	0.14	8.04	6.47

In Table 6 the observed and calculated structure factors are compared for all $0kl$ and $h0l$ reflections within the range of Cu$K\alpha$ radiation. The figures listed represent one fourth of the absolute values. The F_{calc} correspond to the coordinates of Table 1, except for the $0kl$ reflections, where the z coordinates from $\varrho(yz)$, listed above, were employed.

The reliability factor, $R = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$, is 0.15 for the $0kl$ reflections and 0.16 for the $h0l$ reflections. The non-observed reflections are

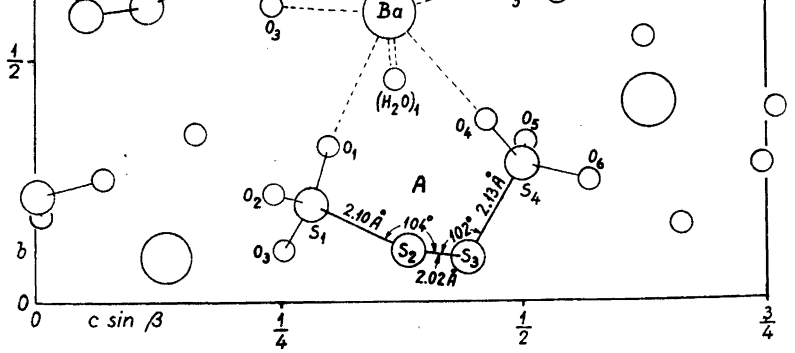

Fig. 5. Arrangement of ions as viewed along the a axis.

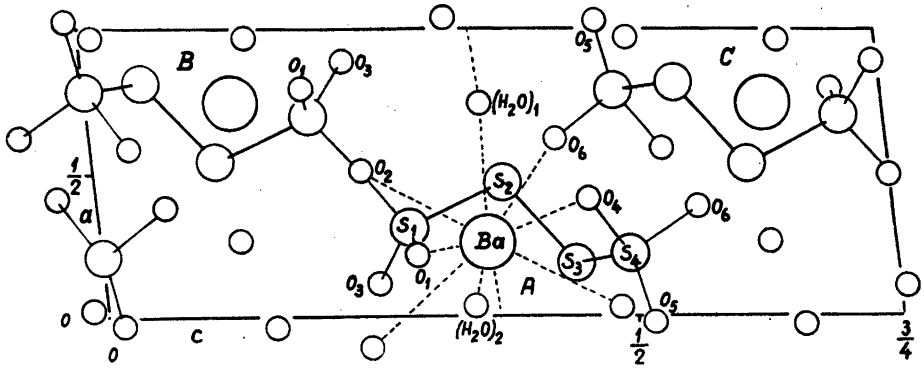

Fig. 6. Arrangement of ions as viewed along the b *axis.*

included in R, as the difference between $|F_{calc}|$ and the lowest observable value of $|F_{obs}|$, only when $|F_{calc}|$ is greater than the smallest value of $|F_{obs}|$. They are not included in $\Sigma|F_{obs}|$.

THE TETRATHIONATE ION

Interatomic distances and angles, calculated on the basis of the coordinates of Table 1, are given below. The numbering of atoms is shown in Figs. 5 and 6.

Table 2. Sulphur-sulphur bond lengths and bond angles.

$S_1-S_2 = 2.10$ Å	$\angle S_1S_2S_3 = 104°$
$S_2-S_3 = 2.02$	$\angle S_2S_3S_4 = 102°$
$S_3-S_4 = 2.13$	*$S_1S_2S_3/S_2S_3S_4 = 90°$

*Dihedral angle

The sulphur-sulphur distances, $S_1—S_3 = 3.25$ Å, $S_1—S_4 = 4.15$ Å, and $S_2—S_4 = 3.25$ Å. It is apparent that no chemical bonds exist between the atoms S_1 and S_3, S_1 and S_4, or S_2 and S_4. The sulphur atoms of the tetrathionate ion thus form an unbranched and non-planar chain.

The terminal sulphur atoms, S_1 and S_4, are each surrounded by three oxygen atoms, in approximately tetrahedral positions. The pertinent data are collected in Table 3. The average S—O distance, 1.41 Å, is in fair agreement with values found in other sulphur-oxy compounds [8].

Table 3. Sulphur-oxygen bond lengths and bond angles.

$S_1-O_1 = 1.45$ Å	$\angle S_2S_1O_1 = 112°$	$\angle O_1S_1O_2 = 101°$
$S_1-O_2 = 1.36$	$\angle S_2S_1O_2 = 105°$	$\angle O_1S_1O_3 = 113°$
$S_1-O_3 = 1.39$	$\angle S_2S_1O_3 = 109°$	$\angle O_2S_1O_3 = 114°$
$S_4-O_4 = 1.38$	$\angle S_3S_4O_4 = 109°$	$\angle O_4S_4O_5 = 114°$
$S_4-O_5 = 1.42$	$\angle S_3S_4O_5 = 108°$	$\angle O_4S_4O_6 = 111°$
$S_4-O_6 = 1.45$	$\angle S_3S_4O_6 = 105°$	$\angle O_5S_4O_6 = 109°$
Average: 1.41 Å	Average: 108°	Average: 111°
*$\delta = 0.04$ Å	$\delta = 2\ ½°$	$\delta = 4°$

*Standard deviation

The average distance from the oxygen atoms to the nearest non-bonded sulphur atom is 2.87 Å, and the average oxygen-oxygen distance within sulphonate groups is 2.32 Å, with standard deviations of 0.06 Å and 0.05 Å, respectively. The shortest oxygen-oxygen distance between sulphonate groups is O_1—O_4 = 3.21 Å.

The sulphur chain of the tetrathionate ion possesses, within experimental accuracy, a twofold axis of symmetry, passing midway between S_2 and S_3 and between S_1 and S_4. The sulphonate groups are rotated out of the relative positions required by a twofold axis.

THE IONIC ENVIRONMENT

The shortest barium-oxygen distances are listed in Table 4. All other such distances exceed 3.7 Å. The letters *A*, *B* and *C* refer to different tetrathionate ions, as shown in Figs. 5 and 6. A prime denotes nearest corresponding atom in adjacent unit cell along the *a* axis.

Table 4. Barium-oxygen distances.

Ba-O_{1A} = 2.77 Å	Ba-O_{6C} = 2.75 Å	Ba-$(H_2O)_1$ = 2.86 Å
Ba-O_{4A} = 2.79	Ba-$O_{3B'}$ = 2.81	Ba-$(H_2O)_{1'}$ = 2.86
Ba-O_{2B} = 2.90	Ba-$O_{5C'}$ = 2.77	Ba-$(H_2O)_2$ = 2.73

It appears that the barium ion is surrounded by nine oxygen atoms at an average distance of 2.80 Å. Six of these are sulphonate oxygen atoms and three are water oxygen atoms. Within a tetrathionate ion, two oxygen atoms, one from each sulphonate group, are coordinated to the same barium ion, whereas the three oxygen atoms of a sulphonate group are all coordinated to different barium ions. The six sulphonate oxygen atoms surrounding a barium ion thus belong to as many different sulphonate groups, and to five different tetrathionate ions.

The water molecule, $(H_2O)_1$, lies equidistant from two barium ions, Ba and Ba′. The oxygen of this water molecule thus probably coordinates to both of the barium ions. The angle between the directions to the barium ions is 125°.

The shortest distances from water molecules to sulphonate oxygen atoms are summarized in Table 5. The error in these distances may be as large as 0.1 Å, as discussed later, and those only are included which, within this limit, have been found shorter than 3.0 Å.

Table 5. Water oxygen-sulphonate oxygen distances.

$(H_2O)_1$-O_{4A} = 2.99Å	$(H_2O)_1$-O_{6C} = 3.03 Å	$(H_2O)_{2'}$-O_{1B} = 2.79 Å
$(H_2O)_1$-O_{3B} = 2.88	$(H_2O)_1$-$O_{1A'}$ = 3.08	$(H_2O)_{2'}$-O_{6C} = 2.94

The lengths of O—H···O bonds in crystalline hydrates usually [9,10] lie in the range 2.75 Å—2.90 Å. In the present structure, hydrogen bonding probably occurs from $(H_2O)_2'$ to O_{1B} and O_{6C}. The angle, O_{1B}—$(H_2O)_2'$—O_{6C}, is 112°. Furthermore, there are possibilities for hydrogen bonds from $(H_2O)_1$ to one or two of the atoms, O_{4A}, O_{3B}, O_{6C} and $O_{1A'}$.

Table 6. Observed and calculated values of structure factors for barium tetrathionate dihydrate. The figures represent one fourth of the absolute values.

l	F_{obs}	F_{calc}	l	F_{obs}	F_{calc}	l	F_{obs}	F_{calc}
	0*kl* zone		12	19	− 21	14	< 7	− 2
	00*l*		13	30	+ 32	15	< 7	+ 9
2	2	+ 1	14	< 6	− 5	16	< 7	− 5
4	28	− 19	15	< 7	+ 2	17	11	− 12
6	21	+ 16	16	< 7	+ 3	18	10	+ 9
8	49	+ 50	17	17	− 18	19	9	+ 8
10	24	− 21	18	10	− 11	20	< 5	+ 3
12	27	− 25	19	< 7	+ 1	21	5	+ 4
14	38	+ 30	20	< 6	− 5	22	4	− 5
16	23	+ 26	21	< 5	+ 8	23	4	− 7
18	9	− 5	22	< 5	+ 4			
20	< 6	+ 2	23	6	− 11		05*l*	
22	8	+ 14	24	3	− 3	1	5	− 3
24	< 4	+ 7				2	8	− 6
				03*l*		3	34	+ 36
	01*l*		1	8	− 10	4	< 6	+ 2
1	20	+ 23	2	16	− 17	5	10	+ 9
2	13	− 15	3	19	− 19	6	< 6	− 3
3	27	− 24	4	42	+ 36	7	21	− 16
4	< 4	+ 1	5	12	− 8	8	< 6	+ 3
5	8	− 6	6	38	+ 35	9	< 7	− 1
6	35	+ 34	7	16	− 15	10	< 7	− 5
7	48	+ 48	8	17	− 14	11	32	+ 27
8	31	− 22	9	< 6	− 4	12	< 7	+ 4
9	7	+ 10	10	18	− 18	13	< 8	− 3
10	7	+ 2	11	8	− 8	14	< 8	+ 3
11	21	− 19	12	19	+ 17	15	14	− 11
12	15	+ 12	13	< 7	− 3	16	8	− 8
13	< 6	+ 7	14	< 8	+ 2	17	24	+ 23
14	7	− 5	15	< 8	− 8	18	< 7	− 1
15	20	+ 22	16	25	− 26	19	14	+ 17
16	16	− 15	17	< 8	− 1	20	< 5	+ 3
17	13	− 14	18	< 8	+ 1	21	< 4	− 2
18	8	− 12	19	< 7	+ 3	22	< 4	+ 2
19	16	− 20	20	17	+ 19			
20	10	+ 13	21	8	− 10		06*l*	
21	10	+ 12	22	< 4	+ 4	0	36	− 37
22	< 4	− 6	23	< 4	− 2	1	8	+ 6
23	5	+ 11				2	10	− 9
24	4	− 5		04*l*		3	16	+ 14
			0	39	− 39	4	28	+ 31
	02*l*		1	20	− 21	5	17	− 17
0	9	− 8	2	8	+ 6	6	< 6	+ 1
1	44	− 50	3	26	− 28	7	11	− 11
2	4	− 1	4	32	+ 31	8	25	− 22
3	26	− 25	5	28	+ 26	9	10	+ 11
4	47	− 46	6	6	− 8	10	24	+ 24
5	18	+ 18	7	5	+ 3	11	< 8	− 2
6	4	0	8	15	− 15	12	14	+ 15
7	27	+ 28	9	20	− 19	13	11	− 9
8	10	+ 9	10	24	+ 20	14	14	− 10
9	38	− 39	11	11	+ 5	15	8	− 7
10	24	− 21	12	24	+ 23	16	14	− 18
11	< 5	− 3	13	19	+ 18	17	7	+ 10

l	F_{obs}	F_{calc}
18	9	+ 11
19	< 5	− 2
20	< 3	+ 1
21	3	− 3
	07l	
1	13	− 9
2	17	+ 18
3	9	+ 8
4	18	− 16
5	< 7	+ 2
6	17	− 13
7	30	− 25
8	< 8	+ 1
9	7	− 6
10	12	+ 12
11	< 8	+ 2
12	12	− 16
13	9	− 10
14	7	− 6
15	12	− 11
16	18	+ 21
17	< 5	+ 1
18	< 4	+ 1
19	5	+ 8
20	4	− 11
	08l	
0	7	− 3
1	28	+ 31
2	8	+ 5
3	17	+ 17
4	< 8	− 5
5	18	− 18
6	< 8	− 4
7	8	− 9
8	< 8	+ 1
9	22	+ 24
10	< 8	− 6
11	< 7	+ 1
12	< 7	+ 4
13	18	− 21
14	< 6	− 8
15	< 6	− 5
16	< 5	+ 3
17	9	+ 14
18	< 4	− 3
19	< 4	− 4
	09l	
1	8	+ 6
2	11	+ 9
3	< 8	− 3
4	< 8	− 6
5	< 8	+ 4
6	21	− 22
7	15	+ 12
8	13	+ 12
9	< 7	+ 1
10	9	+ 11
11	< 6	− 5
12	6	− 5
13	6	+ 8
14	< 5	− 3
15	5	+ 5
16	5	+ 12
	0,10,l	
0	27	+ 27
1	9	+ 7
2	7	− 1
3	7	+ 8
4	10	− 11
5	< 6	− 4
6	< 6	+ 3
7	< 6	+ 1
8	10	+ 17
9	7	+ 8
10	6	− 7
11	< 5	+ 2
12	7	− 14
13	5	− 10
14	3	+ 9
	0,11,l	
1	< 6	+ 1
2	< 6	+ 3
3	13	− 13
4	< 5	+ 1
5	6	− 7
6	< 5	+ 1
7	13	+ 15
8	< 4	− 1
9	< 4	− 2
10	< 3	+ 2
	0,12,l	
0	10	+ 11
1	7	− 13
2	< 4	− 1
3	6	− 6
	$h0l$ zone	
	00l	
2	4	+ 1
4	24	− 21
6	18	+ 15
8	47	+ 48
10	22	− 22
12	26	− 28
14	34	+ 30
16	24	+ 24
18	11	− 8
20	< 8	+ 3
22	10	+ 11
24	< 4	+ 6
	10l	
0	< 4	− 4
2	58	+ 56
4	8	− 6
6	14	+ 8
8	14	+ 6
10	26	+ 29
12	17	− 14
14	14	− 15
16	19	+ 13
18	< 8	− 1
20	10	− 6
22	7	+ 5
	20l	
0	65	− 54
2	< 5	+ 4
4	32	+ 28
6	18	− 13
8	15	− 13
10	7	+ 1
12	13	+ 15
14	30	− 36
16	11	− 11
18	17	+ 16
20	< 6	− 3
22	8	− 14
	30l	
0	13	− 9
2	35	− 30
4	15	+ 10
6	24	+ 20
8	21	− 16
10	12	− 10
12	18	+ 19
14	15	+ 18
16	25	− 30
18	< 6	+ 2
20	7	+ 5
	40l	
0	40	+ 37
2	10	− 9
4	29	− 33
6	21	+ 19
8	24	+ 28
10	< 8	− 1
12	10	− 10
14	16	+ 25
16	9	+ 15
18	6	− 11
	50l	
0	< 8	+ 1
2	28	+ 33
4	< 7	− 5
6	12	− 13

l	F_{obs}	F_{calc}	l	F_{obs}	F_{calc}	l	F_{obs}	F_{calc}
8	9	+ 2	6	27	− 24	6	11	+ 12
10	9	+ 11	8	20	− 18	8	38	+ 34
12	5	− 4	10	22	+ 22	10	15	− 11
14	< 3	− 3	12	29	+ 34	12	19	− 18
	$60l$		14	35	− 35	14	12	+ 8
0	9	− 8	16	11	− 10	16	< 8	+ 7
2	< 6	+ 1	18	11	+ 10	18	9	− 7
4	10	+ 16	20	< 7	− 3	20	< 5	− 2
6	7	− 7	22	23	− 29			
8	5	− 7	24	4	+ 2		$\bar{5}0l$	
	$\bar{1}0l$			$\bar{3}0l$		2	12	− 8
2	43	− 54	2	29	+ 26	4	11	+ 10
4	28	+ 31	4	9	− 11	6	28	+ 30
6	28	+ 26	6	14	− 8	8	12	− 14
8	20	− 20	8	< 7	− 1	10	18	− 15
10	32	− 37	10	16	+ 16	12	17	+ 15
12	37	+ 36	12	26	− 23	14	12	+ 12
14	27	+ 26	14	12	− 9	16	8	− 10
16	22	− 20	16	< 8	+ 4	18	< 4	− 3
18	< 8	+ 3	18	< 8	− 5			
20	18	+ 18	20	18	− 20		$\bar{6}0l$	
22	< 6	+ 4	22	< 5	− 3	2	< 6	+ 5
24	12	− 17				4	10	+ 12
						6	9	− 12
	$\bar{2}0l$			$\bar{4}0l$		8	19	− 22
2	12	− 8	2	< 7	+ 2	10	8	+ 10
4	17	+ 15	4	23	− 16	12	6	+ 6

ESTIMATE OF ERRORS

Errors in the coordinates due to finite termination of Fourier series were estimated from a Fourier synthesis based on the calculated $0kl$ structure factors [11]. The average shift in the y and z coordinates of the sulphur and the oxygen atoms was 0.013 Å and 0.028 Å, respectively, with maximum shifts of 0.02 Å and 0.057 Å.

The accuracy of the results is further influenced by the overlapping of the terminal sulphur atoms with oxygen atoms, and by the obscuring effect of the heavy barium ion, particularly on the oxygen atoms.

It is believed that the S_2—S_3 bond length, 2.02 Å, and the average S_1—S_2 and S_3—S_4 bond length, 2.12 Å, are in error by not more than ± 0.03 Å, with a corresponding error of ± 2° in angles involving sulphur atoms alone. The average S—O bond length, 1.41 Å, is probably correct to ± 0.04 Å, whereas the oxygen-oxygen distances may be in error by as much as ± 0.1 Å.

SULPHUR-SULPHUR BOND LENGTHS

The average length, 2.12 Å, of the two terminal sulphur-sulphur bonds in the tetrathionate ion, is in close agreement with the recent value, 2.11 Å, for a single sulphur-sulphur bond [12]. The middle sulphur-sulphur bond is 2.02 Å and thus shorter by an amount which is outside the experimental error. Two

kinds of sulphur-sulphur bonds, of lengths 2.02 Å and 2.11 Å, also exist in cesium hexasulphide [12].

The crystal structure of a close analogon of tetrathionate, *viz.*, dimethanesulphonyl disulphide, has recently been determined by Sörum [13]. The compound, $S_2(SO_2CH_3)_2$, arises from tetrathionate by substitution of one oxygen atom in each sulphonate group by methyl. The bond lengths reported are, 2.06 Å for the middle sulphur-sulphur bond and 2.10 Å for the two terminal bonds. The difference between the lengths of the middle bond in the two compounds is within the experimental error; however, the larger value in the methyl derivative may be significant in view of the fact that the methanethiosulphonate group is more electronegative than the thiosulphate group [14].

SUMMARY

The crystal structure of barium tetrathionate dihydrate has been determined by two-dimensional Patterson and Fourier methods, by use of the heavy atom technique.

The sulphur chain of the tetrathionate ion is unbranched and non-planar. Three oxygen atoms are arranged in approximately tetrahedral positions around each of the terminal sulphur atoms, at an average distance of 1.41 Å.

The length of the middle sulphur-sulphur bond is 2.02 Å and that of the two terminal sulphur-sulphur bonds is 2.12 Å. The bond angle of the two divalent sulphur atoms is 103° and the dihedral angle is 90°.

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Remarks on the Stereochemistry of Disulphide Compounds

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Theoretical considerations predict [1] that compounds of the disulphide type, X—S—S—X, are non-planar. The dihedral angle, *i. e.*, the angle between the X—S groups as seen projected on a plane normal to the S—S bond, is expected to be around 90°, due to interaction of the electron clouds of the two sulphur atoms.

STRUCTURE OF COMPOUNDS OF THE DISULPHIDE TYPE

Pertinent data are available for hydrogen disulphide [2], disulphur dichloride [3,4], diphenyl diselenide [5,6] which is isomorphous with diphenyl disulphide [5,7], di-*p*-bromophenyl disulphide [8], dimethanesulphonyl disulphide [9] and barium tetrathionate dihydrate [10]. In every case, the disulphide group is non-planar, with a dihedral angle close to the predicted value.

A non-planar, symmetrical X—S—S—X group possesses a twofold axis of symmetry, but no centre, whereas a planar, *trans* group has a centre. Unit cell and space group data, but no detailed structure analysis, are available for dibenzoyl disulphide [5], di-*p*-chlorophenyl diselenide [11], disulphur and diselenium dibenzenesulphinate and di-*p*-toluenesulphinate [12], and potassium, rubidium and ammonium tetrathionate [13,14]. No molecular symmetry is required in any of the crystals. In view of the crystallographic experience that a molecular centre of symmetry, if present, usually will coincide with a crystallographic centre of symmetry, whereas this only occasionally happens with twofold axes, the evidence is thus in favour of non-planar disulphide groups in the above compounds.

The space group of dibenzyl disulphide and diselenide [5] and *bis*(dimethylthiocarbamyl) disulphide [15] is probably C_{2h}^6—$C2/c$, with four molecules per unit cell. Here, a twofold axis or a centre is required as molecular symmetry. By inference, it appears likely that the molecular symmetry element is a twofold axis and not a centre.

It may be stated that no exceptions to the principle of non-planarity have so far been established for compounds of the disulphide type.

The unit cells and space groups of disulphur and diselenium dibenzenesulphinate, referred to above, were determined some time ago, and are reported here. Oscillation and Weissenberg photographs were taken with Fe$K\alpha$ radiation, $\lambda = 1.934$ Å, and densities were measured by a flotation method. The values given below for axial lengths are believed to be accurate to within 0.5 %.

Disulphur dibenzenesulphinate [16], $S_2(SO_2C_6H_5)_2$. Monoclinic prismatic; $a = 12.30$ Å, $b = 6.09$ Å, $c = 20.72$ Å, $\beta = 109°$. There are four molecules per unit cell; density, calc. 1.57, found 1.57. Absent reflections, $h0l$ when l is odd, $0k0$ when k is odd. Space group, $C_{2h}^5 - P2_1/c$. The crystals appear as flat prisms, elongated along the b axis, and with $\{10\bar{2}\}$ predominant.

The crystals have been described by Groth [17]. With Groth's a and c axes doubled, his data correspond to a cell in which the b face is centered. The translation of the glide plane is along Groth's a axis.

Diselenium dibenzenesulphinate [12], $Se_2(SO_2C_6H_5)_2$. Monoclinic prismatic; $a = 5.54$ Å, $b = 14.25$ Å, $c = 23.18$ Å, $\beta = 108°$. Four molecules per unit cell; density, calc. 1.68, found 1.65. Absent reflections, $h0l$ when l is odd, $0k0$ when k is odd. Space group, $C_{2h}^5 - P2_1/c$. The crystals appear as prisms $\{011\}$, and there is a tendency to twinning on (001).

It appears that disulphur and diselenium dibenzenesulphinate are not isomorphous, and crystallize differently from the p-toluene analogues, which are mutually isomorphous [12].

SPACE GROUP AND TWINNING OF SODIUM TETRATHIONATE DIHYDRATE

As has been pointed out recently [18] with reference to organic disulphides, there are two enantiomorphous forms of non-planar disulphide groups. The crystals of diphenyl disulphide and diselenide [5–7] and probably di-p-tolyl disulphide [7] are based on enantiomorphous space groups. The number of molecules in the unit cells do not exceed the number of general positions of the space groups, and a unit cell thus contains only one of the two enantiomorphous forms. This appears to be true also for sodium tetrathionate dihydrate.

As reported earlier [14], the crystals of sodium tetrathionate dihydrate are monoclinic, with the unit cell dimensions, $a = 14.46$ Å, $b = 6.37$ Å, $c = 5.44$ Å, $\beta = 105.5°$. There are two molecules per unit cell. Absent reflections, hkl when $h + k$ is odd. Granted that the sulphur chain of the ion is non-planar in this salt as has been found in barium tetrathionate dihydrate [10], the space group is C_2^3—$C2$, which requires that the tetrathionate ion possesses a twofold axis of symmetry.

The space group, C_2^3—$C2$, is enantiomorphous, and a unit cell contains only one of the two enantiomorphous forms of the tetrathionate ion. The crystals are always submicroscopically twinned [14], with (100) as a twin plane. They appear to consist of submicroscopic lamellae of alternating d and l crystals. On zero layer Weissenberg photographs about the b axis, equivalent $h0l$ reflections from d and l lamellae occur with the same intensity. This type of twinning is analogous to that [19] of inactive β-phenylglyceric acid of m.p. 141°.

POSSIBLE EXISTENCE OF STABLE ISOMERS

The enantiomorphous forms of disulphide compounds should be expected to isomerize very rapidly in solutions at room temperature, the barrier restricting rotation about a sulphur-sulphur bond being [1] in the neighbourhood of

5 kcal./mole. The same should apply to the enantiomorphous *trans* forms of trisulphide and pentathionic compounds [20].

The isomerization of the tetrathionate ion probably occurs by rotation through the planar *trans* form, since in the planar *cis* form, with a dihedral angle of 0°, the approach of the oxygen atoms of the two sulphonate groups would be too close, or require an excessive bending of bonds. In dimethyl disulphide [21], the carbon-carbon distance in the planar *cis* form may be calculated as roughly 3.1 Å. The van der Waals radius of methyl and methylene groups being [22] about 2.0 Å, it appears that isomerization by rotation through planar *cis* forms is unfavourable also for organic disulphides. With large organic groups attached to sulphur, as in the case of di-*tert*-butyl disulphide [23], spatial interference between the groups give rise to an increased value, above 100°, of the dihedral angle, and rotations through planar *cis* forms should not be expected to take place.

In cyclic disulphides with not too large rings, isomerization by rotation through planar *trans* forms would also be impossible. Thus, stable rotational isomers of disulphide compounds might perhaps occur among cyclic disulphides, having, preferrably, bulky substituents on the carbon atoms next to sulphur.

SUMMARY

The available data concerning the structure of disulphide compounds are in accordance with a non-planar distribution of valencies about the sulphur-sulphur bond.

The twinning of sodium tetrathionate dihydrate crystals is discussed in connection with the occurrence of two enantiomorphous forms of the tetrathionate ion.

It is pointed out that stable rotational isomers of cyclic disulphides may exist.

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The Crystal Structure of Barium Pentathionate Dihydrate

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This is the first in a series of papers on the crystal structure of pentathionic compounds. The structure of barium tetrathionate dihydrate has been published recently [1]. The work serves to confirm the old theories of Blomstrand [2] and Mendelejeff [3], that the polythionic acids and their salts are built up of unbranched sulphur chains.

Barium salts of pentathionic acid have been prepared by Lenoir [4], Fordos and Gélis [5], Lewes [6], and Deines and Christoph [7]. The crystal chemistry of barium and strontium pentathionate, selenopentathionate and telluropentathionate is rather complex, especially with regard to solvates with organic solvents. It is intended to make a fuller study of these phenomena later; meanwhile, it may suffice to say that the orthorhombic dihydrate, described here, was obtained from potassium pentathionate by metathesis with barium perchlorate, and recrystallization from water by addition of methanol. Ethanol, acetone, or dioxane can not be used, since in that case solvates, having different unit cells, result.

Preliminary data on the crystals have been reported earlier by one of us [8], and also some remarks on the stereochemistry of the pentathionate ion and other pentathionic compounds [9].

X-RAY DATA

The orthorhombic crystals of the salt, $BaS(S_2O_3)_2 \cdot 2H_2O$, appear as flat prisms, elongated along the a axis and with {001} predominant. {011} also occurs. There is perfect cleavage along (001). As is shown later, the cleavage probably takes place along the glide plane a normal to the c axis, there being no chemical bonds across this plane.

The unit cell dimensions [8] are: $a = 5.00$ Å, $b = 10.30$ Å, $c = 21.78$ Å. There are four molecules per unit cell; density, calc. 2.55, found 2.53 g/cm^3. Absent reflections, $0kl$ when $k + l$ is odd, $hk0$ when h is odd. The habit of the crystals, the Wilson [10] ratio for the $0kl$ reflections [9], and the Patterson projection along the a axis, are in accordance with the centrosymmetric space group D_{2h}^{16}—*Pnma*, and this may be regarded as confirmed by the relatively good agreement obtained between the observed structure factors and those calculated on the basis of this space group, particularly for the $0kl$ reflections.

For intensity measurements, a crystal elongated along a and with a cross-section of 0.05 × 0.08 mm was used to take a series of Weissenberg photographs recording the $0kl$ reflections. The crystal, later kept mounted on the glass fiber in a closed tube, was unchanged after ten months. For the b axis photographs, a crystal was cut to a cross-section of 0.08 × 0.15 mm. The cutting was difficult due to the pronounced cleavage along the c plane, and the b axis photographs were therefore not quite satisfactory.

142 of the 158 $0kl$ reflections and 107 of the 144 $h0l$ reflections theoretically observable with Cu$K\alpha$ radiation were recorded on the films with measurable intensity. The intensities were estimated visually, and corrected for the Lorentz and polarization factors. No corrections for absorption were applied, although this might have been appropriate in the case of the $h0l$ reflections. The resultant structure factors were eventually placed on an approximately absolute scale by comparison with the calculated values.

The calculated structure factors were based on the Hartree atomic scattering curves for sulphur and oxygen, and the Thomas-Fermi curve for xenon in the case of the barium ion. The same curve was used for all sulphur atoms, and no account was taken of the hydrogen atoms of the water molecules. A temperature factor exp $-(B \sin^2\Theta/\lambda^2)$ with $B = 3.0$ Å^2 for the $0kl$ reflections and $B = 2.4$ Å^2 for the $h0l$ reflections was applied to the calculated structure factors. The values of B were obtained by plotting $\log \Sigma|F_{obs}|/\Sigma|F_{calc}|$ against $\sin^2\Theta$ in the ranges of 2 sin Θ = 0—0.5, 0.5—0.6, 0.6—0.7, etc. to 2. The smaller value of B in the case of the $h0l$ reflections may be due to absorption effects.

The Patterson and Fourier summations were made with Beevers-Lipson strips. It was considered accurate enough to sum a 6° intervals along the c axis, even though the length is 21.78 Å. Peaks were located by Booth's method [11], using graphical interpolation from three lines through each atom along each axis.

ANALYSIS OF STRUCTURE

The space group, D_{2h}^{16}—*Pnma*, has eightfold general positions. The four molecules contained in the unit cell must lie in one of the fourfold positions, of which there are three sets, one having a mirror plane of symmetry and the others a centre.

The Patterson projection along the a axis is reproduced in Fig. 1. The barium-sulphur vectors are marked with points, and lines are drawn to indicate the outline of the pentathionate ion. It is apparent that the barium ion and the central sulphur atom of the pentathionate ion lie in the mirror plane.

Approximate values for the y and z coordinates of barium and of the sulphur atoms were derived from the Patterson map, and used to calculate signs of the $0kl$ reflections. 132 terms were included in the first Fourier synthesis, and none of the signs were later found to be incorrect. The first Fourier map revealed the positions of the oxygen atoms and the water molecules, and hence, by double Fourier series refinement in the usual way, the electron density map of Fig. 2 was produced. A line diagram is shown in Fig. 3.

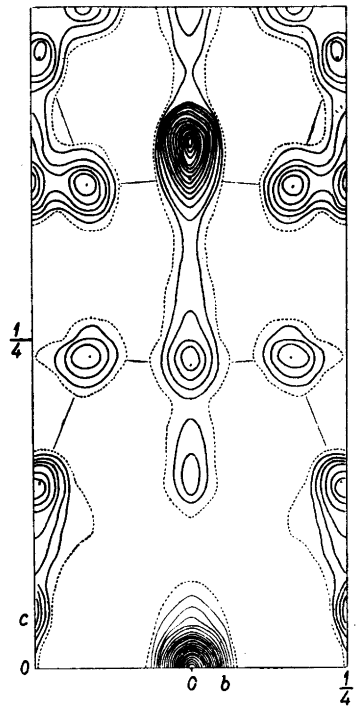

Fig. 1. Patterson projection along the a axis. A quarter of a unit cell (two asymmetric units) are shown. Contours at arbitrary but equal intervals except at the origin peak, where the intervals are doubled. The barium-barium vector overlaps partly with one of the barium-sulphur vectors.

The *h0l* data were used to evaluate the x coordinates. The b axis Patterson projection could be interpreted to show that the barium ion and four of the sulphur atoms lie close to the glide plane at $x = \frac{1}{4}$, but gave no information as to the exact location relative to the glide plane. In this plane group, atoms at $x = \frac{1}{4}$ do not contribute to *h0l* reflections with l odd, whereas for reflections with l even the contribution is large. Inspection of the *h0l* data showed that for larger values of h, reflections with l odd were quite strong relative to those with l even, indicating that the barium ion does not lie exactly at $x = \frac{1}{4}$. Signs were then calculated for the *h0l* reflections with l even on the basis of $x = \frac{1}{4}$ for barium and the four sulphur atoms, and a Fourier series was computed using these even l terms only. Such a projection contains peaks corresponding to the atomic positions, and also images of these atoms produced by the operation of a spurious centre of symmetry. The true peaks were identified by including in the series those reflections with l odd having h indices equal to or larger than three, with signs calculated from the barium contributions alone on the assumption that the ion was a little removed from the glide plane. Successive refinements could thereafter be carried out. The final electron density map, $\varrho(xz)$, is shown in Fig. 4, and an explanatory diagram is reproduced in Fig. 5.

The atomic coordinates, in fractions of cell edges, are listed in Table 1. In the case of resolved peaks, the atomic centres were taken as coincident with

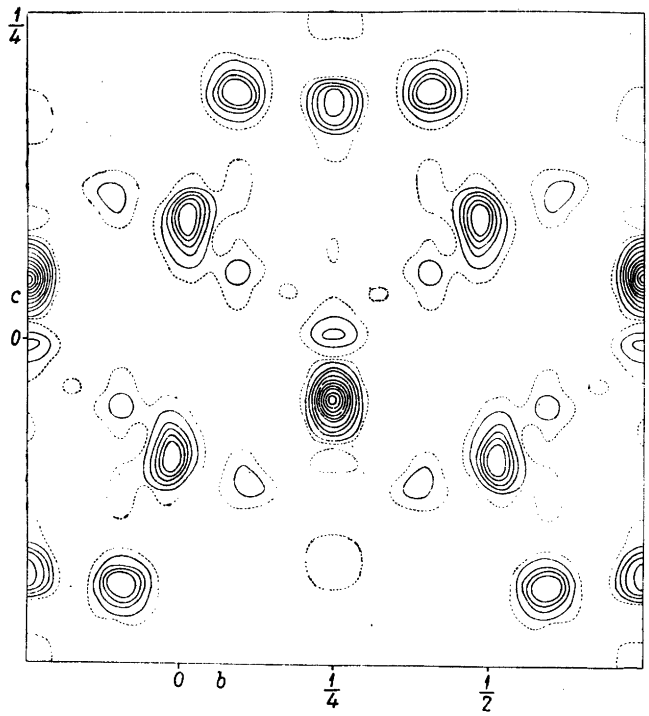

Fig. 2. Projection of $BaS(S_2O_3)_2 \cdot 2H_2O$ along the a axis, ϱ(yz). Half a unit cell (four asymmetric units) are shown. The 4-electron line is dotted. Contour intervals: 10 e.Å^{-2} for the barium ion, and 4 e.Å^{-2} for sulphur and oxygen atoms and water molecules.

the points of highest electron density. In $\varrho(yz)$, the y and z coordinates of S_1 and O_1 could not be read from resolved peaks, due to overlapping of the two atoms. The y coordinates remain unreliable, whereas better values for the z coordinates were obtained from $\varrho(xz)$. The $(H_2O)_1$ peak in $\varrho(yz)$ is excessively high due to diffraction effects from the barium ion, and the $(H_2O)_2$ peak is rather diffuse. In $\varrho(xz)$, overlapping of S_1 and O_2 occurs, and the x coordinates of these atoms may be correspondingly inaccurate. The $(H_2O)_1$ peak is distorted, and $(H_2O)_2$ is obscured by S_3. The x coordinate of $(H_2O)_2$ was therefore arrived at through trial and error calculations.

Table 1. Atomic coordinates, referring to the positions (c) *and* (d) *of the* Int. Tables [12]. *Origin at centre of symmetry.*

	Fourfold positions				*Eightfold positions*		
	x	y	z		x	y	z
Ba	0.220	0.250	− 0.050	S_2	0.285	0.092	0.185
S_3	0.530	0.250	0.179	S_1	0.300	0.013	0.094
$(H_2O)_1$	0.700	0.250	0.002	O_1	0.579	0.008	0.078
$(H_2O)_2$	0.350	0.250	− 0.170	O_2	0.180	− 0.109	0.109
				O_3	0.170	0.094	0.050

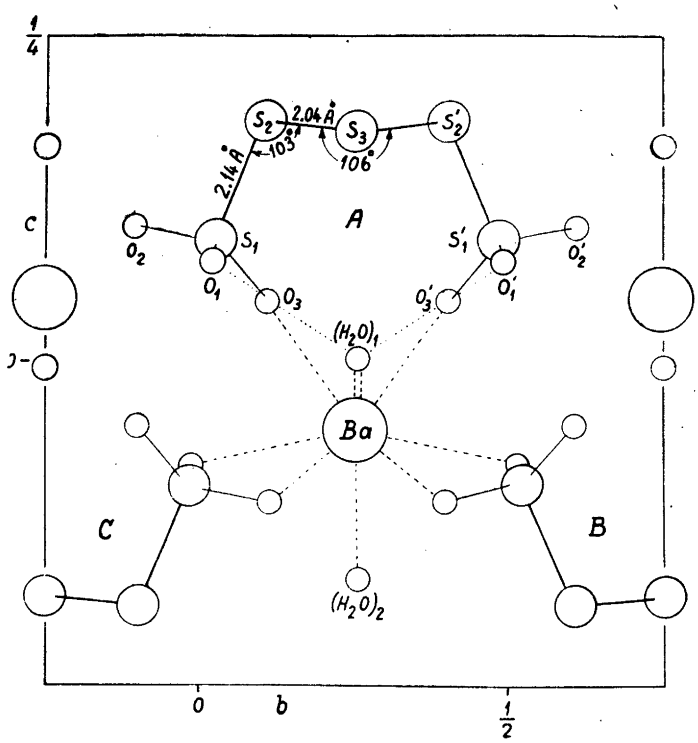

Fig. 3. Arrangement of the ions as viewed along the a *axis.*

In Table 5, the observed and calculated structure factors are compared for all 0*kl* and *h*0*l* reflections within the range of Cu$K\alpha$ radiation. The signs of the 0*kl* data correspond to the origin of the plane group *cmm* of the *Int. Tables*[12] p. 64, situated at $y = \frac{1}{4}$, $z = -\frac{1}{4}$ with respect to the origin of Table 1. The 0*kl* and *h*0*l* data were calculated on the basis of $z = 0.049$ and $z = 0.053$, respectively, for O_3; otherwise, the z coordinates are the same for both zones.

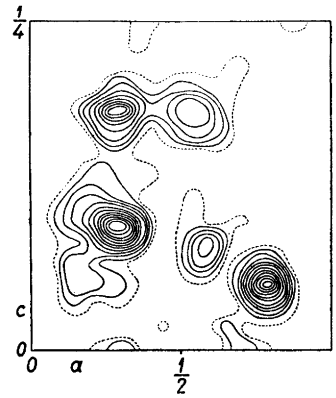

Fig. 4. Projection of $BaS(S_2O_3)_2 \cdot 2H_2O$ *along the* b *axis,* $\varrho(xz)$. *A quarter of a unit cell (one asymmetric unit) is shown. The 6-electron line is dotted. Contour intervals as in Fig. 2, except for the water molecule,* $(H_2O)_1$, *where the intervals are 2 e.Å*$^{-2}$.

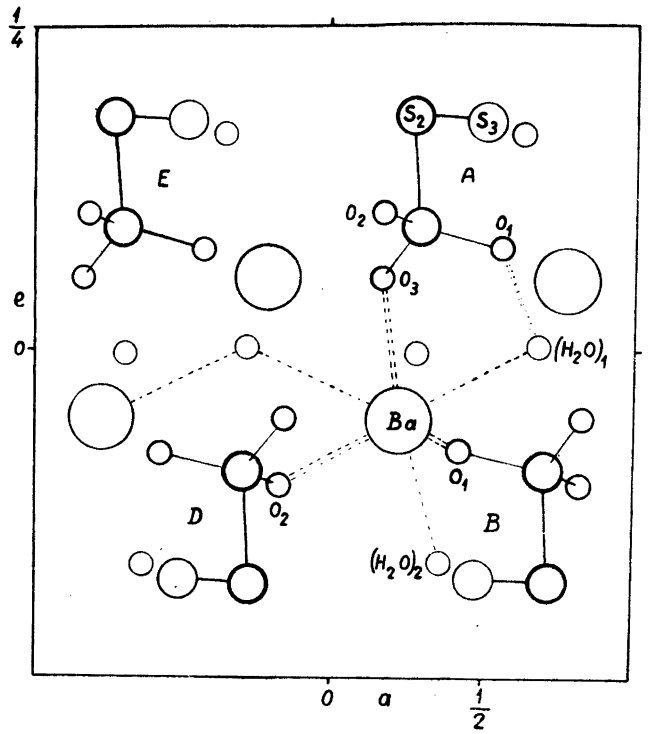

Fig. 5. Arrangement of the ions as viewed along the b *axis. Origin at the midpoint of the diagram.*

The reliability factor, $R = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$, is 0.15 for the $0kl$ reflections and 0.19 for the $h0l$ reflections. For both zones together, $R = 0.17$. The unobserved reflections are included in R, as the difference between $|F_{calc}|$ and the lowest observable value of $|F_{obs}|$, only when $|F_{calc}|$ is greater than the smallest value of $|F_{obs}|$. They are not included in $\Sigma|F_{obs}|$.

The largest discrepancy occurs for the 002 structure factor, the observed value of which is about 70 units smaller than the calculated value. This relatively strong reflection is probably subject to extinction.

THE PENTATHIONATE ION

The ion possesses an exact mirror plane of symmetry, passing through the middle atom of the sulphur chain and not through any other atom of the ion. Distances and angles, calculated on the basis of the coordinates of Table 1, are as follows. The numbering of atoms is shown in Figs. 3 and 5. A prime denotes the image of an atom across the mirror plane.

Table 2. Sulphur-sulphur bond lengths and angles.

$S_1-S_2 = 2.14$ Å	$\angle S_1S_2S_3 = 103°$	$S_1S_2S_3/S_2S_3S_2'$
$S_2-S_3 = 2.04$	$\angle S_2S_3S_2' = 106°$	(dihedral angle) = 110°

Non-bonded sulphur-sulphur distances.

$S_1-S_1' = 4.88$ Å	$S_1-S_3 = 3.27$ Å
$S_1-S_2' = 4.53$	$S_2-S_2' = 3.25$

The length of a single sulphur-sulphur bond [13] being about 2.11 Å and the van der Waals radius of sulphur [14] about 1.60 Å, the data of Table 2 show that the pentathionate ion is built up of an unbranched sulphur chain *.

The terminal sulphur atoms are rotated an angle of 110° out of the plane of the three middle atoms, to the same side of the plane. The pentathionate ion thus occurs in a *cis* form [9] in this salt.

Three oxygen atoms, in approximately tetrahedral positions, surround each of the terminal sulphur atoms. The data are collected in Table 3. The average sulphur-oxygen bond length, 1.43 Å, compares with the corresponding average, 1.41 Å, in barium tetrathionate dihydrate [1].

Table 3. Sulphur-oxygen bond lengths and angles.

$S_1-O_1 = 1.44$ Å	$\angle O_1S_1O_2 = 115°$	$\angle S_2S_1O_1 = 106°$
$S_1-O_2 = 1.43$	$\angle O_1S_1O_3 = 106°$	$\angle S_2S_1O_2 = 96°$
$S_1-O_3 = 1.43$	$\angle O_2S_1O_3 = 118°$	$\angle S_2S_1O_3 = 113°$

The non-bonded sulphur-oxygen distances, $S_2—O_1=2.89$ Å, $S_2—O_2=2.70$ Å, $S_2—O_3 = 3.00$ Å, and the non-bonded oxygen-oxygen distances, $O_1—O_2 = 2.42$ Å, $O_1—O_3 = 2.31$ Å, $O_2—O_3 = 2.47$ Å. The sulphur atom, S_3, lies 3.33 Å, 4.32 Å, and 3.70 Å, respectively, from the oxygen atoms O_1, O_2 and O_3.

The shortest oxygen-oxygen approach between sulphonate groups of the same pentathionate ion is 3.21 Å and occurs in the case of O_3 and its equivalent across the mirror plane.

Regarding the sulphur chain of the pentathionate ion, it is of interest to note that the configuration is similar to that of the S_8 ring of orthorhombic sulphur [15] with three adjacent sulphur atoms removed. The latter three atoms represent the sulphur chain of the trithionate ion [16]. Likewise, the sulphur chain of the tetrathionate ion [1] resembles that of the S_8 ring, divided in two halves of four sulphur atoms each.

THE IONIC ENVIRONMENT

The barium ion is surrounded by nine oxygen atoms, at an average distance of 2.81 Å. Six of these are sulphonate oxygen atoms and three are water oxygen atoms. In Table 4, the letters *A* to *E* refer to different pentathionate

* The significance of variations in sulphur-sulphur bond lengths will be discussed in a later article in connection with data on some analogous pentathionic compounds.

ions, as shown in Figs. 3 and 5. Distances listed in the left column of the Table occur two times, once on each side of the mirror plane.

Table 4. Barium-oxygen distances.

$Ba\text{-}O_{3A} = 2.72$ Å	$Ba\text{-}(H_2O)_{1A} = 2.65$ Å
$Ba\text{-}O_{1B} = 2.91$	$Ba\text{-}(H_2O)_{1E} = 2.84$
$Ba\text{-}O_{2D} = 2.79$	$Ba\text{-}(H_2O)_{2A} = 2.69$

The three oxygen atoms of a sulphonate group are all coordinated to different barium ions. Within a pentathionate ion, two oxygen atoms, one from each sulphonate group, are coordinated to the same barium ion. The coordinating powers of barium towards oxygen are thus, perhaps, responsible for the *cis* form of the pentathionate ion occurring in this salt. The six sulphonate oxygen atoms surrounding a barium ion belong to six different sulphonate groups, and to five different pentathionate ions. The arrangement is like that found in barium tetrathionate dihydrate [1].

The water molecules lie in the mirror plane. One of the molecules, $(H_2O)_1$, is approximately equidistant from two barium ions, situated in adjacent unit cells along the *a* axis, and the water molecule probably coordinates to both of the barium ions. The angle, $Ba\text{-}(H_2O)_1\text{-}Ba$, is 131°. The hydrogen atoms of this water molecule probably form hydrogen bonds to the oxygen atoms, O_1 and its mirror image, belonging to different sulphonate groups of the same pentathionate ion. The $(H_2O)_1\text{-}O_1$ distance is 3.05 Å and the angle, $O_1\text{-}(H_2O)_1\text{-}O_1'$, is 110°. The $Ba\text{-}(H_2O)_1\text{-}Ba$ and the $O_1\text{-}(H_2O)_1\text{-}O_1'$ bonds lie in perpendicular planes. This hydrate structure is similar to that occurring in lithium perchlorate trihydrate [17].

The water molecule, $(H_2O)_2$, is coordinated to one barium ion, at 2.69 Å, and is located 3.07 Å from two oxygen atoms, O_2 and its image, belonging to different pentathionate ions. The angles, however, appear to be unfavourable for hydrogen bonds to exist from this water molecule.

No chemical bonds occur across the glide plane *a* normal to the *c* axis at $z = \frac{1}{4}$ and $-\frac{1}{4}$. The only contacts are the van der Waals ones between the sulphur atoms S_2, S_3 and S_2' and their equivalents, produced by the operation of the glide plane. The approaches are, $S_2\text{—}S_2 = S_2'\text{—}S_2' = 3.78$ Å, $S_3\text{—}S_3 = 3.98$ Å, and $S_2\text{—}S_3 = S_2'\text{—}S_3 = 3.61$ Å. The closest contacts between S_8 molecules in orthorhombic sulphur [15] are 3.28 Å and 3.32 Å, and between hexasulphide chains in the cesium salt [13], 3.39 Å. The lack of strong attractive forces across the glide plane accounts for the ready cleavage of the crystals along the *c* plane.

ESTIMATE OF ERRORS

A Fourier synthesis based on the calculated 0*kl* structure factors was made in order to correct for errors due to finite termination of Fourier series [18]. Average shifts of 0.015 Å for sulphur and 0.04 Å for oxygen atoms, respectively, occurred in the *y* and *z* coordinates. When the 0*kl* structure factors were recalculated on the basis of the corrected coordinates, the reliability factor

Table 5. Observed and calculated values of structure factors for barium pentathionate dihydrate.

l	F_{obs}	F_{calc}
	0kl zone	
	00l	
2	135	− 206
4	55	− 72
6	82	+ 70
8	235	− 270
10	36	+ 14
12	30	− 10
14	112	+ 128
16	42	+ 43
18	53	− 56
20	51	+ 69
22	81	− 104
24	< 10	− 1
26	27	+ 19
28	10	− 15
	01	
1	10	− 13
3	138	− 185
5	158	+ 176
7	20	− 25
9	62	+ 94
11	32	+ 32
13	101	− 106
15	56	+ 53
17	86	− 91
19	32	+ 16
21	39	+ 44
23	< 20	− 11
25	30	+ 39
27	20	− 27
	02l	
0	96	+ 69
2	73	− 76
4	155	+ 149
6	113	− 129
8	82	− 86
10	148	+ 184
12	110	− 105
14	25	+ 26
16	63	+ 66
18	77	− 96
20	35	+ 36
22	23	− 26
24	17	+ 18
26	< 10	+ 8
	03l	
1	125	+ 120
3	149	− 143
5	177	+ 186
7	178	− 207
9	17	− 1
11	62	+ 55
13	81	− 80
15	102	+ 105
17	55	− 46
19	32	+ 43
21	< 17	+ 2
23	49	− 55
25	30	+ 37
27	14	− 32
	04l	
0	254	+ 230
2	170	− 162
4	25	+ 11
6	93	+ 82
8	101	− 104
10	67	+ 66
12	25	+ 17
14	< 17	+ 4
16	27	− 20
18	39	− 38
20	54	+ 50
22	45	− 55
24	17	+ 30
26	17	+ 27
	05l	
1	23	− 16
3	80	− 73
5	143	+ 125
7	79	− 84
9	74	+ 79
11	44	+ 40
13	98	− 100
15	62	+ 53
17	56	− 56
19	17	+ 11
21	29	+ 27
23	14	− 11
25	20	+ 30
	06l	
0	187	+ 195
2	49	− 34
4	72	+ 70
6	85	− 90
8	121	− 138
10	92	+ 94
12	72	− 67
14	66	+ 70
16	72	+ 88
18	62	− 63
20	23	+ 27
22	36	− 45
24	< 10	− 3
1	< 14	+ 9
3	140	− 130
5	110	+ 98
7	36	+ 25
9	30	+ 31
11	< 18	+ 14
13	56	− 44
15	25	+ 14
17	65	− 68
19	36	+ 21
21	27	+ 28
23	14	− 10
	08l	
0	186	+ 166
2	94	− 80
4	< 17	− 12
6	50	+ 42
8	78	− 66
10	45	+ 27
12	25	− 8
14	35	+ 32
16	< 17	+ 4
18	34	− 35
20	35	+ 35
22	25	− 37
	09l	
1	23	+ 26
3	32	− 26
5	66	+ 58
7	86	− 91
9	39	+ 26
11	30	+ 16
13	59	− 55
15	63	+ 67
17	25	− 21
19	< 10	+ 6
	0,10,l	
0	28	+ 13
2	50	− 35
4	44	+ 39
6	< 17	− 16
8	34	− 24
10	65	+ 66
12	45	− 33
14	< 14	0
16	17	+ 14
18	25	− 35
	0,11,l	
1	25	+ 15
3	59	− 55

l	F_{obs}	F_{calc}
5	56	+ 56
7	25	− 20
9	< 14	+ 3
11	17	+ 16
13	25	− 22
15	17	+ 14
	0,12,l	
0	69	+ 84
2	27	− 25
4	≤ 14	+ 4
6	< 10	− 8
8	42	− 53
10	23	+ 16
12	10	− 7
	0,13,l	
1	17	− 15
3	17	− 14
5	20	+ 28
	$h0l$ zone	
	00l	
2	134	+ 206
4	60	− 80
6	83	− 75
8	232	− 281
10	43	− 15
12	38	− 5
14	146	− 130
16	49	+ 62
18	72	+ 66
20	69	+ 76
22	110	+ 116
24	< 29	− 6
26	38	− 25
28	< 13	− 19
	10l	
1	55	− 48
2	84	− 90
3	105	+ 108
4	125	+ 143
5	38	− 31
6	157	+ 147
7	22	+ 11
8	137	+ 160
9	< 22	+ 10
10	59	+ 35
11	107	+ 116
12	136	− 172
13	31	− 32
14	104	− 80
15	< 30	+ 16
16	54	+ 46
17	< 32	0
18	42	− 27
19	58	+ 51
20	47	+ 45
21	< 33	+ 7
22	< 33	+ 2
23	≤ 30	− 17
24	≤ 28	+ 9
25	< 25	+ 7
26	40	+ 55
27	< 17	− 10
	20l	
0	282	− 288
1	< 20	0
2	160	+ 144
3	≤ 20	− 3
4	< 21	− 7
5	25	+ 27
6	65	+ 62
7	27	+ 24
8	100	+ 101
9	< 25	− 2
10	68	+ 33
11	< 27	+ 17
12	60	+ 77
13	< 29	+ 2
14	69	+ 69
15	32	− 21
16	< 33	− 21
17	33	− 19
18	58	− 50
19	54	− 40
20	79	− 96
21	< 31	− 1
22	66	− 65
23	35	+ 34
24	< 25	+ 7
25	22	+ 14
26	< 17	+ 15
	30l	
1	60	− 49
2	38	+ 52
3	69	− 54
4	92	+ 107
5	< 27	+ 5
6	88	+ 65
7	28	+ 28
8	84	− 76
9	82	+ 72
10	56	− 71
11	91	+ 104
12	72	+ 97
13	46	+ 40
14	81	+ 70
15	< 33	− 10
16	38	+ 40
17	42	− 48
18	37	+ 47
19	40	− 39
20	29	− 29
21	< 26	+ 2
22	28	− 19
23	< 22	+ 2
24	18	− 11
25	< 3	− 7
	40l	
0	92	+ 98
1	72	− 78
2	45	+ 23
3	83	+ 63
4	< 32	+ 13
5	59	− 36
6	< 33	+ 17
7	42	− 54
8	60	− 66
9	43	+ 54
10	43	+ 18
11	33	+ 18
12	33	− 24
13	< 33	+ 23
14	32	− 40
15	35	+ 36
16	30	+ 26
17	32	+ 12
18	26	+ 18
19	28	+ 29
20	25	+ 27
21	21	+ 20
22	17	+ 42
	50l	
1	38	+ 24
2	46	+ 34
3	56	+ 60
4	55	+ 54
5	< 32	− 17
6	44	+ 30
7	< 31	+ 18
8	< 30	+ 15
9	54	− 46
10	< 29	− 2
11	62	− 88
12	26	− 23
13	25	− 8
14	27	− 27
15	22	+ 12
16	23	+ 28
17	18	+ 18
	60l	
0	< 23	− 9
1	26	+ 32
2	< 26	− 8
3	41	+ 39
4	< 22	− 10
5	32	+ 34
6	< 20	0
7	19	− 28
8	17	+ 17
9	< 16	− 12
10	14	+ 18

remained the same as was obtained from the coordinates of Table 1. The back-shift corrections would have caused minor changes only in bond lengths, *viz.*, —0.01 Å for S_1—S_2, +0.01 Å for S_2—S_3, and +0.02 Å for S_1—O_2 and S_1—O_3.

As in the case of barium tetrathionate dihydrate [1], the object of the structure determination has been, more to establish the constitution of the polythionate anion, than to arrive at accurate values for bond lengths and angles. The accuracy of the present structure is probably about the same as for the tetrathionate structure [1], *i. e.*, ±0.03 Å for sulphur-sulphur bond lengths, ±0.04 Å for the average sulphur-oxygen bond length, and ±0.1 Å for oxygen-oxygen distances. Errors in angles involving sulphur atoms only are not likely to exceed ±2°.

SUMMARY

The crystal structure of barium pentathionate dihydrate, and the constitution of the pentathionate ion, has been determined by two-dimensional Patterson and Fourier methods.

The crystals are orthorhombic bipyramidal, with the axial lengths, $a = 5.00$ Å, $b = 10.30$ Å, $c = 21.78$ Å. The space group is D_{2h}^{16}—*Pnma*, and there are four molecules per unit cell.

The sulphur chain of the pentathionate ion is unbranched and non-planar. The ion possesses, by space group requirements, a mirror plane of symmetry, and occurs in a *cis* form. Three oxygen atoms, in approximately tetrahedral positions, surround each of the terminal sulphur atoms.

The length of the middle sulphur-sulphur bonds is 2.04 Å and that of the terminal sulphur-sulphur bonds is 2.14 Å. The bond angle of the middle sulphur atom of the chain is 106° and that of the two other divalent sulphur atoms is 103°. The dihedral angles are 110°, and the average sulphur-oxygen bond length is 1.43 Å.

Weak van der Waals contacts only occur between layers normal to the *c* axis, resulting in a ready cleavage of the crystals along the *c* plane.

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On the Isomerism of Hydroxyurea

III. Some Physical Properties of the Isomers

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In Part II of this series an improved method was described for the preparation of the alleged isomers.

The present paper is chiefly devoted to an examination of the products in the solid state.

Crystal form, approximate solubilities and stability of the solids are discussed. The common empirical formula $CH_4O_2N_2$ has been confirmed. The homogeneity of the two products was tested by microthermal analysis and X-ray powder diagrams. The isomers had entirely different X-ray patterns and none of them was contaminated with the other one to an extent detectable by this method.

It has been demonstrated that the lower melting isomer is not detectably converted to the higher melting one on heating in the solid phase.

Whereas all samples of the lower melting hydroxyurea showed the same diffraction pattern, two different patterns were found for the higher melting isomer. This is probably due to polymorphism.

Comparison of the X-ray pattern of the freeze-dried reaction mixture with those of the pure components suggests that the lower melting isomer is the primary product formed by the reaction between hydroxylammonium ion and cyanate ion.

A parallel investigation of the analogous process leading to methoxyurea showed that this compound does not display a similar isomerism, even under favourable conditions. Methoxyurea was found to be monomorphous.

CRYSTAL FORM AND HABIT

At room temperature both hydroxyureas are colourless crystalline substances without any characteristic taste or smell.

The lower melting isomer, isolated as described in a previous paper [1], crystallizes from anhydrous ether solution as rhombical plates. Well developed single crystals up to 0.5 mm were best obtained from the first batches of ether when the solvent was evaporated in a round-bottomed flask connected directly to a water pump. The heat of evaporation was supplied by infra-red radiation in such a way that the temperature was held below 10° C.

It proved less easy to obtain well-developed single crystals of the higher melting isomer. When crystallized from ethanol, ethanol + ether or dioxane it usually formed crystal aggregates, often rosettes or compact spheres of a size up to 0.5 mm or more. A typical crystal form, irregular hexagonal plates, was found in microsublimates (*vide infra*). A similar crystal form has been reported by Dresler and Stein [2] who obtained "mikroscopischen rhombischen Blättchen mit abgestumpften spitzen Winkeln" on addition of ether to an alcoholic solution.

SOLUBILITY

Both substances are very soluble in water and insoluble in benzene at 20° C. Boiling absolute ethanol dissolves approximately 3 % of the higher melting isomer; at 0° C the solubility is less than 0.2 %. Even a small content of water in the solvent raises the solubility considerably. The lower melting hydroxyurea is more soluble in ethanol than the higher melting one.

Boiling anhydrous ether dissolves approximately 0.2 % of the lower melting isomer; at —10° the substance is practically insoluble. The higher melting isomer is insoluble in anhydrous ether at the boiling point.

Boiling anhydrous dioxane dissolves approximately 1.5 % of the higher melting hydroxyurea. The solubility at 20° C is *ca.* 0.2 %. The lower melting isomer shows a higher solubility.

STABILITY IN THE SOLID STATE

The higher melting isomer is relatively stable. Pure samples can be stored at room temperature for at least several weeks before a slow decomposition sets in. On the other hand a sample, which had been stored in a sealed tube for 6 months, had acquired a moist appearance, and the tube exploded violently owing to gas pressure when an attempt was made to open it.

The lower melting isomer is more labile. Pure specimens stored at room temperature in a dry atmosphere usually begin to smell from ammonia after 1—2 days. Impure specimens sometimes decompose within hours.

At low temperatures both isomers show a satisfactory stability for practical work. The lower melting hydroxyurea has been stored at —15° C over phosphorus oxide for 1 week without detectable drop in melting point. All investigations in this and the following papers were carried out within one week from the date of the preparation and applying the above storage conditions.

The spontaneous decomposition process has been investigated in some detail in older literature. Carbon dioxide, ammonia, nitrogen and urea were among the decomposition products found by Dresler and Stein [2] on heating the higher melting isomer to the melting point 130°, and Francesconi and Parrozzani [3] obtained the same products when decomposing the lower melting isomer at 100° C.

MICROTHERMAL EXAMINATION

Freshly isolated, pure and dry specimens of the isomers were examined in a polarising microscope equipped with an electrical hot stage, essentially as designed by F. Halstrøm [4]. The magnification was 50 : 1.

The lower melting hydroxyurea appeared as well-developed rhombical plates, displaying beautiful interference colours between crossed nicols. On heating with a rate of 2°/min no changes whatsoever were observed until a temperature of 68—70°, at which the crystals began to sinter, and gas bubbles appeared in their interior. At 71° C the half-melted substance decomposed rapidly with a brisk effervescens, at 72—73° C all crystalline material had disappeared and the object appeared black between crossed nicols. The general behaviour and decomposition temperature correspond to that given in previous literature [3]. The main object of the investigation was to find out, whether the "isomers" were possibly two forms of one and the same polymorphic substance. There were no indications of such relationship, but unfortunately the lability of both hydroxyureas at and above their melting points prohibited a detailed microthermal analysis. Every attempt to seed one substance with crystals of the other one at appropriate temperatures resulted in immediate decomposition.

When the lower melting hydroxyurea was quickly heated to just under the destruction temperature — about 65° — and this temperature was maintained for some time, a microsublimate was slowly deposited on the cover slip. It was both in appearance and melting point identical with the bulk. Higher melting crystals have never been observed during these experiments. It seems justified to conclude, therefore, that the lower melting hydroxyurea is not transformed into the higher melting isomer (m.p. 140°) simply on heating the solid. The transformation reported [3] earlier must therefore be confined to solutions. Transformation in solid phase is further disproved by X-ray methods (*vide infra*).

The sample of the *higher melting hydroxyurea* consisted of crystal aggregates with poorly developed crystal form. They showed typical anisotropy between crossed nicols. On heating at a rate of 2°/min no visible changes occurred until the crystals began to sinter at approximately 135° with a slight evolution of gas. At 140° the last trace of crystalline material disappeared (crossed nicol field) and there was a rapid decomposition accompanied by evolution of gas. The decomposition temperatures reported in the literature [2,3,5,6], vary from 128 to 141 °C. The absolute values are for both isomers strongly dependent on the rate of heating.

When the higher melting isomer was heated on a slide to 125° C and this temperature was maintained for some time a microsublimate was deposited on the cover slip. It consisted of hexagonal plates which showed the same decomposition temperature as the main crop.

The two hydroxyureas thus behave similarly when heated under the microscope, the only difference being that the sublimation and decomposition occur at different temperatures.

X-RAY POWDER DIAGRAMS

In an attempt to throw further light on the formation, homogeneity and interconversions of the isomers some X-ray powder diagrams were taken.

The radiation, Cr-K_α; $\lambda = 2.2896$ Å, was supplied by a self-rectifying Machlett tube with a chromium target. A vanadium pentoxide filter was used. The diffraction pattern was recorded photographically in a 19 cm

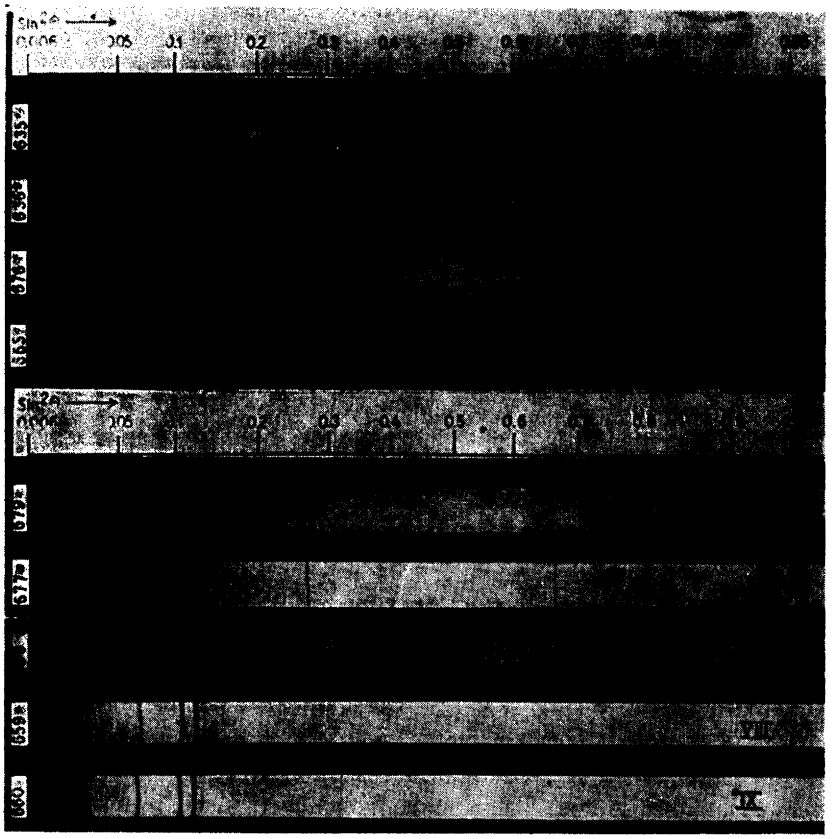

Fig. 1. For explanation see the text and Table 1.

Bradley camera, and the general technique was as previously described by one of us [7]. The time of exposure was *ca.* 6 hours and the temperature was 21—23° C.

The patterns are reproduced in Fig. 1. Detailed analyses, carried out as previously described [7] are given in Table 1. The positions of the various lines in the pattern are described by the term $\sin^2 \Theta$, in which Θ is the glancing angle. The relative intensities (I) of the diffraction lines are estimated by visual examination and described as follows: s+, s, s— (strong), m+, m, m— (medium), w+, w, w— (weak), ? doubtful. Identity of any two patterns with respect to position and relative intensity of the lines may be taken as an indication that the corresponding substances are chemically and crystallographically identical. From non-identity of patterns it is not possible to decide, without thorough investigation, whether the substances differ in chemical structure or only in crystal lattice.

In Fig. 1, *I* is reproduced the pattern of the lower melting isomer, first crop of crystals obtained on cooling the first batch of ether used for extraction (ref. 1, p. 940); *II* represents the pattern of the last crop from the same prepa-

Table 1. Analysis of the characteristic X-ray diffraction patterns. (Roman numerals correspond to those in Fig. 1.)

II Hydroxyurea, lower melting isomer
III Hydroxyurea, higher melting isomer, from ethanol
IV Hydroxyurea, higher melting isomer, sublimate
V Freeze-dried reaction mixture, containing II, IV (?) and VI
VI Potassium chloride
VIII Methoxyurea

II		*III*		*IV*		*V*		*VI*		*VIII*	
I	$\sin^2\Theta$	I	$\sin^2\Theta$	I	$\sin^2\Theta$	I	$\sin^2\Theta$	I	$\sin^2\Theta$	I	$\sin^2\Theta$
.0655	m+	.0297	w	.0679*	w	.0656	w−	.1333	m+	.0269	m+
.0872	m+	.0683*	w+	.0777	m	.0777	w−	.2664	m	.0313	m
.0961	w	.0853*	m+	.0814	w−	.0872	w−	.3982	w	.0511	w
.1023	w	.1050	w	.0856*	m−	.1023	?	.5305	w	.0583	w
.1157	m+	.1143	w	.0937	m−	.1153	w−	.6633	m−	.0691	m
.1233	m+	.1221*	m+	.1015	m	.1231	w	.7954	m−	.0910	w
.1387	m	.1320	m	.1220*	m	.1295	w−			.1026	w
.1558	m	.1462	m	.1287	m+	.1330	m+			.1087	m+
.1604	m−	.1566*	w	.1364	w	.1562	w−			.1235	m+
.1738	m	.1662	m	.1479	w+	.1740	?			.1318	m
.1886	w	.1928	w	.1566*	m	.2653	m			.1391	w
.2290	w+	.2046*	w+	.1687	w+	.3979	w			.1545	w+
.2343	w−	.2181*	w	.2053*	w	.5305	w−			.1679	m−
.2402	w	.2270	w+	.2129	w+	.6626	m−			.1762	w+
.2630	w+	.2445	w	.2186*	w+	.7950	m−			.2010	w
.2761	w	.2660*	w+	.2235	m−					.2100	m
.2945	w	.3111*	w	.2312	w+					.2332	m−
.3019	w	.3299	w−	.2427	w+					.2413	m−
.3194	w+	.3402	w	.2655*	w−					.2492	m−
.3339	w+	.3500	w	.2967	w−					.2572	w+
.3487	w	.3703	w	.3116*	w+					.2644	m−
.3595	w	.4209	w−	.3220	w					.2747	w+
.3889	w−	.4889	w+	.3513	w					.2864	m−
.3992	w	.5354	w−	.3832	w					.2934	w+
.4626	w+	.5642	w−	.4855	w					.3201	w+
.4941	m−			.5171	w+					.3438	w
.5213	w			.5338	w					.3532	w
.5299	?									.3683	w+
.5543	w									.3840	w
.5886	w−									.3897	w+
.6601	w−									.3948	w+
.6685	w−									.4371	w
										.4697	w
										.4807	w
										.4915	w
										.5163	w
										.5279	w
										.5569	w
										.5713	?
										.6175	w
										.6469	w+

ration, obtained on concentration of the mother liquor. The patterns are identical, which is a reasonable proof of the homogeneity of the ether-soluble fraction. The melting points were also identical.

The pattern of the higher melting isomer, crystallized from ethanol is reproduced in *III*. It is entirely different from that of the lower melting hydroxyurea.

The higher melting hydroxyurea (pattern *III*) could be sublimated in quantity at 115° C and 1 mm Hg in Eder's apparatus [8]. The sublimate, about one third, showed the normal decomposition temperature, 135—140°, of the higher melting isomer, but the X-ray diffraction pattern (*IV*), was different from *III*. The possibility could not *a priori* be excluded that the higher melting isomer in fact consisted of two or more closely related chemical species. The difficulties in obtaining well-developed single crystals could be a result of such inhomogeneity. Since sublimation usually implies some purification it might be expected that the sublimate (*IV*) represented one compound or crystal modification and the original substance (pattern *III*) were a mixture of this and a different species. On inspection of the data in Table 1, *III* and *IV* it is seen that a certain number of lines in pattern *IV* (marked with an asterisk) actually reappear in pattern *III*. The $\sin^2 \Theta$ values as well as the relative intensities of corresponding lines fit fairly well. A serious objection, however, is that several of the most intense lines (m and m+) of pattern *IV* are lacking in pattern *III*. It is therefore most reasonable to conclude that *III* and *IV* correspond to two pure modifications of the higher melting isomer, or alternatively that *IV* represents a new isomer.

It is important that pattern *III* does not contain any lines attributable to the lower melting isomer (*II*). This means that the higher melting hydroxyurea

Table 2. Origin of the lines in the pattern of the reaction mixture, V.

Reaction mixture *V*		KCl *VI*		Hydroxyurea lower m. p. *II*		Hydroxyurea higher m. p. *IV*	
$\sin^2 \Theta$	I	$\sin^2 \Theta$	I	$\sin^2 \Theta$	I	$\sin^2 \Theta$	I
.0656	w−			.0655	m+		
.0777	w−					.0777	m
.0872	w−			.0872	m+		
.1023	?					.1015	m
.1153	w−			.1157	m+		
.1231	w			.1233	m+		
.1295	w−					.1287	m+
.1330	m+	.1333	m+				
.1562	w−			.1558	m		
.1740	?			.1738	m		
.2653	m	.2664	m				
.3979	w	.3982	w				
.5305	w−	.5305	w				
.6626	m−	.6633	m−				
.7950	m−	.7954	m−				

is not contaminated with the lower melting isomer. The X-ray powder method is, however, not very sensitive. Less than *ca.* 10 % impurity cannot usually be traced in the patterns of the present type of compounds. The efficiency of the method of separation [1] has, however, been demonstrated by other means, as will be reported in a following paper.

In Fig. 1, *V* is reproduced the pattern of the freeze-dried reaction mixture (ref. 1, p. 940) recorded immediately after the solvent had been removed. Besides the intense lines of potassium chloride (*cf.* the pure potassium chloride pattern, *VI*) it exhibits the major lines of the lower melting hydroxyurea (*I—II*), whereas it is less easy to discern those of the higher melting isomer (*III* or *IV*). The origin of the various lines in *V* is more clearly seen from Table *2*, in which the relevant data have been extracted. All the lines of potassium chloride are easily recognizable. Six of the most intense lines of the lower melting hydroxyurea reappear in the mixed pattern as weak lines, whereas only two, perhaps three of the lines appertaining to the higher melting isomer can be detected in *V*. These lines represent the strongest lines in pattern *IV* of the *sublimate*, whereas there are no common lines with the higher melting hydroxyurea crystallized *from ethanol*, pattern *III*. Although somewhat surprising this is probably an incident, which is readily understood, if the relationship between *III* and *IV* is assumed to be a case of polymorphism. A probable conclusion from Table *2* is that the hydroxyurea formed primarily by the reaction of hydroxylammonium chloride with potassium cyanate in aqueous solution under the given mild conditions [1] is the *lower* melting isomer. During the process of isolation this is partially transformed into the higher melting one, so that the ultimate ratio becomes about 1 : 1 (*cf.* ref. 1, p. 940).

Fig. 1, *VII* shows the pattern of the lower melting isomer, the sample having previously been heated to 50° C for 3 hours. The pattern is identical with *I* and *II*, which confirms that the lower melting hydroxyurea is not appreciably, if at all, transformed into the higher melting isomer on heating the solid, in accordance with the observation, that it could be sublimated unaltered (*I—II*). The rearrangement reported by others is therefore confined to solutions.

A parallel investigation of methoxyurea, which only exists as one chemical individual, showed that the substance is probably monomorphous. Powder diagrams of a sample crystallized from ethanol and of a sublimate are reproduced in Fig. 1, *VIII* and *IX*. It is evident that they are identical.

EXPERIMENTAL

Materials. The preparation of the *hydroxyureas* has been described in a previous paper [1]. Analyses:

Lower melting isomer ($CH_4O_2N_2$):	Calc.	C	15.79	H	5.31	N 36.68
	Found	»	16.12	»	5.20	» 36.34
Higher melting isomer ($CH_4O_2N_2$):	Calc.	N	36.86			
	Found	»	36.3			

Molecular weights in aqueous solution were determined by cryoscopy, using the conventional Beckmann apparatus.

Results:

	Thermom. reading		
Water	2.708 2.709 2.708 2.710	2.709	Depression 0.629° C M. w. 76.8 (calc. 76.0)
Higher melting hydroxyurea 0.762 g dissolved in 29.2 g water.	2.078 2.081 2.083 2.080	2.080	
Water	2.104 2.100 2.099 2.101	2.101	Depression 0.619° C M. w. 78.5 (calc. 76.0)
Lower melting hydroxyurea 0.767 g dissolved in 29.2 g water	2.718 2.722 2.720 2.719	2.720	

The results are in accordance with those obtained by Francesconi and Parrozzani [8]. They clearly indicate that the hydroxyureas exist as monomers in aqueous solution and are only little dissociated. The same conclusion was previously drawn from conductivity measurements [9].

Methoxyurea was synthesized in the following way. Potassium hydroxylamine disulphonate was first prepared according to Raschig's method [10].

$$KNO_2 + K_2S_2O_5 + H_2O \rightarrow HON(SO_3K)_2 + KOH$$

In the original procedure sodium hydrosulphite and sodium nitrite were used and the less soluble potassium salt of the sulphonic acid was precipitated by adding large quantities of potassium acetate. It was found more convenient to use directly potassium salts as starting materials, taking 1 mole (222 g) potassium pyrosulphite (corresponding to 2 moles potassium hydrosulphite) and 1 mole potassium nitrite. The yield of potassium hydroxylamine disulphonate was 240 g or 89 %. This compound was used for the preparation of O-methyl hydroxylammonium chloride by methylation followed by hydrolysis according to Traube [11]. The yield was recrystallized from ethanol and had m.p. 151° C (lit. 151° C [11]). Finally methoxyurea was obtained by a process analogous to that leading to hydroxyurea

$$CH_3ONH_3Cl + KOCN \rightarrow O=C\begin{matrix}NHOCH_3\\NH_2\end{matrix}$$

By following the experimental conditions of Traube, who first prepared this substance, methoxyurea was obtained in good yield, m.p. 84° C, both from absolute ethanol and from benzene. Contrary to the hydroxyureas, methoxyurea is quite stable at the melting point.

A particular preparation of methoxyurea was carried out, using the "mild" conditions of reaction and isolation which were found to favour the yield of the labile, lower melting hydroxyurea. The product was carefully investigated for possible occurrence of isomers, but only one chemical individual could be isolated.

The authors are indebted to Mrs. B. Jerslev for discussion. Thanks are due to A/S Grindstedværket and A/S Dansk Gærings Industri for grants enabling one of us (T-Y. H.) to participate in the work. Analyses by Mr. W. Egger, Chemical Laboratory, University of Copenhagen.

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On the Isomerism of Hydroxyurea

IV. Acid-base Properties of the Isomers

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It is demonstrated by potentiometric titrations in aqueous solution that the higher melting hydroxyurea exhibits weak acidic properties, $pK_a = 10.6$. The lower melting isomer is a weak base; the conjugate acid has $pK_a = 2.3$. The monobasic properties have been confirmed by titration with perchloric acid in glacial acetic acid. In this medium the complete titration curve was accessible. The weak acidic properties of the higher melting hydroxyurea were also observed in a titration with sodium methoxide in pyridine, although a reproducible titration curve could not be obtained. Comparative studies of methoxyurea revealed that this compound is a weak monoprotic acid; it could be titrated with fair accuracy with sodium methoxide in pyridine.

Previous information on the protolytic properties of the alleged isomers of hydroxyurea has been of a purely qualitative nature. According to Dresler and Stein [1] an aqueous solution of the higher melting hydroxyurea is neutral to litmus paper. The substance has, however, weak acidic properties. Hodges [2] succeeded in preparing hygroscopic metal salts of the type $CH_3O_2N_2K$, $CH_4O_2N_2$, analogous to those formed by hydroxamic acids [3].

The lower melting isomer is reported to be a weak base. Francesconi and Parrozzani [4] have prepared a relatively stable mono-hydrochloride. Their attempts to prepare metal salts of the lower melting isomer, on the other hand, were unsuccessful. Potassium oxyfulminate was obtained instead of the anticipated salt, when hydroxyurea was treated with potassium ethoxide in absolute ethanol.

It was desirable to produce some quantitative evidence for the difference in acid-base properties of the two hydroxyureas since a confirmation of this point would strongly support an explanation on grounds of structural isomerism. For this purpose potentiometric titrations have been carried out in aqueous and non-aqueous media.

RESULTS

Titrations in aqueous solution

Fig. 1a illustrates a titration of the higher melting isomer with hydrochloric acid. The original, freshly prepared solution was neutral, its initial pH being approximately 6.5. The titration curve a is identical with the medium curve c showing that the substance has no detectible basic properties. Here and in the following the term "identical" implies that coincidence can be brought about by a simple translation along the ordinate (ml — axis). In order to find out whether acid-catalyzed rearrangements or decomposition took place the solution was retitrated with sodium hydroxide. The acidic branch of the curve (Fig. 1b) is identical with a. Since the above processes would probably influence the acid-base properties of the system, it may be concluded that they are absent or very slow. In the basic region there is a distinct buffer effect, confirming the weak acidic properties of the higher melting isomer. For the same reasons as above the reversibility was investigated, Fig. 2. To an aqueous solution of the higher melting hydroxyurea was added alkali to pH 10.6 and it was retitrated with acid. The titration curve, Fig. 2a, is identical with Fig. 1b on the alkaline side, but shows a slightly larger acid consumption at

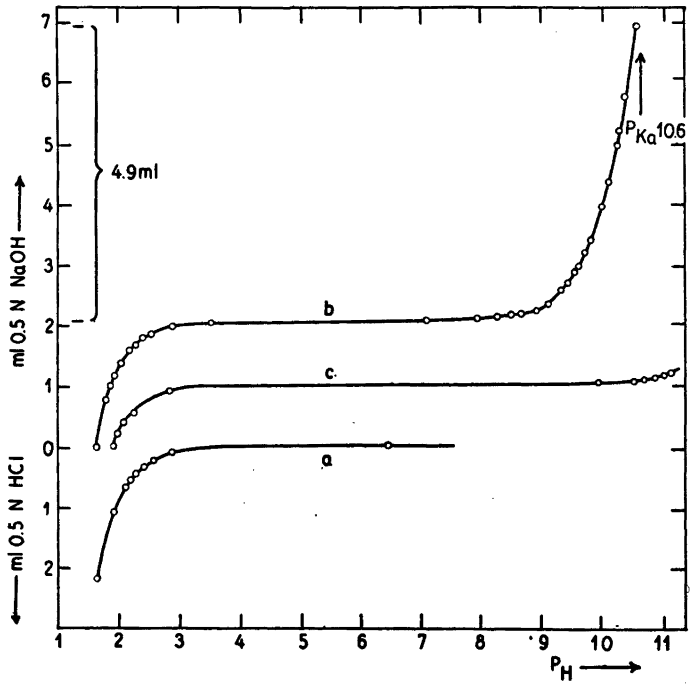

Fig. 1. a. Titration of 4.92 millimoles higher melting hydroxyurea in 40 ml water with 0.5 N hydrochloric acid. b. Retitration with alkali; c. Blank, 40 ml water, added 1 ml 0.5 N hydrochloric acid and titrated.

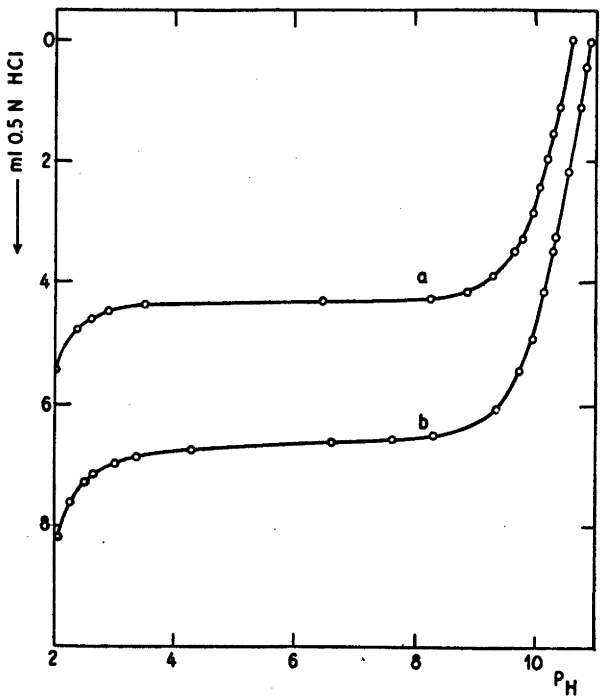

Fig. 2. a. To 4.92 millimoles higher melting hydroxyurea dissolved in 40 ml water was added alkali to pH 10.6 and the solution was titrated with acid; b. Same experiment but the alkalized solution was stored 4 ½ h before retitration.

pH 4—2. This phenomenon is more pronounced in Fig. 2b, in which the same experiment was carried out, except that the alkalized solution was stored for 4.5 h at room temperature before retitration. It may be explained by a base-catalyzed rearrangement to the lower melting hydroxyurea, which has basic properties (*vide infra*), or by a progressive decomposition to *e.g.* ammonia.

The lower melting hydroxyurea was subjected to a similar systematic investigation. An aqueous solution was titrated with hydrochloric acid, Fig. 3 a, and then retitrated with sodium hydroxide, b. The two curves are identical and consequently no irreversible processes seem to occur by change of reaction to the acidic side within the time of observation, *ca.* 45 min. The lower melting hydroxyurea has weak basic properties, as shown by the buffer effect at pH 2—4, *cf.* the medium curve c. There is also a slight buffer effect on the basic side pH 8—10. This does not necessarily mean that the lower melting isomer has acidic properties. It is known that the substance, when heated in alcoholic solution, is partially rearranged to the higher melting hydroxyurea, which has acidic properties *(vide supra)*, and partially decomposed. Similar reactions may proceed slowly in aqueous solution at room temperature, possibly catalyzed by acid or base.

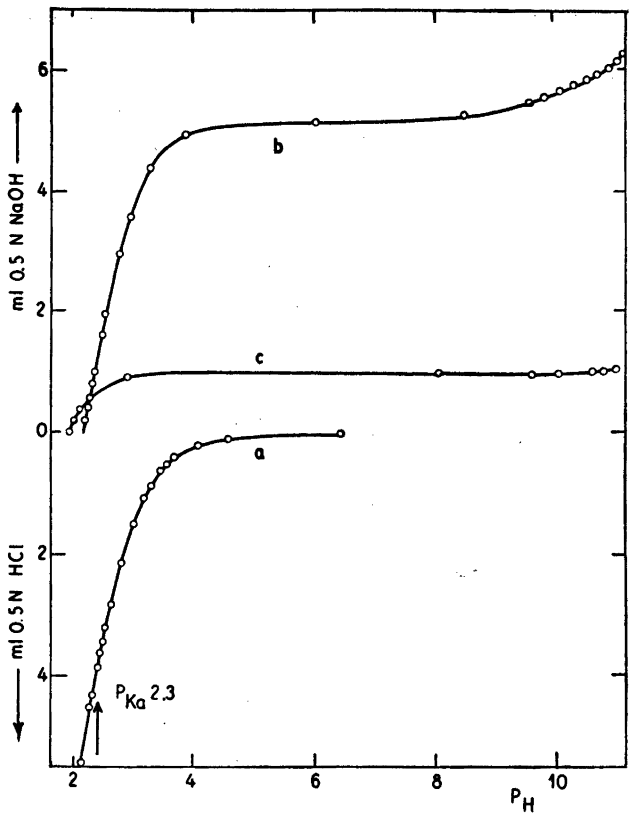

Fig. 3. a. Titration of 4.20 millimoles lower melting hydroxyurea in 40 ml water with 0.5 N hydrochloric acid; b. retitration with alkali; c. Blank; to 40 ml water was added 1 ml 0.5 N hydrochloric acid and the solution titrated.

For the final general discussion of the structural problem the essential result of the above experiments is that the higher melting hydroxyurea is a weak acid and the lower melting one is a weak base. The dissociation constants are so small that a complete titration to the equivalence point is impossible in aqueous medium and the method is inapplicable as an analytical tool. This is in accordance with conductivity measurements reported previously [5]. A rough estimation of the dissociation constants may, however, be attempted. Let it be assumed that both isomers require 1 equivalent titrant per mole for complete "neutralization". The quantity of the higher melting isomer titrated in Fig. 1 represents 4.92 millimoles, which would require 4.92 ml 0.5 *N* sodium hydroxide for "half neutralization". The corresponding pH read on the curve, Fig. 1b, is *ca.* 10.6. The approximate dissociation constant for the higher melting isomer is consequently $K_a = 10^{-10.6}$. In the titration given in Fig. 3 4.20 millimoles of the lower melting isomer was taken. On the assumption

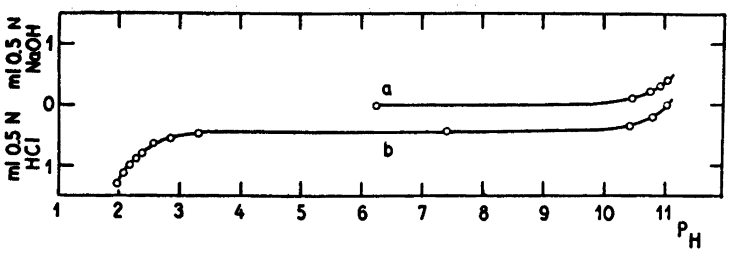

Fig. 4. a. Titration with alkali of 4 millimoles methoxyurea in 40 ml water; b. retitration with acid.

that it is monoprotic, this quantity requires 4.20 ml 0.5 *N* hydrochloric acid for "half neutralization". The corresponding pH is 2.3. Thus $K_a = 10^{-2.3}$ is a probable dissociation constant of the hydroxyuronium ion formed by the lower melting isomer.

As an aid in a structural interpretation of the observed protolytic properties of the hydroxyureas the related substances methoxyurea and urea have been titrated with the same technique. Experimental points are given in Figs. 4—5, and for convenient comparison all the characteristic titration curves, normalized to 4 millimoles substance, have been plotted in Fig. 6, including the blank curve for the solvent (aq). The urea curve (u) is identical with that of the solvent, whereas methoxyurea (m) shows a slight buffer effect at pH 10—11, and should therefore be a very weak acid, much weaker than the higher melting hydroxyurea (h). The weak acidic properties of methoxyurea have been confirmed by titration in non-aqueous medium, *vide infra*. From the results summarized in Fig. 6 it appears that the acid-base properties of the hydroxyureas cannot be attributed to the urea torso, but must be characteristic of the hydroxylamine part of the molecule or at least induced by it. A normal hydroxylamine structure with a free hydroxy group would explain the weak acidic properties of the higher melting isomer, whereas an amine oxide structure would account for the basic properties of the lower melting hydroxyurea. The existence of only one methoxyurea and the much weaker protolytic properties of that compound fit well into the picture. There are, however, other possibilities, and the whole structural problem including the localization of the acid-base properties will be discussed in a larger context in a final paper.

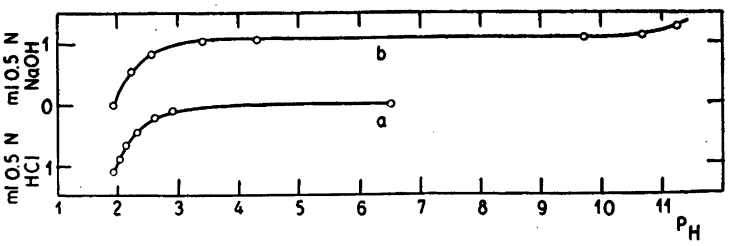

Fig. 5. a. Titration with acid of 4 millimoles urea in 40 ml water; b. retitration with alkali.

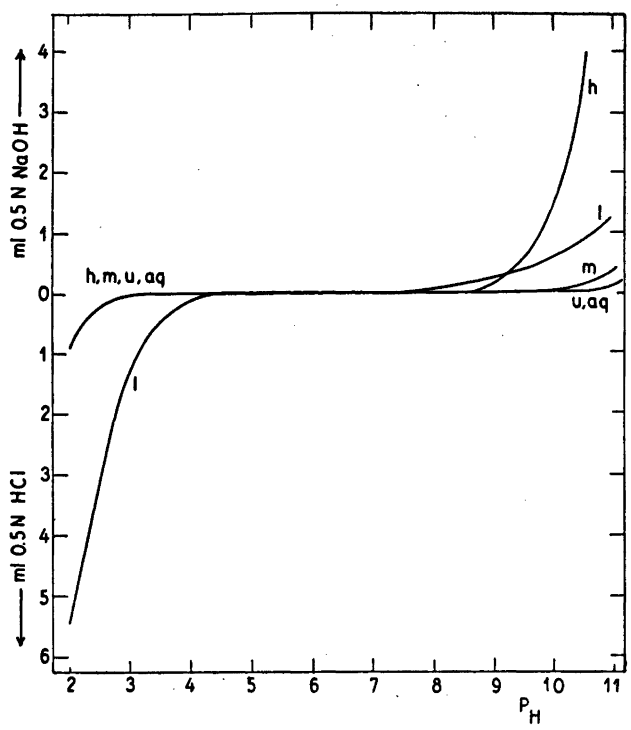

Fig. 6. Titration curves fig. 1—5 converted to 4 millimoles substance.
h. higher melting hydroxyurea l. lower melting hydroxyurea.
u. urea m. methoxyurea aq. solvent blank (water).

Titrations in non-aqueous media

Acid-base titrations in various non-aqueous solvents have in recent years attracted considerable interest because in this way the simple, visual or potentiometric titration technique can often be extended to acids and bases, which are too weak to be titrated in water. Thus in glacial acetic acid many weak bases will be sufficiently strong to permit titration with perchloric acid. A review of this method has recently been given by Riddick [6]. Analogously weak acids may often be titrated in basic media such as pyridine and various aliphatic amines with sodium methoxide as a titrant. For a review of this method see ref. 7.

As discovered in the first part of this paper the hydroxyureas are typical examples of a weak acid and a weak base respectively, the equivalence points of which are beyond reach in aqueous solution. It was, therefore, interesting to try whether more precise and complete information could be obtained in non-aqueous media.

It was found that the lower melting hydroxyurea was soluble and could be readily titrated potentiometrically in glacial acetic acid, using perchloric acid

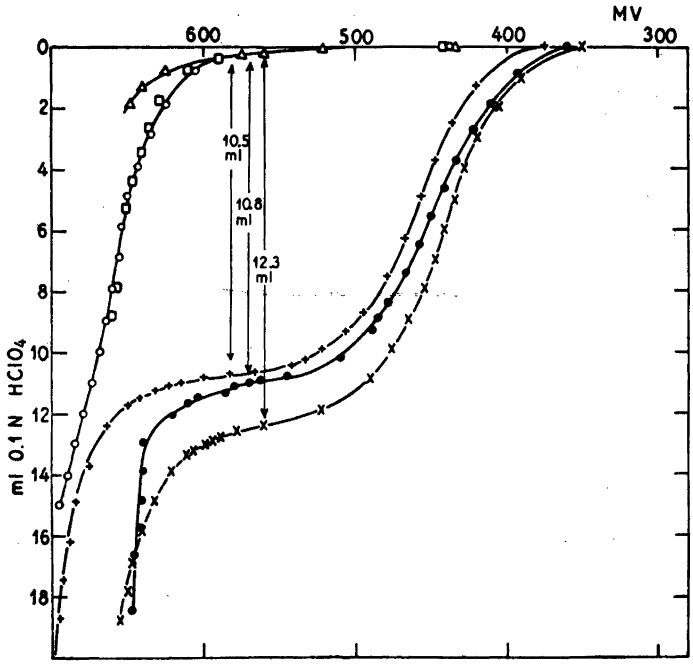

Fig. 7. Titrations with 0.1 N perchloric acid in glacial acetic acid; 0.100 g substance, 40 ml solvent.

× *lower melting hydroxyurea, immediately after isolation.*
● *same sample after 1 week storage at −10° C.*
\+ *same sample after a total of 2 weeks storage at −10° C and 48 h at 23° C.*
○ *higher melting hydroxyurea, immediately after isolation.*
□ *methoxyurea.*
△ *solvent blank.*

in the same solvent as a titrant. A typical titration curve is shown in Fig. 7, notation ×. The first part of the curve, at approximately 350 to 450 MV, corresponds to the incomplete titration curve, Fig. 3 a, obtainable in aqueous medium. In glacial acetic acid the titration can be carried to an end, the lower melting isomer behaves as a monoprotic base. The experimental equivalent weight is 81.3 (1 gram comsumes 12.3 ml 0.1000 *N* perchloric acid). This is slightly more than the theoretical value, 76.0, but leaves little doubt that the substance is monoprotic, which was assumed in the estimation of the aqueous dissociation constants above. For a computation of dissociation constants from the acetic acid data it is necessary to work out a standard curve, relating potential in acetic acid to dissociation constant in water for the particular electrode system, such as carried out by Veibel *et al.*[8] This was not done in the present case.

The high experimental equivalent weight may be due to the lability of the substance (*cf.* ref. 9). This point was further investigated by following the variation of the equivalent weight with time. The solid was stored over

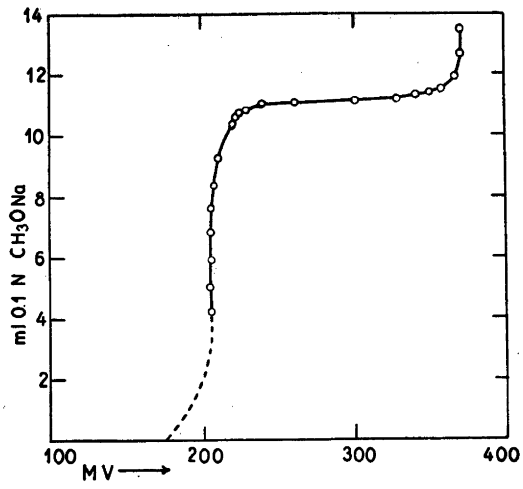

Fig. 8. Titration of 0.100 g methoxyurea in 50 ml "pyridine-bases" with 0.1 N sodium methoxide.

phosphorus oxide at —10° and at room temperature. Further details are given in Fig. 7. The equivalent weight increases with time even when the substance is stored at low temperature. After the last titration, the rest of the sample was stored at room temperature for 2 weeks; the decomposition was then obvious from its appearance.

The titration curves of the higher melting hydroxyurea (○) and of methoxyurea (□) in Fig. 7 seem to suggest that these compounds, besides being weakly acidic, have extremely weak basic properties, *cf.* the solvent blank (△).

The weak acidic properties of the higher melting hydroxyurea, observed in aqueous solution should be more pronounced in basic solvents. Potentiometric titration was attempted in "pyridine-bases", a commercial mixture of homologues successfully employed by Heiz [7] in this laboratory for the titration of barbituric acids. The titrant was sodium methoxide in benzene-methanol as suggested by Fritz and Lisicki [10], whose general procedure was also followed. A glass/antimony electrode combination gave poorly defined potentials. Every addition of titrant caused a change in voltage reading, but the pointer drifted slowly back again, without reaching an equilibrium position. By taking the maximum readings, however, a fairly reproducible titration curve could be obtained for the primary standard, benzoic acid, and for methoxyurea. The latter is given in Fig. 8. The equivalent weight 89.3 agrees satisfactorily with the theoretical value, 90.1. The acidic properties of this substance, faintly suggested in Fig. 6, m, have thus been confirmed.

The higher melting hydroxyurea could not be titrated reproducibly, but it was beyond doubt that it consumed the titrant in accordance with its acidity in aqueous solution, Fig. 6, h.

A calomel/antimony electrode combination suffered from the same instability as the above system. In fact the lack of adequate electrode systems is often a serious limitation to titrations in non-aqueous solvents. There is in this field a pronounced tendency toward an uncritical usage of conventional aqueous electrodes such as glass and calomel electrodes. It must be admitted,

that these arrangements sometimes serve analytical purposes quite well, but from a physico-chemical points of view it would be more satisfactory to set up specific electrode cells based on the relevant solvent and not on water. But the problem of electrode instability in non-aqueous solvents will perhaps eventually be most effectively overcome by the promising new technique of high-frequency titration, in which immersed electrodes are avoided altogether[11].

EXPERIMENTAL

Materials. The preparation of the *hydroxyureas* and of *methoxyurea* have been described in previous papers [9,14].

Glacial acetic acid. To an analytical grade product was added a calculated amount of acetic anhydride in order to reduce the water content to less than 0.2 %. The percentage was checked according to the method of Karl Fischer. Excess of acetic anhydride was carefully avoided, because it might acetylate the compounds to be titrated.

"Pyridine-bases". A commercial mixture of pyridine homologues was carefully dried over potassium hydroxide and distilled. The fraction distilling at 114—150° C was used.

Methanol and *benzene* were dried and distilled over sodium.

Titrations in aqueous solution were carried out in a nitrogen atmosphere in a closed titration vessel, using a glass electrode and a "saturated calomel" electrode. The potential was determined with a Radiometer tube potentiometer. A standard curve voltage *vs.* pH was worked out on the basis of conventional standard buffer solutions. The titration vessel was fitted with a magnetic stirrer.

Titrations in glacial acetic acid. Titration vessel and electrode combination as above. The titrant, 0.1 *N* perchloric acid, was prepared essentially as recommended by Markunas and Riddick [12]. The exact normality was determined against potassium biphthalate. All volume readings were corrected to 0.1000 *N* titrant, taking into account also possible deviation of titration temperature from that of the standardization (thermal expansion coefficient of glacial acetic acid 0.0011).

Titration in "pyridine-bases". Glass/antimony electrode combination. Titrations in a closed vessel with nitrogen blanketing and magnetic stirring. 0.1 *N* sodium methoxide in benzene-methanol was prepared as indicated by Vespe and Fritz [13]. The exact normality was determined against benzoic acid as a primary standard.

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A Study of the Phosphorus-Containing Proteins of Cells

I. The Isolation of Phosphoserine from the Liver Proteins of Calf and Rat

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Within recent years numerous studies have been made of the incorporation of P^{32} into the cells of various body organs (*e. g.*[1–3]). These studies have commonly employed either the method of Schmidt and Thannhauser [4] or that of Schneider [5] for the chemical fractionation of cells. Both methods have proved useful and have disclosed several interesting features of cell metabolism. One of these is the high activity of the "phosphoprotein phosphorus", a property which lends especial interest to this fraction. Nevertheless, before much more can be said about the metabolic significance of cellular phosphoproteins, it is necessary that their chemical nature be more closely defined. In this sense, the work of Hess, Yasnoff, and Lagg, who prepared a protein fraction from lymphocytes containing 0.65 % phosphorus [6], is a step in the right direction.

In this laboratory we have also been interested in the chemical characterisation of phosphoproteins, and have hitherto concentrated our efforts toward the development of methods for the isolation and purification of phosphorus-containing amino acids and peptides, using enzymatic and acid partial hydrolysates of bovine casein as model substances [7,8]. The present paper describes the extension of these methods to the partially hydrolysed Schneider "protein" fraction from calf and rat liver, whereby phosphoserine has been prepared in crystalline form, and several other phosphorus-containing fractions have been obtained.

METHODS

Calf liver * was obtained at the slaughter house and frozen in liquid air within 30—40 minutes after the killing of the animal. After thawing the liver was homogenized in ice-cold acetone in a Waring blendor and subsequently extracted for 20 minutes with cold 10 % TCA. Lipides were extracted with the same series of solvents used by Davidson [1], and nucleic acids were extracted according to Schneider [5] for 15 minutes at 90° C with

* The livers of pasture calves only, were used.

5 % TCA. The residue from this treatment was washed twice with warm 5 % TCA, then with alcohol-ether (3 : 1) and ether, and finally taken as the Schneider protein fraction.

In order to obtain phosphorus-containing amino acids and peptides the protein fraction was partially hydrolysed according to Lipmann [9] with 10 ml 2 *N* HCl per gram protein under reflux on a water bath for 20 hours. Subsequently, after most of the HCl had been removed by repeated evaporation *in vacuo*, the hydrolysate was run through a short column of charcoal, similar to those described previously [8] except that it had been washed with 1 *N* HCl before use. The charcoal removed most of the humin which had been formed during hydrolysis, without adsorbing small phosphorus-containing peptides and amino acids. Thus, the latter were readily washed from the column with water. Finally, the wash water was concentrated to a few ml, applied to a 120 × 4 cm column of Dowex 50 (8 % cross linking, 250—500 mesh), and the phosphorus-containing compounds were eluted with 0.01 *N* HCl.

The fractionation was followed with the aid of an automatic conductivity recording device [10], and the fractions obtained were further studied using two dimensional paper chromatography. The solvent systems found to be the most suitable were *iso*butyric acid-ammonia [11] and benzene-propionic acid [8]. The paper used was Munktell No. OB; and separation was indicated using the ninhydrin and phosphate spot test reagents as in a previous paper [8]. These two reagents could be applied successively to the same chromatogram, and in this way not only ninhydrin positive, but also phosphorus-containing compounds could be detected.

Crystallizing fractions were studied using powder and single crystal diagrams.

Rat livers were obtained from 6 adult male animals of the Wistar strain from the departmental colony. The rats were injected subcutaneously between the shoulder blades with 1 μc P^{32}-phosphate (carrier free) per g body weight. 4 hours after injection the animals were killed by cervical dislocation and the livers were rapidly removed and frozen in liquid air. After pulverizing the livers in a mortar with dry ice, they were extracted and washed with 10 % TCA and then subjected to the same extraction and hydrolysis procedures described for the preparation of the calf liver protein hydrolysate. After hydrolysis and removal of the HCl, the concentrated hydrolysate was applied directly to a 6 × 40 cm Dowex 50 column and eluted with 0.01 *N* HCl.

Fractionation was followed this time with a device for the continuous registration of radioactivity of the effluent [12]. For a more sensitive indication of the fractionation, 1 ml aliquots from each of the test tubes in the fraction collector were evaporated on aluminum discs, and the number of impulses per minute counted with the aid of Tracerlab's SC-18 Superscaler. For further study of the fractions two dimensional paper chromatography again proved useful. Moreover, the P^{32}-containing spots could be indicated by autoradiography.

The phosphorus of the various fractions was determined as in a previous paper [8].

RESULTS

Calf liver. Fig. 1 is a photograph of the essential sections of the conductivity curve obtained when the calf liver protein hydrolysate was eluted from a Dowex 50 column, compared with a similar curve of a casein hydrolysate. Most of the phosphoserine of the latter had previously been removed in order to facilitate recording of the peaks due to other compounds. The peaks corresponding to inorganic phosphate have been omitted from both curves, so that the first peak shown in each case is that corresponding to a mixture of pyroglutamic acid (as indicated by a crystal diagram) and several ninhydrin-positive substances. The second peak of the liver curve has no obvious counterpart on the casein hydrolysate curve. A two dimensional chromatogram of this material has shown the presence of a substance which contains phosphorus as well as several which react with ninhydrin. On the other hand the third peak of the liver hydrolysate may possibly be similar to the second peak of the

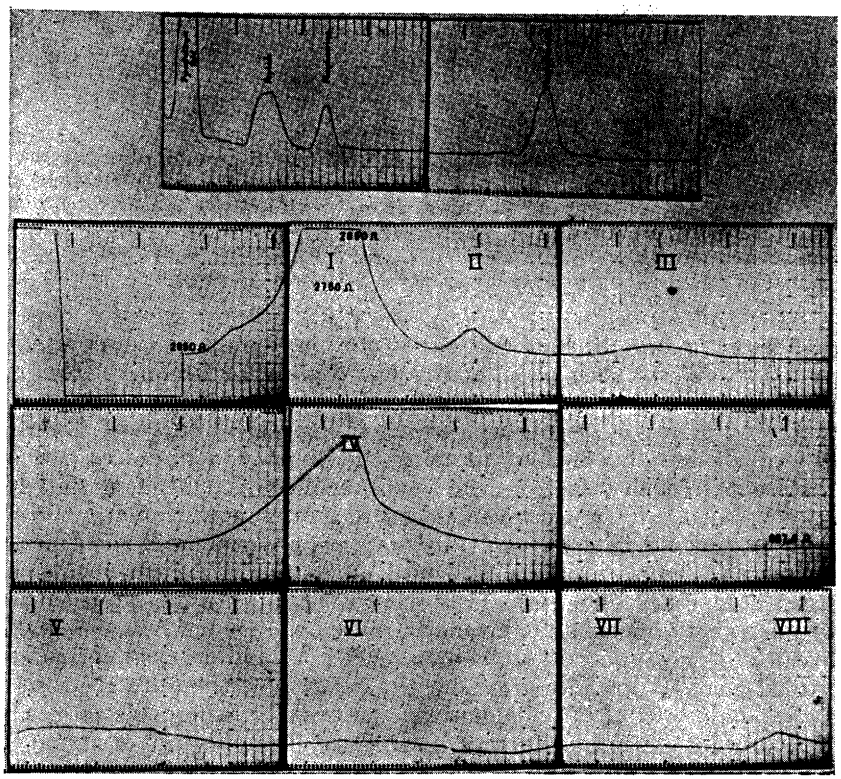

Fig. 1. Conductivity curve from a casein hydrolysate (above), compared with a similar curve from the hydrolysate from the calf liver protein fraction.

casein hydrolysate, which has been found to contain peptides containing phosphoserine and glutamic acid. From peaks IV and III of the liver and casein hydrolysate, respectively, phosphoserine can be obtained in crystalline form. It must be observed that the mother liquor in both instances contains several ninhydrin positive substances. Fractions V and VI from liver contain both ninhydrin positive substances and substances which give a positive phosphate reaction. Fraction VII probably contains phosphothreonine (for reasons to be discussed later with the results from the rat liver protein hydrolysate). Fraction VIII contains several ninhydrin positive substances, two of which give a positive phosphate reaction. One of the latter appears to be similar to the phosphorus-containing spot of fraction VII. Fraction VII and VIII thus probably correspond to fraction IV of the casein hydrolysate.

Fig. 2 is a photograph of the left half of a rotating crystal diagram of the crystals from fraction IV of the calf liver hydrolysate juxtaposed, to facilitate comparison, with the right half of a similar diagram of phosphoserine crystals from casein. The identity of the two crystal preparations is evident.

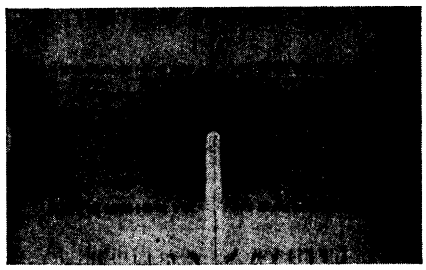

Fig. 2. Rotating crystal photographs of phosphoserine from casein (right) and crystals from peak IV (left). (b-axis rotation, taken with a Weissenberg-goniometer equipped with a cylinder camera, diameter 57.3 mm.)

Fig. 3 shows a one-dimensional paper chromatogram of the crystals from fraction IV compared with phosphoserine and phosphothreonine from casein, using *iso*butyric acid-ammonia as solvent.

Rat liver. Fig. 4 is a photograph of the radioactivity curve of the rat liver protein hydrolysate obtained by plotting the number of impulses per minute in 1 ml aliquots of each tube from the fraction collector. Only four well defined peaks can be seen, the first corresponding to inorganic phosphate, the second to peptide-bound phosphorus, the third to phosphoserine, and the fourth very possibly to phosphothreonine plus small quantities of another phosphorus-containing compound. Preliminary work with the P^{32}-containing substances eluted from a two dimensional chromatogram of the second peak has indicated the presence of glutamic acid as well as serine. When material corresponding to the third peak is chromatographed, the position of the only P^{32}-containing spot corresponds to that of phosphoserine. Moreover, when this spot is eluted from the chromatogram and further hydrolysed, the presence of serine can be shown by conventional amino acid chromatography [8]. The grounds for supposing that the major phosphorus-containing component of the fourth peak is



Fig. 3. One dimensional chromatogram of crystals from peak IV, developed with isobutyric acid-ammonia. For purposes of comparison, phosphoserine (right) and phosphothreonine (left) from casein have been run on the same chromatogram.

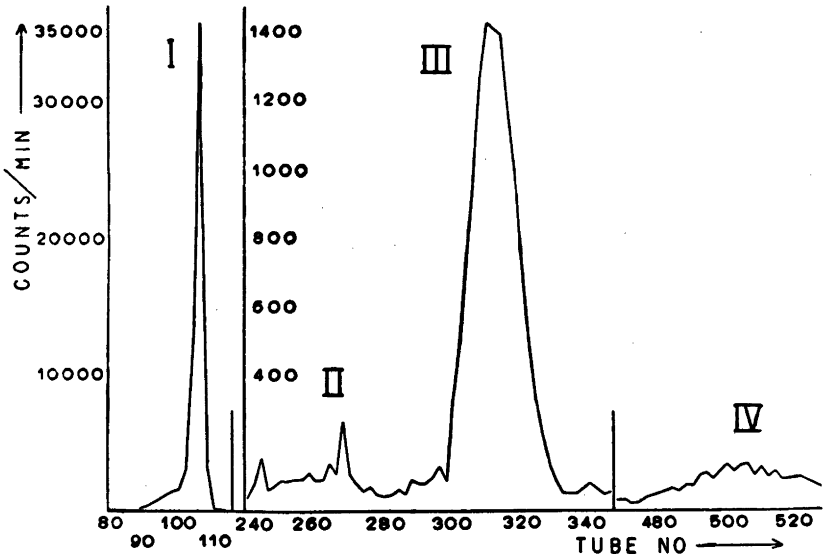

Fig. 4. Radioactivity curve from the hydrolysate of the rat liver protein fraction.

actually phosphothreonine are the following. The fraction is eluted from the Dowex 50 column at about the same position as phosphothreonine. Secondly, the principal phosphorus-containing spot of a two dimensional chromatogram of this fraction also corresponds to the position of phosphothreonine. Fig. 5 is a photograph of a chromatogram of material from the fourth peak which has been sprayed with ninhydrin. In the same figure, for comparison, is an autoradiogram of the same chromatogram.

The test tubes corresponding to the inorganic phosphate and phosphoserine peaks, respectively, were combined and the radioactivity evaluated as the number of counts/min./100 μg P. Whereas the total amount of phosphorus in the fractions was 13.9 mg P and 0.42 mg P, respectively, the specific activities of the two fractions were 9 700 and 52 400 counts/min./100 μg P, respectively. Thus, while the total amount of phosphoserine phosphorus was only

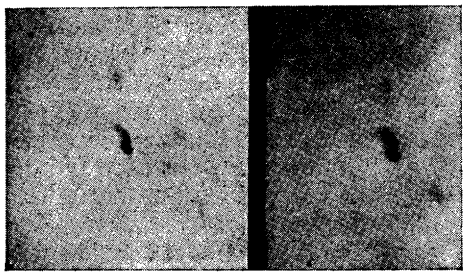

Fig. 5. Two dimensional chromatogram of material from the fourth peak of the rat liver protein hydrolysate, developed with benzene-propionic acid, and isobutyric acid-ammonia (right). To the left in the figure is an autoradiogram of the same chromatogram. (Gevaert Curix X-ray film, 10 day exposure).

1/33rd as great as the total amount of inorganic phosphorus, the specific activity of the phosphoserine phosphorus was 5.4 times greater than that of the inorganic phosphorus.

DISCUSSION

Davidson and co-workers [1,13,14] have amply demonstrated the need for a more rigorous definition of the "RNA" phosphorus obtained by the method of Schmidt and Thannhauser. That the "phosphoprotein" phosphorus of this method, as well as that obtained by the Schneider method, is also in need of a closer characterisation is evident from the varying and divergent results obtained by the several investigators who have used the two procedures [1–3,15,16]. While the Schneider method has consistantly yielded higher values for the total amount of phosphorus, the method of Schmidt and Thannhauser has yielded the higher specific activity values. At the same time in neither of the two methods has it been shown that the phosphorus measured is actually phosphoprotein phosphorus.

To date protein-bound phosphorus is generally thought of in terms of phosphoserine or phosphothreonine [7]. Therefore, until other types of phosphorus-protein linkages are demonstrated, the present method for the isolation of phosphoserine (and possibly phosphothreonine) from cell protein preparations would seem to be of value, especially in studies of protein phosphorus metabolism. Something should be said from the outset, however, about the scope and limitations of the method.

The method is based on the work of Lipmann [9], who isolated phosphoserine from a partial acid hydrolysate of casein. During the course of the hydrolysis, however, a good deal of the phosphoserine itself is hydrolyzed, presumably along with other, more acid labile phosphorus compounds. This constitutes the major limitation of the method.

Another point which should be mentioned is that a part of the phosphoserine isolated from the "Schneider protein residue" may possibly have originated from serine phospholipides bound to the protein residue. That this possibility should not be overlooked is suggested by the experiments of Folch [17], who has shown that some inositol phosphatides are not extracted by the usual lipide solvents. Although Folch has also shown that these phosphatides are split off from the protein residue by treatment with hot TCA, it may be that some additional phosphatides — and among them serine phosphatides — still remain bound to the protein. Should this be the case it is possible that some phosphoserine might be liberated on acid hydrolysis, although this is rendered improbable by the earlier observations of Folch [18] that phosphoglycerine and not phosphoserine is the major product of the partial acid hydrolysis of serine phosphatides.

Preliminary experiments where the activity of the phosphoserine phosphorus of rat liver protein was determined in animals killed 1/2, 4, and 24 hours after injection of radioactive inorganic phosphate indicate that the present method is of value in spite of the limitations noted above. In these experiments the phosphorus bound to serine has exhibited a higher turnover rate than the inorganic phosphorus liberated by the acid hydrolysis. In this respect

at least it would appear to resemble the Schmidt and Thannhauser protein phosphorus. It is expected that enzymatic and milder acid hydrolysis techniques will make it possible to find out more about the source of the inorganic phosphorus.

Other experiments now in progress have demonstrated the presence of phosphoserine in the proteins of all the cell fractions obtained by the Schneider and Hogeboom techniques [19,20].

SUMMARY

A method has been described for the isolation of phosphoserine and some other phosphorus-containing compounds from a partial hydrolysate of the Schneider protein residue of cells.

With the aid of this method crystalline phosphoserine has been obtained from a partial hydrolysate of calf liver protein.

This investigation was supported by a grant from the *Swedish Medical Research Council*. The technical assistance of Mr. T. Fransson and Mr. T. Persson is gratefully acknowledged.

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Electrophoretic Studies of Serum Lipoproteins

I. A Description of Apparatus and Technique for their Separation in Starch Medium

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In the study of serum lipoproteins both physical methods, such as ultracentrifugal and electrophoretical investigations, and pure chemical methods, have been applied. Serological methods have also been used.

The electrophoretical approach was chosen because of the promising results of investigations in free electrophoresis. At the time when these investigations were started (1951) the following ways were open to the study of serum lipoproteins in free electrophoresis: A) Coloring the lipids by some fat soluble dye and observing the position of the color during and after the electrophoretic separation (Bennhold [1]). B) Isolating the electrophoretically separated components and analysing the fractions for various fats (Mellander [2], Blix *et al.*[3]). C) Delipidation of serum by fat extraction without protein denaturation (Blix [4], Kunkel and Ahrens [5]), or by high speed centrifugation (Longsworth *et al.*[6]), prior to the electrophoresis. The resulting decrease of some of the peaks in the electrophoretic diagram is then observed.

Since that time electrophoresis in supporting medium has been greatly developed and won widespread application, *i. a.* in the field of lipoprotein research. All the above-mentioned methods *i. e.* coloring by fat soluble dyes (Fasioli 1952 [8], Kunkel and Slater 1952 [9], Durrum *et al.* 1952 [7], Rosenberg 1952 [10], Swahn 1952 [11]), extracting and analysing the separated components (Kunkel and Slater [9]) and delipidation by centrifugation prior to the electrophoresis (Swahn 1953 [12]) have been applied. Paper, being very easy to handle, has been the most commonly used supporting medium. Due to considerable adsorbtion on to the paper of β-lipoprotein (Kunkel *et al.*[9]) and low density lipoproteins (chylomicrons) (Smith *et al.* 1953 [13], Swahn [12]) in particular, paper is unfortunately not an ideal supporting medium for the study of lipoproteins. The adsorption interferes of course with the resolution of the lipid "spectrum" and with the fat extraction out of the paper. To the author's knowledge no explanation of this adsorption has been found.

In spite of these objections lipoprotein investigations by means of paper electrophoresis have yielded and confirmed earlier (Russ, Barr, and Eder

1951 [14,15]) valuable clinical informations of the lipoprotein distribution in the normal state and in various diseases with disturbed lipoprotein metabolism. (Kunkel *et al.*[9], Malmros and Swahn 1953 [16], Nikkilä 1953 [17], Swahn [12].)

The discovery that serum lipoproteins were not being adsorbed on starch (Kunkel and Slater 1952 [9,18]) seemed to offer new possibilities for the application of electrophoresis in a supporting medium to the lipoprotein research.

The author's investigations of these possibilities started in the summer of 1953. From the beginning it was clear that a different performance of the method than that of Kunkel and Slater [9] was desirable, for two particular reasons: First, the cutting of the starch into segments after finished separation seemed difficult to perform exactly, and secondly it would be of great advantage if one could avoid extracting the proteins from the starch.

This problem was solved by carrying out the electrophoresis containing the starch in a vertical tube and *forcing the separated components out of the starch by simple buffer flow.*

In 1950, Haglund and Tiselius [19] gave a preliminary report of a similar device for zone electrophoresis in glass powder. However, these authors state: "... much more work is necessary to make the apparatus a good analytical instrument."

DESCRIPTION OF THE METHOD

Apparatus. Principally the apparatus consists of a plexiglas tube (*A* in Fig. 1) supported with a glass filter (*B*) (Jenaglass, porosity 40–90 μ) as bottom, upon which the starch column rests. It is surrounded by another plexiglas tube (*C*), serving as a cooling mantle (for further details, see Fig. 1). The top of the tube is fitted with a plate which can be screwed on to it (*D*). Centrally into this plate, a cooling glass tube (*E*) is hermetically inserted. This cooling tube is centered by a plexiglas cross (*F*), resting on the glass filter. The plate is further pierced by two holes (*G*), positions according to Fig. 1. These holes are devoted to the filling of the starch slurry, tamping and smoothing of the starch surface and the addition of buffer and protein solutions to the top of the starch (by means of a pipette). To connect the tube to the electrode vessel during electrophoresis, siphons are inserted into these two holes (Fig. 2). Finally the plate is attached to a pressure-device connection (*H*).

The filterplate is held in position by a screwed ring (*J*). For the collection of the fractions after separation, a special funnel (*K*) is screwed onto the filterholder.

For smaller tubes (up to 30 mm in diameter) the entire cooling system can be excluded. The apparatus then consists of a simple plexiglas tube supported with a glass filter as bottom as described above.

Packing of the tube. It is of utmost importance to pack the starch homogeneously throughout the tube to avoid the spreading and tailing of the zones. The packing method for starch columns described by Stein and Moore [20] has been found superior to all other methods tried. In this method, advantage is taken of the property of starch to absorb water while swelling. Potato starch can thus increase its volume by 55–60 % (from the anhydrous state to complete saturation). As recommended [20], a suspension is made of starch in butanol to which distilled water has been added to make the initial water content 30 % of the dry weight of the starch. An important point is to have all the water dissolved in the butanol before the starch is added, otherwise the starch granules will cluster.

A homogeneous suspension free of lumps is easily obtained by grinding the starch carefully with the butanol in a mortar.

Before filling the tube, the top plate with the cooling tube must be screwed on. A slightly bent glass tube is introduced into the central tube through one of the holes in the top plate. The slurry is then poured through this glass tube. In this way the slurry will flow along the walls of the plexiglas tube and incorporation of air bubbles is avoided.

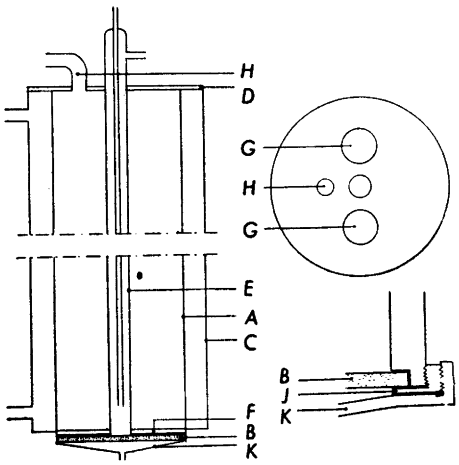

Fig. 1. The principle of the apparatus. In the right top corner the top plate is seen from above to show the position of the holes.

Thereafter the holes are corked with rubber stoppers and air pressure of 4—6 cm of mercury is applied. Now the starch settles slowly for about one hour (height of starch column 30 cm). The butanol above the starch surface is then carefully withdrawn by a pipette and water is poured in. Pressure is applied again (4—6—8 cm of mercury) and the water is driven into the starch. Water is now allowed to pass through the starch for about 48 hours. During this time the starch absorbs water and swells. As pointed out earlier this swelling is of greatest importance because it creates an evenly distributed internal pressure which increases the uniformity of the intergranular spaces. After equilibration with water the desired buffer solution is driven into the starch.

The electrophoresis. To start an experiment, the buffer solution above the starch is removed to within 1 cm above the starch surface. The remaining solution is driven down

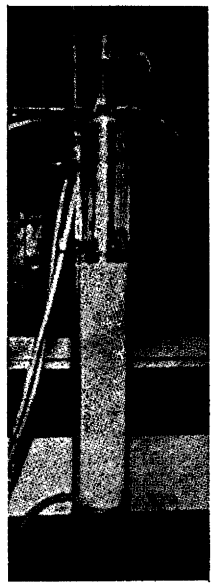

Fig. 2. Photograph of the apparatus.

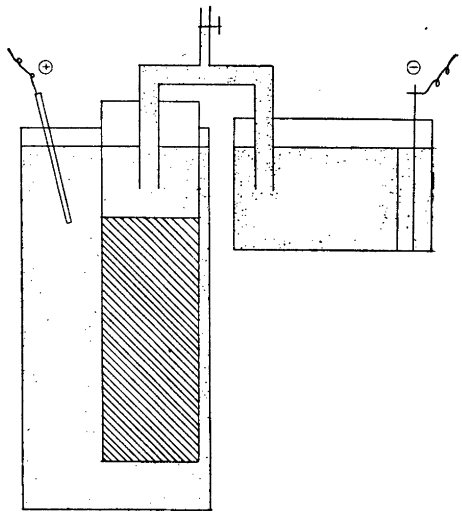

Fig. 3. Principle arrangements for the electrophoresis.

by pressure. Now the protein solution, being of the same temperature as the tube system, is added. It is driven into the starch column by pressure until the very moment when the starch surface is dry. Now just enough buffer to cover the surface is carefully added and pressed down. The tube is then filled up with buffer. The starting position of the protein solution must be chosen with reference not only to the mobilities of the protein components, but also to the electroosmosis. Otherwise one or more of the components may migrate out of the starch. When *e. g.* serum proteins are to be examined in an alkaline medium, the solution must be depressed approximately one third of the distance albumin is assumed to migrate. (These distances are measured on the outside of the column and are of course relative migration distances.) This is due to the fact that the electroosmosis is directed towards the cathode and thus counteracts the migration of the proteins.

When the solution has reached its starting position, the plexiglas tube is immersed in a vessel filled with buffer solution. Thereafter two siphons are inserted through the top plate to connect the buffer solution above the starch with an electrode vessel (see Fig. 2). As during electrophoresis considerable heat is generated in these siphons due to the high current density, they have been equipped with a cooling system by inserting a cooling glass tube through their horisontal part. (The cooled solution in the horisontal part will of course sink, and in this way an effective circulation is obtained inside the siphons.) The electrode vessel is principally constructed as described by Kunkel and Tiselius [21] and furnished with a lid, in which there are holes for the siphons.

The liquid menisces in the vessels are now adjusted to the same level (Fig. 3) so that no liquid streaming due to gravity is obtained.

An electrode is immersed in the external vessel. The electric current is now turned on and the electrophoresis started.

Fraction collection. After finished electrophoretic separation the special funnel is screwed on (with the tube still in the vessel to avoid air bubbles in the funnel). The tube is then taken out of the vessel, and a pressure of about 10 cm of mercury is applied. Fractions of constant volume are then collected, either by some volumetric device or by counting the drops. As yet this has been done manually, but an automatic fraction collector is under construction. It has been advisable to give the fractions the same volume as the applied protein solution had.

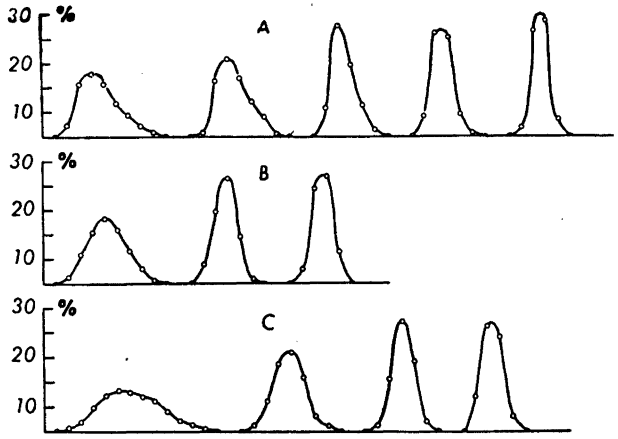

Fig. 4. Effluent diagrams from sera forced through a starch column. Each value represents one fraction (1 ml) and is expressed as percentage protein of the total amount of protein. Fort further explanations see text. A) Normal serum. The concentrations of the applied protein solutions were (from left to right): 7.4 %, 3.7 %, 2.5 %, 1.8 %, 1.2 %. B) Nephrotic serum. Protein concentrations: 4.3 %, 2.2 %, 1.5 %. C) Serum from a case of essential hyperlipemia. Protein concentrations: 7.8 %, 2.0 %, 1.3 %, 1.0 %.

Data for an apparatus suitable for about 4 ml of protein solution. For meaning of the letters see under the description of the apparatus.

A: Internal diameter 44 mm, external diameter 48 mm. Heigth 500 mm.
C: Internal diameter 64 mm, external diameter 70 mm. Heigth 490 mm.
E: External diameter 7 mm.
G: Diameter 14 mm.

Composition of the starch suspension to obtain a 30 cm high column: 300 g starch (calculated as anhydrous), 400 ml butanol and distilled water to make the water content 30 % of the dry weight of the starch.

EXPERIMENTAL

The protein analyses have been carried out either according to the modified biuret-reaction of Goa [22] adapted to the "ultra-micro-scale" or to the Folin copper method [23]. The cholesterol has been determined by the titrimetric method of Schmidt-Thomé [24], which has been slightly modified. The phospholipids have been determined as inorganic phosphate, the phosphorus analysed with ascorbic acid as reducing agent [25]. All analyses, except those concerning the phospholipids, have been carried out in duplicate.

First of all, the possibility of forcing serum through a starch column was investigated. This investigation was carried out in tubes with a diameter of 22 mm, the starch being packed as described above. Ammonium buffer of pH 9.0 and ionic strength 0.1 was used. In each experiment 1 ml protein solution (serum, undiluted or diluted with buffer) was forced through the starch column. 1 ml fractions were collected and analysed for protein. It is of utmost importance to keep the protein zone as narrow as possible when it is forced through the starch column. Otherwise a good electrophoretic separation can not be obtained. In order to avoid spreading the zone, the protein concentration must not be too high, as is evident from Fig. 4. For normal sera as well as for a nephrotic serum the best result is achieved with protein concentrations below 1.5—2.0 %. The greatest spreading of the zone showed the milky serum from a case of essential hyperlipemia. Here the spreading is most probably due to the presence of the large lipid particles, the chylomicrons. In none of these experiments was the recovery below 95 %.

The packing of the starch. Different kinds of potato starch have been tried. The starch manufactured by Morningstar Nicol, Inc., New York, has been found superior to all others. The following ways of packing the starch were tried: A) Suspensions of starch in butanol with the water content adjusted to 10, 20, 25, 30, 35 and 40 % of the dry weight of the starch, B) Suspensions of starch in water, C) Filling the tube with starch powder during simultaneous vibrating of the tube.

The homogeneity of packing was then judged by forcing protein solutions through the starch as described above. Of these methods, the best results were obtained with a suspension of starch in butanol with a water content of 30—35 %. With this degree of packing, the starch occupies about 2/3 of the volume of the column, *i. e.* the "free space" of the starch column is 35 % of the volume.

Possible influence of increasing salt concentrations upon the spreading of the protein zone. The effect of increasing salt concentrations was investigated in ammonium buffers with pH 9.0. No influence was observed, on the spreading of the zones or on the recovery of the proteins, by varying the ionic strength from 0.05 to 0.45.

Influence of pH and of various buffer-systems upon the spreading of the protein zone. Varying the pH from 8.0 to 9.5 of the ammonium buffers did not influence the spreading or recovery of the protein zone. Results obtained with veronal buffer (pH 8.6, ionic strength 0.1) and with Michaelis' veronal-acetat buffer (pH 2.0, ionic strength 0.1) were identical with those obtained with ammonium buffers. On the other side, experiments with phosphate buffers showed unsatisfactory results with considerable spreading of the zone.

Influence of adjusting the salt concentration of the protein solution. Dialysis of the protein solutions against the buffer to be used before they were forced through the starch column, did not show any advantage.

Influence of coloring the proteins with bromophenolblue. It is a great advantage if the proteins are visible, making it possible to follow their migration. Bromophenolblue was added to serum, which was forced through the starch column. The dye itself is adsorbed on to the starch, but when it is added to serum, it is bound to the albumin and not then adsorbed. No influence was observed on the behaviour of the zone. (Because of its adsorption on to the starch the dye must not be added in excess of the serum).

The behaviour of serum lipoproteins. The fractions collected after forcing serum through a starch column were analysed for cholesterol and phospholipids. 0.70 ml of each fraction was extracted with 20 ml boiling alcohol-ether (3 : 1). The extracts were filtered, evaporated and transferred to 10 ml volumetric flasks, and made up to volume with alcohol. Of these extracts 3 ml were evaporated to dryness and extracted with petroleum-ether. The remaining alcohol extract is analysed for phospholipids. The petroleum-ether extracts were transferred to 5 ml volumetric flasks and made up to volume. Aliquots are then taken of these extracts and analysed for cholesterol.

In this way normal sera as well as pathologic sera (nephrosis, diabetes, essential hyperlipemia) have been investigated.

The recovery of the lipids have in all cases been 105—95 %. Furthermore *the percental concentration of protein, cholesterol and phospholipids in each fraction has been almost identical.*

Extraction of lipids from the collected fractions after electrophoretic separation. From the preceding results it is evident that the protein concentration during electrophoresis should be 2 % or lower. Otherwise, due to spreading and overlapping of the zones, no good separation can be obtained. If serum has a protein concentration of between 6 and 8 % it is diluted with three parts of buffer, as a matter of course.

For the study of the lipid distribution of normal sera 1 ml is needed. This gives a volume of 4 ml after dilution with buffer. For this amount a starch column with a diameter of about 45 mm is convenient. The protein zone then measures about 8 mm initially.

Prior to the electrophoresis a few grains of bromophenolblue are added to the serum. This has two advantages: First, it is possible to follow the migration of the fastest moving component, the albumin. Secondly, if the amount of bromophenolblue is determined colorimetrically in the collected fractions after the separation, a differentiation of the albumin and the α_1-globulin-fraction is achieved, if these are not separated.

After the electrophoresis 1 ml serum is distributed on about 35 fractions of 4 ml each, *i. e.* 140 ml totally. Due to this dilution it is technically impossible to extract the lipids

Table 1. Extraction of cholesterol and phospholipids from serum diluted with ammonium buffer. Final volume 3 ml.

Amount of serum per 3 ml final volume ml	Cholesterol calculated γ	Cholesterol found γ	Phospholipids calculated as γ P	Phospholipids found as γ P
		24.6		9.9
		23.8		9.9
		24.6	10.0	9.0
0.20	24.6	24.8		9.5
		12.3		4.8
		12.4		4.9
0.10	12.3	12.8	5.0	4.9
		12.2		5.1
		6.3		2.4
		6.2		2.7
0.05	6.15	6.4	2.5	2.5
		6.2		2.6
		1.4		0.5
		1.5		0.5
0.01	1.2	1.2	0.5	0.6
		1.3		0.6

with alcohol-ether without simultaneous extraction of the buffer salts. Unfortunately these buffer salts interfere in many ways during the lipid analyses.

The problem to get a lipid extract without buffer salts was solved by precipitation of the proteins with trichloroacetic acid (TCA). The precipitate is heated for half a minute on a boiling waterbath and subsequently filtered through filter paper. The filter paper with the precipitate is then extracted with 20 ml boiling alcohol-ether (3 : 1). Before this extraction, however, the TCA must be removed from the filter paper. This is obtained by rinsing twice with distilled water. If the TCA is not removed, substances interfering with the subsequent lipid analyses will be extracted out of the paper. (Pretreatment of the filter paper with alcohol-ether or 1 % TCA in alcohol-ether was not successful.)

To control the accuracy of this method, dilutions of serum were made, all having a final volume of 3 ml. After precipitation of the proteins with 1 ml 10 per cent TCA, cholesterol and phospholipids were determined as described above. As is evident from Table 1, the method gives very satisfactory results.

If bromophenolblue has been added to serum, the precipitate has a yellow color. This color still persists after the first rinsing with distilled water, but during the second rinsing the color changes to blue, indicating that the TCA has been removed.

The protein determination. The advantage of the Folin copper method is its high sensitivity. In a reaction volume of about 4.5 ml 10—700 micrograms of protein can be determined. This dilution makes it possible to use the method in case of highly turbid sera. The great disadvantage is that the amount of color is not related to the amount of nitrogen and on that account varies with different proteins. Furthermore the color reaction does not show agreement with Beer's law. (This is of course easily overcome by means of standard curves.)

The modification of the biuret reaction according to Goa adapted to the "ultra microscale" has the advantages of being directly related to the peptid linkage and of following Beer's law. Unfortunately the sensitivity of this method is only about one tenth of that of the Folin copper method, *i. e.* in a reaction volume of 0.5 ml (10)—20—400 micrograms of protein can be determined. This makes the method more liable to disturbances by turbidity.

In Fig. 5 and Table 2 data are presented from an electrophoretic experiment (normal serum, ammonium buffer of pH 9.0 and ionic strength 0.1), where the fractions have been analysed by both these methods.

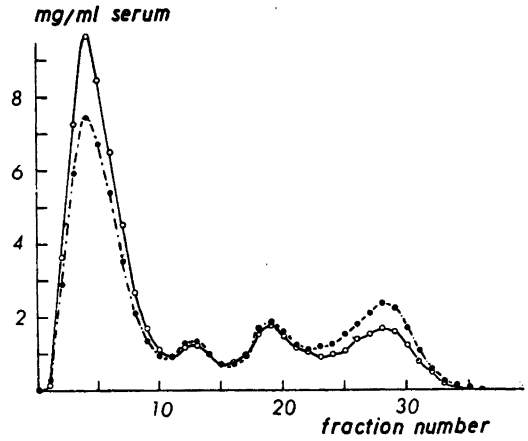

Fig. 5. Electrophoresis of normal serum in ammonium buffer of pH 9.0 and ionic strength 0.1. O—O *protein determined according to the modified biuret reaction.* ●—● *protein determined according to the Folin-copper method.*

In Fig. 6 and Table 3 the results are given from an electrophoretic experiment with 1 ml of normal serum (diluted with three parts of buffer). The electrophoresis was carried out during a period of 18 hours in ammonium buffer of pH 9.0 and ionic strength 0.1, current 100 mA. Fraction volume 4 ml, of which 3 ml was precipitated with TCA, extracted with alcohol-ether and analysed for lipids as described above. The protein analyses were carried out with the modified biuret reaction.

DISCUSSION

Starch must be regarded as an almost ideal supporting medium for the study of serum lipoproteins. Except in phosphate buffer there has not been the slightest adsorbtion of the serum proteins as judged by the complete recovery of protein as well as lipids, when serum is forced through a starch column. Not even chylomicrons are adsorbed, as shown by Carlson and Olhagen [26] in a case of essential hyperlipemia. Factors like pH and increasing salt concentration have not been found to influence upon the behaviour of the

Table 2. Distribution of the serum proteins in a normal serum. Total protein 7.2 per cent. Electrophoresis carried out in ammonium buffer of pH 9.0 and ionic strength 0.1.

Fraction	Protein according to the Folin copper method		Protein according to the modified biuret reaction	
	mg/ml serum	Relative concentration in per cent.	mg/ml serum	Relative concentration in per cent.
Albumin + α_1-globulin	37.2	55.2	45.4	63.9
α_2-globulin	5.2	7.7	4.9	6.9
β-globulin	9.3	13.8	9.2	13.0
γ-globulin	15.8	23.3	11.5	16.2
Total	67.5		71.0	

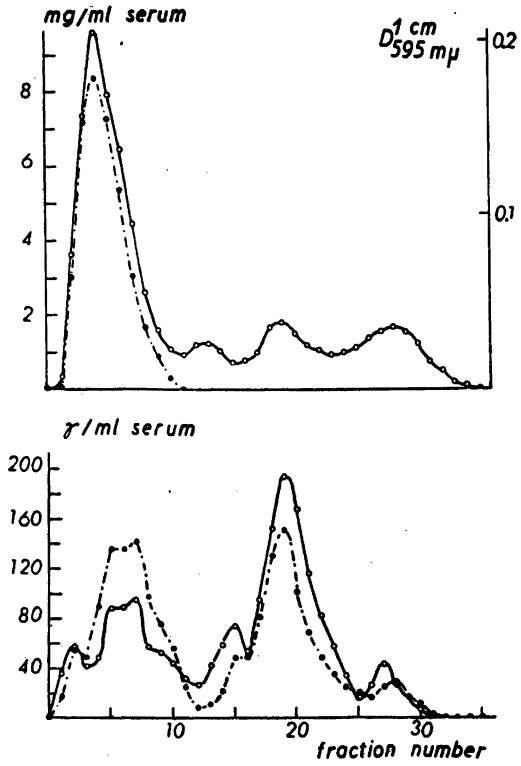

Fig. 6. Electrophoresis of normal serum in ammonium buffer of pH 9.0 and ionic strength 0.1. Upper part: ○—○ protein. ●—●color of bromophenolblue read at 595 mμ. Lower part: ○—○ cholesterol. ●—● phospholipids.

proteins when forced through the starch column. On the other side, phosphate ions seem to interact with the adsorbing power of starch.

However, there are some smaller disadvantages with starch as a supporting medium. Above all, it is the low ratio of "free space" to total volume of the column, which makes it necessary to use large columns. This will necessitate higher currents and thus increase the heat generated. Another disadvantage is

Table 3. Distribution of lipids in the electrophoretically separated lipoproteins of normal serum. (Cholesterol 178 mg/100 ml, phospholipids 172 mg/100 ml.

	Cholesterol γ / ml serum	Phospholipids * γ / ml serum	Ratio Cholesterol / Phospholipids
Albumin	135	120	1.10
α_1-globulin	517	755	0.7
β_1-globulin	211	106	2.0
β_2-globulin	927	684	1.4
γ-globulin	123	91	1.4
Total	1 913	1 756	
Recovery	107 %	102 %	

* Lipid-P is converted to phospholipids by the factor of 25.

the electroosmotic flow, which complicates measurements of the electrophoretic mobilities. Having once been determined, however, it is easily counterbalanced by raising or lowering one of the liquid menisces in Fig. 2. The electroosmosis is more pronounced in starch than in paper and seems to be of the same magnitude as the mobility of the β-globulin in ammonium buffers of pH 9.0. There has been no evidence, that the electroosmosis disturbs the migration of the protein zones.

The choice of method for the protein determinations has been briefly discussed earlier. It can further be added that the Folin copper method gives too low values for the albumin and to high values for the γ-globulin, as is evident from Table 2 and Fig. 5.

The electrophoretic separation of the serum lipoproteins by means of this method has revealed at least 5 distinct lipoprotein components (Fig. 6). The fastest moving lipoprotein has a mobility slightly greater than the mean mobility of the albumin. The next component is the α_1-lipoprotein, which has not separated from the albumin at the ionic strength used. At higher ionic strength, however, the α_1-lipoprotein is separated from the albumin, which has been shown in starch electrophoresis as well as in free electrophoresis. Following this come the β_1-lipoprotein and then the β_2-lipoprotein. Finally there is a smaller component in the γ-globulin fraction. The separation of the lipoproteins and their behaviour at various pH and salt concentrations during electrophoresis as well as their composition and distribution in various diseases are subject to further investigations and will be discussed later on.

SUMMARY

A description and discussion is given of a method for electrophoresis in a starch column. By forcing the electrophoretically separated components out of the starch by means of buffer flow, no extraction of the proteins is needed. The usefulness of the method for the study of serum lipoproteins is shown.

The author is indebted to Dr. B. Olhagen for his valuable criticism during this work. The investigation was financially supported by grants from the "*Statens Medicinska Forskningsråd*". A "Doktorandstipendium" from the *Karolinska Institutet, Stockholm,* is gratefully acknowledged.

During the preparation of this article it has come to the author's knowledge that a method for electrophoresis in supporting medium, based upon the same fundamental principles, i. e. carrying out the electrophoresis in a vertical tube and forcing out the components by buffer-flow, has been developed at the Institute of Biochemistry, Uppsala, Sweden, by Drs. P. Flodin and J. Porath. Their paper will appear in Biochim. et Biophys. Acta **13** (1954) 175.

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Short Communications

A Spot Area Method for Quantitative Determination of Amino Acids on Two Dimensional Paper Chromatograms

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Since Fisher *et al.*[1] showed the logarithmic relationship between concentration and spot area of amino acids on one dimensional paper chromatograms, this fact has been successfully applied by several authors for quantitativ determination of amino acids (*cf.* Block [2]). As only a few amino acids can be completely separated by one dimensional paper chromatography the use of this method has, however, so far been limited.

The present investigation has shown that a logarithmic relationship between concentration and spot area is valid also for two dimensional paper chromatograms. The main difficulty in obtaining accurate quantitative results from spot areas from two dimensional paper chromatograms depends on the fact that the spots often appear irregular as to shape. This difficulty has largely been overcome by dividing the amino acids into two groups as described later in this paper.

For numerical calculations it was found practical to use the following expression:

$$^{10}\log\frac{100 \cdot C}{M} = A \cdot F_p \qquad (1)$$

where C = spot concentration of amino acid in μg; M = mol. weight of amino acid; A = spot area in cm^2; F_p = molar spot area constant.

It appears that F_p within experimental errors is *constant for each amino acid examined within the investigated range of spot concentrations* (10—50 μg). Furthermore F_p is dependent on the solvent and the filter paper used.

Method. For the preparation of two dimensional amino acid paper chromatograms a method by Boissonnas [3] has been adopted in a slightly modified form. The principle of this method is that the amino acids are parted into two groups, each being chromatographed with special solvents. Consequently two paper chromatograms must be prepared for the quantitative determination of all the amino acids.

Group A. Isoleucine, leucine, methionine, phenylalanine, valine and tyrosine.

Group B. Alanine, α-aminobutyric acid, arginine, aspartic acid, glutamic acid, glycine, histidine, lysine, serine, taurine and threonine.

Paper. It is important that the filter paper used gives spots with distinct edges and no sliding. For *group A* Whatman No. 1 was found excellent and has therefore been used. For *group B* the following papers were tested: Whatman No 1 (used by Boissonnas [3]), Whatman No. 4 and Munktell No. 20. The best spots were obtained on Whatman No. 4, which has been used. The size of each paper was adjusted to about 40 × 40 cm^2 before use.

Application of spots. Fisher *et al.*[1] applied the amino acid solution to the paper in *one* drop. The present investigator has found that if the amino acid solution is applied *drop by drop* to the paper no logarithmical relationship between concentration and spot area is obtained on one dimensional chromatograms but a linear relationship of the type:

$$C = k_1 \cdot A + k_2 *$$

where C is spot concentration, A spot area, k_1 and k_2 are constants. However, on two di-

* Guilty for most amino acids between certain limits of spot concentration.

mensional paper chromatograms no such difference can be detected and formula (1) seems to be valid for both methods of application. When the drop by drop method is used, the best results are obtained if not more than 4 drops (each of *ca.* 5 μl) are applied. It is important to dry the paper carefully after each drop has been applied. The recommended spot concentration of each amino acid is 10—30 μg.

Solvents. For *Group A:*

Ketone liquid (first direction), tert. butanol: methylethyl ketone: water, 2 : 2 : 1 (v : v : v).

Methanol liquid (second direction), tert. butanol: methanol: water, 4 : 5 : 1, (v : v : v).

For *group B:*

Propanol liquid (first direction), *n*-propanol: water, 7 : 3, (v : v).

Phenol liquid (second direction), phenol: water, 7 : 3, (v : v).

All chemicals used have been of C.P. quality. Especially important is the purity of the *n*-propanol. Sometimes it was found necessary to distil it before use. The solvents can only be used 1—2 times.

Chromatographic technique. The method with ascending solvents has exclusively been used at a temperature of 20 $\pm$ 1° C. After application of the amino acid solution the filter paper has been folded to a cylinder and the edges of the paper have been kept together with adhesive tape. To obtain a straight solvent front it is recommendable to leave a distance of about one centimeter open between the edges of the paper. After 18 $\pm$ 2 hours in the first solvent the paper has been unfolded and carefully dried. After turning the paper at right angles a new cylinder has been made in the same way. After 18 $\pm$ 2 hours in the second solvent the paper has been unfolded and again carefully dried. It is advisable to use a stream of warm air (*e.g.* from an ordinary hair drier) to dry the papers, especially when the phenol liquid has been used. The order of the solvents is important for good results.

Colour development of spots. The dried chromatograms have been sprayed with a solution of ninhydrine (0.2 % in 96 % ethanol) and the spots have been developed at 100° C during *exactly* 5 minutes.

Estimation of spot areas. The edges of the spots have been marked with a pencil and redrawn on a homogeneous paper. These new spots have been cut out and their areas have been determined by weighing.

Results. The following F_p values have been found for the 17 investigated amino acids:

Group A	F_p	Investigated range (μg)
Isoleucine	0.226	12—37
Leucine	0.230	10—30
Methionine	0.203	7—42
Phenylalanine	0.197	13—39
Tyrosine	0.192	6—45
Valine	0.206	18—53
Group B		
Alanine	0.166	14—47
α-Aminobutyric acid	0.154	16—62
Arginine	0.215	13—92
Aspartic acid	0.176	14—51
Glutamic acid	0.205	10—44
Glycine	0.209	15—58
Histidine	0.140	8—44
Lysine	0.180	15—83
Serine	0.222	9—44
Taurine	0.262	17—53
Threonine	0.170	13—73

The mean error in F_p is about $\pm$ 2 %.

As different observers interpret the spot edges differently (*cf.* Fisher *et al.*[1]) it follows that F_p is a "personal constant". Consequently it is recommended that each investigator determines his own value of the molar spot area constant before this method is employed for quantitative determinations.

The accuracy of this spot area method has been thoroughly investigated and the error range is given in the following table.

Number of determinations	error range
1	5—10 %
2	2— 5 »
3	0— 3 »

The estimation of spot areas, which causes the largest error in the results, might be made more accurate by a photographic technique, but this possibility has not been tested.

The author wishes to thank Professor *Hans von Euler* for his kind interest in this work and for extending to the author the hospitality of his laboratory. This work was financially supported by *Lars Hiertas Minnes Fond.*

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Studies on Mercury(II)oxide Chlorides and Mercury(II)oxide

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Several mercury(II)oxide chlorides have been described in literature, *viz.* $Hg_{n+1}O_nCl_2$ with $n = \frac{1}{2}$, 1, 2, 3, 4, 5, 6, and 7 [1]. In the course of studies of these substances the compounds with $n = \frac{1}{2}$, 2, 3, and 4 have been synthetized and investigated by X-rays.

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It does not seem possible to arrive at a final decision on the arrangement of the chlorine and oxygen atoms from geometrical considerations. Attempts to attack the problem by means of Fourier methods are in progress.

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Acid-Induced Hydrogen Exchange in Benzene Derivatives as Electrophilic Aromatic Substitution

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Qualitative evidence, presented particularly by Ingold *et al.*[1], has shown that the hydrogen exchange induced by acids in benzene derivatives has the characteristics of an electrophilic aromatic substitution, *i. e.* a replacement of H^+ by H^+. As quantitative measurements of the directing power of several substituents have been carried out for the common types of electrophilic aromatic substitution, especially nitration, it should be of interest to investigate the same influence on the hydrogen exchange.

Preparations of toluene, containing tracer amounts of toluene carrying tritium in its *ortho*, *meta*, *para*, and *alpha* positions, respectively, were shaken with 81.8 % sulphuric acid at 25° C and the decay of the tritium content of the toluene was measured as a function of time. The experiments with the *alpha* substituted toluene showed that the methyl hydrogens do not exchange appreciably with the acid during the reaction times in question (maximum about two days). The results obtained with toluene-2-t, -3-t, and -4-t are shown in Figs. 1 and 2. From the initial slopes of the semilogarithmic curves the rates of tritium exchange were obtained [2], as the integrated rate expression cannot be used owing to the lack of

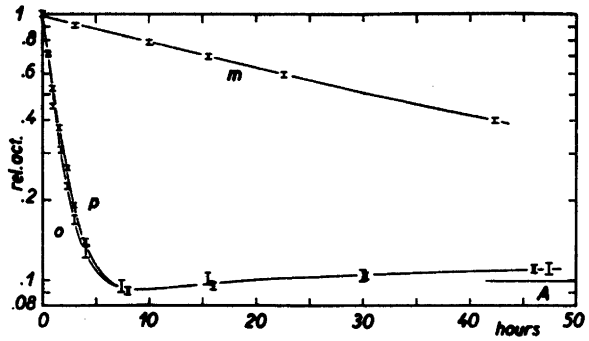

Fig. 1. Semilogarithmic plot of relative specific activity of toluene-2-t (o), -3-t (m), and -4-t (p) as a function of time (3 ml of toluene shaken with 20 ml of 81.8 % sulphuric acid at 25° C). Horizontal line (A) indicates final activity if distribution were uniform between nuclear positions and acid.

knowledge of the exact final equilibrium distribution of tritium in the system. The sulphonation reaction, proceeding with a speed of about 0.5 % of the toluene per hour, makes it impossible to wait for complete equilibrium to be attained. In principle the equilibrium could also at least approximately be found by means of the initial growth rate in a similar experiment with the tritium initially present in the acid. Such experiments were carried out, but for experimental reasons the accuracy was not high enough for an exact determination of the initial slope of the curve.

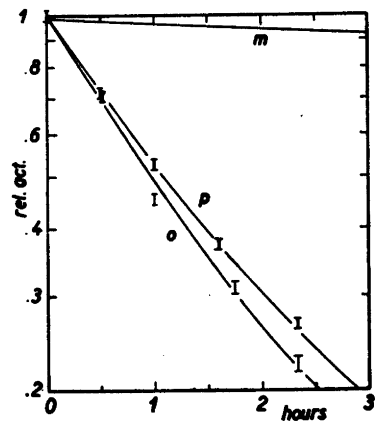

Fig. 2. Part of the same plot as in Fig. 1.

A probable isotope effect in sulphonation [3] will have little influence on the present measurements, since the sulphonation rate given above will be distributed among the different positions in much the same ratio as the exchange rates. Thus the exchange in each position should be very far advanced before an appreciable fraction of the toluene has been sulphonated in that position. Isotopic substitution will certainly have an appreciable effect only on the sulphonation in the same position, and sulphonation in non-triterated positions will not change the specific activity. The only possible influence of importance could be the change in the composition of the acid phase. The acid is present, however, in a tenfold excess over the toluene, and no indications of such an influence have been observed.

The relative exchange rates for tritium in the individual positions of toluene are $o:m:p = (32 \pm 2) : (1.00 \pm 0.06) : (29 \pm 2)$. The growth curve of the activity in toluene (when tritium was initially present in the acid) is in rough agreement with these figures, although no distinction between the *ortho* and *para* positions can be made from this composite curve.

The trend of both kinds of curves for long exchange times seems to indicate that the heavy isotope prefers the position in the aromatic nucleus to that in the acid.

The rate ratio given above is probably approximately valid for all hydrogen isotopes, as spectroscopic data [4,5,6] make it probable that the isotope effect on rate is

not very different for different positions in toluene.

Experiments of the same kind are planned for other benzene derivatives, among which are those containing a bulky alkyl group causing a possible steric hindrance. Homogeneous exchange mixtures should be used in order to enable comparisons between the exchange rates of different kinds of molecules, especially for relating other rates to that of unsubstituted benzene. Experiments with deuterium as tracer are also planned in order to reveal the influence of isotopic mass on the absolute magnitude of the exchange rates and if possible to find differences in isotope effect between different aromatic positions.

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Isolation of α-Methoxy-$\Delta^{\alpha,\beta}$-butenolide from *Narthecium ossifragum* (L.) Huds.

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A new unsaturated γ-lactone has been isolated from a methanolic extract of the flowering parts of *Narthecium ossifragum*. After removal of carotenoids and accompanying substances, *i.a.* 22-hydroxycholesterol [1], by dilution with water and subsequent extraction with petroleum ether, the diluted methanolic extract was evaporated under reduced pressure. From the residual concentrated aqueous solution further impurities were removed by extraction with ether. The lactone could then easily be extracted with chloroform, and, on spontaneous evaporation formed large crystals, which after purification melted at 57° C. (Found: C 52.4; H 5.25. Calc. for $C_5H_6O_3$: C 52.6; H 5.30). *M* (by titration) 114. Calc. 114. $[\alpha]_D^{20} \pm 0°$. The substance contained one methoxyl group; it failed to give an acetyl derivative. The I.R. spectrum indicated a γ-lactone and an $-O-C=CH-$ grouping [2]. The U.V.-spectrum showed a maximum at 224 mμ, log ε 3.88, indicating an α,β-unsaturated lactone, this structure also being supported by the modified Legal reaction of Paist *et al* [3].

The most likely structure for the lactone would be α-methoxy-$\Delta^{\alpha,\beta}$-butenolide or β-methoxy-$\Delta^{\alpha,\beta}$-butenolide. As the previously known β-methoxy compound [4] could be excluded because of its higher m.p. (67°), the synthesis of the α-methoxy compound was attempted.

α-Ketoparaconic acid ethyl ester was prepared by the method of Fischhof [5] and decarboxylated according to Schinz and Hinder [6]. The resulting crude ethereal extract, containing α-keto-butyrolactone, was methylated with diazomethane. The oily reaction product on sublimation (100°, 10 mm), crystallisation from ether and resublimation furnished colourless prisms which melted at 57°, undepressed by the natural substance. The I.R. spectra (in chloroform solution) were identical, thus confirming the identity of the two substances. This lactone is stable and forms a striking contrast to α-methoxy-β-methyl-$\Delta^{\alpha,\beta}$-butenolide, which according to [6] liquefies in two months.

I want to express my thanks to Professor N. A. Sörensen for his continuous interest during this work, and for preparing the I.R. spectra.

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Qualitative evidence, presented particularly by Ingold *et al.*[1], has shown that the hydrogen exchange induced by acids in benzene derivatives has the characteristics of an electrophilic aromatic substitution, *i. e.* a replacement of H^+ by H^+. As quantitative measurements of the directing power of several substituents have been carried out for the common types of electrophilic aromatic substitution, especially nitration, it should be of interest to investigate the same influence on the hydrogen exchange.

Preparations of toluene, containing tracer amounts of toluene carrying tritium in its *ortho*, *meta*, *para*, and *alpha* positions, respectively, were shaken with 81.8 % sulphuric acid at 25° C and the decay of the tritium content of the toluene was measured as a function of time. The experiments with the *alpha* substituted toluene showed that the methyl hydrogens do not exchange appreciably with the acid during the reaction times in question (maximum about two days). The results obtained with toluene-2-t, -3-t, and -4-t are shown in Figs. 1 and 2. From the initial slopes of the semilogarithmic curves the rates of tritium exchange were obtained [2], as the integrated rate expression cannot be used owing to the lack of

not very different for different positions in toluene.

Experiments of the same kind are planned for other benzene derivatives, among which are those containing a bulky alkyl group causing a possible steric hindrance. Homogeneous exchange mixtures should be used in order to enable comparisons between the exchange rates of different kinds of molecules, especially for relating other rates to that of unsubstituted benzene. Experiments with deuterium as tracer are also planned in order to reveal the influence of isotopic mass on the absolute magnitude of the exchange rates and if possible to find differences in isotope effect between different aromatic positions.

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Isolation of α-Methoxy-$\Delta^{\alpha,\beta}$-butenolide from *Narthecium ossifragum* (L.) Huds.

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A new unsaturated γ-lactone has been isolated from a methanolic extract of the flowering parts of *Narthecium ossifragum*. After removal of carotenoids and accompanying substances, *i.a.* 22-hydroxycholesterol [1], by dilution with water and subsequent extraction with petroleum ether, the diluted methanolic extract was evaporated under reduced pressure. From the residual concentrated aqueous solution further impurities were removed by extraction with ether. The lactone could then easily be extracted with chloroform, and, on spontaneous evaporation formed large crystals, which after purification melted at 57° C. (Found: C 52.4; H 5.25. Calc. for $C_5H_6O_3$: C 52.6; H 5.30). *M* (by titration) 114. Calc. 114. $[\alpha]_D^{20} \pm 0°$. The substance contained one methoxyl group; it failed to give an acetyl derivative. The I.R. spectrum indicated a γ-lactone and an $-O-C=CH-$ grouping [2]. The U.V.-spectrum showed a maximum at 224 mμ, log ε 3.88, indicating an α,β-unsaturated lactone, this structure also being supported by the modified Legal reaction of Paist *et al* [3].

The most likely structure for the lactone would be α-methoxy-$\Delta^{\alpha,\beta}$-butenolide or β-methoxy-$\Delta^{\alpha,\beta}$-butenolide. As the previously known β-methoxy compound [4] could be excluded because of its higher m.p. (67°), the synthesis of the α-methoxy compound was attempted.

α-Ketoparaconic acid ethyl ester was prepared by the method of Fischhof [5] and decarboxylated according to Schinz and Hinder [6]. The resulting crude ethereal extract, containing α-keto-butyrolactone, was methylated with diazomethane. The oily reaction product on sublimation (100°, 10 mm), crystallisation from ether and resublimation furnished colourless prisms which melted at 57°, undepressed by the natural substance. The I.R. spectra (in chloroform solution) were identical, thus confirming the identity of the two substances. This lactone is stable and forms a striking contrast to α-methoxy-β-methyl-$\Delta^{\alpha,\beta}$-butenolide, which according to [6] liquefies in two months.

I want to express my thanks to Professor N. A. Sörensen for his continuous interest during this work, and for preparing the I.R. spectra.

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* Research fellow of *Norges Almenvitenskapelige Forskningsråd.*

Separation of Chlorine Isotopes by Ion-exchange Chromatography

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It has been shown [1,2] that different halogen ions can be separated chromatographically in a column of an ion-exchange resin of the strong base type. It seemed, therefore, worth while to investigate the possibility of separating the chlorine isotopes in a similar way. Evidently a very long column would be necessary and so a column of, in principle, infinite length was built in the following manner.

Two glass tubes, A and B, 2.7 cm inner diameter and 150 cm long, were filled with Amberlite I R A 400 in the nitrate form. From a Mariotte bottle the eluent passing a flowmeter is led to the top of column A. Having passed this column the solution flows through a measuring device to the top of column B, and after passage of this it flows out and is discarded. By means of a system of three-way stopcocks in the tube system the eluent can be passed through the columns in the opposite order, *viz.* from the flowmeter through column B downwards to the measuring device and herefrom through column A, likewise downwards. The measuring device contains a silver-silver chloride electrode separated from a calomel electrode by a closed stopcock.

The experiment was started by pouring 15 ml of a 3.5 *M* potassium chloride solution on the top of column A. The eluent was 0.5 *M* potassium nitrate and the flow rate was 5 ml per minute corresponding to a linear velocity of 1.5 cm per minute. The adsorption band moved with about one third of this velocity. When the elution curve obtained by measuring the chloride ion concentration electrometrically showed that the whole adsorption band had passed into column B, the three-way stopcocks were turned so as to send the eluate from B to A and in this way the experiment was continued. By each passage of the elution band from one column to the other an elution curve was obtained.

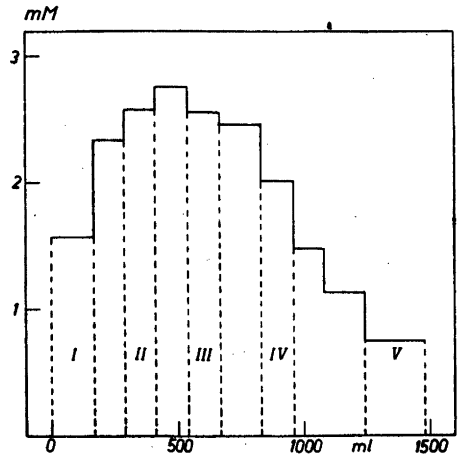

Fig. 1.

Having travelled 43 m the eluate was drawn off the apparatus, collected in fractions and titrated. This last elution curve is shown in Fig. 1 (unfortunately the first fractions were lost). The fractions indicated by roman figures were taken out for mass spectrometric analysis. The silver chloride was centrifuged down, washed and dried. It was then treated with a 25 % excess of methyl iodide in a sealed vessel for 5 hours at 100° C; this procedure resulted in a 65 % conversion. By means of a dry ice-acetone mixture the methyl chloride formed was distilled into an ampoule provided with a stopcock and a ground-glass joint intended for the connection to the mass spectrometer. This was a Metropolitan Vickers type M S 3 instrument. It has a double gas inlet system, and the measurements were carried out in the following manner. The sample was led to the ionizing chamber through one inlet

Table 1. Differences in isotopic composition obtained.

No.	R	n_R	r	n_r	Atomic fraction of ^{37}Cl	$x - 0.246$
I	0.2995±0.0019	3	0.3049±0.0012	3	0.2493±0.0024	+ 0.0033
II	0.3005±0.0005	8	0.3089±0.0006	10	0.2512±0.0009	+ 0.0052
III	0.3031±0.0007	3	0.2966±0.0003	14	0.2420±0.0007	— 0.0040
IV	0.3034±0.0005	6	0.2999±0.0006	7	0.2439±0.0009	— 0.0021
V	0.3031±0.0003	12	0.2938±0.0005	12	0.2403±0.0007	— 0.0057

and the peak heigths of masses 52 and 50 were measured alternately several times. Immediately afterwards the standard, commercial methyl chloride was led through the other inlet and measured in the same way.

The mass spectrum of methyl chloride showed peaks at masses 52, 51, 50, 49, 48 and 47 besides small peaks at 53, 38, 37, 36 and 35. Lower masses were not detectable without altering the magnetic field. The occurrence of masses 53—47 indicates the formation of ions CH_3Cl^+, CH_2Cl^+, $CHCl^+$ and CCl^+ with probably all combinations of isotopic composition. An attempt to calculate the relative amounts of the four ionic species assuming them to be independent of the isotopic composition failed. Neglecting as a first approximation ions containing ^{13}C and ^2H the peak heigth of mass 52 indicates the amount of $^{12}C^1H_3{}^{37}Cl^+$ and that of mass 50 the sum of $^{12}C^1H_3{}^{35}Cl^+$ and $^{12}C^1H^{37}Cl^+$. Measuring in units of the highest peak the total amount of mass 50 is 1, of mass 52 in the standard R and in the sample r. Then the amount of $^{12}C^1H_3{}^{35}Cl^+$ is $1-k$, k being a correction term assumed to be identical in the standard and the sample. The atomic fraction of ^{37}Cl in the standard is 0.246 and in the sample x. For the standard we get

$$\frac{R}{1-k} = \frac{0.246}{0.754}$$

from which $1 - k = 3.065\ R$. For the sample similarly

$$\frac{r}{1-k} = \frac{x}{1-x} \text{ or } x = \frac{r}{3.06\ 5R+r}$$

The results are shown in Table 1. Columns 2 and 4 contain the mean values and the mean errors calculated from the number of observations indicated in columns 3 and 5.

Although the differences are small they are significant, and in order to obtain greater effect the work is being continued.

Thanks are due to *Statens almindelige Videnskabsfond* for a grant.

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Note on the Wet Combustion of Ion Exchange Resins

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In connection with some studies on the exchange kinetics in ion exchange resins [1] it was necessary to determine the content of thorium, and sometimes of other elements, in a resin. Such a determination may in principle be carried out by either 1) elution of the ion from the resin, 2) dry combustion of the resin or 3) wet combustion of the resin. An analysis is then made of the eluate, ashes, or resulting solution respectively.

Elution from the resin is by far the best method when dealing with easily eluted ions in macroquantities. If, however, the ion is highly charged and consequently exhibits a high affinity for the resin and a low exchange rate, a complexing agent is almost unavoidable if complete elution in a reasonable volume of eluant is desired. In many cases the complexing agent interferes with the analysis and must be removed before it is done. As the complexing agent is frequently an organic compound it may be destroyed by either ashing or wet combustion, but if these methods are to be used it would seem to be more advantageous to apply them directly to the resin.

Dry combustion by ignition is limited to ions which do not form any volatile compounds. Even so the conversion of the ashes to a readily analyzed form may often meet with considerable difficulties.

Wet combustion, i. e. complete oxidation of organic matter by means of a liquid oxidant, is frequently employed in inorganic analysis when organic matter may interfere.

Lowen *et al.*[2] decomposed Dowex 50 with concentrated sulfuric acid in the presence of metallic selenium as catalyst. This method seems, however, to be rather time-consuming (see Table 1), and as a relatively high temperature is needed to expel the selenium it is to some extent subject to the same objections as dry ashing. The same authors carried out a combustion by treating the resin with concentrated perchloric acid for 24 hr at 50° C; a good method in principle, but much too time-consuming.

and the peak heigths of masses 52 and 50 were measured alternately several times. Immediately afterwards the standard, commercial methyl chloride was led through the other inlet and measured in the same way.

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Table 1. Time (in minutes) necessary for complete combustion of 1 g resin samples with some different oxidants.

Resin	Particle size mm	Ionic form	$Se+H_2SO_4$	HNO_3+HClO_4 [a]	$H_2SO_4+HNO_3$
Dowex 50	0.5—1.0	Na^+	~200 [b]	20—30	20—30
	0.6—0.8	Th^{4+}	—	~500	20—30
Amberlite IR-100	0.5—1.0	H^+	~150 [b]	10—15	20—30
		Th^{4+}	—	10—15	20—30

a) 6 + 12 ml for Dowex 50-Na^+ (For Th^{4+} the acid mixture had to be renewed twice); 10 + 8 ml for Amberlite IR-100[5].

b) Including time necessary to drive off the selenium.

The most commonly used oxidizing agents which do not introduce any foreign cation are mixtures of concentrated perchloric and nitric acids and sulfuric and nitric acids, respectively. The former mixture has recently been applied to several organic compounds [3,4]. Thus Smith [3] succeeded in decomposing completely a sample of Dowex 50 (ionic form unknown) in 20 minutes. Independently of Smith, Högfeldt *et al.*[5] made a special study of the wet combustion of ion exchange resins of the phenol formaldehyde and polystyrene types in some different ionic forms, and finding decomposition times of the same order as Smith, see also Table 1. When using this method on Dowex 50 saturated with thorium we have found a considerable increase in the time needed for complete combustion. This may be due to the filling of the resin pores with a white precipitate which is formed during the reaction, resulting in mechanical interference with the attack of the resin. The possibility of a catalytically inhibiting effect cannot be excluded. It may be noted, however, that this inhibition was not observed with the more readily oxidized phenolformaldehyde resin, Amberlite IR-100. The latter resin has not such a dense structure as the polystyrene resin Dowex 50, a factor which may influence the oxidation rate, especially when slightly soluble precipitates are formed. The precipitate is dissolved on dilution after complete reaction. However, oxidation of Dowex 50 with concentrated sulfuric and nitric acids was found to be satisfactory even when the exchanger was saturated with thorium. A precipitate, thorium sulfate, which is poorly soluble in concentrated sulfuric acid, was formed in this case also, but it had no measurable effect on the oxidation rate.

Experimental. A resin sample of 1 g dried at 105° C was placed in a 30 ml Kjeldahl flask together with 20 ml conc. sulfuric acid. On heating, the resin becomes increasingly charred, and after boiling for about five minutes a black suspension is obtained. Now 10 ml conc. nitric acid are added dropwise to the boiling suspension at a rate of about 1 ml/min. The reaction is rather vigorous at the beginning, and nitric dioxide fumes are evolved. The suspension changes into a dark red solution, which gradually clears up to light yellow. Boiling is continued for five minutes after the last drop of nitric acid has been added giving a water clear solution on cooling.

A few results are shown in Table 1 in comparison with other methods.

In some cases, as for resins containing sodium and hydrogen, combustion with nitric-perchloric and sulfuric-nitric acids are of the same order of usefulness. In special cases, however, one of the methods may be preferable, *e. g.* when an ion in the exchanger forms insoluble sulfates the nitric-perchloric acid mixture is obviously the most suitable. Often, as for thorium resin, it may not be possible to judge without experiments which method is to be preferred. The nitric-perchloric and sulfuric-nitric acid methods are therefore complementary to each other in a useful manner.

Sincere thanks are due to Dr. E. Högfeldt for lending us his manuscript [5] before publishing.

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Deacylation of N-Acetylated Amines with Alcohol and Boron Trifluoride

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On attempting to prepare 3,4-dinitro-α-naphthylamine [1] from its acetyl derivative it was found that the usual methods of deacylation were unsatisfactory, side reactions accompanying deacylation.

As stated in another investigation [1] 3,4-dinitro-α-naphthylamine is quantitatively transformed to 4-chloro-3-nitronaphthalene diazonium chloride when heated with *hydrochloric acid.*

With *dilute sulphuric acid* it was found that acetaldehyde was formed when the acetyl compound was refluxed in a mixture of equal amounts of ethanol and 50 % sulphuric acid which may suggest the formation of a diazonium compound that in its turn is reduced by the alcohol. The reaction with sulphuric acid is obviously similar to that with hydrochloric acid.

Attempts to split off the acetyl-group by heating the acetyl compound for a short time in *concentrated sulphuric acid* as described by Blanksma [2] also failed, sulphonation apparently taking place.

Roon, Verkade, and Wepster [3] have reported that many aromatic acetyl compounds are converted to the corresponding amine by heating with *absolute alcoholic hydrogen chloride.* However, this method was not successful in the case of 3,4-dinitroaceto-α-naphthalide. A product exploding very violently at ca. 150 °C was formed. It was not investigated further.

Treatment of the acetyl compound with *alcoholic aqueous sodium hydroxide* yielded chiefly products other than 3,4-dinitro-α-naphthylamine. The formation of ammonia indicated that the amino-group was split off.

Attempts were also made to deacylate the acetyl compound in boiling *alcohol with sodium alcoholate as a catalyst* as described by Verkade and Witjens [4] and this method was found to give satisfactory results.

Table 1. The results of deacylation with methanol and boron trifluoride.

Compound	Time min.	Temp. °C	M.p. * of the crude product °C	M. p. of the pure amine °C
Acetanilide	60	65	liquid ($n_D^{20°}$ = 1.586)	liquid ($n_D^{20°}$ = 1.586)
o-Nitroacetanilide	10	65	71—72	71—72
	35 **	65	71—72	
m-Nitroacetanilide	20	65	112—13	114—15
p-Nitroacetanilide	15	65	149—50	150—51
2,3-Dinitroacetanilide	20	65	124—25	126—27
2,6-Dimethylacetanilide	2 160	110	4—6 ($n_D^{20°}$ = 1.556)	9—11 ($n_D^{20°}$ = 1.561)
2,6-Dimethyl-N-methyl-acetanilide	2 160	110	94—95 (unchanged starting material)	liquid
4-Nitroaceto-α-naphthalide	25	65	192—93	196—97
2,3-Dinitroaceto-α-naphthalide [1]	60	65	(unchanged starting material)	232 (decomp.)
3,4-Dinitroaceto-α-naphthalide	65	65	201 (decomp.)	201 (decomp.)

* All melting points are corrected.
** Deacylation in a solution containing 0.01 g BF_3/ml.

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3,4-Dinitroaceto-α-naphthalide	65	65	201 (decomp.)	201 (decomp.)

* All melting points are corrected.
** Deacylation in a solution containing 0.01 g BF_3/ml.

According to Sowa and Nieuwland [5] amides can be esterficated by alcohols and phenols in the presence of boron trifluoride. It might, therefore, be expected that acetylated amines would be deacylated in the acidic solution formed from *boron trifluoride and an alcohol.* This was also found to be the case, and 3,4-dinitroaceto-α-naphthalide and some other acetyl compounds (see Table 1) were easily deacylated when heated in methanol containing boron trifluoride (0.10 g/ml, 4—8 mole / mole acetyl compound) giving the corresponding amine in almost theoretical yield. The time required for complete reaction was determined only approximately. Samples were taken out at suitable intervals and investigated. The relation between the rate of deacylation and the amount of boron trifluoride was not investigated. In the case of *o*-nitroacetanilide, however, an experiment was carried out with one tenth of the above mentioned amount of boron trifluoride; an increase of the time of reaction from 10 to 35 minutes was required.

As can be seen from the Table the sterically hindered 2,6-dimethylacetanilide was very slowly deacylated even at 110 °C, and its N-methyl derivative remained unchanged when heated to this temperature for 36 hours. For comparison it may be pointed out that the former compound can not be hydrolysed in boiling concentrated hydrochloric acid but when heated to 200 °C in 70—75 % sulphuric acid it is hydrolysed in about 6 hours [6]. The N-methyl derivative remains unchanged when boiled in 80 % sulphuric acid for 9 hours or heated to 220° C in concentrated alcoholic potassium hydroxide for 11 hours [7].

Experimental. The acetyl compound (0.30 g) was refluxed in methanol (6 ml) containing boron trifluoride (0.60 g) and at suitable intervals samples were taken out and investigated (m.p. and / or refractive index). When the deacylation was complete the reaction solution was worked up in one of the following alternative ways.

1. After the introduction of ammonia to bind the boron trifluoride as $NH_3 . BF_3$ the methanol was evaporated and the residue extracted with ether. Evaporation of the ether yielded the free amine. By treatment of the ether insoluble residue with sulphuric acid as described by Sowa and Nieuwland [5] practically all the boron trifluoride was liberated.

2. Water insoluble amines forming compounds with boron trifluoride which decompose in aqueous solution (*e. g.* 2,3-dinitroaniline) were precipitated by diluting the reaction solution with water. The precipitated amine was collected and washed with water.

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2. Blanksma, J. J. *Rec. trav. chim.* **27** (1908) 49.
3. van Roon, J. P., Verkade, P. E. and Wepster, B. M. *Rec. trav. chim.* **70** (1951) 1105.
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5. Sowa, J. F. and Nieuwland, J. A. *J. Am. Chem. Soc.* **55** (1933) 5052.
6. Nölting, E. and Pick, B. *Ber.* **21** (1888) 3150.
7. Friedlaender, P. *Monatsh.* **19** (1898) 627 (*cf.* p. 642).

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The Compressibility of Acetylene at 25.0° C

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In connection with equilibrium and solubility determinations earlier described [1], the compressibility of acetylene was measured at pressures below 1.5 atm.

It proved advantageous to use the same type of apparatus as for the solubility determinations, even if more accurate compressibility measurements may be performed by other methods. The apparatus consists of a thick-walled glass flask in a water thermostat; the flask is connected to a manometer and a gas-burette, the latter provided with a separate thermostat. The gas contents of ducts and manometer can be calculated from the pressure and the inner reading of the manometer, using a function which has been determined by separate calibration.

During the measurements V and T are kept constant and the relation between p and n is studied. The measurement of the gas quantity (which is volumetrically performed under suitable standard conditions), however, does not give n but $n\ a$,

According to Sowa and Nieuwland [5] amides can be esterficated by alcohols and phenols in the presence of boron trifluoride. It might, therefore, be expected that acetylated amines would be deacylated in the acidic solution formed from *boron trifluoride and an alcohol.* This was also found to be the case, and 3,4-dinitroaceto-α-naphthalide and some other acetyl compounds (see Table 1) were easily deacylated when heated in methanol containing boron trifluoride (0.10 g/ml, 4—8 mole / mole acetyl compound) giving the corresponding amine in almost theoretical yield. The time required for complete reaction was determined only approximately. Samples were taken out at suitable intervals and investigated. The relation between the rate of deacylation and the amount of boron trifluoride was not investigated. In the case of *o*-nitroacetanilide, however, an experiment was carried out with one tenth of the above mentioned amount of boron trifluoride; an increase of the time of reaction from 10 to 35 minutes was required.

As can be seen from the Table the sterically hindered 2,6-dimethylacetanilide was very slowly deacylated even at 110 °C, and its N-methyl derivative remained unchanged when heated to this temperature for 36 hours. For comparison it may be pointed out that the former compound can not be hydrolysed in boiling concentrated hydrochloric acid but when heated to 200 °C in 70—75 % sulphuric acid it is hydrolysed in about 6 hours [6]. The N-methyl derivative remains unchanged when boiled in 80 % sulphuric acid for 9 hours or heated to 220° C in concentrated alcoholic potassium hydroxide for 11 hours [7].

Experimental. The acetyl compound (0.30 g) was refluxed in methanol (6 ml) containing boron trifluoride (0.60 g) and at suitable intervals samples were taken out and investigated (m.p. and / or refractive index). When the deacylation was complete the reaction solution was worked up in one of the following alternative ways.

1. After the introduction of ammonia to bind the boron trifluoride as $NH_3 . BF_3$ the methanol was evaporated and the residue extracted with ether. Evaporation of the ether yielded the free amine. By treatment of the ether insoluble residue with sulphuric acid as described by Sowa and Nieuwland [5] practically all the boron trifluoride was liberated.

2. Water insoluble amines forming compounds with boron trifluoride which decompose in aqueous solution (*e. g.* 2,3-dinitroaniline) were precipitated by diluting the reaction solution with water. The precipitated amine was collected and washed with water.

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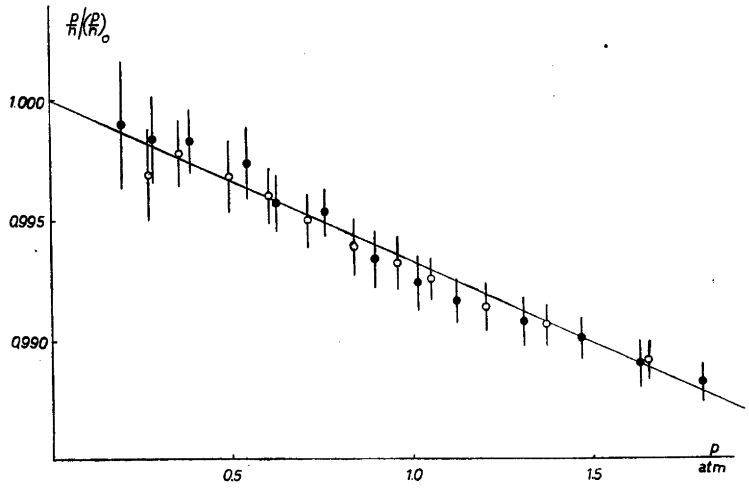

Fig. 1. Compressibility of acetylene.

where a is a constant but unknown factor. The quotient p/na is plotted as a function of p, and by extrapolation to $p = 0$ the limit value $(p/na)_0$ is determined. The results are then expressed by the dimensionless quotient

$$\frac{p}{na} \Big/ \left(\frac{p}{na}\right)_0 = \frac{p}{n} \Big/ \left(\frac{p}{n}\right)_0 \quad (V \text{ and } T \text{ constant})$$

which is identical with the quotient

$$vp \,/\, (vp)_0 \qquad (n \text{ and } T \text{ constant})$$

Fig. 1 illustrates the results from two independent series of measurements. The lengths of the vertical lines through the points correspond to the maximum errors, as derived from the numerical accuracy of the experimental data. The straight line corresponds to the relation $vp \,/\, (vp)_0 = 1 + k \cdot p$.

A statistical treatment, carried out considering the fact that na contains successively accumulated errors, gives for the one series the value $k = -(6.9 \pm 0.3) \cdot 10^{-3}$ and for the other series $k = -(7.2 \pm 0.4) \cdot 10^{-3}$. By insertion of the k-value, $-7.0 \cdot 10^{-3}$ $(\text{atm})^{-1}$, relative vp-values can be computed with a standard error of $\pm\ 3 \cdot 10^{-4}$.

Both the curve and the statistical computation show a deviation tendency, which indicates that in a general compressibility function of the type $vp \,/\, (vp)_0 = 1 + B \,.\, p + C \,.\, p^2 + \ldots\ldots\ldots$ there is a positive *third virial coefficient*, C, and that the correct numerical value for the negative *second virial coefficient*, B, is somewhat higher than the accepted k-value. An accurate determination of C would demand measurements at higher pressures.

Earlier measurements at 25.0° C, performed by Sameshima [2] and falling within the interval 1—8.7 atm, give a fairly constant k-value of $-(7.0$ to $6.6) \cdot 10^{-3}$ $(\text{atm})^{-1}$ in the interval 2.4 to 7.3 atm, but within the interval 1.0 to 2.4 atm his measurements seem to give so high a value as $k = -8.5 \cdot 10^{-3}$. Howarth and Burt [3] have performed compressibility measurements at 0° C and got $k_0 = -8.8 \cdot 10^{-3}$ $(\text{atm})^{-1}$.

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The Crystal Structure of Phosphoric Acid

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The crystal structure of phosphoric acid, H_3PO_4, has been determined, using X-ray diffraction methods. The unit cell is monoclinic with $a = 5.78$ Å, $b = 4.84$ Å, $c = 11.65$ Å, and $\beta = 95.5°$. The space group is $P2_1/c$ and there are four molecules per unit cell, corresponding to a density of 2.01 g.cm^{-3}.

The positions of the phosphorus and oxygen atoms were derived from Patterson- and Fourier-projections along the a and b axes. The reliability factor R is at this stage about 0.23 for both projections and the structure is being further refined. In Fig. 1 the Fourier projection in direction of the b axis is shown.

Although the structure cannot be discussed in detail at this stage, it appears that the four P—O bonds are all of approximately the same length, not far from the value of 1.56 Å found by West [1] in the crystal structure of KH_2PO_4. The PO_4 tetrahedron is fairly regular. Hydrogen bonds hold the molecules together. Two of the oxygen atoms probably take part in two such bonds, whereas the other two seem to participate in only one hydrogen bond. This is indicated by dotted lines in the figure.

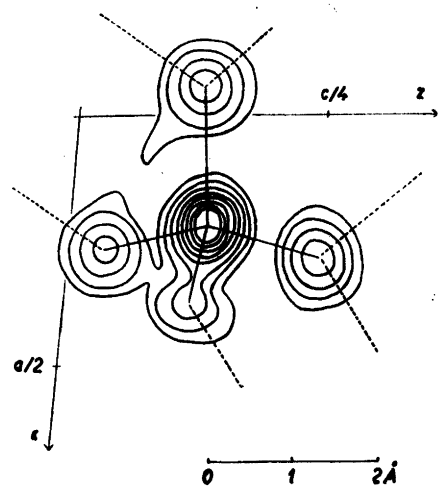

Fig. 1. Electron-density projection along the b axis.

A detailed description of the structure will be published at a later date.

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Cuprous Compounds of Acetylene

IV. Solid Addition Compounds with Cuprous Chloride; Composition and Equilibria

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In the first paper of this series [1] Österlöf has reported on his earlier investigations of two solid addition compounds, for which he suggests the formulae $C_2H_2(CuCl)_3$ and $C_2H_2(CuCl)_2$. These substances were prepared by treating cuprous chloride with acetylene in a reaction medium of ethyl alcohol and hydrochloric acid and collecting the solid reaction products. Analyses of these products, comprising acetylene and copper determinations, gave as a mean 3.2 and 2.1 moles cuprous chloride per mole acetylene. The deviations from the theoretical integers were supposed to be caused by a loss of acetylene during the stage between synthesis and analysis, or possibly by an incomplete conversion; experiments using longer reaction times usually gave values closer to the theoretical ones. In spite of the limited accuracy, the experiments showed with certainty — in contrast to earlier investigations in this field [2] — that there exist two distinct compounds between acetylene and cuprous chloride.

The syntheses were performed at different pressures, which were kept constant during each single experiment. An approximate determination of the dissociation pressures of the compounds was attempted by a stepwise narrowing of the pressure interval within which a change of the composition of the solid occurs. It has later turned out that supersaturation phenomena render such a determination of the dissociation pressures unsatisfactory.

The continued investigations have covered measurements of the dissociation pressures and their temperature coefficients with a manometrical method and accurate determinations of the stoichiometrical composition of the compounds.

It has been confirmed that no reaction occurs within a reasonable time between dry solid cuprous chloride and acetylene, even if the pressure is raised to about 10 atm. Nor do the solid addition compounds decompose in vacuo if the substances are carefully dried, *e.g.* by P_2O_5-dry acetylene. A liquid medium is therefore necessary for these reactions. After tentative comparisons between different media aqueous solutions containing hydrochloric acid were chosen.

If the acetylene pressure is successively raised over aqueous hydrochloric acid solutions containing solid cuprous chloride in excess, the first crystallization of solid addition compound occurs only at a pressure considerably higher than the equilibrium pressure [3]. This supersaturation phenomenon is typical and appears both at the conversion of CuCl into $C_2H_2(CuCl)_3$ and at the further conversion into $C_2H_2(CuCl)_2$. In both cases the crystallization can be initiated by seeding.

In several experiments the crystallization of $C_2H_2(CuCl)_3$ failed to appear and, instead, the cuprous chloride turned directly into $C_2H_2(CuCl)_2$. Therefore it was possible to determine the equilibrium pressure of systems where the substances CuCl and $C_2H_2(CuCl)_2$ are coexisting solid phases, although such systems are unstable with respect to $C_2H_2(CuCl)_3$. It has thus been possible to measure, independent of each other, the following three equilibria:

$$C_2H_2 + 3\ CuCl\ (s) = C_2H_2(CuCl)_3\ (s) \qquad P_3 \qquad (1)$$

$$C_2H_2 + 2\ CuCl\ (s) = C_2H_2(CuCl)_2\ (s) \qquad P_2 \qquad (2)$$

$$C_2H_2 + 2\ C_2H_2(CuCl)_3\ (s) = 3\ C_2H_2(CuCl)_2\ (s) \qquad P_{2,3} \qquad (3)$$

Actually it is easier to study the metastable equilibrium (2) than the stable equilibrium (3) which is approached much more slowly.

There exists a theoretical relation between the three equilibrium pressures P_3, P_2, and $P_{2,3}$. If acetylene is regarded as an ideal gas (and the differences between the volumes of the solid substances are disregarded in comparison to the mole volume of acetylene) the following relation holds:

$$P_{2,3} = P_2^3 / P_3^2 \qquad (4)$$

The manometric method can be used also for the determination of the composition of the solid complexes formed, without the necessity of isolating the substances for analysis. The first step is a *qualitative* analysis, whose purpose is to prove that only C_2H_2 and CuCl enter into the complexes formed, and none of the other components of the reaction medium, *e.g.* hydrochloric acid or water. This is achieved by comparing different experiments which give identical equilibrium pressures, although the composition of the medium has been so changed that the activities of these other components vary.

The *quantitative* analyses are performed by measuring the amount of acetylene required for complete conversion of a given quantity of cuprous chloride. The amount of liquid is small in comparison to the solid phase and even the ratio between the total amounts of cuprous chloride and acetylene gives a good approximate value for the composition of the compound formed. In most experiments the amount of cuprous chloride and acetylene dissolved in the liquid phase can be corrected for by applying empirical functions from earlier investigations [3,4].

APPARATUS AND PROCEDURE

The manometrical apparatus sketched in Fig. 1 has been used for the measurements. The apparatus consists of a gas burette, used both for the addition and removal of gas, an open mercury manometer and a reaction flask,

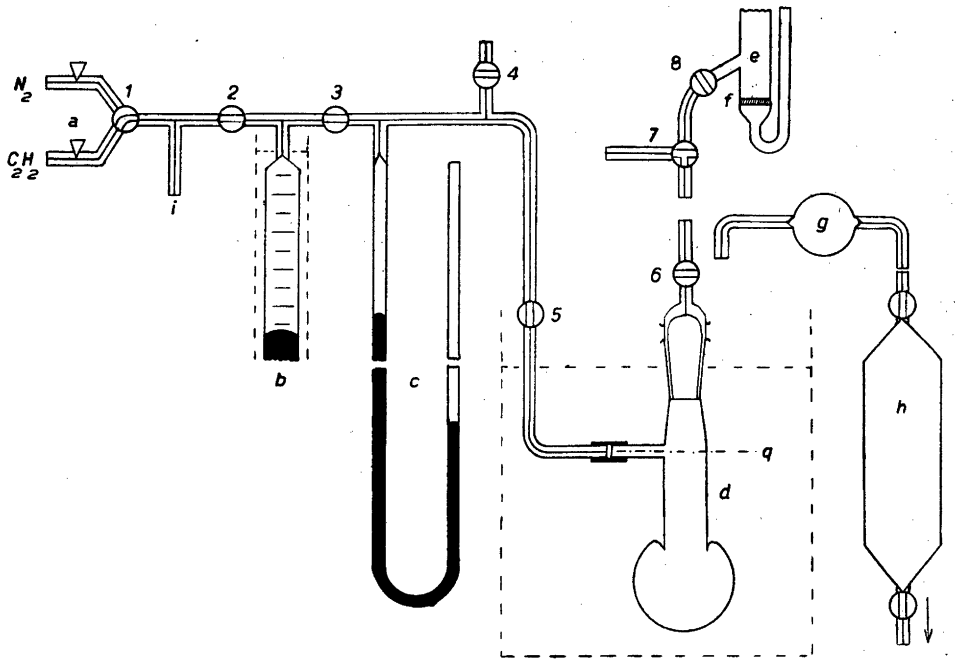

Fig. 1. Manometrical apparatus.
a) Mercury seals. b) Gas burette, water-jacketed. c) Mercury manometer. d) Reaction flask. Sways with line q as axis. e) Burette for liquid. f) Fritted disc. g) Splash trap. h) Evacuation vessel. i) To differential manometer for burette pressure.

immersed in a water thermostat and equipped with a mechanical shaking device. At the beginning of the experiment the flask contains known amounts of solid cuprous chloride and liquid. Measured quantities of acetylene are added under vigorous shaking of the vessel. The pressure momentarily rises with each addition and then slowly sinks towards equilibrium. At suitable points during the experiment, a certain amount of acetylene is sucked out and the rising pressure-time curve is followed. The experiments aim at an empirical following-up of both horizontal and vertical branches of a phase diagram, as in Fig. 6.

In the experiments two types of hydrochloric acid solutions have been used:

a. Hydrochloric acid of concentration from 1 to 4 m.
b. 4 m mixtures of hydrochloric and perchloric acid with a recipe concentration of hydrochloric acid between 0.2 m and 1.0 m.

Lower concentrations of hydrochloric acid prove inconvenient since solid acetylidic compounds may form, whilst higher concentrations must be avoided because of the catalytic formation of vinyl chloride, which can cause a disturbing pressure increase, even if the quantity is stoichiometrically unimportant.

Determination of the equilibrium pressures

The reaction velocity, at which equilibrium is approached, depends on the composition of the liquid and on the ratio between the amounts of liquid and solids. No systematic investigation of the kinetics has been intended. The reaction velocities are irreproducible; changes in particle size etc. of the solid substances may produce considerable variations during one and the same experiment. The impression is, however, that it is advantageous to use a fairly small amount of liquid, which is desirable also in order to depress the effect of by-reactions, which occur in the liquid phase only. However, the mixture should contain so much liquid that it remains mobile on shaking. A quantity of about 1 ml solution per g solid substance has been used in most experiments.

The free gas volume of the reaction flask should be kept small; a large gas volume increases the "buffer capacity" of the system and delays the attainment of equilibrium. This, however, has the disadvantage that insoluble impurities in the acetylene are enriched in the remaining gas. When acetylene is successively added for the synthesis of a larger quantity of addition compound, the total pressure will therefore rise even if the partial pressure of acetylene remains constant. It is difficult to prepare acetylene, which is so pure that this effect disappears entirely. However, if gas is sucked out, a rapid venting of the gas impurities is achieved and the system fills with pure acetylene formed by a partial splitting of the addition compound. If the free volume of the vessel is small and the equilibrium pressure low it is possible to remove all impurities completely by withdrawing a comparatively small portion of the total acetylene content.

The normal routine for measuring an equilibrium pressure comprises the following steps:

a. Synthesis of a suitable quantity of solid addition compound (corresponding *e. g.* to one fourth of the total cuprous chloride) by introducing a large portion of acetylene.

b. One or two withdrawals of gas for the complete removal of gas impurities. To check the reproducibility of the decomposition pressure even further withdrawals may be performed.

c. Addition of a small portion of acetylene which may be the pure gas from the previous withdrawal.

The pressures read under a. are rejected; the readings under b. give the pressure-time curve after splitting (the first one may be rejected if there is reason to suspect noticeable amounts of impurities) and under c. the curve after synthesis.

Determination of the composition of the solid substances

The total amount of cuprous chloride is known by weighing and the amount of acetylene introduced is volumetrically measured. The compressibility of acetylene is nearly ideal; according to measurements in this laboratory [5] at 25.0 °C and within the pressure interval dealt with here, we can use the relation $VP = RT \cdot (1 + k \cdot P)$ where the coefficient k amounts to $-7 \cdot 10^{-3}$ (atm)$^{-1}$. At the volumetric measuring (conditions 20—25° C, 1 atm) the mole volume of acetylene thus is 0.7 % smaller than that for an ideal gas.

The impurities in the acetylene (about 0.4 % in most experiments) need not be corrected for since they are essentially removed (and measured) in the withdrawal steps.

The total amount of acetylene added will be distributed over the gaseous, liquid, and solid phases. The amount of gaseous acetylene is calculated from the pressure, temperature and volume; the free volume of the reaction vessel is determined in each experiment by calibration with nitrogen.

For the computation of dissolved acetylene and cuprous chloride the following empirical functions are used, derived from earlier investigations [3,4] of inorganic complexes of cuprous chloride and of dissolved acetylene compounds.

$$[C_2H_2]_t = [\alpha + \{CuCl\} \cdot (A + B \cdot HCl_t)]P_A \quad (5a)$$

$$CuCl_t = \{CuCl\}\ [S + (A + B \cdot HCl_t) \cdot P_A] \quad (5b)$$

$\{CuCl\}$ is the activity of cuprous chloride, with the solid substance as standard state, and P_A the partial pressure of acetylene in atm. If the solvent is hydrochloric acid only

(of concentration HCl_t), the constants below give the desired concentrations in moles/1 000 g H_2O at 25.0° C.

$a = 0.0424 - 2.62 \cdot 10^{-3} \cdot HCl_t \cdot (1 + 0.116\ HCl_t)^{-2}$
$A = 0.046 \qquad B = 0.0759$
$S = 0.048$ (1 m HCl), 0.225 (2 m HCl), 0.450 (3 m HCl), 0.744 (4 m HCl)

For 4-molal mixtures of hydrochloric and perchloric acid hold:
$a = 0.0509 - 3.5 \cdot 10^{-3} \cdot HCl_t$
$A = 0.016 \qquad B = 0.086$
$S = 0.0435 \cdot HCl_t + 0.0294 \cdot HCl_t^2$

In a system containing solid cuprous chloride, *i. e.* when measuring the pressures P_2 and P_3, these expressions — with $\{CuCl\} = 1$ — are valid, provided the system is in equilibrium.

After complete conversion into solid addition compound, and on measurement of the equilibrium pressure $P_{2,3}$, the cuprous chloride activity sinks below unity. In a system which is in equilibrium with a solid addition compound of the composition $C_2H_2(CuCl)_n$, the cuprous chloride activity depends on the acetylene pressure, P_A, according to

$$\{CuCl\} = (P_n/P_A)^{1/n} \tag{6}$$

where P_n represents the dissociation pressure of the solid addition compound, constant at a given temperature. (Expression (6) presupposes ideal compressibility of acetylene, but no correction is necessary in this case or on the whole when applying any of expressions (5)—(7).) If (6) is inserted into (5a) and (5b) the following expressions result:

$$[C_2H_2]_t = P_A\ [a + (A + B \cdot HCl_t) \cdot (P_n/P_A)^{1/n}] \tag{7a}$$

$$CuCl_t = [S + (A + B \cdot HCl_t) \cdot P_A] \cdot (P_n/P_A)^{1/n} \tag{7b}$$

These expressions have been used in Tables 2—4 for computing the composition of the liquid. For the equilibrium pressures P_n the values given later on have been used.

The quantities of acetylene and cuprous chloride which are dissolved in the liquid are fairly small (maximum 10 %) in comparison with the total amounts. The values computed are only corrections (which partly compensate each other in the computation of the composition of the solid phase). When applying the formulae (7a) and (7b), one may therefore start from an assumption concerning the value of n, *i. e.* the mole ratio cuprous chloride / acetylene in the solid compound, although the aim of the whole calculation is just an empirical determination of this number.

The details of the manometrical experiments are given in the type experiment section; other experiments are only briefly summarized. The type experiment has been chosen so that it comprises measurements of all three equilibria.

For the microscopical examination of the solid addition compounds a technique has been devised which permits sampling into a closed, acetylene-filled microscope chamber. A simple sampling with pipette and standard slide with cover glass, however, normally gives a specimen sufficiently stable for photographing etc.; $C_2H_2(CuCl)_3$ can be treated at room temperature, $C_2H_2(CuCl)_2$ at + 10° C or less. It is easy to observe when disintegration, caused by evaporation of acetylene, occurs.

Type experiment; reaction medium 0.865 m HCl, 3.135 m $HClO_4$

The experimental method, observations and data appear in Table 1, in the time-pressure curves of Fig. 2 and in the following account.

The formulae in the first and second columns are those which have been verified by the experiment. V_f denotes the total amount of gas which has been introduced into the reaction vessel *d* (including the tubing up till stopcock *5*). The value is obtained from the sum of the burette readings minus the gas content enclosed between stopcocks *3* and *5*; this volume is given by a linear function of the reading of the inner meniscus of the manometer. After withdrawal, the gas in the burette will contain water vapour. The volumes

Table 1. Readings and computed data from type experiment: 0.865 m HCl, 3.135 m $HClO_4$.

Charge: 100.4 millimoles cuprous chloride
18.89 g acid mixture, with 14.03 g water

Symbols: V_f-total amount of gas in the reaction vessel, in ml at 21.0° C and 1 atm
p_t-total pressure in mm Hg, uncorrected = $(761.5)^{-1}$ atm.

Stage of experiment	Solid phase	No.	V_f	Temp. °C	p_t	Time hours
Determination of vapour pressure	CuCl	Ev 1	0.0	25.0	19.1	—
		2	0.0	10.0	7.4	—
Nitrogen calibration	CuCl	N_2 1	14.9		247.3	—
		2	30.9		491.9	—
		3	43.6	25.0	686.6	—
		4	60.6		946.5	
Evacuation	CuCl	Ev 3	0.0	25.0	19.1	—
		a 1	12.6		119.2	¼
Acetylene absorption before formation of solid addition compound (and overall check)	CuCl	2	32.5		275.2	¼
		2	32.5		274.1	24
		3	60.5	25.0	496.5	¼
		4	75.8		617.8	¼
		5	93.7		758.9	¼
		6	103.8		838.0	¼
Equilibrium (2) metastable	CuCl + $C_2H_2(CuCl)_2$	b 1	267.1		(484.8)	16
		2	231.9		(455.1)	1
		3	198.1	25.0	453.0	1
		4	165.3		452.9	1 ½
		5	195.9		455.2	1 ½
Equilibrium (1), first series	CuCl + $C_2H_2(CuCl)_3$	c 1	453.2		(337.1)	17
		2	422.2		(309.6)	1 ½
		3	390.8	25.0	306.1	1
		4	359.1		305.6	1 ½
		5	391.8		306.4	1 ½
Equilibrium (1), second series	CuCl + $C_2H_2(CuCl)_3$	d 1	764.5		(336.8)	16
		2	734.3		(309.0)	1 ½
		3	703.4		304.7	1 ½
		4	673.8	25.0	306.2	4
		5	642.2		304.3	4
		6	673.3		309.4	4
Absorption after complete conversion to C_2H_2 $(CuCl)_3$	$C_2H_2(CuCl)_3$	e 1	881.5		767.4	90
		2	846.6		418.7	1
		3	861.0	25.0	556.6	1
		4	883.0		772.6	1
	$C_2H_2(CuCl)_3$	f 1	887.2		682.4	16
		2	853.2		378.4	1
		3	877.4	10.0	592.1	1
		4	893.7		738.2	1
		5	920.2		970.7	1
Equilibrium (3), first series	$C_2H_2(CuCl)_3$ + $C_2H_2(CuCl)_2$	g 1	1038.4		(409.5)	30
		2	1001.3	10.0	383.4	50
		3	965.2		378.7	50
		4	996.5		406.8	50

Stage of experiment	Solid phase	No.	V_f	Temp. °C	p_t	Time hours
Equilibrium (3), second series	$C_2H_2(CuCl)_3$ + $C_2H_2(CuCl)_2$	h 1	1181.9	10.0	(445.3)	40
		2	1149.5		380.0	50
		3	1120.1		373.8	40
		4	1085.9		367.5	50
		5	1115.7		396.9	50
Absorption after complete conversion to $C_2H_2(CuCl)_2$	$C_2H_2(CuCl)_2$	i 1	1286.8	10.0	834.2	75
		2	1252.0		498.1	1
		3	1263.5		603.4	1
		4	1277.2		731.5	1
		5	1295.0		896.3	1
	$C_2H_2(CuCl)_2$	k 1	1290.9	25.0	967.4	16
		2	1282.2		875.2	1
		3	1270.8		757.7	1
	$C_2H_2(CuCl)_2$	m 1	1298.2	10.0	886.2	14
		2	1284.4		752.9	1

read are then reduced to dry gas, the mixture being regarded as ideal. The partial pressure of the water vapour is computed from the vapour pressure of the solution, taking into consideration the compression which takes place before the reading of the burette. The total pressure p_t is obtained from the barometer pressure and the manometer readings. In the last column of the table, the time which has passed between the alteration in V_f (addition or removal) and the reading of the p_t-value in the preceding column is given; the times are approximate.

Charge: Chemicals, *cf.* paper II of this series [3], p. 428. The reaction flask is charged with coarse crystalline cuprous chloride and is evacuated to about 0.01 mm with shaking. Oxygen-free nitrogen is then introduced to a pressure of approximately 50 mm. A mixture of acids of the composition stated is made from 4 molal stock solutions; dissolved oxygen is removed by stripping with nitrogen in a burette device *e*, provided with a fritted disc *f* for gas distribution and a liquid seal at the top. The stopcock of this burette is connected to the stopcock *6*. The desired amount is measured volumetrically; the density of the solution is determined separately.

Determination of the vapour pressure: The system is evacuated through stopcock *6*. To avoid unnecessary evaporation a closed vessel *h* is employed — volume about 5 times that of the reaction flask — and is alternatively connected to the flask and the vacuum pump. After five evacuations the pressure becomes constant. The splash trap *g* is then flushed with water; titration with NaOH shows that splash losses of acid are negligible. Nitrogen remaining in the manometer duct is removed by repeated evacuations of the tubing between *3* and *5*, through *4*. The temperature of the glass tubes is high enough to prevent condensation.

The acetylene absorption before the formation of a solid addition compound is measured by adding acetylene with brisk shaking of the reaction flask. Equilibrium is rapidly attained and a constant pressure reached after about 5 min. As an example, the pressure-time curve of mom. *a 5* is reproduced in Fig. 2 : I.

Overall check of the pressure constancy of the system is inserted in mom. *a 2*. After 15 min, p_t equals 276.2 and 24 hours later 275.1 mm. This small change may depend on a slow irreversible acetylene reaction and / or deficiencies in the apparatus. The pressure constancy can be regarded as better than 0.05 mm / h.

Equilibrium (2), metastable. After *a 6*, one more portion of acetylene is added and the pressure rises to 970 mm. This pressure remains constant for about 15 min and then suddenly drops when crystallization of addition compound begins. Simultaneously the solution becomes turbid. After 10 min the pressure has sunk to 500 mm,

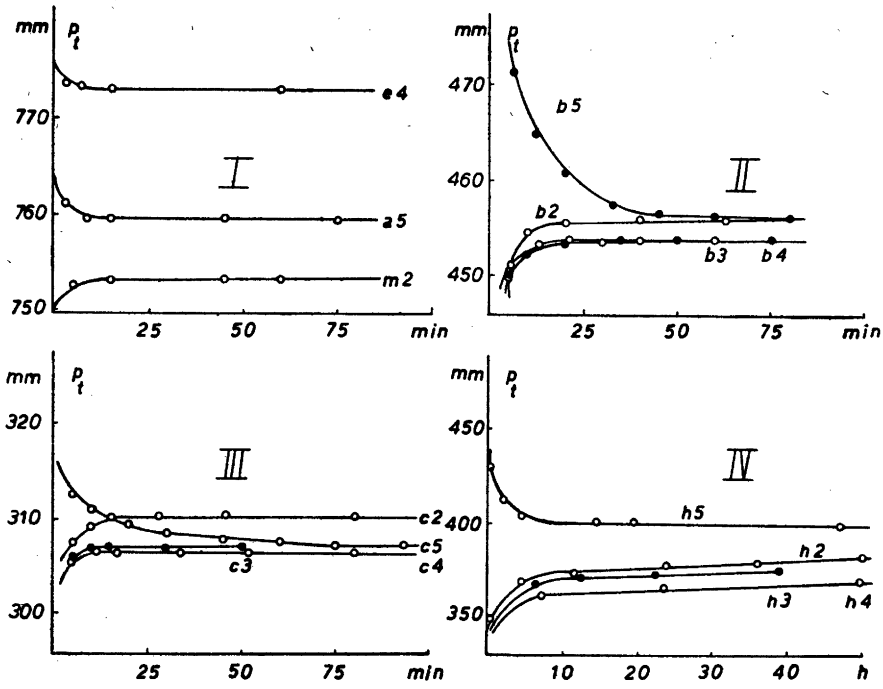

Fig. 2. Pressure-time curves from the type experiment.

I. *One single solid phase*
- *a 5:* CuCl 25.0° C
- *e 4:* $C_2H_2(CuCl)_2$ 25.0° C
- *m 2:* $C_2H_2(CuCl)_2$ 10.0° C

II. *Solid phases:* CuCl *and* $C_2H_2(CuCl)_2$ — *metastable equilibrium* — *25.0°* C
- *b 2—4 after successive removals of acetylene*
- *b 5 after addition of acetylene*

III. *Solid phases:* CuCl *and* $C_2H_2(CuCl)_2$; *25.0° C*
- *c 2—4 after successive removals*
- *c 5 after addition*

IV. *Solid phases:* $C_2H_2(CuCl)_2$ *and* $C_2H_2(CuCl)_2$; *10.0° C*
- *h 2—4 after successive removals*
- *h 5 after addition*

and after 20 min to 490. The pressure decrease then slows down and after another half hour the pressure still exceeds 480 mm. This shows — according to earlier experiments — that the compound formed is $C_2H_2(CuCl)_2$ and that the system is still supersaturated with respect to $C_2H_2(CuCl)_2$. More acetylene is added before recording the time curve *b 1*; during this operation the pressure is not allowed to rise above 550 mm. — Then follows three withdrawals, *b 2, 3* and *4*. In each step the main portion is sucked out during a period of about 20 min at 425 mm, a pressure which will then be the initial one for these three time curves. Finally there is a small addition in mom. *b 5*. The main part is added during 10 minutes at 500 mm, which is the starting point for the time-curve. For this addition, the same gas as has been sucked out in *b 4* is used. The time curves of Fig. 2 : II show that equilibrium is reached within about 30 minutes after decomposition, after synthesis this requires about 60 min. In the time curve *b 5*, there is one more reading after 4 hours and the pressure still remains unchanged.

After *b 5*, a further portion of acetylene is added at about 550 mm. The pressure then suddenly plunges, indicating that $C_2H_2(CuCl)_2$ begins to crystallize and that the previously formed $C_2H_2(CuCl)_2$ disappears.

Equilibrium (1), first series comprises one larger addition *c 1*, three evacuations *c 2, 3, 4* which are performed at 275 mm (10 – 15 min), and finally an addition, *c 5*, at 350 (10 min) of gas which was sucked out in mom. *c 4*. The time curves starting from these two pressures are given in Fig. 2 : III and show that equilibrium is reached after about 20 min (decomposition) and 60 min (synthesis) respectively.

Equilibrium (1), second series is performed in the same manner, but includes one more withdrawal. The time curves are of the same type as in the *c*-series.

Absorption after complete conversion to $C_2H_2(CuCl)_2$. To ensure complete conversion of the cuprous chloride, the pressure is kept at 760 mm during 90 hours of shaking. The acetylene absorption, however, comes to a standstill at an earlier stage, and for the last 24 hours of *e 1* no acetylene absorption of importance is observed. To remove gas impurities an evacuation *e 2* is inserted, when the pressure is lowered to just above the dissociation point. Then follows four small additions, *e 3, 4, 5, 6*. The time curves *e 2 – 6* reach equilibrium rapidly — see Fig. 2 : I where values from *e 4* are used as examples. An analogous series *f 1 – 5* is performed at 10.0° C, as a basis for the measurements at this temperature.

Equilibrium (3), first series. Towards the end of period *f 5*, after 1 h shaking at 970 mm, crystallization of $C_2H_2(CuCl)_2$ begins, causing a rapid pressure drop and a turbidity in the solution. The series comprises one larger addition *g 1*, two removals *g 2* and *3* at 300 mm, and a small addition *g 4* at 400 mm. Initially the equilibrium seems to be approached rapidly, but the reaction then proceeds very slowly and when the readings are interrupted after 50 hours, equilibrium has not yet been reached. The reaction velocity, however, decreases so much that a prolongation of the experiment becomes impracticable.

Equilibrium (3), second series. Identical with the first series but with one more withdrawal; time curves in Fig. 2 : IV.

Absorption after complete conversion to $C_2H_2(CuCl)_2$. Complete conversion is effected by 75 hours shaking at about 850 mm; after 20 hours there is no absorption of importance. Then follows removal *i 2* and three small additions *i 3, 4* and *5*. Some measurements — *k 1, 2* and *3* — of the acetylene absorption at 25° in this system are inserted; in *k 3*, at a pressure of 750 mm, a minor pressure increase is observed after 1 ½ h, indicating a commencing of the re-formation of $C_2H_2(CuCl)_2$. The experiment is concluded by some further readings at 10°, *m 1* and *m 2*. In *m 1*, the re-formed amount of $C_2H_2(CuCl)_2$ is again dissolved by 45 h shaking at about 950 mm.

All time curves in the series *i*, *k* and *m* (except *i 1* and *m 1*) reach equilibrium rapidly. *Cf.* Fig. 2 : I where curve *m 2* is given as an example.

The total duration of the experiment was 41 days.

Calculations: The partial pressure of acetylene — and nitrogen — is calculated as the difference between the measured total pressure, p_t, and the vapour pressure of the liquid at the prevailing temperature, as determined in stages *Ev 1* and *2*.

The acetylene content of the free gas volume is calculated according to $V_g = p \cdot Ø$ where p is the partial pressure (in mm) and the constant Ø is determined by nitrogen calibration in stage N_2 *1 – 4*, where $V_f = V_g$. The four measurements give Ø = (0.065), 0.0654, 0.0653, 0.0653, mean 0.0653 at 25°. At 10° the computed value Ø = 0.0688 has been used. The same Ø-values can be used for acetylene — no correction for the compressibility is required.

From the difference $V_f - V_g$, the total numbers of acetylene millimoles in the liquid and solid phases are calculated, considering the non-ideal mole volume of acetylene at 21° and 1 atm, *cf.* p. 536.

The results calculated are found in Tables 2 A, 3 A and 4 A.

THE REACTION: $C_2H_2 + 3\ CuCl\ (s) = C_2H_2\ (CuCl)_2\ (s)$

The measurements which have bearing upon this reaction are experimentally very satisfactory. The equilibrium is reached quickly and the agreement between the values for synthesis and decomposition is good. Also,

Table 2 A. $CuCl(s)-C_2H_2(CuCl)_2(s)$; reaction medium 0.865 m HCl, 3.135 m $HClO_4$; 25.0° C.

System: 100.4 millimoles CuCl
18.89 g acid mixture with 14.03 g water

No.		C_2H_2 total	P_A atm	Liquid phase C_2H_2	Liquid phase CuCl	Solid phase $C_2H_2/(CuCl)_6$	Solid phase Substance
c	1	18.04	(0.4176)	0.81	1.37	1.04	
	2	16.83	(0.3815)	0.74	1.32	0.97	CuCl +
	3	15.53	0.3769	0.73	1.32	0.90	$C_2H_2(CuCl)_2$
	4	14.20	0.3762	0.73	1.32	0.82	
	5	15.57	0.3773	0.73	1.32	0.90	
d	1	31.04	(0.4172)	0.81	1.37	1.83	
	2	29.85	(0.3807)	0.74	1.32	1.76	
	3	28.58	0.3750	0.73	1.32	1.69	CuCl +
	4	27.34	0.3770	0.73	1.32	1.61	$C_2H_2(CuCl)_2$
	5	26.02	0.3745	0.72	1.31	1.53	
	6	27.30	0.3812	0.74	1.32	1.61	
e	1	34.74	0.9827	1.56	1.51	2.013	
	2	34.24	0.5248	0.94	1.34	2.016	$C_2H_2(CuCl)_2$
	3	34.46	0.7058	1.25	1.45	2.015	
	4	34.79	0.9895	1.71	1.65	2.011	

Experimental data see Table 1

it is easy to achieve complete conversion of all solid cuprous chloride for the determination of the composition of the addition compound.

Table 2 A contains the results of the type experiment. Under the heading, C_2H_2, in the second column, the total quantity of acetylene in the liquid and solid phases is given and, in the third column, the equilibrium pressure P_A is given in atm. Pressure values which must be rejected because of the presence of gas impurities are bracketed. The third and fourth columns contain the calculated values for the amounts of acetylene and cuprous chloride in the liquid phase. (It should be observed that all quantities are totals — in millimoles — not concentrations.) In the last column is found the calculated composition of the precipitate, expressed by the quotient $C_2H_2/(CuCl)_6$, which should approach 2, when the reaction goes towards complete conversion into $C_2H_2(CuCl)_2$.

Other experiments on this reaction at 25.0° are similarily accounted for in Tables 2 B—E; in Table 2 F are summed up the results from an earlier series at 20.0° C. — The synthesis values *e 5* and *f 5* in Table 2 B had to be rejected, as marked by the parentheses, since the time of reaction was too short; the pressures were still decreasing. — In Table 2 C no information is given concerning the composition of the liquid phase because the constants of formulae (5)—(7) have not been determined. The value stated in the table for the quotient $C_2H_2/(CuCl)_6$ refers to the entire system, including both liquid and solid phases. — In the experiment of Table 2 F (20.0° C) there is only one acetylene withdrawal in each series. The amount sucked out is however fairly large and the venting of foreign gas components is as good as in other experiments.

Table 2 B. $CuCl(s)-C_2H_2(CuCl)_3(s)$, reaction medium 0.2 m HCl, 3.8 m $HClO_4$; 25.0° C.

System: 101.0 millimoles CuCl
11.98 g acid mixture with 8.62 g water

No.		C_2H_2 total	P_A atm	Liquid phase C_2H_2	Liquid phase CuCl	Solid phase $C_2H_2/(CuCl)_6$	Solid phase Substance
a	1	0.11	0.1891	0.14	—	0.000	CuCl
	2	0.28	.4036	.29	—	.000	
	3	0.49	.7033	.50	—	.000	
b	1	8.73	(0.4053)	0.29	0.20	0.50	
	2	7.51	(.3807)	.27	.19	.43	CuCl +
	3	6.47	.3776	.27	.19	.37	$C_2H_2(CuCl)_3$
	4	7.80	.3796	.27	.19	.44	
c	1	15.58	(0.4075)	0.29	0.20	0.91	
	2	14.42	(.3808)	.27	.19	.84	
	3	13.88	.3779	.27	.19	.81	CuCl +
	4	12.57	.3761	.27	.19	.73	$C_2H_2(CuCl)_3$
	5	11.27	.3766	.27	.19	.66	
	6	12.30	.3789	.27	.19	.72	
d	1	20.20	(0.3979)	0.29	0.20	1.18	
	2	19.01	(.3792)	.27	.19	1.11	CuCl +
	3	17.73	.3764	.27	.19	1.04	$C_2H_2(CuCl)_3$
	4	16.44	.3758	.27	.19	0.96	
	5	17.52	.3808	.27	.19	1.03	
e	1	25.41	(0.4026)	0.29	0.20	1.50	
	2	24.16	(.3802)	.27	.19	1.42	CuCl +
	3	22.88	.3786	.27	.19	1.35	$C_2H_2(CuCl)_3$
	4	21.55	.3787	.27	.19	1.27	
	5	22.46	(.3863)	.28	.19	1.32	
f	1	31.87	(0.4160)	0.30	0.20	1.89	
	2	30.78	(.3853)	.28	.19	1.81	CuCl +
	3	29.50	.3807	.27	.19	1.74	$C_2H_2(CuCl)_3$
	4	28.24	.3794	.27	.19	1.66	
	5	29.49	(.3857)	.28	.19	1.74	
g	1	33.74	0.9080	0.59	0.26	1.976	
	2	33.53	.4524	.32	.20	1.977	$C_2H_2(CuCl)_3$
	3	33.73	.8602	.56	.25	1.977	

In Tables 2 B, D and E the acetylene absorption b e f o r e the crystallization of the addition compound has been accounted for and the results are found in the *a*-series of the tables. During this stage all acetylene absorbed should remain in the liquid phase. The good agreement between the values in the second and fourth columns confirms equation (5) and its constants.

The equilibrium pressure at 25.0° is independent of the ratio between the solid phases and independent of the composition of the reaction medium, as is shown by an inspection of Tables 2. In the dispersion diagram of Fig. *3 a*, all accepted measurements are given. There is a significant difference between the means for decomposition, 0.3769 ± 0.0003 atm, and synthesis, 0.3785 ± 0.0005 atm. During the synthesis experiments there is a risk

Table 2 C. $CuCl(s) - C_2H_2(CuCl)_2$ (s); reaction medium 0.30 m HCl, 5.70 m $HClO_4$; 25.0° C.

System: 93.60 millimoles CuCl
12.26 g acid mixture

No.		C_2H_2 total	P_A atm	$C_2H_2/(CuCl)_2$ total	Substance
b	1	9.66	(0.4205)	0.62	
	2	8.74	(0.3812)	0.54	CuCl +
	3	7.52	0.3775	0.48	$C_2H_2(CuCl)_2$
	4	6.60	0.3775	0.42	
	5	7.76	0.3786	0.50	
c	1	19.12	(0.3856)	1.23	
	2	18.16	(0.3782)	1.16	CuCl +
	3	17.10	0.3774	1.10	$C_2H_2(CuCl)_2$
	4	15.97	0.3762	1.02	
	5	17.52	0.3783	1.12	
d	1	17.48	(0.4187)	1.76	CuCl +
	2	26.55	(0.3853)	1.70	
	3	25.30	0.3773	1.62	$C_2H_2(CuCl)_2$
	4	24.12	0.3779	1.55	
	5	25.01	0.3790	1.60	

for small amounts of gas impurities in some of the experiments and the decomposition values should thus be given preference, But, mainly, the difference seems to be real, the dissimilar shapes of the pressure-time curves of Fig. 2 : III

Table 2 D. $CuCl(s) - C_2H_2(CuCl)_2(s)$; reaction medium 2 m HCl; 25.0° C.

System: 95.5 millimoles CuCl
12.55 g acid with 11.70 g water

No.		C_2H_2 total	P_A atm	Liquid phase C_2H_2	Liquid phase CuCl	Solid phase $C_2H_2/(CuCl)_2$	Solid phase Substance
a	1	0.33	0.1187	0.33	—	0.00	
	2	0.94	0.3309	0.92	—	.00	
	3	1.79	0.6385	1.78	—	.00	CuCl
	4	2.54	0.9031	2.52	—	.00	
	5	2.95	1.0504	2.93		.00	
b	1	18.54	(0.3808)	1.06	3.52	1.14	CuCl +
	2	17.44	0.3749	1.04	3.50	1.07	
	3	18.31	0.3769	1.05	3.51	1.13	$C_2H_2(CuCl)_2$
c	1	28.25	(0.3829)	1.07	3.52	1.77	CuCl +
	2	27.14	0.3778	1.05	3.51	1.70	
	3	26.03	0.3774	1.05	3.51	1.63	$C_2H_2(CuCl)_2$
	4	27.01	0.3785	1.05	3.51	1.69	
d	1	32.46	0.9497	1.83	3.06	1.981	
	2	31.75	0.5297	1.28	3.27	1.982	
	3	32.07	0.7183	1.54	3.13	1.982	$C_2H_2(CuCl)_2$
	4	32.27	0.8445	1.71	3.09	1.984	
	5	32.44	0.9526	1.83	3.06	1.988	

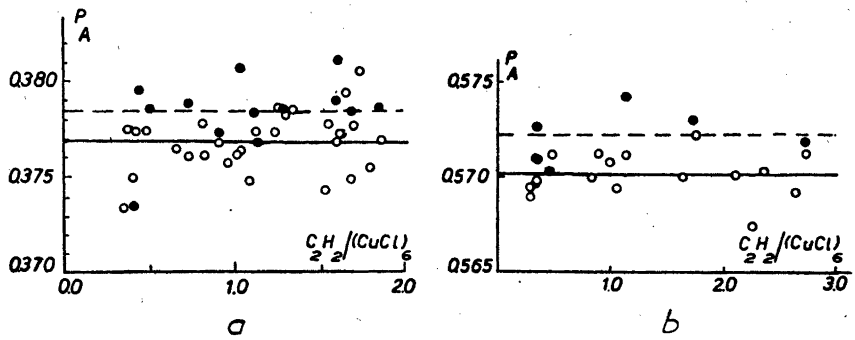

Fig. 3. Dispersion diagrams for the measurements of the equilibrium pressures P_a and P_b at 25.0° C.

● *After synthesis of addition compound*
○ *After decomposition*
a) $C_2H_2 + 3\ CuCl\ (s) = C_2H_2(CuCl)_2\ (s)$ P_a
b) $C_2H_2 + 2\ CuCl\ (s) = C_2H_2(CuCl)_2\ (s)$ P_b

also favours this assumption. Probably there are minor variations in the activity of the solid substance. The accepted value for the equilibrium pressure is:

$$P_3 = 0.377 \pm 0.001 \text{ atm.} \qquad \text{(at 25.0° C)}$$

Table 2 E. CuCl (s) + $C_2H_2(CuCl)_2$ (s); reaction medium 4 m HCl; 25° C.

System: 109.62 millimoles CuCl
14.38 g acid with 12.55 g water

No.	C_2H_2 total	P_A atm	Liquid phase C_2H_2	Liquid phase CuCl	Solid phase $C_2H_2/(CuCl)_6$	Solid phase Substance
a 1	0.60	0.1255	0.61	—	0.00	
2	1.35	0.2762	1.34	—	.00	
3	3.69	0.7534	3.67	—	.00	CuCl
4	5.24	1.1064	5.19	—	.00	
b 1	9.48	(0.3942)	1.92	11.10	0.46	CuCl +
2	7.89	0.3750	1.82	10.97	0.40	
3	7.45	0.3735	1.82	10.97	0.34	$C_2H_2(CuCl)_2$
4	8.52	0.3736	1.82	10.97	0.41	
c 1	24.76	(0.4082)	1.99	11.12	1.38	CuCl +
2	23.12	0.3782	1.84	10.99	1.30	
3	22.30	0.3774	1.84	10.98	1.24	$C_2H_2(CuCl)_2$
4	23.05	0.3786	1.85	10.99	1.29	
d 1	33.81	(0.4174)	2.03	11.16	1.93	
2	32.60	0.3770	1.83	10.98	1.87	CuCl +
3	31.44	0.3756	1.83	10.98	1.80	$C_2H_2(CuCl)_2$
4	32.39	0.3787	1.85	10.99	1.86	
e 1	37.11	1.1057	3.82	9.92	2.003	
2	35.30	0.4654	2.14	10.62	2.010	$C_2H_2(CuCl)_2$
3	35.84	0.6469	2.70	10.18	1.998	
4	36.73	0.9391	3.41	9.94	2.006	
5	37.18	1.1099	3.92	9.92	2.001	

Table 2 F. CuCl (s) + $C_2H_2(CuCl)_2$ (s); reaction medium 2 m HCl; 20.0° C.

System: 131.6 millimoles CuCl
a—c 6.895 g acid with 6.43 g water
d—f 12.05 g acid with 11.23 g water

No.	C_2H_2 total	P_A atm	Liquid phase C_2H_2	Liquid phase CuCl	Solid phase $C_2H_2/(CuCl)_6$	Solid phase Substance
b 1	11.78	(0.2864)	0.54	1.76	0.52	CuCl +
2	10.05	0.2653	0.47	1.73	0.44	$C_2H_2(CuCl)_2$
3	11.36	0.2676	0.48	1.73	0.50	
c 1	18.44	(0.2842)	0.51	1.76	0.83	CuCl +
2	16.75	0.2650	0.47	1.73	0.75	
3	17.83	0.2677	0.48	1.73	0.80	$C_2H_2(CuCl)_2$
d 1	25.28	(0.2842)	0.51	1.76	1.14	CuCl +
2	20.67	0.2637	0.47	1.73	0.93	$C_2H_2(CuCl)_2$
e 1	33.96	(0.2893)	0.90	3.09	1.54	CuCl +
2	32.08	0.2671	0.83	3.03	1.46	
3	32.97	0.2718	0.85	3.05	1.50	$C_2H_2(CuCl)_2$
f 1	44.76	0.6764	1.64	3.01	2.012	
2	43.37	0.3035	0.92	3.00	1.981	
3	43.85	0.4129	1.14	2.95	1.993	
4	44.27	0.5538	1.42	2.97	2.000	
5	44.35	0.6848	1.65	3.02	1.993	$C_2H_2(CuCl)_2$
6	44.48	0.8196	1.89	3.09	1.988	
7	44.61	0.9528	2.11	3.17	1.986	
8	44.86	1.0803	2.31	3.25	1.990	
9	45.02	1.1608	2.44	3.30	1.992	
10	45.13	1.2419	2.56	3.35	1.992	

The composition of the liquid phase has been calculated according to (5) — (7), using the constants $a = 0.0429$; $(A + B \cdot HCl_t) = 0.235$; $S = 0.207$

In the synthesis experiments of Österlöf [1], a pressure of 0.62 atm was required to effect a conversion of CuCl into $C_2H_2(CuCl)_2$ within 24 h at 25° C, which can easily be explained by supersaturation. Decomposition is said to take place at 0.48 atm, and this value is derived from two experiments where the complex $C_2H_2(CuCl)_2$ is decomposed at 0.92 and 0.48 atm, $C_2H_2(CuCl)_2$ and CuCl respectively resulting. In the CuCl-case a stepwise decomposition was assumed. But even 0.48 atm is considerably higher than the equilibrium pressure. A reasonable explanation seems to be that a direct decomposition has occurred, according to reaction (2) whose equilibrium pressure is 0.57 atm.

The temperature dependence of the equilibrium pressure has been determined in an experiment illustrated by Fig. 4. (The measurements are part of the experiment from which the data of Table 2 F have been taken.) The experiment was performed by varying the temperature without any change in the composition of the system, and has been supplemented by determinations of the vapour pressure of the solvent at different temperatures. The points in the diagram have been numbered according to the experiment; the agreement between the rising and falling series is good. The linear relation can be given by the formula:

$$\log P_3 = \log 0.377 - 2.68 \cdot 10^3 \cdot \left(\frac{1}{T} - \frac{1}{298.2}\right) \qquad (8)$$

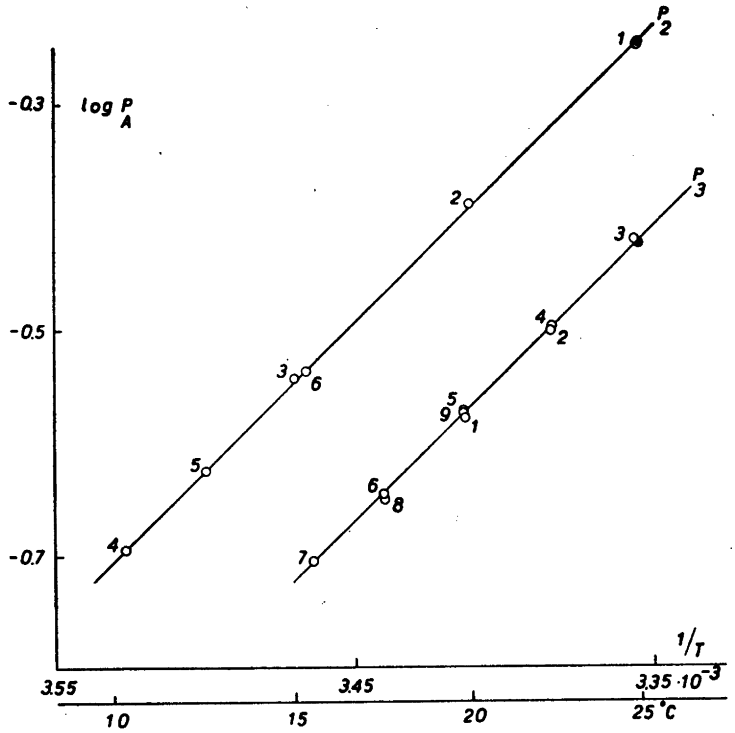

Fig. 4. Temperature dependence of the equilibrium pressures.

P_2: *Reaction* $C_2H_2 + 3$ CuCl (s) = $C_2H_2(CuCl)_2$ (s); *reaction mixture as in Table 2 F;* $C_2H_2/(CuCl)_6$ *for solid phases about 0.8.*

P_3: *Reaction* $C_2H_2 + 2$ CuCl (s) = $C_2H_2(CuCl)_2$ (s) *reaction mixture as in Table 3 C;* $C_2H_2/(CuCl)_6$ *for solid phases about 0.8.*

The dots ● correspond to mean values for other measurements at 25.0° C.

The first term in this expression corresponds to the mean stated above for all measurements at 25.0° while the temperature coefficient has been obtained graphically from the diagram.

The composition of the complex formed is given by the quotient, $C_2H_2/(CuCl)_6$, which after complete conversion reaches values very close to the theoretical one, *i.e.* 2.000. The composition of the addition compound is established with an accuracy better than 1 %.

The coinciding equilibrium pressures of experiments 2 A and 2 B show that hydrochloric acid does not enter into the compound. The reaction medium consists of 4 m mixtures of hydrochloric and perchloric acid, with a hydrochloric acid concentration of 0.20 m (2 B) and 0.87 m (2 A). A comparison between experiments 2 D and E, where the medium consists of hydrochloric acid of different concentrations, gives the same result. Water, also, can be excluded, as appears very clearly from a comparison between Tables 2 C and 2 D where the media are 0.3 m HCl—5.7 m $HClO_4$, and 2 m HCl solely. The



a b c d e

Fig. 5. Crystals of $C_2H_2(CuCl)_3$, CuCl *and* $C_2C_2(CuCl)_2$.
a) $C_2H_2(CuCl)_3$, *freshly formed, 185* ×
b) —»— *after 24 h undisturbed, growth, 90* ×
c) —»— *after equilibrium experiment with prolonged brisk shaking, 45* ×
d) CuCl, *formed by decomposition of* $C_2H_2(CuCl)_2$, *50* ×
e) $C_2H_2(CuCl)_2$, *freshly formed, 60* ×

activity of H_2O is approximately 20 % less in the firstmentioned system, but the equilibrium pressures agree within 0.5 %; consequently the compound formed does not contain water, anyway not in an appreciable molar amount.

A microscopical examination shows that the addition compound $C_2H_2(CuCl)_3$ is well crystallized, but after an equilibrium experiment the crystals have been rather abraded by the shaking — *cf.* Fig. 5 c. In experiments without or with only slight shaking, the freshly formed crystals are as a rule thin six-sided plates with edge angles of nearly 60° — *cf.* Fig. 5 a. They show, however, a double refraction (with extinction parallel to the long edge) even under perpendicular light and thus are only pseudo-hexagonal. After about 25 h the plates grow thicker and assume a shape — *cf.* Fig. 5 b — which makes an orthorhombical symmetry seem most likely.

Earlier powder X-ray photograms [1], indicating a preservation of the CuCl-structure in $C_2H_2(CuCl)_3$ must be misleading, since optical double refraction is incompatible with cubic symmetry. Probably a surface layer of cuprous chloride has formed during the exposure. Later x-ray investigations by another technique have shown interferences different from those of CuCl.

More complete information on crystallographic data will be given in a separate paper.

THE REACTION: $C_2H_2 + 2\ CuCl\ (s) = C_2H_2\ (CuCl)_2\ (s)$

The direct conversion of cuprous chloride into the complex $C_2H_2(CuCl)_2$ has occurred spontaneously in most of the experiments. Only in the experiment corresponding to Table 3 C was the reaction initiated by seeding which was effected by adding a small amount of acid mixture containing a suspension of $C_2H_2(CuCl)_2$, prepared in a stepwise synthesis according to reactions (1) and (3). The addition causes a rapid pressure drop from the original 0.80 atm to the pressure of *b 1* 0.57 atm.

Generally the measurements pertaining to this reaction are interrupted by crystallization of the stable complex, $C_2H_2(CuCl)_3$. Often the supersaturation

Table 3 A. CuCl(s) — $C_2H_2(CuCl)_2(s)$, reaction medium 0.865 m HCl, 3.135 m $HClO_4$; 25.0° C.

System: 100.4 millimoles CuCl
18.89 g acid mixture with 14.03 g water

No.	C_2H_2 total	P_A atm	Liquid phase C_2H_2	Liquid phase CuCl	Solid phase $C_2H_2/(CuCl)_6$	Solid phase Substance
a 1	0.25	0.1315	0.25	—	0.00	
2	0.66	0.3363	0.65	—	.00	
3	1.22	0.6269	1.21	—	.00	CuCl
4	1.53	0.7862	1.52	—	.00	
5	1.89	0.9715	1.88	—	.00	
6	2.10	1.0754	2.08	—	.00	
b 1	9.88	(0.6116)	1.18	1.61	0.53	
2	8.49	(0.5726)	1.11	1.56	0.45	CuCl +
3	7.09	0.5698	1.10	1.56	0.36	$C_2H_2(CuCl)_2$
4	5.72	0.5697	1.10	1.56	0.28	
5	6.99	0.5727	1.11	1.56	0.36	

Experimental data see Table 1.

ceased before it was possible to take any readings. One then only observes that the falling pressure-time curve pauses for a longer or shorter period at 0.57—0.60 atm (25.0° C) and then drops to the vicinity of 0.377 atm, *i.e.* the equilibrium pressure for reaction (1). The crystallization of $C_2H_2(CuCl)_3$ is usually initiated by the momentary pressure increase upon the addition of acetylene, but it has occurred also that the supersaturation has ceased on de-

Table 3 B. CuCl(s) — $C_2H_2(CuCl)_2(s)$, reaction medium 0.2 m HCl, 3.8 m $HClO_4$; 25.0° C.

System: 73.18 millimoles CuCl
10.62 g acid mixture with 7.65 g water

No.	C_2H_2 total	P_A atm	Liquid phase C_2H_2	Liquid phase CuCl	Solid phase $C_2H_2/(CuCl)_6$	Solid phase Substance
a 1	0.11	0.1766	0.11	—	0.00	
2	0.23	0.3551	0.23	—	.00	CuCl
3	0.43	0.6954	0.44	—	.00	
4	0.75	1.1782	0.75	—	.00	
b 1	7.43	(0.6140)	0.39	0.91	0.58	CuCl +
c 1	27.20	(0.6321)	0.40	0.92	2.23	$C_2H_2(CuCl)_2$
2	25.95	0.5702	0.36	0.90	2.12	
d 1	34.83	(0.6123)	0.39	0.91	2.86	
2	33.41	0.5712	0.36	0.90	2.74	CuCl +
3	32.22	0.5692	0.36	0.90	2.64	$C_2H_2(CuCl)_2$
4	33.05	0.5718	0.36	0.90	2.71	
e 1	36.67	1.1869	0.67	0.73	2.980	
2	36.35	0.6437	0.40	0.86	2.983	$C_2H_2(CuCl)_2$
3	36.41	0.7395	0.44	0.82	2.983	
4	36.55	0.9928	0.57	0.76	2.981	

Table 3 C. CuCl (s)—$C_2H_2(CuCl)_2$ (s), reaction medium 0.30 m HCl, 5.70 m $HClO_4$; 25.0° C.

System: 90.23 millimoles CuCl
10.77 g acid mixture

No.	C_2H_2 total	P_A atm	$C_2H_2/(CuCl)_6$ total	Substance
b 1	6.36	(0.5733)	0.42	CuCl + $C_2H_2(CuCl)_2$
2	5.22	0.5708	0.35	
3	4.15	0.5690	0.28	
4	5.41	0.5710	0.36	
c 1	14.65	(0.6203)	0.97	CuCl + $C_2H_2(CuCl)_2$
2	13.52	0.5712	0.90	
3	12.38	0.5700	0.82	

Table 3 D. CuCl (s)—$C_2H_2(CuCl)_2$ (s), reaction medium 2 m HCl; 25.0° C.

System: 53.18 millimoles CuCl
8.69 g acid with 8.10 g water

No.	C_2H_2 total	P_A atm	Liquid phase C_2H_2	Liquid phase CuCl	Solid phase $C_2H_2/(CuCl)_6$	Solid phase Substance
b 1	6.30	(0.6008)	1.15	2.78	0.61	CuCl + $C_2H_2(CuCl)_2$
2	5.14	0.5712	1.09	2.74	0.48	
3	3.93	0.5697	1.09	2.73	0.34	
4	4.99	0.5704	1.09	2.74	0.46	
c 1	11.07	(0.6112	1.17	2.80	1.18	CuCl + $C_2H_2(CuCl)_2$
2	9.51	0.5709	1.09	2.74	1.00	
d 1	14.83	(0.6103)	1.17	2.80	1.63	
e 1	17.16	(0.6290)	1.20	2.83	1.90	CuCl + $C_2H_2(CuCl)_2$
2	15.93	0.5723	1.10	2.74	1.76	
3	14.69	0.5701	1.09	2.73	1.62	
4	15.63	0.5731	1.09	2.74	1.73	
f 1	27.15	1.1139	1.63	2.58	3.027	$C_2H_2(CuCl)_2$
2	26.62	0.6532	1.18	2.68	3.021	
3	26.86	0.8068	1.35	2.62	3.026	
4	27.02	0.9431	1.48	2.59	3.030	
5	27.21	1.2491	1.74	2.58	3.021	

Table 3 E. CuCl (s)—$C_2H_2(CuCl)_2$ (s), reaction medium 3 m HCl; 25.0° C.

System: 94.13 millimoles CuCl
10.76 g acid with 9.70 g water

No.	C_2H_2 total	P_A atm	Liquid phase C_2H_2	Liquid phase CuCl	Solid phase $C_2H_2/(CuCl)_6$	Solid phase Substance
b 1	19.97	(0.6183)	1.86	6.00	1.23	CuCl + $C_2H_2(CuCl)_2$
2	18.70	0.5711	1.72	5.87	1.15	
3	17.28	0.5694	1.72	5.87	1.06	
4	18.50	0.5742	1.74	5.91	1.14	
c 1	37.63	(0.6210)	1.87	6.00	2.43	CuCl + $C_2H_2(CuCl)_2$
2	36.07	0.5704	1.72	5.87	2.34	
3	34.80	0.5673	1.71	5.85	2.25	

creasing the pressure or after a period when the pressure has been constant for some time. Normally the crystallization of $C_2H_2(CuCl)_3$ causes a pressure drop, but in experiment 3 E we observed the somewhat paradoxical phenomenon that the system shifted to a more stable state with a spontaneous pressure increase. The explanation is that the total amount of acetylene present is so large — $C_2H_2/(CuCl)_6 > 2$; *cf.* Fig 6 — that the formation of $C_2H_2(CuCl)_3$ consumes all CuCl while $C_2H_2(CuCl)_2$ is still present. Thus both complexes will be present as solid phases and the pressure therefore rises towards that value which characterizes equilibrium (3) at 25.0° C.

If one disregards the practical inconveniences arising from the instability of the equilibrium, the measurements on this reaction are experimentally very satisfactory. The equilibria are reached somewhat slower than in reaction (1), but rapidly enough to render disturbances from by-reactions etc. negligible.

In Table 3 A are given the results corresponding to the two first stages of the type experiment. The *a*-series contains values for the acetylene absorption b e f o r e the formation of addition compound; the agreement between the measured absorption (second column) and the absorption calculated according to (5 a) (fourth column) is quite satisfactory also in this case. Series *b* comprises the equilibrium measurements which correspond to the pressure-time curves of Fig. 2 : II. The other experiments, referring to this reaction at 25.0° C, are likewise summarized in Tables 3 B—E; in Table 3 C, however, information is lacking concerning the composition of the liquid phase.

T h e e q u i l i b r i u m p r e s s u r e a t 25.0° C is independent of the ratio between the solid phases and independent of the reaction medium. In Fig. 3 b the accepted measurements are summarized. The means for the decomposition pressures are 0.5702 ± 0.0003 and for the synthesis pressures 0.5722 ± 0.0006. The accepted value for the equilibrium pressure is:

$$P_2 = 0.571 \pm 0.001 \text{ atm} \qquad \text{(at 25.0° C)}$$

T h e t e m p e r a t u r e d e p e n d e n c e o f t h e e q u i l i b r i u m p r e s s u r e has been determined in the experiment illustrated by Fig. 4. The procedure has been the same as described for the corresponding experiment in the preceding section. It is evident that the agreement between the rising and the falling series is satisfactory. After the sixth measurement the experiment is interrupted by crystallization of $C_2H_2(CuCl)_3$. The temperature dependence is expressed by

$$\log P_2 = \log 0.571 - 2.60 \cdot 10^3 \cdot \left(\frac{1}{T} - \frac{1}{298.2}\right) \qquad (9)$$

T h e c o m p o s i t i o n of the complex can be determined after complete conversion, but the synthesis procedure must give evidence that no crystallization of $C_2H_2(CuCl)_3$ has occurred. To achieve this, one should follow the dotted horizontal line of Fig. 6 as far as possible before going over to measurements on the vertical branch. Only in two experiments, Tables 3 B and D, it has been possible to carry the experiment to complete conversion in this way. To diminish the risk of crystallization of $C_2H_2(CuCl)_3$ the duration of the experiment has been kept as short as possible. In order to save time, some series of

readings, *e.g.* *b* and *c* in Table 3 B are incomplete and aim only at checking that supersaturation still exists.

The empirical composition, resulting from these measurements, agrees within 1 % with the theoretical. The fact that the equilibrium pressure is independent of the reaction medium shows that no other components except C_2H_2 and CuCl enter into the compound.

Under the microscope is observed that samples of $C_2H_2(CuCl)_2$ which have not been subjected to violent shaking consist of needle-shaped crystals, double-refracting with parallel extinction — *cf.* Fig. 5 e. They can easily be distinguished from crystals of $C_2H_2(CuCl)_3$, *cf.* Fig. 5 a and b, and from the characteristic single-refracting tetrahedrons of cuprous chloride, *cf.* Fig. 5 d.

THE REACTION: $C_2H_2 + 2\ C_2H_2\ (CuCl)_3\ (s) = 3\ C_2H_2\ (CuCl)_2\ (s)$

When calculating the equilibrium pressure $P_{2,3}$ for this reaction from the values for P_2 and P_3 by inserting (8) and (9) in (4), the following expression results:

$$\log P_{2,3} = \log 1.31 - 2.44 \cdot 10^3 \left(\frac{1}{T} - \frac{1}{298.2}\right) \qquad (10)$$

Applying the earlier stated errors in the P_2- and P_3-values at 25° C, the $P_{2,3}$ value for this temperature will have an accuracy corresponding to 1.31 ± 0.01 atm. Expression (4) presupposes an ideal gas. The deviation of acetylene from ideal compressibility — *cf.* p. 536 — can be accounted for by including in the expression the approximate correction factor $1 + k \cdot (3\ P_2 - 2\ P_3 - P_2^3/P_3^2)$. At 25.0° C the correction amounts to + 0.003 atm and may be neglected.

The direct measurement of $P_{2,3}$ does not give quite satisfactory results. The equilibria are approached substantially slower than in the other two reactions. The time curves of Fig. 2 : IV are typical. (Note that the scales differ from the other diagrams of Fig. 2.) Initially the reaction proceeds quite rapidly, but after 5—10 h a stage enters in which the pressure change takes place with a low but almost constant speed. After successive withdrawals of acetylene time curves are obtained which do not coincide but seem to be parallelly displaced towards lower pressures. (A similar displacement occurs in the other equilibrium measurements as a result of successive removals of gas impurities. In the present case the effect is, however, more pronounced and cannot be attributed to gas impurities.) The parallel displacement need however not indicate that the unattained final values of the curves are different.

One has the impression that the reaction velocity during the later, slow stage of the conversion is particularly low if the duration of the experiment has been very long. (In some prolonged experiments, not described here, the reaction velocities are still lower; also the parallel displacement of the decomposition curves is more marked.) Therefore the duration of the experiments has been limited and the reading of the time curves was in some cases interrupted before the velocity of the pressure change had dropped so much that interferences from by-reactions etc. could be feared.

Table 4 A. $C_2H_2(CuCl)_3(s)$ — $C_2H_2(CuCl)_2(s)$, *reaction medium 0.865 m HCl, 3.135 m* $HClO_4$

System: 100.4 millimoles CuCl
18.89 g acid mixture with 14.03 g water

No.	Temp.	C_2H_2 total	P_A atm	$C_2H_2/(CuCl)_6$ total	Liquid phase C_2H_2	Liquid phase CuCl	Solid phase $C_2H_2/(CuCl)_6$	Solid phase Substance
f 1		35.09	0.8864	2.10	—	—	—	
2		34.54	0.4969	2.06	—	—	—	
3	10.0	34.94	0.7678	2.08	—	—	—	$C_2H_2(CuCl)_3$
4		35.20	0.9597	2.10	—	—	—	
5		35.64	1.2650	2.13	—	—	—	
g 1		42.18	(0.528)	2.52	—	—	—	
2	10.0	40.71	0.494	2.43	—	—	—	$C_2H_2(CuCl)_3$
3		39.21	0.488	2.34	—	—	—	+
4		40.44	0.525	2.41	—	—	—	$C_2H_2(CuCl)_2$
h 1		48.06	(0.575)	2.87	—	—	—	
2		46.90	0.489	2.80	—	—	—	$C_2H_2(CuCl)_3$
3	10.0	45.69	0.481	2.73	—	—	—	+
4		44.28	0.473	2.64	—	—	—	$C_2H_2(CuCl)_2$
5		45.44	0.512	2.71	—	—	—	
i 1		51.32	1.0858	3.06	—	—	—	
2		50.84	0.6444	3.04	—	—	—	
3	10.0	51.01	0.7827	3.05	—	—	—	$C_2H_2(CuCl)_2$
4		51.22	0.9509	3.06	—	—	—	
5		51.49	1.1673	3.08	—	—	—	
k 1		51.29	1.2453	3.06	1.90	1.63	2.999	
2	25.0	51.17	1.1242	3.06	1.77	1.61	2.999	$C_2H_2(CuCl)_2$
3		51.02	0.9699	3.05	1.59	1.58	3.001	
m 1	10.0	51.65	1.1540	3.04	—	—	—	$C_2H_2(CuCl)_2$
2		51.46	0.9790	3.08	—	—	—	

Experimental data see Table 1.

In Table 4 A are given the results from the type experiment. The composition of the system before the lowering of the temperature from 25.0° to 10.0° is evident from Table 2 A, *e 1—4*, which shows that $C_2H_2(CuCl)_3$ is the sole solid phase. A lower temperature is necessary if the crystallization of $C_2H_2(CuCl)_2$ is to be initiated by a moderate acetylene pressure. Data for the calculation of the composition of the liquid phase at 10° are missing; in the fourth column the quotient, $C_2H_2/(CuCl)_6$, is given for the whole system, including both liquid and solid phases. In the equilibrium measurements, moments *g 2—4* and *h 2—5*, the five decomposition pressures vary from 0.473 atm to 0.494 and the two synthesis pressures are 0.512 and 0.525 atm. The pressure which can be calculated from (10) is 0.482 atm.

There is no difference of importance between the *g*- and *h*-series and the experiment thus indicates that the pressure is independent of the molar ratio of the solid phases.

Table 4 B. $C_2H_2(CuCl)_2(s) - C_2H_2(CuCl)_2(s)$, *reaction medium 2 m HCl; 20.0° C.*

System: 131.6 millimoles CuCl
12.05 g acid with 11.23 g water

No.	C_2H_2 total	P_A atm	$C_2H_2/(CuCl)_6$ total	Liquid phase C_2H_2	Liquid phase CuCl	Solid phase $C_2H_2/(CuCl)_6$	Solid phase Substance
g 1	51.5	(1.022)	2.35	—	—	—	$C_2H_2(CuCl)_2$
2	49.3	0.977	2.25	—	—	—	+
3	50.1	1.007	2.29	—	—	—	$C_2H_2(CuCl)_2$
h 1	55.2	(1.011)	2.52	—	—	—	$C_2H_2(CuCl)_2$
2	53.1	0.944	2.42	—	—	—	+
3	54.4	0.974	2.48	—	—	—	$C_2H_2(CuCl)_2$
i 1	56.3	(1.048)	2.57	—	—	—	$C_2H_2(CuCl)_2$
2	53.5	0.960	2.44	—	—	—	+
3	52.6	0.964	2.40	—	—	—	$C_2H_2(CuCl)_2$
4	53.7	1.002	2.45	—	—	—	
k 1	60.5	(1.051)	2.76	—	—	—	$C_2H_2(CuCl)_2$
2	57.9	0.946	2.64	—	—	—	+
3	55.9	0.923	2.55	—	—	—	$C_2H_2(CuCl)_2$
4	57.2	0.967	2.61	—	—	—	
m 1	63.1	(1.035)	2.88	—	—	—	
2	61.5	0.930	2.81	—	—	—	$C_2H_2(CuCl)_2$
3	60.4	0.924	2.76	—	—	—	$C_2H_2(CuCl)_2$
4	60.9	0.972	2.78	—	—	—	
n 1	67.6	1.532	3.09	2.81	3.27	3.03	
2	67.0	0.980	3.06	2.12	3.15	3.03	$C_2H_2(CuCl)_2$
3	67.4	1.364	3.08	2.61	3.22	3.03	

In Table 4 B are found the results from equilibrium measurements at 20.0° with a medium of 2 m hydrochloric acid. The experiment is a continuation of the one included in Table 2 F. Here too the crystallization has been initiated by a pressure rise at a lower temperature, which was then again raised to 20.0° before the measurements. The mean of the decomposition pressures is 0.946 ± 0.008 and the mean of the synthesis pressures 0.984 ± 0.009. It seems permissible to draw the conclusion that the correct equilibrium pressure must lie within the interval 0.96 ± 0.02, which is in good agreement with the value of 0.95 ± 0.01 calculated according to (10).

The composition of the complex formed can be stated without difficulties in this case also. If the pressure is sufficiently increased beyond the equilibrium pressure there is a complete conversion. And in systems with one single solid phase the equilibrium is attained rather quickly after addition and removal of acetylene. In Table 4 A, mom. *i 1—5*, the measurements of the acetylene absorption after complete conversion at 10.0° are given. The temperature is then raised to 25.0° and, from the measurements *k 1—3*, the composition of the solid phase can be computed exactly. The pressure prevailing in the *k*-series is lower than $P_{2,3}$ at 25° and the system thus is supersaturated with regard to $C_2H_2(CuCl)_3$. The circumstance that no crystallization of this substance occurs until the end of moment *k 3* — see end of account of type experiment — further emphasizes that the earlier conversion was complete.

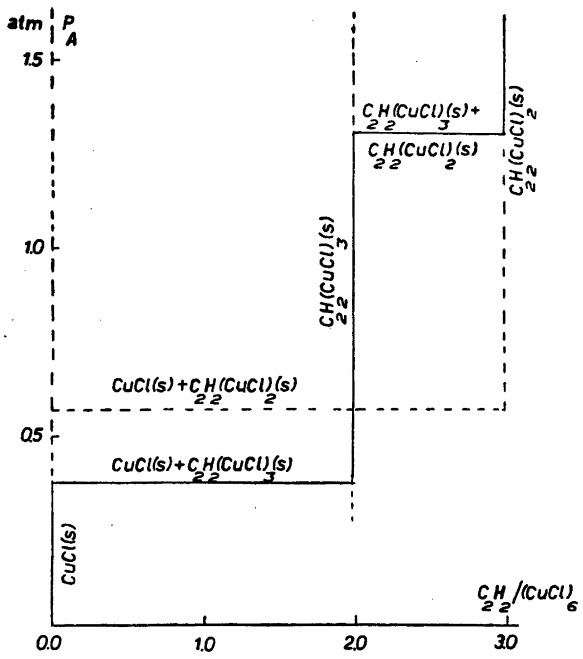

Fig. 6. Phase diagram for the system C_2H_2 + CuCl *at 25.0° C. Dotted lines indicate measurable metastable states.*

The results from this experiment and the corresponding series, *n 1—3*, of Table 4 B show that the composition of the compound formed agrees with $C_2H_2(CuCl)_2$ within 1 %.

THERMODYNAMIC DATA

The formulae (9), (10) and (11) give the following thermodynamic data for the three reactions at 25.0° C.

Acetylene at atmospheric pressure is chosen as the standard state for acetylene.

Reaction	$\Delta G°$ cal	$\Delta H°$ kcal	$\Delta S°$ cal/degree
C_2H_2 + 3 CuCl(s) = $C_2H_2(CuCl)_3$(s)	−575 ± 2	−12.23	−39.1
C_2H_2 + 2 CuCl(s) = $C_2H_2(CuCl)_2$(s)	−330 ± 1	−11.85	−38.6
C_2H_2 + 2 $C_2H_2(CuCl)_3$(s) = 3 $C_2H_2(CuCl)_2$(s)	+160 ± 4	−11.1	−37.7

For the determination of these data, the non-ideal compressibility of acetylene has been corrected for by computation of $\Delta G°_n$ and $\Delta H°_n$ for the first two reactions according to the approximate formulae $\Delta G°_n/RT = \ln P_n + k(P_n - 1)$ and $\Delta H°_n/R = (1 + k \cdot P)_n \cdot d\ln P_n/d(1/T)$ where k, according to p. 536, equals $-7 \cdot 10^{-3}$ (atm)$^{-1}$. However, the corrections amount only to +3 cal for $\Delta G°_2$, +2 cal for $\Delta G°_3$, and −0.03 and −0.05 kcal for the corresponding $\Delta H°$-values and are thus of the same magnitude as the errors in these values. The remaining data are derived from the thus corrected values.

The connection between the three reactions is illustrated by the phase diagram of Fig. 6 which shows the equilibrium pressures at 25.0° C as functions of the composition of the mixture of the solid phases, as expressed by the quotient $C_2H_2/(CuCl)_6$.

The mole entropy S°_{25} for solid cuprous chloride is stated by Watanabe [6] to be 20.66 cal/degree; the best value for acetylene is 48.029, given by Gordon [7]. Using the ΔS° values given above the results are

$$\text{for } C_2H_2(CuCl)_3 \text{ (s)} : S^\circ_{25} = 70.9 \text{ cal/degree}$$
$$\text{for } C_2H_2(CuCl)_2 \text{ (s)} : S^\circ_{25} = 50.7 \text{ cal/degree}$$

SUMMARY

1. The stoichiometrical composition of two solid compounds between acetylene and cuprous chloride has been determined by manometrical experiments, measuring the quantity of acetylene required for a complete conversion of a given amount of cuprous chloride.

The composition agrees within 1 % with the formulae $C_2H_2(CuCl)_3$ and $C_2H_2(CuCl)_2$ respectively, which were suggested by Österlöf in the first paper [1] of this series.

2. The equilibrium pressure for the reaction

$$C_2H_2 + 3\ CuCl\ (s) = C_2H_2(CuCl)_3\ (s)$$

has been determined between 15.0° and 25.0° C and is given by formula (8). Its value at 25.0° is 0.377 ± 0.001 atm.

3. The metastable equilibrium

$$C_2H_2 + 2\ CuCl\ (s) = C_2H_2\ (CuCl)_2\ (s)$$

can be measured with good accuracy, although the system is supersaturated with regard to $C_2H_2(CuCl)_3$ (s). The results at 10.0°—25.0° are given by (9). At 25.0° C the equilibrium pressure is 0.571 ± 0.001 atm.

4. The equilibrium

$$C_2H_2 + 2\ C_2H_2(CuCl)_3\ (s) = 3\ C_2H_2(CuCl)_2\ (s)$$

can be calculated from these data; direct measurements agree with the computed values.

5. Thermodynamic data for the reactions and mole entropies for the solid compounds have been calculated.

6. Both compounds are colourless and crystalline. In contrast with cuprous chloride they show optical double refraction and their structure thus is non-isometric.

This work forms part of a series of investigations which has been supported by grants from *Statens Tekniska Forskningsråd*. The author wishes to express his very best thanks to Professor Arne Ölander and Docent Sven Brohult for their great and unvailing interest. At various periods of the experiments valuable assistance has been received from Claes Löfman and Igor Cedergren, Siv Edelö-Laurent and Karl-Axel Johansson, and from Elisabeth Ralf-Vestin, who also made the translation.

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ACTA CHEMICA SCANDINAVICA 8 (1954) 558—562

The Non-radical Nature of the Barium salts of Alloxantin, Tetramethyl-alloxantin and Hydrindantin. Studies in Magnetochemistry 10

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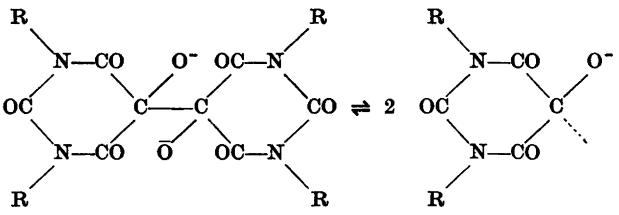
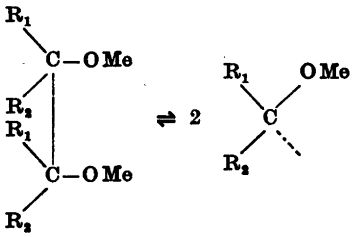
A magnetochemical investigation of the blue and blue-violet bariumsalts of alloxantin, tetramethyl-alloxantin and hydrindantin has proved that these compounds in contrast to the proposal of Hantzsch are not free radicals related to the metal-ketyls. The difference between the molar susceptibilities measured and calculated shows the existence of a small temperature-independent paramagnetism.

The barium salts of alloxantin, tetramethyl-alloxantin and hydrindantin are deep blue or blue-violet coloured and according to A. Hantzsch [1] rather instable towards oxygen and carbon dioxide. Hantzsch explained the halochromism of these compounds "durch die Bildung von dreiwertigem Kohlenstoff als wesentlichsten Bestandteil des chromophoren Komplexes der violetten Salze — also formell einfach durch Halbierung der aus den symmetrischen pinakon-ähnlichen Formeln der Alloxantine abzuleitenden Salzformeln".



R=H, salt of alloxantin; $R=CH_3$, salt of tetramethyl-alloxantin.

Hantzsch also points out that these salts are nearly related to the metal-ketyls of W. Schlenck [2]. The metal-ketyls may be considered as partly dissociated pinacolates:



R_1 and R_2 equal or different aromatic radicals.

The normal state of the pinacolate molecule is a singlet state. The ketyl-form having one uncompensated electron is in a doublet state. The magnetic measurement gives directly the multiplicity of the molecule in the normal state in the crystal. In case of an equilibrium between the pinacolate molecule and the ketyl molecule it will be possible to calculate the degree of dissociation α of the pinacolate molecule by means of:

$$\alpha = \frac{\chi_M}{2 \cdot 1\,270 \cdot 10^{-6}}$$

where χ_M is the observed molar magnetic susceptibility and $1\,270 \cdot 10^{-6}$ is the molar magnetic susceptibility of a molecule in a doublet state ("spin only" value).

Our first magnetic measurements gave at room temperature for the barium salt of alloxantin a molar magnetic susceptibility $= + 396 \cdot 10^{-6}$ (corrected for diamagnetism). This corresponds to an α-value of 15 %. We might then conclude that the interpretation of Hantzsch be correct. However, the measurements of further two different preparations of the bariumsalt of hydrindantin gave at room temperature diamagnetic susceptibilities of practically the same value. Furthermore our experiences concerning the stability of these compounds are in contrast to those of Hantzsch. After isolation from the mother liquor and drying are the salts rather stable. They may be kept unaltered for several days without protection. When suspended in water their colour will only fade very slowly when oxygen or carbon dioxide is passed through the suspension. The salts behave in these respects very differently from the metal-ketyls, which are highly sensible to moisture and oxygen. We then measured the magnetic susceptibilities of the barium salt of alloxantin and the two samples of the barium salt of hydrindantin at different values of the magnetic field strength (4 000—8 000 Ørsted). The results are given in Fig. 1, curve A_1 and H_1, respectively. The paramagnetism of the barium alloxantin salt originates in a ferromagnetic impurity, which also is present in the two samples of barium hydrindantin. The preparations of the salts were then repeated after having recrystallized the barium hydroxide 3 times (the salts themselves can not be recrystallized). These highly purified prepara-

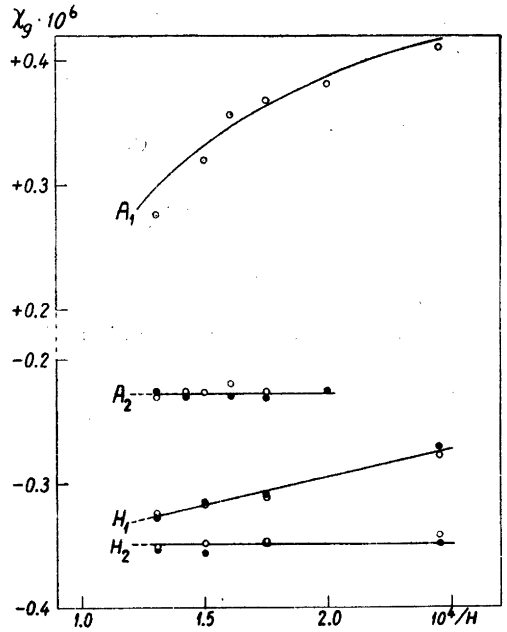


Fig. 1. Magnetic susceptibilities per gram plotted against the reciprocal of the magnetic field strength. The curves A_2, H_1 and H_2 give the results for 2 different samples of barium alloxantin and barium hydrindantin (○ and ● respectively).

tions were magnetically pure and the susceptibilities were all negative and independent of the magnetic field strength as shown in Fig. 1, curves A_2 and H_2. The magnetic susceptibilities of the barium salts of alloxantin, tetramethyl-alloxantin and hydrindantin are all negative and practically independent of the temperature in the interval: liquid-air temperatures to room temperature (Table 1). The organic components and the barium hydroxide used were also diamagnetic.

The difference between the measured molar susceptibilities of the barium salts and the molar susceptibilities calculated by means of Pascal and Pacaults increments (for Ba and H respectively —38.2 and —2.93) results in all cases in a small paramagnetic increment (Table 2). A similar small temperature-independent paramagnetism is well known in other deeply coloured compounds, which have a total molar magnetic susceptibility of negative value.

The barium salts here investigated do consequently not contain radicals and are not to be compared with the metal-ketyls. In the case of the metal-ketyls Eugen Müller and co-workers [3] have shown that the amount of free radical ranges from practically zero and to nearly 96 %, *e.g.* flavone potassium and Michler's ketone potassium respectively. The barium salts of alloxantin, tetramethyl-alloxantin and hydrindantin may be considered either as pinacolates or possibly as being derived from acetal like configurations. The magnetic method alone can not distinguish between these two possibilities.

EXPERIMENTAL

Magnetic measurements

We have applied the Gouy-method using a microbalance for the force determinations. The magnetic field strength may be varied from 4 000 to 8 000 Ørsted and the magnetic susceptibilities may be determined in the temperature interval 85 °K to 673 °K. The results of the measurements are given in Fig. 1, Table 1 (gram susceptibilities χ_g) and Table 2.

Table 1.

Substance	Sample No.	Analysis % Ba	$\chi_g \cdot 10^6$ 295 °K	195 °K	85 °K
Barium alloxantin		calc: 49.34			
$C_8H_2O_8N_4Ba_2$	2	found: 51.41	− 0.225	− 0.232	−
	3	found: 49.36	− 0.228	− 0.259	− 0.279
Barium tetramethyl-alloxantin		calc.: 28.76			
	1	found: 29.50	− 0.320	− 0.354	− 0.341
$C_{12}H_{12}O_8N_4Ba$	4	found: 28.84	− 0.326	− 0.371	− 0.351
Barium hydrindantin		calc.: 30.02			
$C_{18}H_8O_6Ba$	4	found: 29.71	− 0.347	− 0.356	− 0.366
	5	found: 29.82	− 0.351	− 0.344	− 0.381

Table 2 gives the molar susceptibilities measured (χ_{exp}) and the calculated molar susceptibilities (χ_{calc}) above referred to. In the two cases where the preparation directly gives a dihydrate, these were also measured magnetically together with the anhydrides. The results are in good agreement.

Table 2.

Substance \ Molar Susceptibilities	$\chi_{exp} \cdot 10^6$	$\chi_{calc} \cdot 10^6$	$(\chi_{exp} - \chi_{calc}) \cdot 10^6$
Alloxantin dihydrate	− 130	− 128	− 2
Alloxantin	− 104	− 102	− 2
Barium alloxantin	− 126	− 168	+ 42
Tetramethyl-alloxantin	− 149	− 150	+ 1
Barium tetramethyl-alloxantin	− 155	− 181	+ 26
Hydrindantin dihydrate	− 169	− 179	+ 10
Hydrindantin	− 141	− 153	+ 12
Barium hydrindantin	− 160	− 174	+ 14

Preparation of the compounds

Alloxantin, tetramethyl-alloxantin and hydrindantin were prepared following literature methods of Nightingale [4], Biltz [5] and Ruhemann [6], respectively.

$Ba(OH)_2 \cdot 8H_2O$ was an ordinary c.p. product. This compound was purified by recrystallization 3 times. An aqueous solution (0.1 molar) was applied for the preparation of the barium salts. The hydroxide solution was refluxed for half an hour and filtered before use.

Barium alloxantin according to Hantzsch [1] by mixing a solution of alloxantin with the equivalent amount of a 0.1 *M* barium hydroxide solution.

Barium tetramethyl-alloxantin and *barium hydrindantin* were prepared by shaking the finely powdered organic component with a 5 % excess of a 0.1 *M* barium hydroxide solution for 4—5 hours.

The salts were in all cases collected on filter-crucibles, washed three times with ethanol then dried in vacuum. All preparations and packing of the powdered salts in the magnetic measuringtubes were undertaken in a nitrogen atmosphere. The tubes were evacuated and then sealed off.

Quantitative analysis. Barium was determined as barium sulphate (micro-method).

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The Magnetic Properties of the Elements Niobium, Tantalum and Rhenium. Studies in Magnetochemistry 11

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The magnetic susceptibilities of niobium, tantalum and rhenium have been measured at varying temperatures (79° K to 578° K) and at different values of field strength at each temperature. These metals exhibit feeble paramagnetic susceptibilities, which are nearly temperature independent. The χ_g,T curves for niobium and tantalum show that χ_g decreases slowly and linearly with increasing temperature, the temperature coefficents being $-\ 0.0003_5$ and $-\ 0.00006_8$ respectively. The χ_g,T curve for rhenium increases extremely slowly and linearly with the temperature (interpreted as temperature independence). All susceptibilities measured are field strength independent at all the temperatures investigated. As average values for the atomic magnetic susceptibilities for niobium, tantalum and rhenium in the temperature interval 79° K to 578° K may be accepted the values: $+\ 207 \cdot 10^{-6}$, $+\ 151 \cdot 10^{-6}$ and $+\ 56 \cdot 10^{-6}$ respectively.

The accuracy of magnetic measurements on metals and alloys is severely limited by the difficulties in preparing samples of the highest degree of purity. Ferromagnetic impurities are easy to detect and correct for on account of the field strength variation of the susceptibility. Paramagnetic contaminations in metals may in some cases be detected and corrected for because of the temperature dependence of the susceptibility. Diamagnetic impurities are difficult to detect because the diamagnetic susceptibility is independent of field strength and temperature variations; these foreign substances must be eliminated by chemical methods.

The measurements here reported have been carried out on spectroscopically pure metals in which the amounts of paramagnetic and diamagnetic impurities are extremely small. Ferromagnetic substances are not present, because the susceptibilities measured are independent of the field strength at all temperatures.

Niobium

The first magnetic measurements concerning niobium were published in 1910 by Honda [1]. The specimen used was a metal lump ("regulus") obtained from Dr. v. Bolton having the specific gravity 12.7, a value which is very dif-

ferent from that of our sample 8.60. The susceptibility was dependent on the field strength and had at room temperature the value $1.29 \cdot 10^{-6}$ for $H \to \infty$ (gram suscept). The value was not altered by heating to 450° C. Owen [2] published 2 years later results on a niobium lump from Prof. Freund containing 0.25 % iron and having the specific gravity 12.7 (compare Honda's sample). Owen reports the value $1.3 \cdot 10^{-6}$ for the susceptibility. The results of Honda and Owen are in agreement but are rather different from recent values, probably a consequence of impurities present in the specimens used by these authors. In 1933, however, de Haas and van Alphen [3] at Leiden measured a spectroscopically pure sample from Brand Hilger containing small traces of iron and tin. The presence of these metals did not influence the result. The gram susceptibilities are at 289° K, 20.4° K and 14.2° K $2.28 \cdot 10^{-6}$, $2.34 \cdot 10^{-6}$ and $2.34 \cdot 10^{-6}$ respectively. In 1948 Brauer [4] measured powdered niobium sheet from Siemens having $\chi_g = 2.15 \cdot 10^{-6}$ (18° C) and another specimen ("sinterpresling" powdered) giving $\chi_g = 2.20 \cdot 10^{-6}$ (18° C) and $2.28 \cdot 10^{-6}$ (— 183° C). In Fig. 1 our measurements are plotted against temperature (°K). The straight line has been determined by means of the method of least squares and has the form:

$$\chi_g \cdot 10^6 = 2.350 - 0.000353 \cdot T \qquad (1)$$

In Fig. 1 we have also plotted the 3 susceptibilities measured at Leiden (●) and the 3 values (◑) reported by Brauer. It is significant that these 6 points lie nicely on our susceptibility curve and its extrapolated part. This may be attributed in the main to the high degree of purity of the 4 samples of different origin used in the 3 laboratories. We also consider this agreement to be a support to our statement that the susceptibilities of our specimen at all temperatures are independent of the field strength (see exptl. part); Brauer *e. g.* used considerable higher field strengths than we (up till 15 350 Ørsteds).

It is seen from Fig. 1 and equation (1) that the susceptibility decreases very slowly with increasing temperature. As the niobium sample is spectroscopically pure and most paramagnetic metals are absent, this suggests that this slow decrease with temperature may be attributed to the niobium metal. The following average values valid for the temperature interval 79° K to 578° K may be accepted for niobium:

$$\bar{\chi}_g = + 2.225 \cdot 10^{-6} \text{ and } \bar{\chi}_A = + 207 \cdot 10^{-6}$$

Tantalum

Honda [1] also studied tantalum using a sheet of the metal from Dr. v. Bolton having the specific gravity 16.6 (our value is 16.7). Honda gives the value $0.93 \cdot 10^{-6}$ for the gram susceptibility at room temperature decreasing to nearly $0.7 \cdot 10^{-6}$ at 800° C. Owen [2] measured a sheet of tantalum from Dr. v. Bolton and reports the value $0.8 \cdot 10^{-6}$ decreasing slightly with the temperature. The susceptibilities given by Honda and by Owen differ rather much (nearly 15 %). The magnetic properties of tantalum have not received attention since 1910—12.

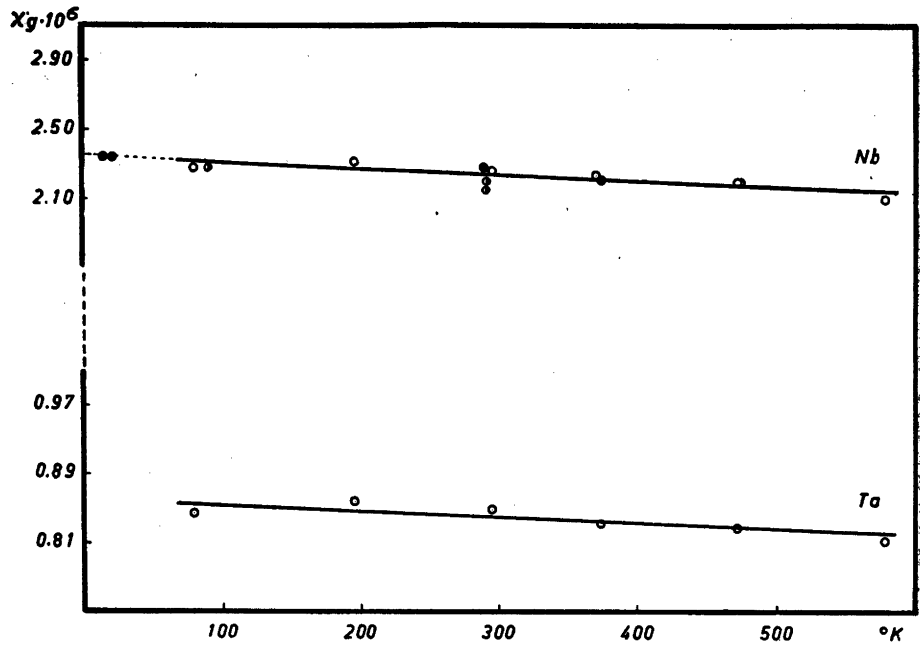

Fig. 1. Temperature dependence of the magnetic gram susceptibilities of niobium and tantalum. Our measurements ○, *Leiden measurements* ●. *G. Brauer's results* ◑.

The results of our measurements are given in Fig. 1. Like the susceptibilities for niobium these may be represented by a straight line having the equation:

$$\chi_g \cdot 10^6 = 0.860 - 0.000068 \cdot T \qquad (2)$$

The slope of this line is lesser than that for niobium (1). For reasons given above for niobium one may conclude that this slight decrease with temperature probably is a property of the metal. The average values of the gram susceptibilities and of the atomic susceptibilities in the temperature interval 79° K to 578° K are:

$$\bar{\chi}_g = + 0.83_7 \cdot 10^{-6} \text{ and } \bar{\chi}_A = + 151 \cdot 10^{-6}$$

Rhenium

Six years after the discovery of rhenium Albrecht and Wedekind [5] published the first magnetic measurement on the element. The gram susceptibility was determined to be $+ 0.046 \cdot 10^{-6}$ at room temperature. 1933—35 Perakis and Capatos [6] studied rhenium samples from Riedel-de Haën, Kahlbaum and Hilger at room temperature and the sample from Kahlbaum also at 0°, —24°, —79° and —180° C. The average value for all samples was $+ 0.366 \cdot 10^{-6}$. The susceptibility was practically temperature independent in the interval

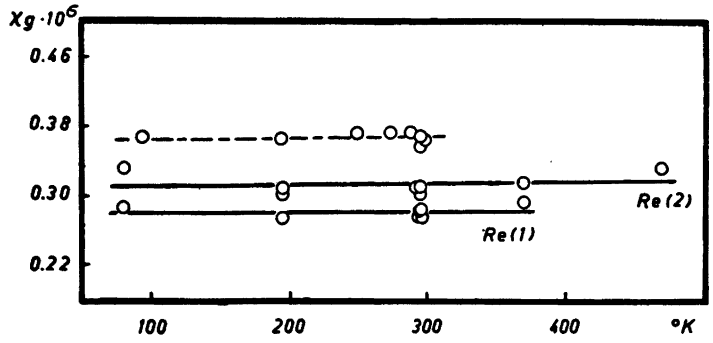

Fig. 2. Temperature dependence of the magnetic gram susceptibility of rhenium. Our measurements given in the two lower curves for the 2 samples Re (1) and Re (2). Results of Perakis and Capatos, upper curve.

93° K to 298° K. In Fig. 2 we give our results for our 2 specimens Re (curve 1) and Re (curve 2) studied in the temperature interval 79° K to 471° K. The diameter of the dots correspond to the experimental error (± 2 %) in these particular cases. It is possible by means of the method of least squares to fix a straight line through the points for each sample. These lines have the equations:

$$\text{Re (1)}: \quad \chi_g \cdot 10^6 = 0.280 + 0.0000075 \cdot T \tag{3}$$

$$\text{Re (2)}: \quad \chi_g \cdot 10^6 = 0.310 + 0.000017 \cdot T \tag{4}$$

These equations and the curves in Fig. 2 show that the susceptibilities increase extremely slowly with increasing temperature. For comparison we also plot the values of Perakis and Capatos [6] tabulated in their paper. We have drawn a straight line through these points by judgment (upper curve). The 3 curves demonstrate that the paramagnetic susceptibility of rhenium is independent of the temperature (79° K to 471° K). We are not convinced that the very small positive temperature coefficent is more than apparent. Another question is however: which of the 3 curves (respectively mean susceptibilities) must be preferred? The average values for our samples are $\bar{\chi}_g = + 0.282 \cdot 10^{-6}$ and $+ 0.314 \cdot 10^{-6}$. Perakis and Capatos give $\underline{\chi}_g = + 0.366 \cdot 10^{-6}$. Our sample Re (1) is spectroscopically pure and free for paramagnetic and most diamagnetic elements (see exptl. part). We have no reason for preferring Re (1) for Re (2) and can not explain why our mean values are 22.9 % and 14.2 % lower than that of Perakis and Capatos. Only the presence of rather considerable amounts of diamagnetic components in our samples can account for this discrepancy, and this is not probable. The presence of paramagnetic impurities in the specimens of Perakis and Capatos is only possible if these foreign substances have a paramagnetism independent of the temperature. It is well known that the reduction of the perrhenates with hydrogen for preparing metallic rhenium gives samples with higher susceptibilities than those of Perakis and Capatos until the reduction is completed. Schüth and Klemm [7] have measured the susceptibilities of Re_2O_7, ReO_3 and ReO_2. The gram sus-

ceptibility for Re_2O_7 is $-0.08 \cdot 10^{-6}$ at 2 050 Ørsteds and $-0.05 \cdot 10^{-6}$ at 3 640 Ørsteds, the decrease is nearly 37 %. The gram susceptibilities of ReO_3 is at 2 050 Ørsteds $+0.16 \cdot 10^{-6}$ and at 3 640 Ørsteds $+0.13 \cdot 10^{-6}$; the decrease is nearly 19 %. The oxide ReO_2 gives $+0.2 \cdot 10^{-6}$ independent of the field strength. Both these oxides have metallic character and their susceptibilities seem to be temperature independent. We do not consider these oxides to be present in our samples, because they do not show any significant field strength dependence at the values 2 657, 3 346, 3 822 and 4 194 Ørsteds at all temperatures measured (see Table 1). We also state that our samples were measured and kept in evacuated, sealed off measuring tubes (gas pressure 10^{-4}—10^{-5} mm Hg).

We give the average value of all our measurements of the susceptibilities of Re (1) and Re (2) as our accepted value for the susceptibility for rhenium metal in the temperature interval 79° K to 471° K:

$$\bar{\chi}_g = +0.29_8 \cdot 10^{-6}, \qquad \bar{\chi}_A = +56 \cdot 10^{-6}$$

The susceptibility $+0.046 \cdot 10^{-6}$ given by Albrecht and Wedekind [5] received some attention because this value does not lie between the susceptibilities of the neighbour elements wolfram and osmium having the values $+0.33 \cdot 10^{-6}$ and $+0.074 \cdot 10^{-6}$ respectively; compare, however, our determination: $+0.30 \cdot 10^{-6}$.

EXPERIMENTAL

1. The metal samples used for the measurements

Niobium. This metal was prepared by The Fansteel Corp. of North Chicago in very high purity for use as a spectroscopic standard. It contained 99.99 % Nb. A spectroscopic examination has proved that the sensitive lines of Si, Fe, Ti, Al and Cu were only faintly visible. The lines of the elements Ag, Ca, Co, Cr, K, Li, Mg, Mn, Mo, Na, Pb, Sb, Sn, Ta, V, W, Zn and Zr were not observed. The metal contained a trace of Ni (< 0.01 %), which however had no influence upon the measurements (field strength dependence of the magnetic susceptibility). The specific gravity was determined to 8.60 (Landolt-Börnstein's tables give 8.60). The shape of the specimen was cylindrical (diameter 4.71 mm and length 10.01 cm).

Tantalum originated from the firm above mentioned. The metal contained more than 99.98 % tantalum. The spectrografic analysis has shown that the sensitive lines of Nb, Mg, Si and Cu were faintly visible. The elements Ag, Al, Ca, Co, Cr, Fe, K, Li, Mn, Mo, Na, Ni, Pb, Sb, Sn, Ti, V, Zn and Zr were not present. The specific gravity was determined to be 16.7 (Landolt-Börnstein's tables give 16.6). The cylindrical rod had the dimensions: diameter 4.48 mm and length 9.77 cm.

Rhenium. Two rhenium samples of different origin were used for the magnetic measurements.

Re (1) was prepared by Johnson, Matthew and Co., London for use as a standard in spectrografic analysis. The sensitive lines of Ca, Mg and Cu were faintly visible and the lines of Al, Si and Fe were only very faintly visible. The elements Ag, Ca, Cr, K, Li, Mn, Mo, Na, Ni, Pb, Sb, Sn, Ti, V, Zn and Zr were not detected.

Re (2) was prepared in this laboratory from purified potassium perrhenate. The salt was reduced in hydrogen at 400° C. Potassium hydroxide was removed by extraction with water; drying in oxygenfree nitrogen. Re (1) and Re (2) were measured as powders.

2. Magnetic measurements

The magnetic susceptibilities were measured at the four field strengths 782, 1 500, 2 138 and 2 657 Ørsteds (Nb and Ta). The rhenium samples were measured at 2 657, 3 346, 3 822 and 4 194 Ørsteds. The dependence upon temperature was studied in the temperature interval 79° K to 578° K. The higher temperatures were produced by means of a small electric tube-furnace with bifilar wiring. A copper-constantan thermoelement placed in a defined position between the sample and the protection tube indicated the temperature (constant force). The magnetic force was determined by weighing (microbalance, Gouy's method).

The results of the magnetic measurements are given in Figs. 1 and 2. The field strength dependence was investigated at each temperature applied. The susceptibilities were constant in the interval 782—2 657 Ørsteds for niobium and tantalum, and in the interval 2 657—4 194 Ørsteds for rhenium. The measurements arbitrarely selected for Table 1 exemplify this constancy.

The determinations of the susceptibilities of the rhenium samples at all temperatures and field strengths were carried out with 4 and 3 independent packings in the measuring tube for Re(1) and Re(2) respectively.

Table 1. Magnetic gram susceptibilities at different temperatures and field strengths.

	Niobium		Tantalum			Rhenium (1)		
Temp. °K / H(Ørsteds)	79	472	79	472	Temp. °K / H(Ørsteds)	79	295	371
782	2.27_3	2.23_8	0.84_3	0.81_7	2 657	0.29_2	0.28_6	0.29_5
1 500	2.27_5	2.17_6	0.84_1	0.82_5	3 346	0.29_5	0.28_6	0.29_2
2 138	2.28_1	2.18_5	0.84_6	0.83_3	3 822	0.28_6	0.28_3	0.29_4
2 657			0.84_3	0.83_2	4 194	0.27_5	0.28_3	0.29_2
Mean value	2.28	2.20	0.84_5	0.82_7	Mean value	0.28_7	0.28_5	0.29_4

The results plotted in Figs. 1 and 2 are mean values calculated as in the table. We have not determined the susceptibilities at $H \rightarrow \infty$ by extrapolation of the χ_g, H^{-1} curves to the zero value of H^{-1}, because our measurements do not indicate any significant variation of χ_g in the field strength interval applied.

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Separation of Methylated Sugars on Carbon Columns

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The fractionation of mixtures of methylated sugars is a problem of great importance in structural polysaccharide chemistry. The distillation techniques, developed and successfully employed by the Haworth school, have been replaced in recent years by chromatographic methods. Partition chromatography on silicagel columns [1], paper strips and cellulose columns [2] has proved to be of great value in the separation of methylated sugars, but often fails to separate isomeric substances.

Following the observation that mannitol monoacetate moves at about the same rate as a disaccharide on a carbon column [3], we have investigated the possibility of separating methylated sugars by this means **.

Initially, the separation of glucose, 3-*O*-methyl-glucose, 2,3-di-*O*-methyl glucose, 2,3,6-tri-*O*-methyl-glucose and 2,3,4,6-tetra-*O*-methylglucose was investigated. A mixture of these sugars, 0.5 g of each, was added to the top of a carbon-Celite column (20 × 3 cm) and subjected to linear gradient elution with aqueous ethanol. The optical rotation-volume curve (Fig. 1 a) indicated a good separation of the substances, which were recovered chromatographically pure and with the exception of the tetramethyl ether, in good yields, by combining and concentrating suitable fractions. Between the maxima, corresponding to the di- and trimethyl ethers, there is a small peak obviously due to an impurity in the dimethyl ether, as our sample was known to be not pure. The optical rotation of the dimethyl ether used, agrees fairly well with that of the sum of the dimethyl ether and the impurity fractions. Thus no material appears to have been lost or modified during the separation. The gradient chosen seemed suitable for the first four substances but the tetramethyl-glucose was spread over a rather large volume, which is inconvenient. In order to elute the tetra-ether in a smaller volume, a steeper gradient was chosen for this concentration range (Fig. 1 b).

A disadvantage of aqueous ethanol as eluant is the increase in viscosity with concentration causing a retardation in the rate of flow through the

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** During the course of this investigation we learnt from Dr. W. J. Whelan of Bangor that he and Mr. Morgan are pursuing similar independent studies. Dr. Whelan kindly sent us the manuscript of their publication [4], and where the investigations overlap, the results agree.

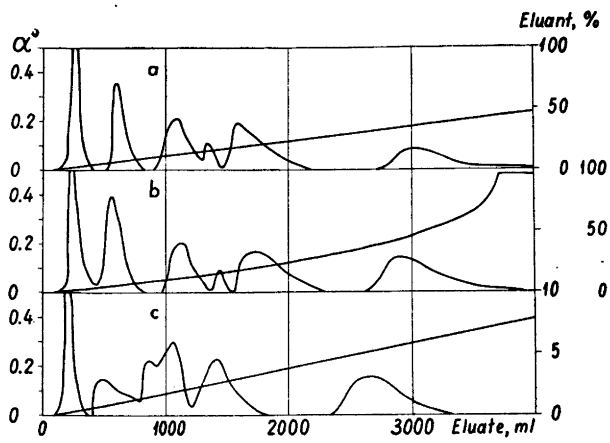

Fig. 1. Chromatographic separations of glucose and some of its methyl ethers. The peaks, from left to right, correspond to glucose, 3-O-methyl-glucose, 2,3-di-O-methyl-glucose, an unknown impurity, 2,3,6-tri-O-methyl-glucose and 2,3,4,6-tetra-O-methyl-glucose.

column. Alm [5] has suggested the use of ethyl methyl ketone, which he found to be about five times more efficient than ethanol as an eluant. A run with this solvent showed that the flow was almost independent of the concentration in the range selected. The tri- and tetra-ethers were separated well, but for the lower members the picture was complicated (Fig. 1 c). This may be due to strong adsorbtion of the ketone itself upon the column, so that its concentration in the initially weak solution is reduced considerably, but when the column is almost saturated it breaks through as a steep front.

The next step was to investigate whether isomeric, methylated sugars could be separated in the same way, and for this, the pair 2,3,6- and 2,3,4-tri-*O*-methyl-glucose were chosen. For the second substance, which is a non-crystalline syrup, different values for the specific rotation between 43—67° (water), are recorded in the literature. According to Irvine and Oldham [6], the substance cannot be purified by distillation. We prepared the substance by acid hydrolysis of 2,3,4-tri-*O*-methyl-levoglucosan, and obtained a product with the specific rotation of + 86° in water. This high value and a satisfactory analysis suggest that our sample has a higher degree of purity than previous preparations. For the separation of the two trimethyl ethers (0.4 g of each) a larger column (43 × 3.5 cm) was selected and ethyl methyl ketone used as eluant. A complete separation was achieved (Fig. 2) and the substances were recovered pure, in almost quantitative yields.

From the results of the present investigation it is evident that chromatography on carbon columns offers a good method for the separation of monosaccharides with different degrees of methylation. One advantage over other methods is the high capacity of the carbon columns, thus permitting the quantitative isolation of a minor component of a mixture in reasonable amounts, *e.g.* a low percentage of a dimethyl hexose from the hydrolysate of a

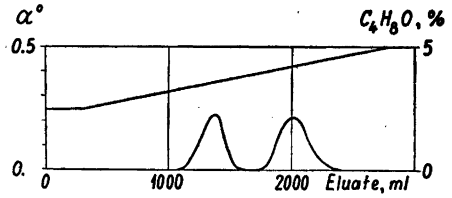

Fig. 2. Separation of 2,3,6-tri-O-methyl-glucose (left peak) and 2,3,4-tri-O-methyl-glucose (right peak) on a carbon column.

methylated polysaccharide. It is also evident from our experiments with the two trimethyl ethers that the method may be useful in the separation of isomeric methylated sugars.

EXPERIMENTAL

Substances (All melting points uncorrected)

Glucose. Merck & Co. analytical reagent $[\alpha]_D^{20}$ + 52.5° (water, c = 2).

3-O-Methylglucose was prepared according to Glen, Myers and Grant [7], except that the methylation of di*iso*propylidene glucose was performed in dry dioxane as described below for 2,3,4-tri-*O*-methyllevoglucosan. Yields were somewhat lower than those obtained by Glen *et al.* M. p. 159—161°, $[\alpha]_D^{20}$ + 55.5° (water, c = 2).

2,3-Di-O-methylglucose was prepared according to Evans *et al.*[8] but methylation of 4,6-benzylidene-*α*-methyl glucoside was performed with methyl sulphate and powdered sodium hydroxide in dry dioxane (yield of methylated derivative 80 % of theory), and further the hydrolysis to 2,3-di-*O*-methylglucose of the product so obtained was made in one step, omitting the isolation of the corresponding methyl glucoside. The product, however, was noncrystalline and was shown by hypoiodite oxidation [9] to contain 83 % reducing sugar calculated as dimethyl ether. The existence of one impurity with high optical rotation is revealed by the chromatographic experiments. $[\alpha]_D^{20}$ +60.8° (water, c = 2).

2,3,6-Tri-O-methyl glucose. A sample prepared from methylated starch was purified by crystallisation from *iso*propyl ether-chloroform. M. p. 112—116°, $[\alpha]_D^{20}$ +67° (water, c = 2).

2,3,4-Tri-O-methyl glucose was prepared by hydrolysis of levoglucosan trimethyl ether [6] using a slight modification of previous methods. In a flask fitted with a reflux condenser and an effective stirrer, containing levoglucosan (8 g), powdered sodium hydroxide (24 g), and dry peroxide-free dioxane (150 ml), methyl sulphate (29 ml) was slowly added with vigorous stirring. The temperature was raised to 60° and kept there during the addition of the methyl sulphate (1 hour) and for the following 5 hours. After standing at room temperature overnight the mixture was filtered, care being taken not to let the filter cake crack. The residue was washed with dry benzene and the dioxane-benzene solution concentrated. The residue was distilled *in vacuo*. B. p. 8 mm 127—128° (7.5 g) and 127—132° (1.7 g), mobile oils. After a few hours the remainder in the distillation flask crystallised, and after inoculation the distillates also were obtained crystalline. M. p. 58—60° (first fraction) and 55—58° (second fraction). Total yield was 9.2 g or 91 % of theory.

For the preparation of 2,3,4-tri-*O*-methylglucose trimethyl levoglucosan (2.5 g) was dissolved in 1 *N* sulphuric acid (50 ml) and the solution heated on a boiling water bath for 17 hours. The aqueous solution obtained by neutralisation of the hydrolysate with $BaCO_3$ and filtration (90 ml) was extracted with chloroform in 10 ml portions until a drop of the chloroform phase gave a negligible residue on evaporation (9 extractions). From the combined chloroform solutions trimethyl levoglucosan (0.65 g), m. p. 58—60°, was recovered.

The extracted aqueous solution was concentrated under reduced pressure, the residue extracted with chloroform and the chloroform solution after filtration concentrated to dryness *in vacuo*, yielding 2,3,4-tri-*O*-methyl glucose (1.76 g) as a faintly yellow syrup. $[\alpha]_D^{20}$ + 86° (water, c = 2.3).

The purity of the trimethyl glucose was estimated at 99 % by hypoiodite oxidation.

2,3,4,6-Tetra-O-methyl glucose. A sample, m. p. 76—90°, was recrystallised twice from light petroleum (b.p. 90—100°), but no change in the melting point could be observed. However, according to hypoiodite oxidation data the product was 100 % pure. $[\alpha]_D^{20}$ + 82.5° (water, c = 2).

Procedure

Columns. Equal parts of animal charcoal "zur Analyse" (J. D. Riedel-E. de Haen AG) and Celite No 535 (Johns-Manville) were mixed and treated with conc. hydrochloric acid. The mixture was then washed with large quantities of water and ethanol and then poured into the columns as a thick slurry. Two columns, with the dimensions 20 × 3 cm and 43 × 3.5 cm, were used in this investigation. The adsorbent in the first column was treated with cold, that in the second with boiling hydrochloric acid. This treatment probably involves a desirable deactivation of the carbon, in addition to the removal of inorganic material.

Gradient elution. Two open, cylindrical flasks, connected by a siphon, were filled to hydrostatical equilibrium with the required initial and final concentrations of the eluant. The content of the flask containing the lower concentration was slowly stirred and siphoned directly on to the column, three meters below the flasks. All connections were made of glass or polyethene tubings. When the two flasks have the same diameter a linear gradient is obtained, and by altering the diameter ratio, gradients of different types may be obtained. A more detailed treatment of gradient elutions will be published elsewhere.

Separation experiment. In the first three runs, the small column (20 × 3 cm) was used. The substances to be separated, in 10 % aqueous solutions, were added to the top of the column, washed down with a small amount of water and the elution started. Fractions of equal volume (26.5 ml) were collected, and the optical rotation of the fractions determined (2 dm tube). Appropriate fractions were combined and concentrated to dryness under reduced pressure. The amount of sugars present was determined by hypoiodite titration[9] and by determination of the optical rotation. The purity was checked by paper chromatography. The optical rotation-volume curves are given in Fig. 1 and the actual data for the separations in Table 1.

Table 1. Separation of glucose, 3-mono, 2,3-di, 2,3,6-tri and 2,3,4,6-tetra-O-methylglucose.

			Glucose	Mono-	Di- [a]	Tri-	Tetra-
Flask A 2 500 ml 1 % EtOH [b]	Added mg		500	443	492	511	500
Flask B 2 500 ml 60 % EtOH	Recovered %	By rotation	97	100	95	99	86
Diameter ratio A : B = 1		By titration	96	96	100	99	89
Flask A 2 620 ml 1 % EtOH	Added mg		499	497	456	501	497
Flask B 940 ml 96 % EtOH	Recovered %	By rotation	98	100	95	102	98
Diameter ratio A : B = 1.8		By titration	97	96	95	98	98
Flask A 2 500 ml water	Added mg		499	493	490	503	494
Flask B 2 500 ml 10 % C_4H_8O	Recovered %	By rotation	98	—	—	96	96
Diameter ratio A : B = 1		By titration	93	—	—	100	100

[a] As mentioned above this substance was not pure. The figures given in this column are based upon rotation and titration determinations on the impure starting material, and include the almost completely separated impurity.

[b] By volume.

In the last run, 2,3,6- and 2,3,4-tri-*O*-methyl-glucose, 410 mg and 392 mg respectively, were dissolved in 2.5 % aqueous ethyl methyl ketone (10 ml), added to the top of the larger column (43 × 3.5 cm), prewashed with 2.5 % ethyl methyl ketone and the sugars washed down with the same solvent mixture. The eluant was taken from two flasks with the same diameter, containing 1 500 ml of 2.5 % and 5.5 % aqueous ethyl methyl ketone and the eluate worked up as described above. The recovery of chromatographically pure starting materials were, by rotation 97 % and 90 % and by titration 96 % and 95 %, respectively.

SUMMARY

Glucose and a mono-, di, tri and tetra-methyl ether of this sugar have been separated on carbon columns, using the gradient elution method with aqueous ethanol or ethyl methyl ketone as eluents. The isomeric pair 2,3,6- and 2,3,4-tri-*O*-methyl glucose was also separated by the same method. The separations are complete and the recoveries quantitative, hence the method may be of value in structural carbohydrate chemistry.

An improved method for the preparation of 2,3,4-tri-*O*-methylglucose is reported.

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Bestimmung der Beeinflussung der Viscositätsherabsetzung des Systems Testikel- bzw. *Clostridium perfringens*-Hyaluronidase und Hyaluronsäure durch Pharmaka

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In Orientierungsversuchen wurde viscosimetrisch geprüft, ob die contraceptive Wirkung von Phenylmercuriazetat, Chinosol und Chininsulfat (Baker [1]) mit einer Hemmung des Testikelhyaluronidasesystems und ob die antiseptische Wirkung von Phenylmercuriazetat, Mercurochrom, Eosin, Chinosol, Chininsulfat mit einer Hemmung des Clostridium-Hyaluronidase-Systems einhergeht.

Die genannten Substanzen, sowie Cocain und die Antimonpräparate Kalium-Antimonyltartrat und Antimosan wurden zwecks Ermittlung einer Hemmwirkung auf beide bzw. einer eventuellen spezifischen Hemmwirkung auf eines der beiden Enzymsysteme bei deren pH-Optima (pH ≈ 7 bzw. ≈ 6,0; McClean [2]) geprüft.

Redoxmessungen wurden durchgeführt, um eventuelle Zusammenhänge zwischen rH und Hyaluronidasewirkung bzw. Hemmung festzustellen.

MATERIAL UND METHODIK

Für die Viscositätsbestimmungen wurden Ostwald-Viscosimeter mit Auslaufzeiten für Aqua dest. (20° C) von 20, 40, 60 und 80 Sec. verwendet. Für jede Versuchsreihe wurden die gleichen Viscosimeter benützt.

Als Puffer wurden folgende Lösungen benützt:

a) 0,1 *M* Azetat, pH = 6,2, 0,15 *M* NaCl, rH ≈ 28 (20° C)

b) 0,1 *M* Phosphat, pH = 6,9, 0,15 M NaCl, rH ≈ 29 (20° C)

Die Hyaluronsäure * war ein reines und relativ niedermoleculares Produkt. Sie wurde mit Puffer als Lösungsmittel als 0,3 %ige Lösung verwendet.

Folgende Enzymsysteme fanden Verwendung:

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* Wir danken Herrn Prof. G. Blix, Vorstand des Institutes für Medizinische Chemie der Universität Uppsala, für deren Überlassung.

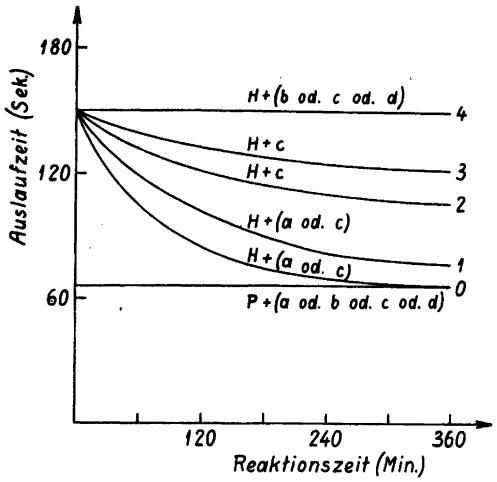

Abb. 1.

0, 1, 2, 3, 4 = *Viscositätsmass (rel.)*
a, b, c, d = *Lösungen*
H = *Hyaluronsäure*
P = *Puffer.*

a) Das Stierhodenhyaluronidasepräparat »Hyalas» (AB Leo, Schweden) mit 1 mg Methylgelatine als Stabilisator pro 2,5 VRE. Die Versuchslösung enthielt 50 VRE/100 ml Puffer.
b) Autolysat von *Clostridium perfringens*, zell- und sporenfrei, pH korrigiert auf 6,2 bzw. 6,9.

Folgende Substanzen wurden mit Ausnahme von Antimosan, das in 6,3 %iger Lösung Verwendung fand, in 4 %iger oder gesättigter pH-kontrollierter Lösung geprüft: Phenylmercuriazetat (technisch), Chinosol (Chinosolfabriken A.G., Hamburg), Chininsulfat (Pharmakopoeware), Mercurochrom (technisch), Eosin gelblich (Bayer), Cocainchlorid (Pharmakopoeware), Kaliumantimonyltartrat (Pharmakopoeware), Antimosan (Behring).

Die Viscosimeterversuche wurden im Thermostaten bei 37,0 ± 0,1 °C ausgeführt: Es wurden 3 ml einer 0,3 %igen Hyaluronsäurelösung eingebracht und 2 ml von einer der Lösungen a, b, c, und d (s. u.) mit gleichzeitigem mässigen Durchblasen von Luft zugesetzt. Die Auslaufzeiten wurden nach 2, 15, 30, 45, 60, 120, 180, 240 und 360 Minuten bestimmt. Lösung a bestand aus 1 ml Enzymlösung und 2 ml Puffer, Lösung b aus 1 ml einer Lösung mit der zu prüfenden Substanz und 2 ml Puffer, Lösung c aus 1 ml Enzymlösung, 1 ml einer Lösung der zu prüfenden Substanz und 1 ml Puffer. Lösung d war Pufferlösung. Als Kontrolle wurden die Auslaufzeiten einer Mischung aus 3 ml Puffer und 2 ml der a-, b-, c- und d-Lösung verwendet. Sämtliche Lösungen waren vorgewärmt, der pH-Wert wurde bei 20° C elektrometrisch kontrolliert.

Die Redoxmessungen wurden mit den gleichen Lösungen, mit denen die Viscositätsbestimmungen vorgenommen wurden, ausgeführt. Die Lösungen wurden mit Paraffinum liquidum überschichtet, die EMK wurde bei 20° C mit einem Radiometerapparat, Typ PHM 3 [System: (−) gesättigte Kalomelelektrode / gesättigte KCl-Lösung/ (/4 %iger Agar-Agar mit 16 %iger NH_4NO_3-Brücke/) Probelösung / blanke Platin-Elektrode (+)] ermittelt.

Die Platinelektroden verblieben während der Versuchszeit in der Versuchslösung. Sie waren durch eine 0,1 *M* $FeCl_3$-Lösung kontrolliert. Die Messungen wurden nach

1, 2, 3, 4, 6 und 24 Stunden oder bis zur EMK-Änderung von ca. 6 mV/Stunde durchgeführt.

Die Werte der Redoxmessung wurden in E_H-Werte umgerechnet. Die rH-Werte wurden mittels Jörgensens Nomogramm ermittelt (Jörgensen [3]).

ERGEBNISSE

Zur qualitativen Beurteilung der Viscosimeterversuche wurden die Ergebnisse in Diagrammform aufgezeichnet (s. Abb. 1 als Beispiel für eine Auslaufzeit für Aqua dest. (20° C) von 60 Sec.) und dann in der Tabelle 1 zusammengefasst.

Tabelle 1.

	pH 6,2						pH 6,9					
	Hyaluronsäure											
	ohne Enzym		Clostr. enzym		Testik. Hyaluronidase		ohne Enzym		Clostr. enzym		Testik. Hyaluronidase	
	Visc.	rH	Visc.	rH	Visc.	rH	Visc.	rH	Visc.	rH	Visc.	rH
ohne Pharmaka	4	28	0	32	1	31	4	34	1	31	0	29
Phenylmercuriazetat	4	28	3	27	1	30	4	32	1? 2	26	0	31
Chinosol	4	30	0	30	2	33	4	31	1	31	2	30
Chininsulfat	4	31	0	29	1? 2	30	4	31	1	—	1? 0	31
Mercurochrom	4	26	4	29	4	26	4	30	4	30	4	27
Eosin	4	30	4	32	4	31	4	35	4	31	4	31
Cocainchlorid	4	31	0	—	1	29	4	30	1	31	0	35
Kaliumantimonyltartrat	4	27	0	—	1	29	4	28	1	—	0	29
Antimosan	4	28	0	28	1? 2	27	4	28	1 ?	30	0? 1	31

Die Untersuchungen liessen erkennen, dass nur Mercurochrom und Eosin beide Enzymsysteme sowohl bei pH 6,2 als auch bei 6,9 hemmen. Ein mehr differenzierter Effekt wurde mit Phenylmercuriazetat erhalten. Diese Substanz hemmte nur das Clostridiumenzymsystem bei pH 6,2 deutlich, eine weniger deutliche Hemmwirkung übte sie bei pH 6,9 aus. Chinosol hemmte nur das Testikelenzymsystem und zwar ausgeprägter bei pH 6,9 als bei pH 6,2. Die Wirkungen der übrigen Substanzen waren schwierig zu beurteilen. Es scheint aber so, dass Antimosan und Chininsulfat das Testikelenzymsystem hemmten.

Cocain und Kaliumantimonyltartrat hemmten die Viskositätsherabsetzung nicht.

Die rH-Werte schwankten in sämtlichen Systemen zwischen 26 und 35.

BESPRECHUNG DER ERGEBNISSE

Die contraceptive Wirkung von nitrierter und azetylierter Hyaluronsäure ging nach Pincus [4] mit einer Hemmung von Testikelhyaluronidase einher. In Übereinstimmung mit Mc Cullagh u. Mitarbeitern [5] ging jedoch die contraceptive Wirkung der geprüften Phenylmercuriverbindung nicht mit einer Hemmung der Testikelhyaluronidase einher; es scheint deshalb die genannte Wirkung auf andere Weise als durch einen Antihyaluronidaseeffekt zustandezukommen. Mit der antiseptischen Wirkung von Phenylmercuriazetat scheint eine Hemmung des Clostridiumenzymsystems besonders bei pH 6,2 einherzugehen. Die unterschiedliche Beeinflussbarkeit der beiden Enzyme differenter Genese beleuchtet deren Verschiedenheit.

Da Chinosol und ev. Chininsulfat, wenn auch nur schwach das Testikelhyaluronidasesystem und nur dieses hemmten, kann diese Wirkung zu deren nicht sehr ausgeprägten contraceptiven Wirkung möglicherweise beitragen. Die antiseptische Wirkung der beiden Substanzen geht nicht mit einer Hemmung des Clostridiumenzymsystems einher.

Die antiseptisch wirkenden Substanzen Mercurochrom und Eosin zeigten keine spezifische Hemmwirkung. Beide Enzymsysteme wurden bei pH 6,2 —6,9 gehemmt. Die antiseptische Wirkung von Mercurochrom steht möglicherweise im Zusammenhang mit dieser Hemmung.

Cocainchlorid und Kaliumantimonyltartrat waren bei pH 6,2 und 6,9 ohne spezifische Wirkung, da die Systeme unbeeinflusst blieben. Dagegen zeigte Antimosan eine schwache spezifische Hemmwirkung auf das Testikelhyaluronidasesystem bei pH 6,2 und 6,9, nicht aber auf das Clostridiumenzymsystem.

Die Redoxmessungen zeigten, dass Testikel- und Clostridiumhyaluronidase bei rH 29—32 (pH 6,2—6,9; 20° C) wirkten. Die rH-Werte liegen höher als die, die man bei der Spermieneindringung ins Ei (rH 2—14, pH 7, 37° C; Moricard [6]) und beim Wachstum und bei der Vermehrung von *Clostridium perfringens* findet (rH 6—9, pH 6,5—7,6, 37° C; Reed u. Mitarbeiter [7]). Mit Rücksicht auf die Schwierigkeiten der rH-Bestimmungen, die rH-Werte von 26—35 (pH 6,2—6,9; 20° C) ergaben, kann man keinen bindenden Zusammenhang zwischen Hemmung und rH erkennen.

ZUSAMMENFASSUNG

Die Contraceptiva und Antiseptica Phenylmercuriazetat, Chinosol, Chinin und die Antiseptica Mercurochrom und Eosin sowie Cocain, Kaliumantimonyltartrat und Antimosan wurden viscosimetrisch auf Testikel- und Clostridium perfringens-Hyaluronidasesystemwirksamkeit bei pH 6,2 bis 6,9 geprüft. Auch Redoxmessungen wurden vorgenommen.

Das Testikelhyaluronidasesystem wurde nur durch Chinosol, Chinin, Mercurochrom, Eosin und ev. Antimosan gehemmt. Das Clostridiumhyaluronidasesystem wurde nur von Phenylmercuriazetat, Mercurochrom und Eosin gehemmt.

Die rH-Werte (26—35) liessen keine Beziehung zur Hemmung erkennen.

Wir danken Herrn Prof. K. Sjöberg und Herrn Prof. H. Hedström für ihr Interesse und Entgegenkommen.

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Polymerization of Acrylonitrile in Suspension Systems

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Present manufacturing methods for polyacrylonitrile generally involve *solution* or *quasi-emulsion* polymerisation in water due to the high solubility of acrylonitrile in the latter at elevated temperature. Continuous polymerization in solution is carried out in the presence of a water soluble catalyst and a large excess of water [1–4]. Ammonium persulphate is the preferred catalyst combined with a reduction activator, such as sodium bisulphite [1,3]. The reaction vessel should be flushed with nitrogen before the addition of freshly distilled monomer. In this way the deleterious effect of oxygen [1,6,7] on the initiation is avoided.

Bulk polymerization is also reported in literature [5], but at present it seems to have gained only secondary importance. As the polymer of acrylonitrile is insoluble in its monomer even at low degrees of polymerization, a stiff slurry is obtained immediately after initiation, obviously resulting in a difficult problem on heat transfer.

Suspension polymers of acrylonitrile are not earlier described, as far as known. However, polymerization of acrylonitrile in a suspension system involves no pronounced difficulties provided the following factors are considered:

1. The polymerization catalyst should be *insoluble*, in any case *inactive* in the aqueous phase, even when the water is saturated with monomer at the polymerization temperature.

2. The catalyst should be active below the boiling point of the azeotrope acrylonitrile-water (b.p. 71° C) in order to avoid working under pressure. Furthermore, increasing reaction temperature decreases the yield (conversion) of the polymer due to increasing solubility of the monomer in the aqueous phase [8].

3. The catalyst residues remaining in the polymer particles should be volatile at the drying temperature. It is, of course, desirable that the heat stability of the polymer be affected neither by unchanged catalyst nor by compounds by side reactions in the growing polymer-monomer particle.

Most of the conditions, cited above, seem to be satisfied by the *azo* catalysts; even by the simplest one: α,α'-azo-*bis*(*iso*butyronitrile). This compound may be prepared from acetone, hydrazine sulphate and sodium cyanide, as described

in the literature [9,10]. It is soluble and active in acrylonitrile, saturated with water, above 50° C, but inactive in water, saturated with acrylonitrile. The same applies to the analogous azo compound prepared from methyl *iso*butyl ketone: *a,a'*-azo-*bis*(*a,γ*-dimethylvaleronitrile) [11–13].

In order to obtain an adjusted polymer particle size distribution during the reaction, it is necessary as usual to add a stabilizer to the aqueous phase. Almost any strongly hydrophilic polymer may be used, but three principally different cases have been observed:

1. Water soluble, non-ionic polymers, showing surface activity, for instance mixed ethers of cellulose (ethyl hydroxyethyl cellulose), act as powerful dispersing agents, resulting in small particles in the finished polymer (generally less than 0.1 mm).

2. Polyelectrolytes, such as sodium carboxymethyl cellulose and sodium polyacrylic acid, mainly function as dispersing agents only by increasing the viscosity of the water. The particle size always increases in such systems at least a tenfold compared with an analogous non-ionic, surface active system.

3. Even an *emulsifying* agent can be added to the aqueous phase, in combination with the stabilizer, to obtain a combined effect of increase in viscosity and decrease of surface tension — and still a pure *suspension* polymer is obtained. (Polymerization does not proceed in the dissolved monomer below at least 60° C.) The size of the polymer particle can in this way be regulated within wide limits.

Table 1. Polymerization of acrylonitrile with an azo activator (a,a'-azo-bis(a,γ-dimethylvaleronitrile)) in aqueous suspension in the presence of a non-ionic, surface active stabilizer (ethyl hydroxyethyl cellulose).

Apparatus: (identical for all experiments):
Three-necked flask, volume 1 000 ml, equipped with thermometer, agitator and reflux condensor. Rate of stirring 300–400 r.p.m.
Composition of reaction mixture:

Acrylonitrile	*200 g*
Water	*600 »*
Stabilizer	*1.2 »*
Activator	*See below*

Prep. No.	1	2	3	4	5	6	7	8
Activator added, g	0.5	1	2	3	0.5	1	2	3
Temp. of reaction, °C	50	50	50	50	65	65	65	65
Time of reaction, min.	240	240	210	180	180	180	180	180
Yield, g	125	145	150	142	140	155	150	140
* Average viscosity, M.W.10^{-3}	224	194	112	95	211	196	108	87

* K_m-value used in this table and in the following ones = $1.5 \cdot 10^{-4}$ (compare U.S.P. 2404714).

Table 2. Polymerization of acrylonitrile with an azo activator (azo-bis(dimethylvaleronitrile)) in the presence of an organic diluent.

Composition of reaction mixture:

Acrylonitrile	*200 g*
Water	*600 »*
Stabilizer: ethyl hydroxyethyl cellulose	*1.2 »*
Activator:	*See below*
Diluent:	*See below*

Prep. No.	1	2	3	4	5	6	7	8	9	10
Activator, g	1	2	3	3	1	1	1	1	2	3
Diluent	Kerosene	←	CCl_4	→	←	—	Xylene	—	—	→
Amount of diluent, g	200	20	100	200	50	75	100	200	200	200
Temp. of reaction, °C	50	60	60	60	60	60	60	60	60	60
Time of reaction, min.	240	150	150	150	150	150	150	150	150	150
Yield, g	180	185	155	155	183	152	172	159	164	173
Average viscosityM.W.10^{-3}	176	106	100	84	71	53	52	40	32	31

Medium polymer particle size: 0.08 mm.

The azo catalysts are soluble in acrylonitrile. The weight ratio catalyst: monomer is the main factor influencing the molecular weight, it overshadows every other variable having an effect on the scope of the reaction, even the temperature in the limited region of practical importance (Table 1). The ratio monomer : water is, of course, only of secondary importance as no reaction proceeds in the aqueous phase, but one should adjust it bearing in mind the problems connected with agitation and heat transfer during the main time of the propagation step. A ratio of 1 : 3 has been used during the present investigation.

As is seen from Table 1, the average viscosimetric molecular weights are quite high even when the ratio catalyst : monomer is increased (up to 3 : 200). In the vinyl series considerably lower degrees of polymerization would have been expected. Addition of diluents in the monomer phase (Table 2), such as hydrocarbons or carbon tetrachloride, does not principally change this state of matters. Xylene of technical grade is an astonishing exception from this rule, however. It decreases the molecular weight of the polymer in a regular way as the concentration of the hydrocarbon in the organic phase is increased. The reason for this modifying action is not known, but it might be attributed to existing impurities in the crude isomeric mixture. The effect is not found in toluene and benzene.

Table 3. Polymerization of acrylonitrile with an azo activator (azo-bis(isobutyronitrile)) in the presence of a modifying agent (tert. dodecylmercaptan).

Composition of reaction mixtures:

Acrylonitrile	*200 g*
Water	*600 »*
Stabilizer (cellulose ether)	*1.2 »*
Activator	*See below*
Modifier	*See below*
Temp. of reaction	*60° C*
Time of reaction	*150 min.*

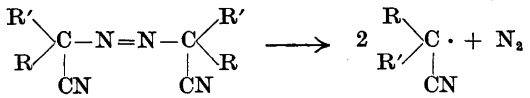
Prep. No.	Activator, g	Modifier, g	Yield, g	Av.visc. M. W. 10^{-3}
1	0.5	1	180	122
2	0.5	2	172	85
3	0.5	3	172	70
4	0.5	5	168	55
5	0.5	7	171	51
6	0.5	10	142	42
7	1.0	1	192	103
8	1.0	2	183	74
9	1.0	3	178	69
10	1.0	4	185	59
11	1.0	5	190	49
12	1.0	7	180	49
13	1.0	10	178	44

Medium polymer particle size = 0.10 mm.

As high ratios of catalyst : monomer severely influence on the heat stability of the finished polymer, a modifying agent (regulator) has, in general, to be added when polymers of "medium" and "low" molecular weights are to be prepared by the suspension technique. Fortunately the modifiers known in the synthetic rubber field [14] are also applicable in this case (Table 3); tertiary dodecyl mercaptan shows a regular influence on the average viscosimetric molecular weight, being generally represented by a continuous function of the amount of modifier in the polymerization system (Fig. 1).

An extraordinary property of acrylonitrile is the inability to polymerize in a basic medium. Favourable working conditions are found on the slightly acid side [1–4] in the case of emulsion polymers. In suspension systems the mentioned circumstances are still valid, but as a general rule minor variations of the acidity of the aqueous phase show no greater influence on the reaction rate. Polymerization at pH 7 indicates no conceivable drawbacks as far as initiation, propagation and reproducible molecular weights are concerned. Most of the present investigations were carried out at said pH value.

The initiating action of the azo catalysts imply a dissociation into free radicals and nitrogen according to the type formula



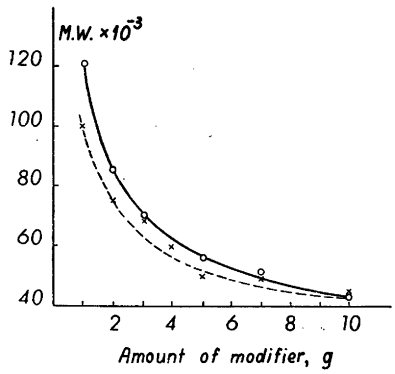
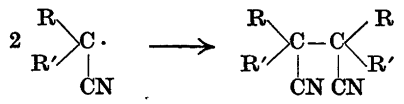


Fig. 1. Relation between the average viscosity molecular weight (= M.W.) of polyacrylonitrile and the amount of modifier in the reaction mixture.
× : *Ratio catalyst / monomer = 1 : 200*
O : *Ratio catalyst / monomer = 1 : 400*

The rate of this molecular dissociation is known to be essentially dependent only on the temperature so far as the energy of activation is approximately constant in a wide range of solvents [15]. The dissociation is not complicated by secondary or induced decomposition, but a side reaction, however, is known[9], competing with initiation of polymerization. Part of the free radicals always coalesce forming tetrasubstituted succinonitrile:



In the ideal case when the polymer chains are initiated and terminated by identical radicals, a principal difference between suspension polymers and redox polymers should exist in the meaning that the suspension polymer, prepared by the azo catalyst, is definitely hydrophobic contrary to a redox polymer (emulsion polymer) showing strongly hydrophilic (ionic) end groups. This may be the main reason for the differences in solubility between emulsion and suspension polymers. Most solvents for polyacrylonitrile [16], such as dimethyl formamide, dimethyl sulfoxide, ethylene carbonate, are hydrophilic and show negative heats of dilution in other hydrophilic low molecular weight compounds. In fact, if solvent action on the two grades of polymers is studied as recently described [17], it is immediately stated that the suspension polymers are substantially *insoluble* at room temperature (solvent : dimethyl formamide) when the emulsion polymer, showing identical average viscosity molecular weight, dissolves almost *completely*, regardless of the differences in molecular weight distribution. On heating (slow heating essential, due to slow rate of dissolution) the suspension polymer dissolves completely in a narrow range of temperature situated at least ten degrees centrigrade above the dissolution range of the emulsion polymer. Principally this difference exists even when the most powerful solvent, dimethyl sulfoxide, is used, but the effect cannot be observed, because every polymer investigated dissolves at 20° C — two centigrades above the melting point of the mentioned solvent!

SUMMARY

Suspension polymerization of acrylonitrile in the true sense, can be carried out, in spite of the high solubility of the monomer in water. The azo catalysts α,α'-azo-*bis*(*iso*butyronitrile) and α,α'-azo-*bis*(α,γ-dimethylvaleronitrile) initiate polymerization in the dispersed monomer, but not in the dissolved monomer. As dispersing agents may be used water soluble non-ionic cellulose ethers, showing surface activity, for instance ethyl hydroxyethyl cellulose. At low values of the ratio catalyst : monomer, the average viscosity molecular weights are fairly high, but they can be adjusted almost at want in a broad range by adding tertiary dodecylmercaptan to the reaction mixture. Xylene of technical grade serves the same purpose.

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Wet Combustion, an Alternative to Elution when Analysing Ion Exchange Resins

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The method of wet combustion has in recent years proved to be useful when analysing organic materials for metallic constituents [1]. For the analysis of ion exchange resins for small amounts of various ions, we have found wet combustion to be practicable and a valuable aid in our experiments [2-3]. As this method might be of value even in many other cases, the following results concerning the wet combustion of different ion exchange resins may be of interest. *

The ion exchange resins

Most of the experiments deal with cation exchangers, but a few experiments with an anion exchanger were also made because metals can be retained on anion exchangers as anionic complexes.

The cation exchange materials studied all contained sulfonic acid groups. They could be divided into two groups with respect to the resin structure: one consisting of phenol formaldehyde resins and the other, polystyrene resins.

As examples of the first group, Wofatit KS and Duolite C-3 were used and of the second group, Dowex 50 and Lewatit S 100. The last-mentioned was kindly placed at our disposal by Dr. H. Löffler.

The anion exchanger used was Amberlite IRA-400, which is a polystyrene divinylbenzene copolymer containing quaternary ammonium groups.

* *Note added in proof:* Unfortunately we did not recognize, before submitting the paper for publication, that Smith [7] as well as Argersinger *et al.*[8] used wet combustion for destroying ion exchange resins. Smith only showed the possibility of dissolving a sample of Dowex 50 of unknown composition in a mixture of nitric and perchloric acids. Argersinger *et al.* used sulfuric acid and metallic selenium as catalyst for the combustion of ion exchange resins. This method has recently been tested by Holm and Svensson [9] who found it too time-consuming in comparison to the nitric-perchloric and nitric-sulfuric acid methods studied by them.

The experiments with the phenol formaldehyde resins

The two groups of resins behaved very differently during the wet combustion, depending upon the differences in the resin structure. The experiments with Wofatit KS and Duolite C-3 were carried out with 1 g portions of air-dried resin. Both hydrogen-, sodium- and potassium-saturated resins were used. The particle size was about 1—1.5 mm for both exchangers.

The wet combustion was carried out in tall, 200 ml Pyrex beakers in which were placed 10 ml conc. nitric acid and 8 ml conc. perchloric acid. The beaker was placed on a hot plate with a temperature of about 200—250° C. Fumes of nitrogen dioxide were immediately expelled and after about 5 minutes the reaction began with gas evolution and the solution became dark red. Within 8—10 minutes the reaction was completed and about 5 ml of a colourless or light yellow solution had been obtained. The reaction was quantitative, even stray particles adhering to the walls of the beaker were destroyed by the vapours and finally dissolved. No difference in time between the sodium and hydrogen forms could be observed.

For potassium saturated exchanger the reaction took about 15—20 minutes and a precipitate of potassium perchlorate was obtained. The formation of a precipitate seems thus to make the dissolution of the resin more difficult.

Some experiments with moist resins proved that they were nearly as easily dissolved as air-dried ones.

To study the possibility of using the method for larger amounts of resin, experiments were made with 10 g samples of air dried hydrogen-saturated Duolite C-3. For such an amount, 50 ml conc. nitric and 40 ml conc. perchloric acids proved to be suitable, and the dissolution was complete within 20 minutes.

It was also shown that a Bunsen burner was as useful as a hot plate and that the proportion, 10 ml nitric acid to 8 ml perchloric acid, could be used equally well as the proportion, 12 ml nitric acid to 6 ml perchloric acid.

As the proportions of acids mentioned above and a total volume of 18 ml for 1 g of resin gave good results no experiments were made to find out if these proportions and total amounts of acids are the most useful ones.

The experiments show that the kind of ion in the resin plays no important role (at least for the ions studied in this paper) — only if a precipitate is formed does the dissolution seem to take a longer time than usual.

In connection with this work it deserves to be mentioned that the risk of explosion seems to be very small as long as the wet combustion is carried out with a mixture of nitric and perchloric acids [1]. As it might be dangerous to carry out the wet combustion only with perchloric acid it is wise as pointed out *e.g.* by Wåhlin [1] to have a mixture of nitric and perchloric acids in suitable proportions ready for use.

The experiments with the polystyrene resins

The particle size of the three resins was: Dowex 50 0.5—1.0 mm, Lewatit S 100 about 0.2—0.4 mm and Amberlite IRA-400 0.5—1.0 mm. All experiments using Dowex 50 and Lewatit S 100 were made with hydrogen-saturated exchanger.

For the cation exchange resins the proportions, 6 ml nitric acid to 12 ml perchloric acid for 1 g of resin proved to be suitable. For this case the proportions were more critical because too much nitric acid caused the solution to foam too vigorously, but it was not proved whether the proportions used are the most useful ones.

Experiments with both dry and moist Dowex 50 showed that it is necessary to dry moist resins in an oven at 100—110° C before the wet combustion, because water causes the solution to bump. When the resin had been properly dried, the reaction began after 15—20 minutes if the temperature on the plate was kept at about 150° C. When the reaction took place the solution foamed and by lowering the temperature the foaming was lessened so that the foam did not rise over the edge of the beaker. The reaction was finished within a few minutes, but it was, however, not quantitative. A small amount (about 1 %) of the resin, partly charred, was still undissolved. When a few drops of nitric acid were added the charred part dissolved and the residue was clear brown. After a while the particles charred again and a few more drops of nitric acid were added. This process was repeated until the residue had dissolved completely. For Lewatit S 100 this process took about 20 minutes and for Dowex 50 about 30 minutes.

For Amberlite IRA-400, 10 ml conc. nitric acid and 8 ml conc. perchloric acid for 1 g of resin proved to be suitable. The reaction was quantitative and finished within 15 minutes.

The material balance

In order to show that all ions taken up by the exchanger can be recovered after the wet combustion the following material balance studies were undertaken with silver, thorium and cadmium ions on Wofatit KS.

Silver. It has been shown by Högfeldt *et al.*[4] as well as Bonner and Rhett [5] that the last traces of silver are difficult to remove from some ion exchangers, and therefore for such a case wet combustion is a valuable method.

The following example is taken from an investigation of the Ag^+—H^+ equilibrium on Wofatit KS at low silver concentrations using Ag^{110} as tracer [2]. The radioactive silver was supplied from A.E.R.E. Harwell, Didcot, Berkshire, England. The experiments were carried out as follows. One g air-dried, hydrogen-saturated resin was shaken to equilibrium with 100.0 ml of a solution containing 10^{-7} C silver nitrate and sufficient nitric acid to give an ionic strength of 0.100. When equilibrium had been attained, the resin was separated from the equilibrium solution. The resin was wet combusted as described above. After dilution of the solution from the wet combustion to 10 ml in a 10 ml measuring flask the silver activities in both the equilibrium and this solution were measured in a liquid β-counter and these activities compared with the activity in the solution with which the resin was shaken to equilibrium. The results are given in Table 1.

Table 1. Material balance of Ag^+ on Wofatit KS.

Sample No.	Activity of equil. soln. cpm	Activity of resin / 10 cpm	Σ activity cpm	Introduced activity cpm	Difference cpm
1.	4 ± 1	15.1 ± 0.3	19 ± 1	20 ± 1	$-1 \pm \sqrt{2}$
2.	3 ± 1	16.9 ± 0.3	20 ± 1	20 ± 1	$\pm 0 \pm \sqrt{2}$

The term introduced activity means the activity in the 10^{-7} C silver nitrate solution with which the resin was shaken. The term Σ activity is the sum of the activity in the equilibrium solution and the activity in the resin after wet combustion. This latter activity is measured in a 10 ml volume after the wet combustion process and must therefore be divided by 10 so that it may be compared with the activity of the original 100 ml solution. As is seen from the last column the agreement is good and within the limits of experimental error.

During the wet combustion some chloride ions were formed but, because of the low silver concentration, no precipitate could be observed.

Thorium. In order to demonstrate the material balance for some more ions experiments were carried out with the thorium ion, since this ion is easily studied by radiochemical methods.

In a small column, 6×100 mm, Wofatit KS (H^+, 0.5—1.0 mm) 5.00 ml of a 0.05 C uranyl nitrate solution in radioactive equilibrium was introduced at such a low flow rate that nearly all of the activity was retained in the resin. After washing with about 10 ml of water, which was collected together with the solution from the sorption step, the resin was transferred to a beaker and wet combusted as described above. After combustion, the solution was transferred to a 10 ml measuring flask and diluted to 10 ml with water. The activities in this solution and the wash solution were measured in a liquid β-counter thus measuring the UX_1 activity and compared with the activity in 5 ml of the original solution diluted to 10 ml. The results are given in Table 2.

Table 2. Material balance of Th^{4+} on Wofatit KS.

Sample No.	Activity of wash soln. cpm	Activity of resin cpm	Introduced activity cpm	Difference between columns 2 and 3 cpm
1.	11 ± 1	$3\,806 \pm 33$	$3\,799 \pm 33$	$+ 7 \pm 33\sqrt{2}$
2.	17 ± 2	$3\,778 \pm 33$	$3\,799 \pm 33$	$- 21 \pm 33\sqrt{2}$

The agreement is good and within the limits of experimental error.

Cadmium. In order to show that not only radiochemical methods are useful when analysing wet combusted resins, some material balance studies were undertaken with cadmium using polarographic methods. The column was the same as in the experiments with uranyl nitrate and the experiments were

carried out in an analogous manner. Through the column was run various amounts of a 10^{-3} C cadmium sulfate solution but no efforts were made to regulate the flow rate so that all material was retained in the resin. At the end of the process the solution was heated to dryness to expel the acids. If the solution is too acid it is impossible to run the polarograms. To the dry residue was added 0.5 C sodium chloride solution and in addition water in such proportions that 100 ml of 0.1 C sodium chloride were obtained. The wash water was also made 0.1 C in sodium chloride. The solutions were polarographed on a Radiometer Polarograph, Type: PO 3 e, and the polarograms compared with that from a standard solution run under the same experimental conditions.

For further information concerning polarography of wet combusted samples the reader is referred to the paper by Wåhlin [1].

The results are given in Table 3.

Table 3. Material balance of Cd^{2+} on Wofatit KS.

Introduced amount μ moles	Wash water μ moles	Resin μ moles	Σ resin + wash water μ moles	Difference μ moles	Difference %
5.00	0.00	5.00	5.00	$\pm$ 0.00	0.0
5.00	0.00	5.10	5.10	+ 0.10	2.0
10.00	0.51	9.22	9.73	− 0.27	2.7
10.00	0.50	9.10	9.60	− 0.40	4.0
15.00	0.90	14.00	14.90	− 0.10	0.7
15.00	0.90	13.70	14.60	− 0.40	2.7
20.00	0.28	20.00	20.28	+ 0.28	1.4
20.00	0.28	19.60	19.88	− 0.12	0.8

The agreement is good and the experimental error seems to be what may be expected under these experimental conditions.

Suggested applications

The experiments mentioned above permit a number of suggestions for the application of wet combustion.

The experiments with silver demonstrate clearly the usefulness of the method when working with ions which can only be eluted with difficulty. Instead of obtaining a large volume we got the material in the resin concentrated in a volume of 10 ml. For all ions that are difficult to elute with or without complexing agents, wet combustion offers an attractive alternative to elution when working with small amounts (gram quantities) of resin.

It is also possible that wet combustion may be a useful alternative to elution when analysing waste waters etc. for small amounts of metals. It has been shown [6] that it is possible to obtain the amount in the resin in a small volume by eluting from the opposite direction to the sorption step and at such a low flow rate that equilibrium is attained. This procedure, however, takes such a long time that wet combustion may successfully compete since this process is rapid if resins of the phenol formaldehyde type are used.

For radiochemists the separation of radioactive tracers on ion exchange resins followed by wet combustion permits the determination of the specific activity of the radioactive constituents.

Other possibilities surely exist and this small paper only points out the possibility of solving analytical problems not only in terms of elution but also in terms of wet combustion.

SUMMARY

It is shown that it is possible to destroy the organic constituents of an ion exchange resin by wet combustion using a mixture of conc. nitric and perchloric acids. At the end of the reaction a clear solution is usually obtained. This fact makes it possible to use wet combustion as an alternative to elution when analysing small amounts of ion exchange resins for metals. Resins of the phenol formaldehyde type are more easily dissolved than resins of the polystyrene type and are to be preferred.

Material balance studies show that wet combustion does not affect the accuracy of the analyses in the cases studied.

A number of applications are suggested and it is stressed that, when analysing for ions, which can only be eluted with difficulty, wet combustion is an attractive method compared to elution.

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Derivatives of Trichlorolactic Acid

I. The Reactions of Trichlorolactic Acid with Phosphorus Pentachloride and Thionyl Chloride

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In a paper by R. Anschütz and A. Haslam [1] it was reported that α,β,β,β-tetrachloropropionyl chloride is the main product of the reaction between β,β,β-trichlorolactic acid and phosphorus pentachloride:

$$CCl_3CHOHCO_2H + 2\ PCl_5 \rightarrow CCl_3CHClCOCl + 2\ POCl_3 + 2\ HCl \qquad (1)$$

The boiling point given for the chloride, 140—142°/12 mm, appeared to us to be too high. Böeseken [2], who later used the same method, obtained the boiling point 150°/18 mm. For comparison it may be noted that pentachloropropionyl chloride has a much lower boiling point, 91—92°/12 mm[3], although the reverse should be true.

It has been even more surprising to note that Anschütz and Haslam state that the α-chlorine in tetrachloropropionyl chloride is very reactive. According to these authors, the chloride is readily hydrolyzed to trichlorolactic acid and in alcoholic solution forms the ethyl ester of α-ethoxy-β,β,β-trichloropropionic acid. These reactions do not seem possible; all other known α,β,β,β-halogenated propionic acids are stable to water [4].

These suspicions led us to renew the experiments of Anschütz and Haslam. After careful fractionation a reaction product was obtained that evidently was identical with that described by Anschütz and Haslam since its hydrolysis gave trichlorolactic acid, but the compound had a different density and above all, its analysis gave different results. The compound contained phosphorus and both the analytical data and the physical and chemical properties prove that the reaction between phosphorus pentachloride and trichlorolactic acid leads to the formation of β,β,β-trichlorolactyl chloride dichlorophosphate:

$$CCl_3CHOHCO_2H + PCl_5 \rightarrow CCl_3CH(OPOCl_2)COCl + 2\ HCl \qquad (1')$$

The analytical data of Anschütz and Haslam differ considerably from those of the product of this reaction, but their data must be in error or their compound

must have been impure. Table 1 allows a comparison of the average analytical data.

Table 1. Comparison of analytical data.

Element	Calculated		Found	
	C_3HOCl_5	$C_3HO_2PCl_6$	A & H	L & R
C	15.63	10.96	15.45	
H	0.43	0.31	0.68	
Cl	76.96	64.71	77.22	64.45
P		9.42		9.34

The structure we propose for the compound is consistent with the fact that the products of the hydrolysis of the compound were identified as trichlorolactic, phosphoric and hydrochloric acids. In addition the amount of liberated hydrochloric acid determined by argentometric titration corresponded to half the amount of the total chlorine in the compound. On the other hand, when dissolved in alcohol, the compound did not yield the ethyl ester of α-ethoxy-β,β,β-trichloropropionic acid as Anschütz and Haslam have reported, but the triethyl ester of β,β,β-trichlorolactic acid dihydrogen phosphate. This reaction has been confirmed by Leimu and Kivisaari [5].

The action of thionyl chloride on trichlorolactic acid was also studied. The reaction yielded β,β,β-trichlorolactic acid chlorosulphite:

$$CCl_3CHOHCO_2H + SOCl_2 \rightarrow CCl_3CH(OSOCl)CO_2H + HCl \qquad (2)$$

Several attempts were made to replace the chlorosulphite group with chlorine using methods earlier employed in corresponding exchange reactions[6, 7], but the experiments were unsuccessful.

It is, however, possible to prepare α,β,β,β-tetrachloropropionic acid and its chloride starting from β,β-dichloroacrylic acid. This synthesis is described elsewhere [8].

The described reactions of trichlorolactic acid are analogous with the reactions of several other hydroxyacids. Hydroxyarylcarboxylic and hydroxyarylsulphonic acids react in general with phosphorus pentachloride in the same manner as trichlorolactic acid [9]. Thionyl chloride reacts similarly with certain other aliphatic hydroxycarboxylic acids as with trichlorolactic acid [10].

The reaction of salicylic acid with phosphorus pentachloride L. Anschütz [11] has explained to occur *via* the intermediate $C_6H_4(OPCl_4)CO_2H$ which yields the compound $C_6H_4(OPOCl_2)COCl$ by a transfer of chlorine and elimination of hydrogen chloride. A similar mechanism is possible for the reaction between trichlorolactic acid and phosphorus pentachloride:

$$\begin{aligned} CCl_3CHOHCO_2H + PCl_5 &\rightarrow CCl_3CH(OPCl_4)CO_2H + HCl \\ CCl_3CH(OPCl_4)CO_2H &\rightarrow CCl_3CH(OPOCl_2)COCl + HCl \end{aligned} \qquad (3)$$

It should be noted that the second stage may also involve the interaction of two molecules by a more complex mechanism.

An alternative mechanism is that phosphorus pentachloride first reacts with the carboxyl group and the phosphorus oxychloride formed reacts with the hydroxyl group to yield the final reaction product:

$$\begin{aligned} CCl_3CHOHCO_2H + PCl_5 &\rightarrow CCl_3CHOHCOCl + POCl_3 + HCl \\ CCl_3CHOHCOCl + POCl_3 &\rightarrow CCl_3CH(OPOCl_2)COCl + HCl \end{aligned} \qquad (3')$$

A decision between these two mechanisms is rendered difficult by the fact that the reaction proceeds only partly according to Eq.(1') to give trichlorolactyl chloride dichlorophosphate and hydrogen chloride. Considerable amounts of phosphorus oxychloride and other byproducts with higher boiling points than that of the main fraction are formed, but the isolation of the latter by distillation is difficult. Phosphorus oxychloride is shown as one of the reaction products in Eq. (1) and as an intermediate compound in Eq. (3'). The occurrence of reaction (1) is questionable since tetrachloropropionyl chloride was not detected among the reaction products. The presence of phosphorus oxychloride among the final products of the reaction may be explained by assuming that another reaction takes place instead of the second stage of reaction (3'), *i. e.* the trichlorolactyl chloride formed in the first stage does not react with phosphorus oxychloride but, for example, with itself with the formation of a di- or polymeric lactide, or with phosphorus pentachloride whereupon the compound $CCl_3CH(OPCl_4)COCl$ is formed. This latter compound, the chloride of the acid formed as an intermediate in reaction (3), may also be produced by the reaction of this intermediate with phosphorus pentachloride, the latter changing into phosphorus oxychloride. The acid $CCl_3CH(OPCl_4)CO_2H$ or its chloride may also react with the α-hydroxyl group of trichlorolactic acid to form a compound containing two trichlorolactic acid residues linked together by the chlorophosphate residue. The elucidation of these different possibilities may require further investigation.

The literature contains numerous investigations which deal with the reactions between organic hydroxycompounds and phosphorus and other non-metal halides but the opinions presented are not concordant with each other in all respects [12]. In only a few of these studies has it been considered to what extent the structure of the hydroxycompound favours the fission of the carbon-oxygen bond in preference to the oxygen-hydrogen bond [13]. It may be noted, however, that among the simple alcohols the tertiary alcohols exhibit the smallest tendency to retain carbon-oxygen bonds and in general they yield the corresponding alkyl halides [13, 14]. These are followed by secondary alcohols which usually give higher yields of alkyl halides than the primary alcohols [13, 15]. β-Chloroethanol [16] and the phenols [13, 17] favour oxygen-hydrogen bond fission and yield ester halides (compounds which are both halides and esters of inorganic acids). These observations can be explained by the fact that an increase in the electronegativity of the carbon atom to which the hydroxyl group is attached strengthens the bond between carbon and oxygen. Accordingly, the formation of ester chlorides as the only detectable products of the reaction of trichlorolactic acid with phosphorus pentachloride and thionyl chloride is readily understood when it is noted that both the trichloromethyl and carboxyl groups increase the electrophilic properties of the adjacent carbon atom carrying the hydroxyl group.

EXPERIMENTAL

The reaction between trichlorolactic acid and phosphorus pentachloride. Trichlorolactic acid was prepared from chloral hydrate by the cyanohydrin synthesis developed by Kölln [18]. The samples of phosphorus pentachloride used in some of the experiments were

commercial products from different manufacturers, whereas the chloride used in other experiments was prepared from pure phosphorus trichloride and chlorine. In accordance with Anschütz and Haslam [1] one mole of trichlorolactic acid and 2 moles of phosphorus pentachloride were heated together for 5—6 hours on an oil bath, first slowly to 70° and then to 120—130°. The phosphorus oxychloride was distilled from the reaction product either under normal pressure or, more advantageously, under reduced pressure, after which the residue was fractionated under reduced pressure. The main fraction obtained after the removal of the phosphorus oxychloride was found to contain phosphorus. The yield from the fractionation was 40—45 % of the theoretical and boiled at 120—140°/10 mm. The distillate was a yellow liquid. The residue, a dark viscous syrupy mass, was fairly large. In a few cases it was possible to distill from the residue a fraction with a higher boiling point than that of the main fraction. The main fraction was redistilled from a Claisen flask two or three times at a pressure of 1—2 mm, whereupon its weight diminished by one half. It boiled at 101—102°/2 mm and had only a very slight yellow colour. When the synthesis was begun with equimolar quantities of trichlorolactic acid and phosphorus pentachloride in accordance with Eq. (1'), the fractionation was more difficult and the yield of the pure compound poor. An even smaller yield of the pure compound was obtained when trichlorolactic acid was first allowed to react with an equimolar amount of phosphorus oxychloride after which an equimolar amount of phosphorus pentachloride was added to the reaction mixture.

To determine the composition of the isolated compound, analyses were conducted for total chlorine, phosphorus, chlorine liberated by hydrolysis, and the molecular weight was determined cryoscopically (benzene). Parallel determinations were performed on the same and different preparations. β,β,β-Trichlorolactyl chloride dichlorophosphate is a viscous fluid slightly yellow in colour, the latter presumably due to impurities. Qualitative tests showed that the compound decomposed in water to trichlorolactic acid, phosphoric acid and hydrogen chloride. Its density at 20° was 1.7682 (d_4^{20}).

Calc. for $C_3HO_3PCl_6$ (329):	Cl	64.71,	P	9.42,	Cl/2	32.36,	Mol.wt.	328.8
Found	»	64.94,	»	9.32,	»	32.42,	»	340.2
	»	64.22,	»	9.13,	»	33.42		
	»	64.20,	»	9.21,	»	32.36		
	»	64.44,	»	9.70,	»	31.92		

The reaction of trichlorolactic acid with thionyl chloride. An excess of thionyl chloride was added to the trichlorolactic acid and the mixture refluxed on an oil bath during five days. Forty grams of trichlorolactic acid yielded 30 grams of a substance boiling at 107—109°/8 mm. When fractionated it boiled at 110°/10 mm. The compound, trichlorolactic acid chlorosulphite, was a colourless liquid of pungent odour which gradually solidified on standing. It dissolved in water with the formation of trichlorolactic acid, sulphur dioxide and hydrogen chloride.

$C_3H_2O_4SCl_4$ (276): Calc. for	Cl	51.40,	S	11.62
Found	»	51.22,	»	11.50

Attempts to transform β,β,β-trichlorolactic acid chlorosulphite into tetrachloropropionic acid or its chloride [6, 7] (in collaboration with P. Niemi). Several experiments were performed in which the relative amounts of thionyl chloride and trichlorolactic acid were varied. When the reaction had continued several days, a few drops of pyridine were added to the reaction mixture the temperature of which was then gradually increased to 110° during several hours. It was not possible to extract α,β,β,β-tetrachloropropionic acid from the reaction mixture with petroleum ether.

Five grams of ethyl trichlorolactate prepared according to Pinner [19] were heated on an oil bath at 80° with thionyl chloride (1.8—3 ml) and pyridine (6 drops) for several days. On distillation under reduced pressure the ester was obtained unchanged, but no ester of tetrachloropropionic acid.

Thirty-five grams of trichlorolactic acid chlorosulphite (0.13 mole) and 54 grams of phosphorus pentachloride (0.26 mole) were heated three days at 130°. The excess of phosphorus pentachloride was filtered off on a glass filter and the filtrate fractionated under reduced pressure. Seven grams of a substance boiling at 115—117°/10—12 mm were obtained. It was found to contain phosphorus and yielded 29.1 per cent hydrolyzable chlorine. In a second experiment the amount of phosphorus pentachloride was

reduced to one half of the above. The reaction product was divided into three fractions on distillation; all three fractions gave qualitative reactions for phosphorus and sulphur. The percentages of hydrolyzable chlorine were 26.7, 26.8 and 27.6. It is probable that the products of these reactions were mixtures of trichlorolactyl chloride dichlorophosphate and trichlorolactyl chloride chlorosulphite, $CCl_3CH(OSOCl)COCl$, since the two compounds liberate 32.4 and 24.1 % chlorine, respectively, on hydrolysis.

SUMMARY

The reaction between β,β,β-trichlorolactic acid and phosphorus pentachloride does not yield, as assumed previously, α,β,β,β-tetrachloropropionyl chloride, but gives β,β,β-trichlorolactyl chloride dichlorophosphate. Thionyl chloride reacts with trichlorolactic acid to give β,β,β-trichlorolactic acid chlorosulphite. The chlorosulphite-group in the latter compound could not be replaced by chlorine.

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Derivatives of Trichlorolactic Acid

II. The Triethyl Ester of β,β,β-Trichlorolactic Acid Dihydrogen Phosphate

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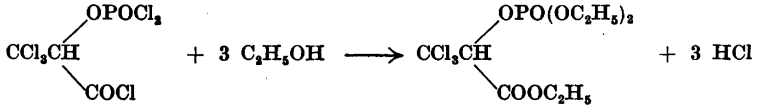
In a publication of Anschütz and Haslam [1] it has been mentioned that α,β,β,β-tetrachloropropionyl chloride is formed when β,β,β-trichlorolactic acid reacts with phosphorus pentachloride. Leimu and Ronkainen [2], however, have shown that the product of the reaction is the dichlorophosphate of β,β,β-trichlorolactyl chloride. According to Anschütz and Haslam, the reaction product reacts with ethyl alcohol with the formation of the ethyl ester of α-ethoxy-β,β,β-trichloropropionic acid, $CCl_3CH(OC_2H_5)COOC_2H_5$. The formation of this latter compound is possible, although less probable, from the parent compound with the composition proposed by Leimu and Ronkainen. The relatively high boiling point, 128—130°/12 mm reported by Anschütz and Haslam for the product of the reaction with alcohol is not in accordance with the structure suggested by them. For this reason it was considered necessary to reinvestigate the reaction. It was established that the reaction product is the triethyl ester of β,β,β-trichlorolactic acid dihydrogen phosphate:



It thus appears that also in this case the analytical data of Anschütz and Haslam are considerably in error, as shown by the comparison of the data in Table 1.

Table 1. Comparison of analytical data.

Element	Calculated		Found	
	$C_7H_{11}O_3Cl_3$	$C_9H_{16}O_6PCl_3$	A & H	L & K
C	33.68	30.23	33.29	
H	4.41	4.51	4.73	
Cl	42.66	29.75	42.86	29.63
P		8.66		8.48

EXPERIMENTAL

β,β,β-Trichlorolactyl chloride dichlorophosphate was prepared as described earlier [1,2]. This compound was reacted with ethanol under conditions identical with those in the experiments of Anschütz and Haslam, but an excess of ethanol was found advantageous. The product of the reaction was distilled twice under reduced pressure from a Claisen flask. Twelve grams of trichlorolactyl chloride dichlorophosphate yielded 4 g of the ester which boiled at 140—142°/1—2 mm. The ester does not immediately form hydrogen chloride when added to water.

Calc. for $C_9H_{16}O_6PCl_3$ (358):	Cl	29.75,	P	8.66
Found	»	29.35,	»	8.48
»	»	29.87		
»	»	29.64		
»	»	29.43		
»	»	29.85		

SUMMARY

When the reaction product of β,β,β-trichlorolactic acid and phosphorus pentachloride is reacted with ethyl alcohol, the reaction product is not the ethyl ester of α-ethoxy-β,β,β-trichloropropionic acid, as assumed previously, but the triethyl ester of β,β,β-trichlorolactic acid dihydrogen phosphate.

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*iso*Thiocyanates VIII. Synthesis of *p*-Hydroxybenzyl *iso*Thiocyanate and Demonstration of its Presence in the Glucoside of White Mustard (*Sinapis alba* L.)

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Sinalbin, the *iso*thiocyanate glucoside in seed of white mustard (*Sinapis alba* L.), is enzymatically hydrolyzed to glucose, the sulphate of the base sinapine and a non-volatile *iso*thiocyanate. The latter has never been isolated in the pure state or adequately identified. Will and Laubenheimer [1] obtained upon treatment of the silver salt of sinalbin with hydrogen sulphide a nitrile, which on acid hydrolysis yielded what they believed to be *o*-hydroxyphenylacetic acid. Salkowski [2] later revised this structure to the corresponding *para*-isomeride and accordingly formulated the mustard oil of sinalbin as *p*-hydroxybenzyl *iso*thiocyanate (I). His attempts to substantiate this assumption by a synthesis of (I) resulted in the production of an oily, vesicant substance for which neither analyses nor other proper characterisation were presented. The extraordinary lability of (I) may explain this as well as more recent failures to synthetise and characterise the *iso*thiocyanate of white mustard seed, as pointed out in the review of André [3]. The fact that seed of *Sinapis alba* constitutes an important ingredient in the manufacture of table mustard seemed to us to warrant a reëxamination of the *iso*thiocyanate contained therein.

A few preliminary synthetic experiments sufficed to indicate the great difficulties attending the preparation of (I) in analytically pure form, due to oxidation, polymerization and maybe other secondary reactions. Therefore, attention was turned to the possibility of removing the desired *iso*thiocyanate immediately after its formation by means of an amine, thereby transforming the labile compound into an equally characteristic but more tractable thiourea-derivative. Eventually, this technique proved successful although a great many experiments were needed in order to establish the rather special conditions required for the reaction to proceed as desired.

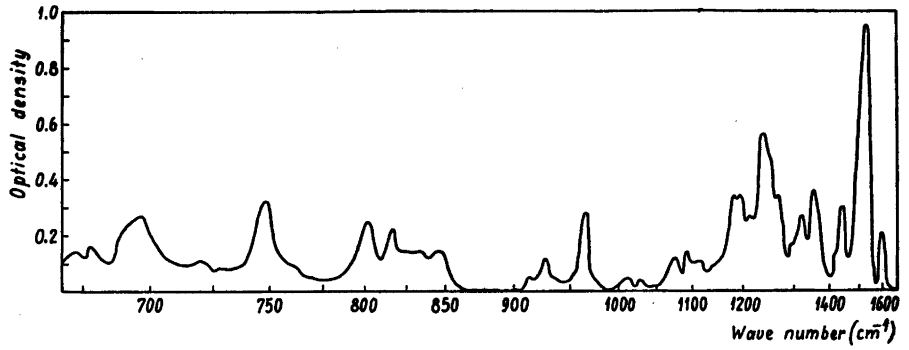
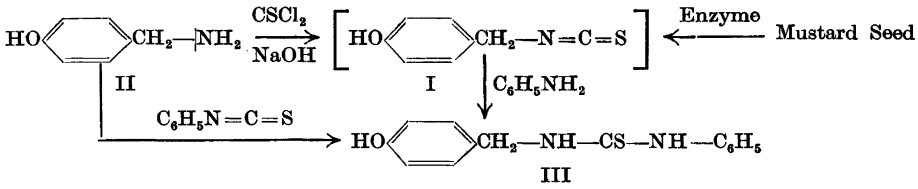


Fig. 1. Infra-red absorption spectrum of N-p-hydroxybenzyl-N'-phenyl-thiourea (III) dispersed in a potassium bromide plate.

p-Hydroxybenzylamine, needed as a starting material, was prepared by catalytic reduction of *p*-hydroxybenzaldoxime, essentially as described by Ott and Zimmermann [4]. Various methods were investigated for the transformation of the amine (II) into the corresponding *iso*thiocyanate (I). These included the Hofmann synthesis, the modified Andreasch-Kaluza procedure (*cf.* Ref. [5]) as well as a great number of modifications of Dyson's method [6,7], employing thiocarbonyl chloride. Most attempts proved completely abortive and resulted in intractable, dark and gummy products which showed no signs of yielding crystalline derivatives upon treatment with a rather wide selection of amines. Intermolecular condensations, in which both the *iso*thiocyanate- and hydroxy-grouping are involved, seem to be mainly responsible for the formation of secondary products, mostly of polymeric nature. It was eventually found, however, that the reaction between (II) and thiocarbonyl chloride, when conducted under special circumstances as described in the experimental part below, proceeded in the desired way to give (I). Its presence in the solution appeared from the isolation of a crystalline thiourea-derivative (III) upon reaction with aniline.



To support the structure (III) another specimen was independently prepared from the amine (II) and phenyl *iso*thiocyanate. Finally, the connection to the natural product was made when it was observed that the *iso*-thiocyanate, liberated upon enzymatic hydrolysis of defatted, pulverised seed of white mustard, could be continuously removed by ether extraction and directly transformed into a phenylthiourea-derivative on reaction with aniline.

After careful purification the three, independently prepared derivatives proved identical, having the same melting point undepressed on mutual admixture. Further proof of the identity was furnished through determination of the infra-red absorption spectra of the three specimens of N-*p*-hydroxybenzyl-N'-phenyl-thiourea (III). The identical infra-red spectrum of the three thioureas is presented in Fig. 1. The very intensive line at 1 532 cm^{-1} is attributable to the C = S-grouping, while the band exhibited at 1 595 cm^{-1} is due to the benzene rings. A rather strong absorption at 1 437 cm^{-1} may be assigned to the CH_2-deformation vibration while the bands at 748 cm^{-1} and 800—815 cm^{-1} probably arise from the mono- and *p*-disubstituted benzene rings, respectively.

Thus it has been definitely proved that *p*-hydroxybenzyl *iso*thiocyanate (I) is the mustard oil liberated on enzymatic hydrolysis of seed of white mustard as suggested many years ago by Salkowski [2], although its extraordinary labile character makes it rather doubtful that an analytically pure sample could be prepared.

EXPERIMENTAL *

p-*Hydroxybenzaldoxime*. The following procedure was found more convenient than the one previously reported [4]. To a suspension of 95 g of *p*-hydroxybenzaldehyde and 81.5 g of hydroxylamine hydrochloride in 500 ml of water was added a solution of 40.5 g of sodium hydroxide in 320 ml of water and enough alcohol to give a clear solution. This was boiled under reflux for one hour, the main part of the ethanol removed *in vacuo* and the aqueous phase successively extracted with one 200 ml and three 100 ml portions of ether. The ethereal phase was dried over sodium sulphate and the ether removed whereupon the oxime separated. It recrystallised from a large volume (6 l) of benzene as beautiful, slightly pink crystals (80 g, 75 %), m.p. 115—116°. (Found: N 10.18. Calc. for $C_7H_7O_2N$; N 10.22).

From the mother liquor an additional crop (15 g) could be obtained which melted at 112—113°. Ref.[4] reports the m.p. 112° for the anhydrous oxime.

p-*Hydroxybenzylamine* (II). The oxime was catalytically reduced by a modification of the procedure of Ott and Zimmermann [4].

To a solution of 15 g of the oxime in 750 ml of 96 % ethanol were added 300—400 mg of Adam's PdO-catalyst and 10 ml of concentrated hydrochloric acid. The mixture was shaken at ordinary temperature and pressure in a hydrogen atmosphere. Within 6—7 hours a total amount of 6.6—6.7 l of hydrogen had been absorbed whereupon the uptake suddenly stopped. The catalyst was removed by filtration through a layer of Celite and the filtrate concentrated to a small volume *in vacuo*. Upon addition of ether, *p*-hydroxybenzylamine hydrochloride separated as a colourless, crystalline powder (12.9 g, 74 %), m. p. 190—191°. (Found: Cl 22.34. Calc. for $C_7H_{10}ONCl$: Cl 22.21). Ref.[4] reports the m.p. 195° for the hydrochloride.

p-*Hydroxybenzyl* iso*thiocyanate* (I) (in solution). After many experiments the following procedure was eventually adopted as the most suitable for the preparation of a dilute ether solution of (I). A three-necked flask, to the bottom of which was sealed a wide-bore stopcock, was equipped with a separatory funnel, in- and outlets for a nitrogen stream and an effective, mercury-sealed stirrer. In the flask were placed 1.08 g of thiocarbonyl chloride dissolved in 25 ml of ether and 1.5 g of *p*-hydroxybenzylamine hydrochloride in 20 ml of water. The apparatus was flushed with nitrogen and a slight nitrogen pressure was kept in the system throughout the following operations.

* All melting points are uncorrected and determined in capillary tubes in an electrically heated block.

The flask was now immersed in an ice-bath and a solution of 1.12 g of sodium hydroxide in 10 ml of water added from the separatory funnel under vigorous stirring in the course of 10 minutes. After stirring for another 10 minutes, the lower phase was drained off through the stopcock whereas the desired *iso*thiocyanate remained in the ether phase as shown by the following reaction.

N-p-*Hydroxybenzyl-N'-phenyl-thiourea* (III) *from* p-*hydroxybenzyl* iso*thiocyanate*. To the ether phase above was dropwise added from the separatory funnel a solution of 0.72 g of aniline in 5 ml of ether. Stirring was continued at room temperature for 24 hours whereupon the clear solution was concentrated *in vacuo* at ordinary temperature. A crystalline mass embedded in a brownish oil remained. The latter was carefully removed with a little ethyl acetate and the crystalline residue was recrystallised several times from dilute ethanol and finally once from aqueous *n*-propanol. The colourless crystals (40 mg) melted at 166°, undepressed on admixture with the independently synthetised specimens reported below. (Found: N 10.92; S 12.31. Calc. for $C_{14}H_{14}ON_2S$ (258.3): N 10.85; S 12.41).

(III) from p-*hydroxybenzylamine*. To a concentrated solution of 0.53 g of *p*-hydroxybenzylamine in ethanol was added 0.64 g of phenyl *iso*thiocyanate and the mixture was refluxed for half an hour. Upon cooling and addition of water an oil separated which soon solidified in the ice-box. Beautiful colourless needles separated on recrystallisation from aqueous ethanol, m.p. 167—168° alone or in admixtures with the two other preparations. (Found: N 10.83; S 12.40).

(III) from seed of Sinapis alba. White mustard seed was thoroughly ground in a mill and the powder exhaustively defatted with hot ether. Fifty-seven grams of the resulting material were shaken with 400 ml of water for four hours. The suspension was separated in a high-speed centrifuge and the supernatant completely cleared upon filtration through Celite. It was next placed in a liquid-liquid extractor and continuously extracted with ether for 48 hours. Before starting the extraction, 0.26 g of aniline was placed in the flask containing the ether to be cyclised, thereby securing immediate reaction between the amine and the labile *iso*thiocyanate carried over by the solvent.

Upon concentration of the ether phase a crystalline material separated in a brownish oil which could be removed by careful treatment with ethyl acetate. Repeated recrystallisations from aqueous ethanol afforded the thiourea as beautiful colourless needles, m.p. 167—168°, undepressed again upon admixtures with the samples above. (Found: N 10.82; S 12.21).

Infra-red absorption spectra. The infra-red spectra of the three thiourea samples were determined in potassium bromide plates on a Beckman IR 2 instrument.

SUMMARY

p-Hydroxybenzyl *iso*thiocyanate has been synthetised in ether solution from *p*-hydroxybenzylamine and thiocarbonyl chloride. Its identity has been established upon comparison of its phenylthiourea-derivative with an authentic specimen of N-*p*-hydroxybenzyl-N'-phenyl-thiourea, prepared from *p*-hydroxybenzylamine and phenyl *iso*thiocyanate.

The *iso*thiocyanate formed during enzymatic hydrolysis of the glucoside of white mustard (*Sinapis alba* L.) has been definitely proved to be *p*-hydroxybenzyl *iso*thiocyanate through its reaction with aniline, giving a thiourea identical with the two independently prepared derivatives above.

The authors are indebted to Dr. Clauson-Kaas and his associates for the preparation of the potassium bromide plates and to Dr. Bak and Mr. Rastrup Andersen for their assistance in determining and discussing the spectra. Microanalyses were made in this laboratory by Mr. W. Egger.

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The Sugar Component of Deoxypentosenucleic Acid from *Mycobacterium phlei*

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In a previous paper [1] one of us with another colleague described a convenient method for the isolation of the sugar component of the purine nucleosides of deoxypentosenucleic acids (DNA). In the case of DNA from soft herring roe and cod roe the sugar component was shown to be 2-deoxy-D-ribose. Very little is known about the sugar of DNA from microorganisms. Only in the case of DNA from *Mycobacterium tuberculosis* has chromatographic data provided by Chargaff *et al.*[2], suggested the presence of 2-deoxy-D-ribose in the purine nucleosides. Consequently it was considered of great interest to identify conclusively the sugar in a microbial DNA. The present work is concerned with the qualitative and quantitative determination of the sugar component of purine nucleosides of DNA from *Mycobacterium phlei*. This organism was chosen because it provided a fairly convenient source for the preparation of sufficient quantities of DNA.

The possibility of the existence of linkages of the type: sugar(1)-phosphate in the DNA under investigation, as suggested by Overend, Stacey and Webb [3] for herring roe DNA has also been examined.

EXPERIMENTAL

Analytical methods

Nitrogen was estimated by means of a micro-Kjeldahl method.

For the phosphorus estimations the colorimetric method of Allen modified by Jones, Lee and Peacocke [5] was used.

The pentosenucleic acid (RNA) content of the nucleic acid was determined by the method of Euler and Hahn [6] using yeast RNA as standard.

Enzymes

The deoxyribonuclease (DRNase) was prepared from oxpancreas according to the procedure of McCarthy as modified by Overend and Webb [7] (For the freeze dried preparation, Found: P 2.0 %). The alkaline phosphatase was isolated from calf small intestine by the method of Klein [8]. The enzymes were stored in the freeze dried state.

The isolation and purification of DNA from *Mycobacterium phlei*

Nucleic acid was isolated from *M. phlei* by the method of Jones [9]. The bacterial cells were disintegrated in the presence of sodium arsenate (0.01 *M*) by shaking with ballotini glass beads. The suspension was diluted and allowed to stand at 0° C for 24 hours. The nucleoproteins were precipitated from solution by the addition of cetyltrimethylammonium bromide and purified by four reprecipitations from 1 *M* sodium chloride solution by dilution with water (3 vols.). The protein, cell debris and cetyltrimethylammonium bromide were removed by treatment with chloroform and octanol, the nucleic acid precipitated with ethanol, dissolved in distilled water, dialyzed and dried *in vacuo* in the frozen state. The white fibrous product contained after extensive drying *in vacuo* at 110° C: N 12.2, P 7.6 and RNA 12 %.

To remove the RNA it was decided in this case to use the charcoal method (Chargaff and Zamenhof [10], Dutta, Jones and Stacey [11]) as this is convenient when the RNA content of the nucleic acid is small.

Impure DNA (0.55 g) afforded 0.26 g of a highly fibrous product. It contained after extensive drying *in vacuo* at 110° C: N 13.2, P 7.6 and RNA <1 %.

The purine and pyrimidine composition of DNA from *M. phlei* has been reported in a previous paper [13].

Enzymic degradation of DNA

Purified DNA (moist product, 0.26 g) was dissolved in water (15 ml) and 0.1 *N* NaOH (3.5 ml) added. The solution immediately became less viscous and was kept at room temperature for 30 minutes. It was subsequently neutralized by the addition of 2 *N* sulphuric acid, and magnesium sulphate (0.196 g with water of crystallisation) in water (4.5 ml) and DRNase (0.0025 g) in water (0.5 ml) added. The mixture was kept in a stoppered flask at 37° C for 24 hours. At intervals the pH of the solution was adjusted to 6 by addition of minute amounts of conc. ammonia. Aliquots (0.06 ml) were withdrawn with a micropipette and the amount of phosphorus present determined. From the volume of the digest the total amount of phosphorus was calculated to be 0.0184 g. (The amount of phosphorus present in the DRNase could be ignored.)

To the DRNase digest were added ammonium sulphate (0.196 g), phosphatase (0.01 g), and the pH adjusted to 9 by addition of conc. ammonia. To prevent growth toluene (2 ml) was added. The mixture was kept for 10 hours at 37° C and the pH again adjusted to 9. The digest was kept for further 38 hours at 37° C, the precipitated magnesium ammonium phosphate filtered off quantitatively on a sintered-glass filter after the mixture had been kept at 0° C for 12 hours, washed with icecold water (10 ml), redissolved on the filter in 2 *N* sulphuric acid and the solution made up to 50 ml with water. The phosphorus content of the solution was determined. By applying a correction for the solubility of magnesium ammonium phosphate at 0° C in the digestion mixture, the percentage of dephosphorylation of the DNA was calculated to be 98.3 %.

The filtrate containing the nucleosides was dried in vacuum in the frozen state. The mixture of salts and nucleosides was then dissolved in water (40 ml) (the pH of the solution was 4.8), insoluble phosphatase removed at the centrifuge, and the precipitated enzyme washed with acetate buffer, pH 4.8 (2 × 2.5 ml). The protein precipitate contained negligible amounts of DNA nucleosides as shown by the negative Dische test. The solution containing the nucleosides and the buffer used for washing the precipitate were transferred quantitatively to a volumetric flask and the total volume made up 50 ml.

Determination of the amounts of thymidine and 2-deoxy-D-ribose of the purine nucleosides in an aliquot of the nucleoside mixture

The mixed bed ion exchange resin technique described in a previous paper [1] for the isolation of a mixture of thymidine and 2-deoxy-D-ribose was applied. A suitable mixed bed ion exchange resin was prepared by mixing with stirring in a glass column wet Amber-

lite IR-120 (H) with an equal volume of Amberlite IR-4B (OH) (the particle size of the resins was 40—60 mesh). An aliquot (5 ml, equal to 1/10 of original digest) of the solution (total volume 50 ml) containing the nucleosides was transferred to the top of the column (diameter 2 cm, length 4 cm) and washed through slowly with water until the eluate gave a negative Dische reaction and contained no ultra-violet absorbing material. (100 ml of water was necessary and the eluate passed through the column at a rate of 0.5 ml/min.). The eluate was concentrated by evaporation in the frozen state in vacuum and then quantitatively transferred to a volumetric flask and the volume made up to 50 ml. The amount of thymidine and 2-deoxy-D-ribose present in the solution was determined in the following way.

(I) Quantitative determination of thymidine. An aliquot of the above solution was diluted suitably with water (2 ½ times) and the ultra-violet absorption was determined in the Beckman spectrophotometer in 1 cm cells using water as blank.

(II) Quantitative determination of 2-deoxy-D-ribose. The colour produced with an aliquot of the solution to be tested (2 ml) and the Dische reagent (5 ml) was measured at 590 mμ in a Colman spectrophotometer. The Dische reagent employed was that described by Deriaz *et al.*[14].

The solution being examined contained as the only components thymidine and 2-deoxy-D-ribose (see later chromatographic evidence) and hence a standard solution consisting of known amounts of thymidine and 2-deoxy-D-ribose has been used. The standard solution was prepared in the following way.

Thymidine (prepared from cod roe DNA by the method of Laland and Overend [1]) was recrystallized twice from acetone and had m.p. 183—184° C. 2-Deoxy-D-ribose was a synthetic product recrystallized twice from ethyl acetate and had m.p. 78—80° C. Approximately 0.005 g of each was accurately weighed and dissolved in water (25 ml). Three solutions were prepared: (1) 2-deoxy-D-ribose solution (5 ml) + water (5 ml), (2) thymidine solution (5 ml) + water (5 ml), (3) 2-deoxy-D-ribose solution (5 ml) + thymidine solution (5 ml).

The optical densities of the colours developed with the Dische reagent and equal volumes of solution (1) and (3) were 0.33 and 0.37 respectively, thus showing that the presence of thymidine had only a small effect on the Dische colour developed by 2-deoxy-D-ribose.

By examining the ultra violet absorption spectrum of solution (2) and (3) it was shown that the presence of 2-deoxy-D-ribose approximately equal to that of thymidine had no effects upon its spectrum.

Solution (3) was employed as the standard solution for determining the amounts of 2-deoxy-D-ribose and thymidine in the eluate from the mixed bed resin. The quantities of these two components in 1/10 of the DNA digest (nucleoside mixture) was thymidine, 1.87 mg and 2-deoxy-D-ribose, 3.25 mg.

Paper chromatography of the eluate containing 2-deoxy-D-ribose and thymidine

An aliquot of the concentrated eluate containing 37 μg of thymidine and 64 μg of 2-deoxy-D-ribose was put on a paper strip and the chromatogram developed with the top layer of a *n*-butanol-ethanol-water-ammonia (conc. aqueous) mixture (40 : 10 : 49 : 1, v/v/v/v). The strip was photographed in the ultra-violet light and only one spot with R_F value identical to a reference spot of thymidine was visible. On subsequent spraying with a phenylene diamine reagent [15] one spot only was visible when examined under the ultra-violet lamp. It had R_F value identical to a reference spot of 2-deoxy-D-ribose.

Method for detection of "free" 2-deoxy-D-ribose in the mixture of salts and nucleosides (from the DNA digest)

The remainder of the solution containing the nucleoside mixture (45 ml, representing 9/10 of the total DNA digest) was dried in the frozen state in vacuum. The residue was dissolved in water, (10 ml), two aliquots (0.5 ml, representing 9/200 of the original mixture of nucleosides) were withdrawn and transferred to small flasks (25 ml). To one of the

flasks was added an aqueous solution (10 μl) containing 2-deoxy-D-ribose (50 μg). The solutions in the two flasks were evaporated to dryness in the frozen state in vacuum. To each of the residues were added absolute acetone (5 ml) (2-deoxy-D-ribose is easily soluble in acetone) and left at room temperature for 48 hours with occasional shaking. The insoluble material was filtered off and washed with acetone (3 ml). The filtrates were concentrated to dryness in vacuum and the two residues were each dissolved in water (300 μl; pH of these solutions was 5.5). On a strip of paper was put the following four spots: 10 μg and 20 μg of synthetic 2-deoxy-D-ribose and 120 μl of each of the above solutions. The solution which contains 2-deoxy-D-ribose added initially, is designated 1) and the other designated 2). The paper strip was developed with the *n*-butanol-ethanol-water-ammonia mixture described previously. The dried strip was photographed in the ultra-violet light. The chromatographic runs of solutions 1) and 2) exhibited on the photographic print three well defined spots with R_F values respectively 0.19, 0.29 and 0.50 (the last spot being thymidine). The paper strip was then sprayed with the phenylene diamine (as hydrochloride) reagent and examined under the ultra-violet lamp. Solution 1) exhibited three spots with the following R_F values: 0.19, 0.29 and 0.40. Solution 2) gave only two spots with R_F values: 0.19 and 0.29. The reference runs of respectively 10 μg and 20 μg of 2-deoxy-D-ribose gave only one spot each with R_F value 0.40. Consequently 2-deoxy-D-ribose was only present in solution 1) to which synthetic sugar had been added originally. An inspection of the chromatogram also revealed that the aliquot (120 μl) of solution 2) on the chromatogram contained less that 10 μg of 2-deoxy-D-ribose (since the 10 μg reference spot could be detected). The appearance of the two slowest moving spots in solution 1) and 2) showing up with the sugar spraying reagent (R_F values 0.19 and 0.29) and having R_F values identical with the two slowest moving nucleosides, is caused by the hydrolysis of the nucleosides with the acidic spraying reagent.

Isolation and characterization of thymidine and 2-deoxy-D-ribose

The remainder of the solution containing the mixture of the nucleosides (9 ml, representing 8/10 of the original nucleoside mixture) was passed down a mixed bed resin as previously described using proportional quantities of resins and eluate, combined with what was left of the 2-deoxy-D-ribose and thymidine obtained from 1/10 of the original digest. The combined solutions were evaporated to dryness in the frozen state in vacuum and the syrupy residue treated with dry acetone (1 ml) for a week at 0° C. The precipitated crystalline material (12 mg) was filtered off, washed with dry, cold acetone (2 ml) and recrystallized from acetone. The highly crystalline material had m.p. 183—185° C, alone and in admixture with thymidine isolated from cod roe DNA. (Found. C 49.6, H 5.7; Calc. for $C_{10}H_{14}O_5N_2$ (242): C 49.6, H 5.8.)

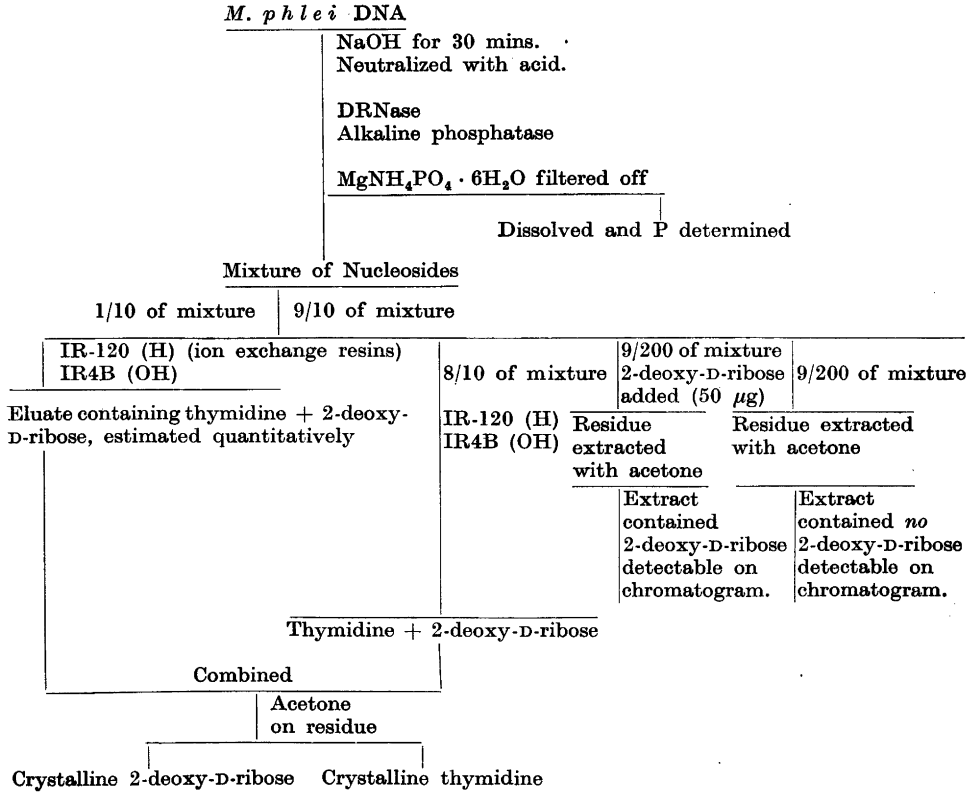
The combined acetone filtrate was concentrated to dryness *in vacuo* and the residue dissolved in water (1.5 ml). Estimation of the amounts of 2-deoxy-D-ribose and thymidine present, was carried out as already described and amounted to respectively 21 mg and 2.1 mg. The optical rotation of the solution (at equilibrium) was determined in a micro-rotation tube (0.5 dm). Taking into account the rotation of the small amount of thymidine ($[\alpha]_D^{20} = +62°$) present, the observed rotation for the 2-deoxy-D-ribose was: $[\alpha]_D^{20} = -64°$. Deriaz *et al.*[16] quote for synthetic 2-deoxy-D-ribose, $[\alpha]_D^{20} = -58°$.

The solution of the 2-deoxy-D-ribose used for the determination of the optical rotation was evaporated to dryness in the frozen state in vacuum. The resulting syrup was seeded with 2-deoxy-D-ribose and when crystallization had terminated ethyl acetate (1—2 ml) was added and the solid filtered off after 24 hours. The faintly yellow product (9 mg) had m.p. 70—80° C (micro melting point) alone or in admixture with authentic 2-deoxy-D-ribose (synthetic 2-deoxy-D-ribose had m. p. 78—80° C). Because of the small amount of sugar isolated no attempt was made to recrystallize it in order to obtain a higher m.p. Instead the anilide was prepared by the procedure given by Deriaz *et al.*[16]. The shiny crystals of the anilide so obtained had m.p. 163—164° C (micro melting point) alone or in admixture with authentic 2-deoxy-D-ribose anilide.

RESULTS AND DISCUSSION

The purified DNA from *M. phlei* employed in the present experiments gave negative protein tests (Millons and Sakaguchi) and contained less than 1 % RNA. Its fibrous nature indicated that extensive degradation has not occurred during the isolation procedure. The fairly low nitrogen and phosphorus values suggest the presence of impurity in the nucleic acid preparation. This "impurity" is not removed by prolonged dialysis or by exhaustive drying *in vacuo* at 110° C and therefore does not appear to be inorganic material or water. The presence of acidic polysaccharide is a possibility (see Dutta, Jones and Stacey [11] and Zamenhof *et al.*[17]), but so far no definite evidence of the presence of polysaccharide in the DNA from *M. phlei* has been obtained. Attempts to raise the nitrogen and phosphorus values by fractionation with cetyltrimethylammonium bromide, calcium and ethanol, copper precipitation and by digestion with sodium hydroxide at 37° C followed by precipitation with dilute acid have been unsuccesful. The presence of impurity in no way invalidates the values of the present investigation, although the presence of phosphorus in the "impurity" may explain why less than 4 moles of base per 4 atoms of phosphorus were found [13].

Table 1. Fractionation scheme of M. phlei DNA digest.



A general outline of the enzymic breakdown of the DNA and the various manipulations of the obtained mixture of nucleosides is seen in Table 1. The method and conditions employed for the degradation of DNA and the isolation of the mixture of 2-deoxy-D-ribose (the recovery of the sugar from a mixed bed resin as employed in this investigation is 100 %) and thymidine permitted a quantitative determination of these two compounds to be made in relation to the DNA phosphorus. Thus it was possible to calculate the moles of thymidine and 2-deoxy-D-ribose per 4 atom of DNA phosphorus. The figures for thymidine and 2-dexoy-D-ribose should agree respectively with that for thymine and total purines obtained by the quantitative chromatographic analysis of *M. phlei* DNA (reported in an earlier paper [13]). All these figures are presented in Table 2.

Table 2. The quantities of various constituents of M. phlei DNA. The figures denote moles per 4 atom of phosphorus.

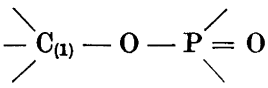
From chromatographic analysis		Present investigation	
Thymine	0.49	Thymidine	0.53
Total purines	1.57	2-Deoxy-D-ribose	1.66

It will be seen that the agreement between the figures in the horizontal rows is fairly good. The figure obtained for the total purines by a method [18] developed by one of us (S.G.L.) is 1.64 and hence the agreement with the present investigation is better.

When attempting to explain the discrepancies between the results of the two methods represented in Table 2, it should be borne in mind, that in the chromatographic analysis of the purines and pyrimidines the total nitrogen of these accounted for only 92.5 % of the total nitrogen of the DNA. The figures quoted from the chromatographic method in Table 2 could therefore possibly be too low.

It is important to note that the method described in this paper for the determination of the purine bound 2-deoxy-D-ribose and thymidine per 4 atom of phosphorus is general applicable to DNA's.

The initial treatment of DNA with alkali (preceding the enzymic degradation) was intended to break linkages of the type (Overend, Stacey and Webb [3]):



In the formulae $C_{(1)}$ is the carbon No. 1 of the 2-deoxy-D-ribose moiety. It would be expected that 2-deoxy-D-ribose molecules involved in the original DNA in linkages of the above type should appear in the final enzymic digest as "free" 2-deoxy-D-ribose. "Free" 2-deoxy-D-ribose would also be present if the enzyme preparation contained some nucleoside-cleaving enzyme. The interpretation of the presence of the "free" sugar could therefore be uncertain

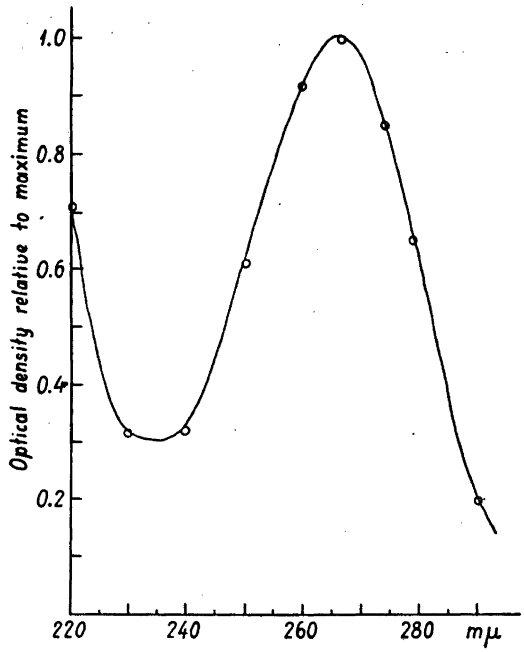


Fig. 1. Ultra-violet absorption spectra in distilled water. The drawn curve is the spectrum of thymidine isolated from DNA cod roe and the open circles represent the spectrum of thymidine from DNA isolated from Mycobacterium phlei.

unless the presence of such an enzyme could be excluded. However, in the absence of the "free" sugar the results could be interpreted with absolute certainty, and this was the case in the present investigation. A chromatographic method was adopted for the detection of any "free" sugar. This method was shown to be capable of detecting the presence of as little as 10 μg of 2-deoxy-D-ribose in an aliquot of the digestion mixture. As no 2-deoxy-D-ribose could be detected, the aliquot of the mixture of nucleosides contained less than 10 μg of 2-deoxy-D-ribose. Thus it could be calculated that the presence of "free" 2-deoxy-D-ribose in the mixture of nucleosides occurred to an extent of less than 1 % of the total sugar content. Consequently the presence of linkages of the $C_{(1)}$—phosphate type in the original DNA would occur in less than 1 % of all the sugar molecules.

The presence of thymidine and 2-deoxy-D-ribose in the nucleic acid from *M. phlei* was finally proved by the isolation and characterization of these substances. On the basis of mixed melting point of the isolated sugar and its anilide with the corresponding synthetic products, analysis, the optical rotation and its chromatographic behaviour it is concluded that the sugar component of the purine nucleosides in *M. phlei* DNA is 2-deoxy-D-ribose. The crystalline thymine nucleoside which was isolated was shown to be identical with thymidine isolated from cod roe DNA on the basis of analysis,

mixed melting point, chromatographic behaviour and ultra-violet absorption properties. A comparison between the two thymine nucleosides is seen in Fig. 1.

SUMMARY

1. A quantitative estimation of the amounts of thymidine and 2-deoxy-D-ribose (originating from the purine nucleosides) per 4 atom of phosphorus in DNA from *Mycobacterium phlei* has been carried out. These figures agreed fairly well with what could be expected from the figures of the chromatographic purine and pyrimidine analysis of the DNA under examination.

2. The presence in *Mycobacterium phlei* DNA of 2-deoxy-D-ribose-(1)-phosphate linkages occurs in less than 1 % of the total sugar molecules.

3. The sugar component of the purine nucleosides in DNA from *Mycobacterium phlei* has been isolated in the crystalline state. It has been shown to be identical with synthetic 2-deoxy-D-ribose. The nucleoside containing thymine was isolated and shown to be identical with the corresponding nucleoside from cod roe DNA.

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Occurrence and Metabolism of Chenodesoxycholic Acid in the Rat. Bile Acids and Steroids 13

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Byers and Biggs [1] and Bergström [2] have recently shown that cholesterol is the precursor of cholic acid in the rat, as had earlier been demonstrated in the dog by Bloch, Berg and Rittenberg [3]. Cholic acid is the main bile acid present in rat bile but it has been observed [2,4] that small amounts of an acid regularly occurred in the chromatograms at the place where dihydroxycholanic acids appeared. This material also became labelled when cholesterol-4-^{14}C had been administered as shown in the curves published earlier [2,4].

The main part of this fraction has now been found to be chenodesoxycholic acid. In order to investigate if this 3α,7α-dihydroxy-cholanic acid is an intermediate in the conversion of cholesterol into 3α,7α,12α-trihydroxy-cholanic acid (cholic acid) we have also studied its metabolism with the aid of chenodesoxycholic acid-24-^{14}C.

A general survey of the bile salts present in rat bile has also been made by paper chromatography.

EXPERIMENTAL

Chenodesoxycholic acid-24-^{14}C was prepared according to Bergström, Rottenberg and Voltz [5].

The chromatographic procedures used have been described in several earlier publications [4,6,7,8].

The systems of phases used have been prepared by mixing the volumes indicated below:

System A: Methanol 180; Water 120; Chloroform 45; Heptane 5.
B: Methanol 150; Water 150; Sec. octanol 15; Chloroform 15.

Of the less hydrophilic phase 4 ml was taken for 4.5 g of hydrophobic Hyflo Supercel. The maximum amount of material for a column of this size is 30—50 mg of bile acids.

The technique of insertion of a bile fistula for the collection of the bile, followed by injection of labelled material intraperitoneally, and finally alkaline hydrolysis and isolation of the free bile acids has also been described earlier [4,9]. Paper chromatography of free and conjugated bile acids has been described by Sjövall [14,15] and by Haslewood and Sjövall [16].

RESULTS

Fractionation of normal rat bile. The combined bile that had been collected in ethanol from different rats, corresponding to the bile secreted from one rat during thirty days, was filtered and evaporated to dryness. Of this crude material 20 mg was dissolved in one ml of water for paper chromatography. A paper chromatogram was run with *iso*propylether: heptane 75/25 as moving phase and 70 % acetic acid as stationary phase in which glycine conjugates are separated. A strong spot appeared at the starting line where taurine conjugates stay and a very weak spot was found at the position of glycocholic acid. No free bile acids could be detected. In work with bile acid conjugation in rat liver slices indications of a slight conjugation of desoxycholic acid with glycine have also been obtained [17].

100 γ of the crude bile salts were run in the butanol system for taurine conjugated acids [15]. After spraying two spots were detected, the stronger one running at the same speed as taurocholic acid and the weaker one at the same speed as taurochenodesoxycholic acid or taurodesoxycholic acid.

The main part of the crude bile salts was hydrolyzed in 1 *N* sodium hydroxide at 110° for 5 hours. Acidification and extraction with ether yielded 1.3 g of residue. 20 mg of this residue was dissolved in 1 ml alcohol and used for paper chromatography.

Ascending chromatography of 100 γ hydrolyzed bile acids with *iso*propyl ether : heptane 60 : 40 as moving phase gave two spots, the weaker one corresponding to chenodesoxycholic acid or desoxycholic acid and the stronger one appearing at the same place as cholic acid. When more than 100 γ was run a very weak spot appeared between the two just mentioned. The compound causing this is as yet unknown.

In order to differentiate between desoxycholic acid and chenodesoxycholic acid 400 γ of hydrolyzed bile acids were run descending for 20 hours with *iso*propyl ether : heptane 20 : 80 as moving phase. Spots appeared at the same places as cholic acid and chenodesoxycholic acid. There was a very weak coloration at the position of desoxycholic acid. It is uncertain whether any substance moving as desoxycholic acid is present. If there is, it must be less than 10 % of the chenodesoxycholic acid (see below) corresponding to 1 or 2 % of the total bile acids present.

For the further purification of the hydrolyzed bile acids the fatty acids were removed from the main part of the hydrolysate. The material was subjected to a two stage countercurrent extraction between light petroleum and 70 % ethanol, with 50 ml of each phase. The combined ethanolic phases yielded a residue of 1.0 g.

For a separation on a preparative scale 500 mg of this material was subjected to reversed phase partition chromatography in phase system A on a column containing 85 g of hydrophobic Supercel. The titration curve of this run is shown in Fig. 1.

The main band (150—272 ml effluent) contained mainly cholic acid that was obtained pure after one crystallisation from aqueous acetic acid and one from aqueous ethanol. Melting point 195—197°, no depression with authentic cholic acid. Infra-red spectrum identical with that of cholic acid.

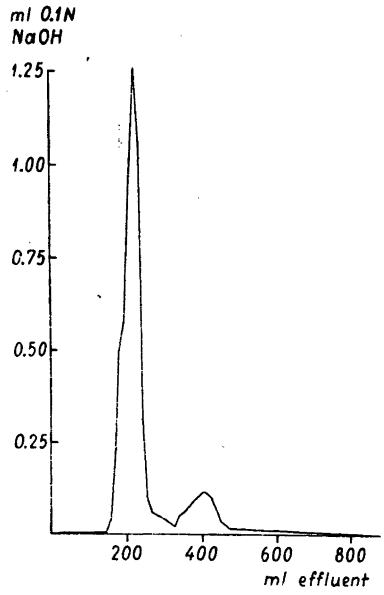

Fig. 1. Chromatography of acids (0.5 g) from hydrolysed rat "crude bile salts". Column 85 g hydrophobic Supercel. Phases: Type A.

The fractions of the second band (325 to 500 ml effluent) were combined, most of the methanol was evaporated off on the water bath. The remaining aqueous solution was acidified with hydrochloric acid and extracted three times with three volumes of ether. The combined ether solutions were washed with water until free from hydrochloric acid and the indicator (bromothymol blue) and then evaporated to dryness. The residue was dissolved in 1 ml of ethyl acetate and was left at 5°, when the gelatinous appearing clusters of crystals formed that are typical for chenodesoxycholic acid. One further crystallization from ethyl acetate and light petroleum yielded 15 mg of crystals melting at 140—42°, undepressed by admixture of authentic chenodesoxycholic acid. For analysis a sample was dried at 110° at 0.001 mm over P_2O_5 for 12 h. (Found: C 73.2; H 10.27; Eq.wt. 393. Calc. for $C_{24}H_{40}O_4$: C 73.4; H 10.27; Eq.wt. 392.6). The mother liquor was subjected to paper chromatography and only showed one spot at the position of chenodesoxycholic acid.

Professor Einar Stenhagen has kindly compared the long X-ray spacing of the isolated material with that of authentic chenodesoxycholic acid and found them to be identical. The IR spectrum of corresponding material was also found to be identical with that of chenodesoxycholic acid.

As mentioned above, it has earlier been found in this laboratory that when ringlabelled cholesterol was administered to rats, some labelled material regularly appeared in the "dihydroxycholanic acid band" [2,4] which has now been found to consist mainly of chenodesoxycholic acid.

That the labelled compound also was chenodesoxycholic acid has been shown by isotope dilution. When labelled material from the "dihydroxy band" was diluted with unlabelled desoxycholic acid the activity was rapidly lost on

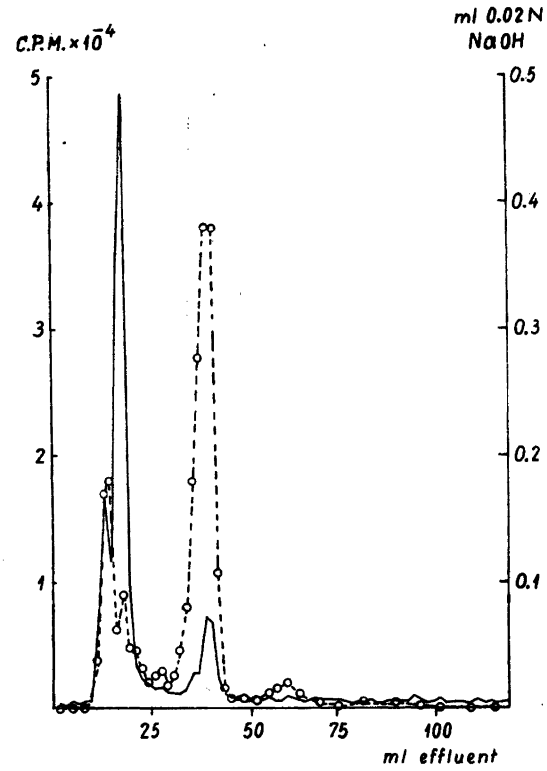

Fig. 2. Chromatography of acids from hydrolysed bile, excreted after intraperitoneal administration of chenodesoxycholic acid-24-^{14}C. Column: 4.5 hydrophobic Supercel. Phases: Type A.

recrystallization. When it was diluted with chenodesoxycholic acid the specific activity of the original mixture remained constant during extensive crystallizations from aqueous ethanol, acetic acid and ethyl acetate.

Metabolism of intraperitoneally administered chenodesoxycholic acid-24-^{14}C. When 2.5 mg of chenodesoxycholic acid-24-^{14}C was injected intraperitoneally into rats with a bile fistula the isotope was excreted in the bile as rapidly as when similar experiments were performed with labelled lithocholic [9] or cholic acid [10], *i. e.* more than two thirds in the three hours following the injection, while practically all of it had appeared in 24 hours. As with the other acids Norman has found that practically all of the labelled material is excreted in the bile conjugated with taurine [11]. Hydrolysis of the bile and chromatography of the free bile acids in system A is shown in Fig. 2. Although most of the activity appeared at the position of chenodesoxycholic acid, about one quarter of the activity has been shifted to the place where cholic acid and other more polar acids appear. The band shows some evidence of inhomogeneity. The material in this first band was then dissolved from the planchets, acidified and extracted

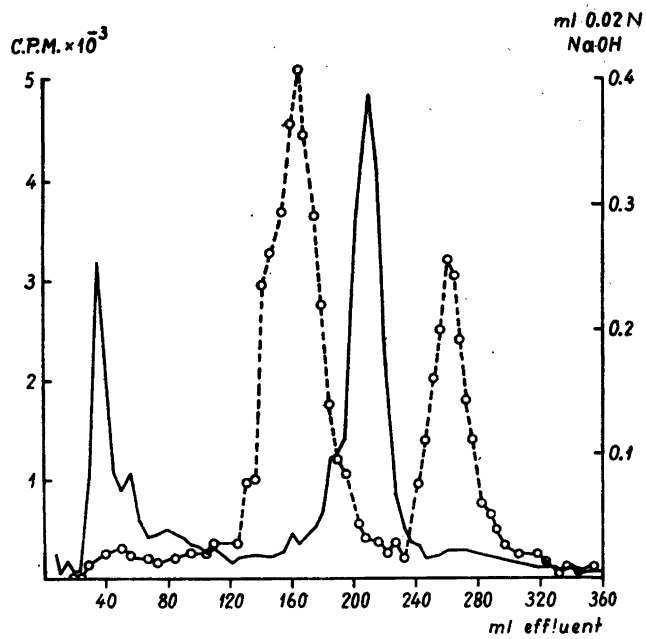

Fig. 3. Sample of the first band (Fig. 2) rerun with unlabelled cholic acid. Column 9 g hydrophobic Supercel. Phases: Type B.

with ether and the residue from the washed ether extract chromatographed in phase system B together with some added cholic acid. The curves shown in Fig. 3 were obtained. It is seen that the main part of the activity is contained in two bands of which one appears before and one after the titrated cholic acid band. In an earlier paper by Bergström and Norman [4] it was found that after administering ring-labelled cholesterol, cholic and chenodesoxycholic acids were the most strongly labelled acidic compounds in the bile. However, two minor components were observed which also appeared just before and after cholic acid at the same place as the two metabolic products found in this work (cf. Fig. 4 in [4]). It appears possible that the former also contain secondary metabolic products formed from the chenodesoxycholic acid. The structure of these metabolic compounds has not yet been determined.

The metabolic transformations of chenodesoxycholic acid take place decidedly more slowly than those of lithocholic [9], desoxycholic [10] or even cholanic acid which is presumably the reason why some chenodesoxycholic acid is present in the bile.

It has resently been shown that the rat transforms desoxycholic acid into cholic acid, *i. e.* a 7α-hydroxyl is introduced whe the 3(α)- and 12(α)-hydroxyl are present. The work reported here indicates that if the 3(α)- and 7α-hydroxyls are present, a 12α-hydroxyl is not introduced, but that other metabolic changes transforming chenodesoxycholic acid into more hydrophilic compounds take place.

Thus chenodesoxycholic acid does not appear to be an intermediate in the normal conversion of cholesterol into cholic acid in the rat. Possibly some of the chemical changes in the ring system occur before the cholesterol side chain has been completely degraded into that of the bile acids.

It might finally be pointed out that in all these experiments [9,10] the labelled acids have been injected intraperitoneally so that they have not been in contact with the intestinal microorganisms before being excreted in the bile fistula. The complicated metabolic changes of the bile acids that take place in the intestinal content are being studied [4,12,13].

SUMMARY

Chenodesoxycholic acid has been identified as a minor component of the bile acids normally present in rat bile. The labelled material appearing in this band after administering cholesterol-24-^{14}C has also been found to be chenodesoxycholic acid.

With the aid of chenodesoxycholic acid-24-^{14}C it has been found that this acid is transformed into more hydrophilic acids by the tissues of the rat. These products are not identical with cholic acid and it thus appears unlikely that chenodesoxycholic is an intermediate in the conversion of cholesterol into cholic acid.

The IR spectra have been recorded by Dr. Rosenberg on a Perkin-Elmer double beam instrument at the Institute of Biochemistry at Uppsala through the courtesy of Professor A. Tiselius.

The technical assistance of Miss Kerstin Hyllén is gratefully acknowledged.

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On the Existence of a Metallic Molybdenum Oxide

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Studies of the molybdenum oxides carried out by Magnéli have revealed the existence of three phases, Mo_4O_{11}[1], Mo_8O_{23}[2] and Mo_9O_{26}[2], besides MoO_2 and MoO_3.

The present investigation was started in order to look for the existence of molybdenum oxides with low oxygen content. Finely powdered molybdenum metal (Lindbergh, 99.6 %) was carefully oxidized in different ways. The following method caused the formation of an oxide phase, not described in the literature. MoO_2, obtained by reduction of MoO_3 (Kahlbaum, *puriss.*) with hydrogen, and Mo powder were mixed in proportions corresponding to the gross compositions $MoO_{0.25}$, $MoO_{0.50}$, $MoO_{0.75}$, $MoO_{1.00}$. The samples were packed in small alumina tubes, placed in silica tubes, which were then evacuated, sealed and heated at 1 000° C after which the temperature was lowered to 400° C at a constant rate of 10° C per day.

The phase analysis of the specimens was performed by means of powder photographs taken in focussing cameras of the Guinier type using Cu$K\alpha$ radiation. For measurement of the high-angle reflections additional photographs were taken in Hägg-Phragmén focussing cameras.

Besides the diffraction patterns of Mo and MoO_2 the photographs contained the lines of a face-centered cubic phase with the axial length $a = 5.549$ Å ($V = 170.9$ Å^3). It was not possible to prepare the new oxide in a pure state. The samples of the compositions $MoO_{0.25}$ and $MoO_{0.50}$ only contained small amounts of Mo and MoO_2, however. The amount of MoO_2 was considerably greater for the composition $MoO_{0.75}$ and $MoO_{1.00}$, indicating a probable oxygen content of between 20 and 30 atom-% for the metallic oxide. The formula is formally given here as Mo_3O, although the oxygen content may differ to some extent from 25 atom-%.

As only reflections with $h + k = 2n$, $k + l = 2n$, $h + l = 2n$ appeared in the photographs, the atoms have a face-centered arrangement. Space is available for the metal atoms only in the following positions: (0,0,0; $0,\frac{1}{2},\frac{1}{2}$; $\frac{1}{2},0,\frac{1}{2}$; $\frac{1}{2},\frac{1}{2},0$) + 4 (*a*) 0,0,0; 4 (*b*) $\frac{1}{2},\frac{1}{2},\frac{1}{2}$; 4 (*c*) $\frac{1}{4},\frac{1}{4},\frac{1}{4}$; 4 (*d*) $\frac{3}{4},\frac{3}{4},\frac{3}{4}$. The reflections with $h + k + l = 4n$ are considerably stronger than those with $h + k + l \neq 4n$. From the $p|F|^2$ values calculated for different combinations of atomic positions it is evident (Table 1) that the metal atoms are situated in 4 (*a*) 0,0,0 + 4 (*c*) $\frac{1}{4},\frac{1}{4},\frac{1}{4}$ + 4 (*d*) $\frac{3}{4},\frac{3}{4},\frac{3}{4}$.

Table 1. Intensities calculated and observed for the Mo_3O phase.

hkl	$I_{calc.}$	$I_{obs.}$
1,1,1	13	15
2,0,0	7	10
2,2,0	44	45
3,1,1	7	10
2,2,2	2	2
4,0,0	12	10
3,3,1	5	5
4,2,0	4	5
4,2,2	28	25
3,3,3	1	3
5,1,1	3	
4,4,0	11	10
5,3,1	5	5
4,4,2	2	2
6,0,0	1	
6,2,0	19	15
5,3,3	2	2
6,2,2	2	2
4,4,4	11	10

For pure Mo the measured value of the lattice constant was $a = 3.126$ Å meaning a volume per metal atom of 15.28 Å^3. The corresponding volume is obtained for a cell content of 11.2 Mo atoms in the oxide phase. As the presence of oxygen (20—30 atom-%) most likely causes an expansion, the unit cell cannot contain more than 11 Mo atoms. The density observed for the Mo_3O samples was 8.6. As they contained small amounts of Mo and MoO_2 this value is not reliable but indicates a cell content of approximately 3 formula units Mo_3O. The density calculated for this case will be 8.86.

With the above presumptions the configuration of Mo_3O is 3 Mo in 4 (*a*) 0,0,0 and 6 Mo in 8 (*c*) $\frac{1}{4},\frac{1}{4},\frac{1}{4}$ in the space-group O_h^5-*Fm3m*. The atoms are distributed at random over these positions. A Mo atom in 8 (*c*) is surrounded by 3 Mo atoms in 4 (*a*) and 6 Mo atoms in 8 (*c*) at the distances of 2.40 Å and 2.77 Å respectively giving a mean value of 2.65 Å. A Mo atom in 4 (*a*) is surrounded by 6 Mo atoms in 8 (*c*) at a distance of 2.40 Å, which value is remarkably small as compared with the interatomic distances in the pure metal, 2.71 Å. The discrepancy is partially due to the lowering of the coordination number from 8 to 6 and may also be partially due to the degree of Mo-O ionisation.

The oxygen atoms (approximately three atoms) can be situated in the remaining 4 (*a*) and 8 (*c*) positions or the 4 (*b*) $\frac{1}{2},\frac{1}{2},\frac{1}{2}$ positions of the space-group *Fm3m*. As the observed O—O distances, even in oxides with metallic character, have always been found to exceed the value of 2.5. Å the former grouping is excluded. The Mo-O distances, with the oxygen atoms in 4 (*b*), are as great as the Mo—Mo distances — close to the radius sum $r_{Mo} + r_{O^{2-}} = 1.35$ Å $+ 1.32$ Å $= 2.67$ Å. The corresponding abnormally great metal-oxygen distances have been observed for the metallic oxides Cr_3O[3] and W_3O[4] of the "ordered" and "disordered" A 15 type respectively. This cannot with the

greatest probability be a contingency in all three cases, but can be explained by an electron displacement from the metal atoms — at most 2/3 electrons per metal atom — to the oxygen atoms. The decrease of the radius value of the metal atom will therefore be very small, and there are still "free" electrons available in the outer *s* and *d* shells for the formation of metal-metal bonds.

The atomic arrangements of the three metallic oxides in group VI a (Cr_3O, Mo_3O, W_3O) form intermediate stages between the cubic close-packing (A 1) and the cubic body-centered lattice (A 2).

Attempts to prepare Mo_3O by oxidation of Mo powder at temperatures between 600° C and 800° C by means of steam in the presence of hydrogen in excess failed owing to the ease with which MoO_3 and MoO_2 forms. Oxidation of Mo by means of Cu_2O in evacuated and sealed silica tubes at 650° C for two weeks only caused the formation of small amounts of Mo_3O. No trace of the oxide was obtained in Mo-MoO_2 samples heated in vacuum furnaces at various temperatures between 1 100° C and 1 600° C. It may be, therefore, that Mo_3O is unstable at these high temperatures.

SUMMARY

A metallic molybdenum oxide with the probable ideal formula Mo_3O has been prepared by the reaction between Mo powder and MoO_2 at a comparatively low temperature. The structure of the oxide is related to that of the Cr_3O and W_3O phases of the "ordered" and "disordered" A 15 type respectively.

9 Mo atoms are distributed at random over the positions 4 (*a*) 0,0,0 + 8 (*c*) $\frac{1}{4},\frac{1}{4},\frac{1}{4}$ in the space-group O_h^5-*Fm3m* and the O atoms are most likely located in the position 4 (*b*) $\frac{1}{2},\frac{1}{2},\frac{1}{2}$ implying a crystal lattice of a defective BiF_3 type.

The magnitude of the Mo—O distances seems to indicate a fairly large ionisation of the O atoms.

The author wishes to thank Professor G. Hägg for his encouraging interest in the investigation. A grant from *Statens Tekniska Forskningsråd* is gratefully acknowledged.

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Ternary Metallic Phases in the Ta—C—N, Ta—C—O, and Ta—N—O Systems

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During the last thirty years or so a great amount of data about tantalum carbides has been published. On the basis of microscopic and X-ray investigations and of melting point determinations on tantalum carbide specimens, Ellinger [1] established a phase diagram of the Ta—C system. The Ta—N and Ta—O systems have recently been studied by the present author [2].

The structures and compositions of the metallic binary phases are thus known, and the present study was undertaken in order to discover possible metallic ternary phases. Tantalum metal powder (Fansteel) and tantalum hydride (Metal Hydrides Inc.) were used for the preparation of carbides and nitrides as starting materials. The carbide samples — Ta_2C and TaC — were obtained from the reaction between the metal or metal hydride and carbon (soot) in a graphite tube vacuum resistance furnace [3] at a temperature of about 1 800° C. The nitride samples — Ta_2N (γ), $TaN_{0.80-0.90}$ (δ), and TaN (ε) — were prepared by nitriding with dry oxygen-free ammonia at various temperatures between 700° and 1 100° C.

The phase analysis was performed by means of powder photographs taken in focussing cameras of Guinier type using monochromatized Cu$K\alpha$ radiation. The carbon and nitrogen contents of the samples were determined by means of combustion and Kjeldahl methods respectively, and the metal content by weighing the pentoxide formed after oxidation of the samples with oxygen.

The Ta — C — N system.

Tantalum carbides were nitrided with dry ammonia, and the nitrides were carburized with dry methane at about 1 100° C. No ternary phase was found to exist.

The unit cell dimensions of Ta_2C were found to vary between $a = 3.094$ Å, $c = 4.918$ Å, $c/a = 1.590$ ($V = 40.78$ Å^3) and $a = 3.111$ Å, $c = 4.948$ Å, $c/a = 1.590$ ($V = 41.46$ Å^3) and those of Ta_2N between $a = 3.041$ Å, $c = 4.907$ Å, $c/a = 1.614$ ($V = 39.29$ Å^3) and $a = 3.048$ Å, $c = 4.918$ Å,

$c/a = 1.614$ ($V = 39.57$ Å^3). The lattice constant values of $Ta_2(C,N)$ specimens indicate a continuous solid solution series Ta_2C—Ta_2N.

The solubility of TaC in the $TaN_{0.80-0.90}$ phase (δ) is of the order of 5 at.-%. Whereas the unit cell dimensions of the δ-nitride are $a = 2.925$—2.938 Å, $c = 2.876$—2.883 Å, $c/a = 0.983$—0.981 ($V = 21.31$—21.55 Å^3), those of a sample with the approximate composition $TaN_{0.85}C_{0.15}$ were found to be $a = 2.938$ Å, $c = 3.071$ Å, $c/a = 1.045$ ($V = 22.96$ Å^3).

The solubility of TaC in TaN could not be determined because of the difficulty of obtaining homogeneous samples. It is probably fairly small. The replacement of some nitrogen with carbon in the TaN lattice causes an increase of the cell dimensions from $a = 5.158$ Å, $c = 2.908$ Å, $c/a = 0.561$ ($V = 67.70$ Å^3) to $a = 5.206$ Å, $c = 2.914$ Å, $c/a = 0.560$ ($V = 68.38$ Å^3). A volume decrease would be expected, however, as the corresponding volumes per metal atom of the TaN and TaC phases are 22.57 and 21.34—22.15 Å^3 (for TaC, $a = 4.403$—4.456 Å) respectively. The solubility of TaN in TaC is not known.

The Ta — C — O system.

The samples were prepared in two ways:

A. Tantalum carbides were oxidized by means of steam in the presence of hydrogen in great excess at about 700° C.

B. Tantalum pentoxide was reduced with carbon (soot) in a graphite tube vacuum furnace at about 1 800° C.

One metallic ternary phase and two phases of unknown properties and compositions were obtained. One of the latter compounds has a face-centered cubic unit cell with an axial value of $a = 10.41$ Å. The ternary metallic phase was only obtained in the presence of rather great amounts of Ta_2C or TaC and has a tetragonally deformed NaCl structure with the lattice constants $a = 4.303$ Å, $c = 4.097$ Å, $c/a = 0.952$ ($V = 75.53$ Å^3). A comparison between the cell volumes of Ta_4O, TaO, Ta_2C, and TaC makes the approximate formula $Ta_2(C,O)$ probable. The C/O ratio is unknown. Phases with an analogous structure have earlier been found in the Mo—N [4] and Nb—N—O [2] systems.

The Ta — N — O system.

Tantalum nitride samples were oxidized with steam in a similar manner to that used for the preparation of Ta—C—O samples. They could be prepared in fairly pure states. The compound most rich in nitrogen was only formed by the oxidation of TaN, while three other compounds were obtained when TaN (ε) or $TaN_{0.80-0.90}$ (δ) were used as starting materials.

The volumes per metal atom of the different oxide-nitrides are compared in Fig. 1.

1. $TaN_{\sim 0.90}O_{\sim 0.10}$. Besides the strong reflections of the ε phase a series of weak superlattice reflections were visible in the powder photograph, indicating a doubling of the a-axis as well as the c-axis of the hexagonal TaN sublattice: $a = 10.34$ Å, $c = 5.802$ Å, $c/a = 0.561$ ($V = 537.1$ Å^3, $Z = 24$). The weakness of the "extra" lines shows that the deviation from the "ideal" atomic positions (in TaN) is very small.

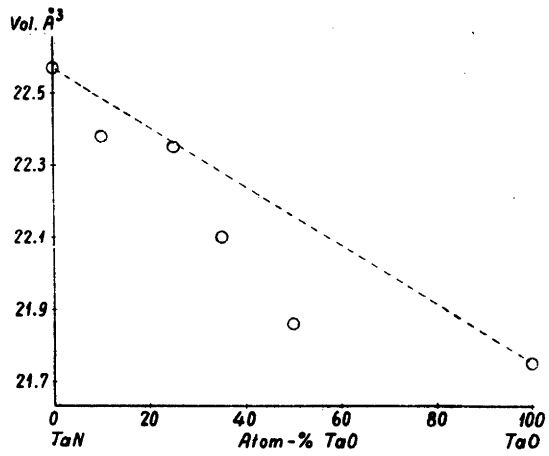

Fig. 1. The volumes per metal atom of the phases in the pseudo-binary TaN—TaO *system.*

2. $TaN_{\sim 0.75}O_{\sim 0.25}$ ($Z = 4$). This phase is closely related to the hexagonal δ phase in the Ta-N system, as indicated by the intensity distribution in the diffraction pattern. Comparatively weak reflections indicate a superlattice with $a \sim 2a_\delta$ and $c \sim c_\delta$. The lengths of the axes are: $a = 5.988$ Å, $c = 2.879$ Å, $c/a = 0.481$ ($V = 89.39$ Å^3; $Z = 4$).

3. $TaN_{\sim 0.65}O_{\sim 0.35}$ ($Z = 12$). This compound, like the last one, has a δ phase superstructure. But in this case $a \sim 2\sqrt{3}\ a_\delta$ and $c \sim c_\delta : a = 10.34$ Å, $c = 2.864$ Å, $c/a = 0.277$ ($V = 265.2$ Å^3).

4. $TaN_{\sim 0.50}O_{\sim 0.50}$ The strongest reflections in the powder photograph of this phase correspond to a hexagonal sublattice with the dimensions: $a = 5.939$ Å, $c = 2.866$ Å, $c/a = 0.483$ ($V = 87.54$ Å^3), containing four formula units $TaN_{0.5}O_{0.5}$. The intensity distribution shows that the atomic arrangement is different both from that in the δ and from that in the ε phase in the Ta—N system. All reflections can be indexed, if a hexagonal superlattice with $a' = 2a$ and $c' = 6c$ is chosen. But the absence of a great many lines in the pattern makes such a big unit cell ($Z = 96$) unlikely. The symmetry may be lower and the cell content then smaller that that corresponding to 96 metal atoms.

The colour of the oxide-nitrides changes continuously from black for $TaN_{\sim 0.90}O_{\sim 0.10}$ to reddish black for $TaN_{\sim 0.50}O_{\sim 0.50}$. The interatomic distances probably differ very slightly from those observed for the nitrogenrich tantalum nitrides [2].

The solubility of N in the TaO phase is not known.

The approximate outline of the "metallic" corner of the Ta—N—O system is drawn in Fig. 2.

If ammonia was led over Ta_2O_5 samples at 1 100° C, besides small amounts of the ε phase in the Ta—O system a black ternary phase was obtained. Its structure is complicated, as indicated by the powder photograph. Metallic oxide-nitrides could not be prepared in this way.

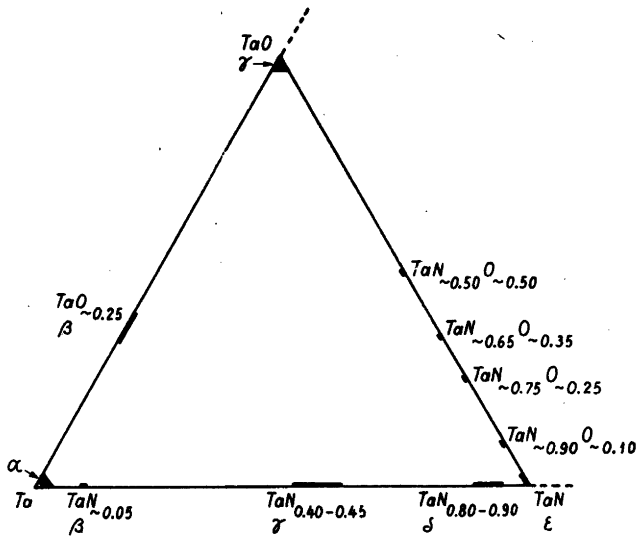

Fig. 2. An outline of the Ta—N—O *system with the* (N,O) / Ta *ratio* ≤ 1.

SUMMARY

Ta—C—N, Ta—C—O, and Ta—N—O samples with the nonmetal / metal ratio $\leq$ 1 have been prepared with different methods.

No ternary phase has been found in the Ta—C—N system. Solid solubility ranges have to some extent been determined in this system.

At least one metallic phase exists in the Ta—C—O system: a compound with the approximate composition $Ta_2(C,O)$ has a tetragonally deformed NaCl structure.

Four metallic phases with the following approximate compositions have been prepared in the Ta—N—O system: $TaN_{0.90}O_{0.10}$, $TaN_{0.75}O_{0.25}$, $TaN_{0.65}O_{0.35}$, and $TaN_{0.50}O_{0.50}$. The stucture for the nitrogen-richest compound is closely related to that of ε-TaN, and the following two oxide-nitrides have δ-TaN superstructures.

An outline of the part of the Ta—N—O system with the (N,O) / Ta ratio $\leq$ 1 has been drawn.

The author wishes to express his gratitude to Professor G. Hägg for his encouraging interest throughout the coarse of the work. A grant from *Statens Tekniska Forskningsråd* which has made this investigation possible is gratefully acknowledged. Also, I wish to thank Mr. S. Hedling and Mr. H. Lindholm for their valuable assistance in the experimental work.

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The Composition of the Phases in the Vanadium-Carbon System

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The intermediate phases V_5C, V_2C, V_4C_3, VC, and V_2C_3 have been reported to exist. Because of the difficulty in avoiding contamination by oxygen and nitrogen, the methods of preparation in many cases cast doubt on the validity of the results. X-ray diffraction and microscopical studies have established the existence of two intermediate phases, although the data about the compositions given by the different authors differ essentially. They are of the common "interstitial" types with the metal atoms arranged in hexagonal and cubic close-packing respectively. Analogous carbides exist in, for instance, the Nb—Ta—C system, with the ideal formulas $(Nb,Ta)_2C$ and (Nb,Ta)C.

A brief summary of earlier investigations has been given by — among other authors — Schwarzkopf and Kieffer (34 references) [1], Rostoker and Yamamoto (15 references) [2].

EXPERIMENTAL

Finely powdered vanadium metal (Vanadium Corporation of America, 99.7 % Grade) and carbon (soot) were carefully mixed in different amounts and moulded into brickettes weighing 1—5 g. The sintering (melting) of the specimens was performed in graphite crucibles, in some cases in a high frequency vacuum induction furnace [3] and others in a graphite tube vacuum resistance furnace [4] (1 900° C). The samples were then cooled to room temperature during a time of about twenty minutes.

X-ray powder photographs of the samples taken in a Guinier focussing camera with monochromatized Cr-$K\alpha$ radiation revealed the existence of three phases *viz.* vanadium, the V_2C phase with the metal atoms in the hexagonal close-packed arrangement, and the VC phase with the sodium chloride structure. The amount of "total" carbon in the samples was determined by combustion and the amount of "free' carbon after dissolving the carbide phase in a mixture of dilute hydrochloric and hydrofluoric acid. The vanadium content of some samples was controlled by potentiometric titration with ceric sulphate [5].

The homogeneity limits of the phases were determined from Fig. 1, where the lattice constants of the three phases have been plotted against the composition.

THE COMPOSITION OF THE PHASES

V-phase. For the pure metal (99.7 %) the cubic axial length was found to be $a = 3.026$ Å ($V = 27.71$ Å^3), and for the vanadium phase in equilibrium with the V_2C phase the value obtained was $a = 3.037$ Å ($V = 28.00$ Å^3).

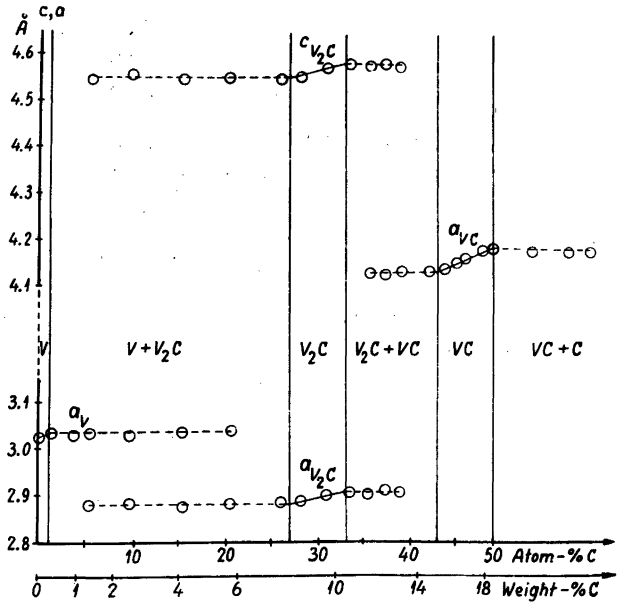

Fig. 1. The lattice constants of the phases in the V—C system v s. the composition.

The latter value of the parameter was also obtained for a sample containing 0.33 weight-% (1.4 atom-%) of C. Accordingly the solubility of carbon in vanadium is assumed to be as low as about 0.2 weight-% (1 atom-%) at a temperature of about 1 000° C.

Tammann and Schönert [6] found that carbon does not diffuse into vanadium at 800° to 900° C. This suggests that the solubility of carbon in vanadium is very small.

V_2C-phase. The hexagonal unit cell dimensions were found to vary continuously between $a = 2.881$ Å, $c = 4.547$ Å, $c/a = 1.578$ ($V = 32.68$ Å^3) at the composition $VC_{0.37}$ (8.0 weight-% = 27 atom-% of C) and $a = 2.906$ Å, $c = 4.597$ Å, $c/a = 1.576$ ($V = 33.50$ Å^3) at the composition $VC_{0.50}$ (10.5 weight-% = 33 atom-% of C). It is seen that the c/a value remains practically constant, although the homogeneity range is comparatively broad.

The mean interatomic distances are: V—6V = 2.83 Å, V—6V = 2.89 Å, C—6V = 2.02 Å, as compared with the intermetallic distances in pure vanadium: 2.62 Å (8-coordination).

VC-phase. The formula of this carbide has been disputed in the literature. Some authors have given it as V_4C_3 and others as VC. From the present investigation it is evident that these two formulas closely correspond to the lower and upper homogeneity limits of the phase. By analogy with the great number of related carbide and nitride phases, the ideal formula VC seems preferable (compare V_2C).

At the lower phase limit, with the composition $VC_{0.75}$ (15.1 weight-% = 43 atom-% of C), the lattice constant is $a = 4.136$ Å ($V = 70.75$ Å^3). Samples

with a carbon content up to the approximate composition $VC_{0.8}$ could be prepared. But on increasing the amount of total carbon, this did not in its entirely react with the vanadium. As a consequence, a small fraction of free carbon was always obtained in the samples. The theoretical carbon content for the composition VC is 19.08 weight-%. Even with a great excess of carbon — up to 40 weight-% — it was not possible to introduce more carbon into the phase than corresponding to the composition $VC_{0.96}$ (18.5 weight-% = 49 atom-% of C). The corresponding lattice constant is $a = 4.182$ Å ($V = 73.14$ Å^3). If a homogeneous phase with the composition VC exist, it is at least difficult to prepare it in this state.

The mean interatomic distances are: V—12V = 2.94 Å and C—6V = 2.08 Å.

SUMMARY

The compositions of the phases in the V—C system have been determined by X-ray methods.

The solubility of carbon in vanadium is about 0.2 weight-% (1 atom-%) at about 1 000 °C.

The V_2C phase of the L'3 type is homogeneous between the limits $VC_{0.37}$ (8.0 weight-% = 27 atom-% of C) and $VC_{0.50}$ (10.5 weight-% = 33 atom-% of C).

The VC phase of the B1 type is homogeneous between the limits $VC_{0.75}$ (15.1 weight-% = 43 atom-% of C) and $VC_{0.96}$ (18.5 weight-% = 49 atom-% of C). The fact that the formula VC could not be attained may be due to low reaction velocities.

The author wishes to thank Professor G. Hägg for his kind interest in this investigation. A grant from *Statens Tekniska Forskningsråd* is gratefully acknowledged. I also wish to thank Mr. L. Westlund for his valuable assistance in the experimental work.

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The Structure of the Metallic Quarternary Phase ZrTaNO

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The structures and approximate compositions of the binary metallic phases in the Zr—N, Zr—O, Ta—N, and Ta—O systems are known. The present author has recently published a study of the metallic corner of the Ta—N—O system, where four ternary phases were found [1]. Zr—N—O samples with the (N, O)/Zr ratio $\leq$ 1 were also prepared. The results obtained indicate a solid solubility of nitrogen and oxygen in the α-Zr lattice up to the approximate limit Zr_2(N, O), as well as that a continuous solid solution series ZrN—ZrO exists*/.

The products were investigated by taking X-ray powder photographs in a focussing camera of Guinier type using monochromatized Cu-$K\alpha$ radiation.

Powdered ZrTa alloys with the compositions $ZrTa_{0.33}$, $ZrTa_{0.67}$, ZrTa, $Zr_{0.67}Ta$, $Zr_{0.33}Ta$ were used as starting materials for the preparation of ternary nitrides and oxides and quarternary nitride-oxides. These alloys were obtained by heating zirconium hydride and tantalum hydride (both from Metal Hydride Inc.) in zirconia crucibles in a high frequency vacuum induction furnace [2] at a temperature of about 1 700° C. Ammonia nitriding with oxygen-free dry ammonia at various temperatures between 700° C and 1 100° C caused the formation of binary nitride phases. Analogously, binary oxide phases were formed, when the samples were oxidized with steam in the presence of hydrogen in great excess at a temperature of 700° C.

When a mixture of steam and ammonia (in great excess) was passed over the alloys at 700° C a new phase was formed. It was obtained in a fairly pure state (traces of ZrO_2 as impurity) starting with the sample of the composition ZrTa and after a reaction time of about three hours. The formula of this compound may thus be written $ZrTaN_xO_y$. The presence of hydrogen in the lattice is most unlikely, as heating *in vacuo* at 1 100° C for one hour did not cause any structural change. The nitrogen content was determined by the Kjeldahl method. The oxygen content was determined indirectly by weighing the ZrO_2 and Ta_2O_5 formed after oxidation of the specimen with oxygen.

* Published data on the lattice constants of ZrN differ considerably. The results obtained by the present author are therefore reported here. The cube-edge of the ZrN phase was found to be a = 4.537—4.562 Å and that of the ZrO phase a = 4.584—4.620 Å. The high value of a = 4.63 Å for zirconium nitride given by some authors is most likely due to the presence of oxygen in the lattice. Both phases have broad homogeneity ranges, approximately 10 atom-%.

Table 1. Calculated and observed intensities for the ZrTaNO phase.

hkl	$I_{calc.}$	$I_{obs.}$	*hkl*	$I_{calc.}$	$I_{obs.}$
0,0,1	40	50	2,0,3	98	110
1,0,0	224	250	0,0,4	18	15
1,0,1	251	250	1,0,4	76	75
0,0,2	69	75	3,0,2	97	110
1,1,0	184	160	2,2,0	48	50
1,0,2	96	110	2,2,1	12	15
1,1,1	32	35	2,1,3	148	160
2,0,0	46	50	3,1,0	33	35
2,0,1	199	160	1,1,4	102	110
1,1,2	235	250	3,1,1	155	160
0,0,3	4	0	2,0,4	41	35
2,0,2	63	75	2,2,2	114	110
1,0,3	128	110	3,0,3	19	15
2,1,0	55	50	3,1,2	121	110
2,1,1	229	250			
1,1,3	16	15			
3,0,0	67	75			
2,1,2	86	75			
3,0,1	16	15			

Because of the unfavourable weight ratio $y\mathrm{O}/\mathrm{ZrTaN}_x\mathrm{O}_y$, this method gives only an approximate value for y. The results closely corresponded to $x = y = 1$, however, corresponding to the ideal formula ZrTaNO. The phase has a greyish black metallic lustre.

From powder photograph interpretation the unit cell was found to have hexagonal symmetry with the lattice constants $a = 3.645$ Å, $c = 3.881$ Å, $c/a = 1.065$ ($V = 44.66$ Å^3). The calculated density for one formula unit ZrTaNO is 11.24. The observed value was 10.6. The Zr and Ta atoms occupy ordered positions in an arrangement of hexagonal close-packing. This is evident from Table 1, where the observed intensities are compared with the intensities calculated for this case.

Each metal atom is surrounded by six metal atoms of the same kind at a distance of 3.645 Å and six metal atoms of the other kind at a distance of 2.863 Å. For 12-coordination of atoms Pauling gives the radius values: $r_{Zr} = 1.60$ Å and $r_{Ta} = 1.49$ Å; $r_{Zr} + r_{Ta} = 3.09$ Å. As the mean interatomic distances are 3.25 Å, these are 5 % greater than those given for the pure metals for the same coordination number. This is in agreement with the corresponding increase of metal-metal distances for most metallic carbides, nitrides, and oxides of "interstitial" structure types.

At first sight the nonmetal atoms would be expected to be situated at the centres of metal atom octahedra, *i.e.* in the positions 2/3,1/3,1/4 and 2/3,1/3,3/4, if the Zr and Ta atoms occupy the positions 0,0,0 and 1/3,2/3,1/2, respectively, as this configuration is typical for nitrides and oxides with metallic character. Each nonmetal atom would then be surrounded by six metal and two nonmetal atoms at distances of 2.32 Å and 1.94 Å respectively. However, the observed

N—N,O—O, and N—O distances *always* exceed the value 2.5 Å in the compounds mentioned above. This can be explained by a displacement of electrons from the transition metal to the nonmetal atom, so that the metal-nonmetal bonds have a partial ionic character. If the N—O distances are accordingly assumed to be greater than 2.5 Å, the only possible placing is with the nonmetal atoms in the positions 1/3,2/3,0 and 2/3,1/3,1/2. The resulting N—O distances are 2.863 Å (6-coordination). From space considerations and from the consideration that the metal-oxygen bonds are stronger than the metal-nitrogen bonds, the oxygen atom is most likely to be in the position 1/3,2/3,0 and the nitrogen atom in 2/3,1/3,1/2. The oxygen atom in this arrangement is at the centre of a bipyramid of 3 Zr and 2 Ta atoms, and the nitrogen atom has a triangle of 3 Ta atoms around it, in the same plane.

The interatomic Zr—Zr and Ta—Ta distances are so great — 3.645 Å — that the metal atoms can be considered to have 6-coordination: the Zr—Ta distances are 2.863 Å. If Pauling's correction factor for 6-coordination is used in this case, assuming that the Born exponent $n \sim 9$[3], the resulting atomic radii are: $r_{Zr} = 1.47$ Å, $r_{Ta} = 1.37$ Å; $r_{Zr} + r_{Ta} = 2.84$ Å. This value is in good agreement with the observed one, 2.86 Å. The O—Zr (3 atoms), O—Ta (2 atoms), and N—Ta (3 atoms) distances are 2.11 Å, 1.94 Å, and 2.11 Å respectively. The calculated radius value for the O atom is then 0.64 Å and 0.57 Å, and that for the N atom 0.74 Å, closely corresponding to the radii given by Goldschmidt and Pauling: 0.60 Å and 0.71 Å respectively.

In the structure suggested here, the configurations of the Zr—Ta and O—N lattices are identical. The lattices are displaced 1/3,2/3,0 from each other. The space-group is D_{3h}^1—$P\bar{6}m2$ with the following atomic positions: 1 Zr in 1 (*a*) 0,0,0, 1 Ta in 1 (*d*) 1/3,2/3,1/2; 1 O in 1 (*c*) 1/3,2/3,0, and 1 N in 1 (*f*) 2/3,1/3,1/2.

SUMMARY

Some sections of the quaternary Zr—Ta—N—O system have been investigated by X-ray methods to find whether metallic ternary and quaternary phases exist.

A metallic compound ZrTaNO has been prepared. The metal atoms occupy ordered positions in a hexagonal close-packed arrangement. The c/a value is close to unity, differing widely from the ideal value of $\sqrt{8/3} = 1.63$. The positions of the nonmetal atoms have been discussed. A structure is suggested which is not related to that of metallic compounds of "interstitial" structure types.

The author wishes to express his gratitude to Professor G. Hägg for his never-failing interest. A grant from *Statens Tekniska Forskningsråd* is gratefully acknowledged.

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On the Existence of Metallic Ternary Oxides Me′Me″O with the Metal Atoms in Hexagonal Close-packing

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During the last three decades, a comprehensive investigation of Me—X systems, where Me = a transition metal and X = one of the nonmetals carbon or nitrogen, has revealed the existence of a group of metallic phases with the ideal formula Me_2X. The nonmetal atom X is located at the centre of a metal atom octahedron. The systematic study of these phases was initiated by the work of Hägg [1,2]. The structure type of the Me_2X phase has been designated by L′3 to indicate the analogy to the A 3 type. Some authors refer to this structure as "Hägg-type". The phases commonly have comparatively broad homogeneity ranges with a highest nonmetal content of 33 atom-%.

No binary oxide phases of the L′3 type have been found to exist, although the maximum solid solubility of oxygen in the α-titanium and α-zirconium lattices seems to correspond to the composition Me_2O.

The present investigation forms part of a study of metallic double-oxides (Me′, Me″)O_x among the transition metals, with Me = Ti, Zr, Th, V, Nb, Ta, Cr, Mo, W, Mn, Re, Fe, Co, Ni, Cu. (Cu is here regarded as a transition element.)

The metals used for the preparation of the oxides were of fairly high purity, about 99.5 %. The samples were prepared in three ways:

1. The powdered metals and a suitable oxide of one of the metals were carefully mixed, moulded into brickettes and heated in zirconia crucibles in a high frequency vacuum induction furnace at various temperatures between 1 400° C and 1 800° C.
2. The same mixture of metals and oxide as above was heated in evacuated silica tubes at 1 000° C for times varying between one hour and four weeks.
3. Binary alloys were prepared in a vacuum furnace starting from the powdered metals. The powdered alloys were oxidized by means of steam in the presence of hydrogen in great excess at a temperature of about 700° C.

The best results were obtained with preparation method 1, although the reaction in some cases was of an explosive nature — for instance, between Ti and FeO. The heating of the samples in silica tubes (2) often caused reactions with the inner walls of the tube, especially in the presence of metals in the groups IVa and Va. The samples from method 3 were often heterogeneous.

Table 1. Unit cell dimensions and interatomic distances for the Me'Me"O phases.

Me'	Me"	c, Å	a, Å	c/a	V Å³	(Me-Me)dist., Å obs.	(Me-Me)dist., Å calc.	(O-Me)dist., Å obs.	r_0, Å calc.
Mo	Fe	4.32	2.75	1.57	28.31	2.72	2.65	1.92	0.60
	Co	4.25	2.74	1.55	27.65	2.70	2.64	1.91	0.59
W	Fe	4.31	2.75	1.57	28.23	2.71	2.67	1.92	0.58
	Co	4.29	2.75	1.56	27.96	2.71	2.66	1.91	0.58
Re	Ti	4.61	2.85	1.62	32.41	2.84	2.84	2.01	0.59
	V	4.46	2.76	1.62	29.45	2.75	2.73	1.95	0.58
	Cr	4.45	2.75	1.62	29.20	2.74	2.67	1.94	0.61
	Mn	4.38	2.72	1.61	28.05	2.71	2.64	1.91	0.59
	Fe	4.37	2.71	1.61	27.26	2.70	2.63	1.91	0.59
	Co	4.32	2.69	1.61	27.05	2.68	2.62	1.89	0.58
"Ti_2O"		4.81	2.96	1.63	36.50	2.96	2.98	2.01	0.60
"Zr_2O"		5.24	3.27	1.61	48.45	3.25	3.20	2.30	0.70

For the X-ray analysis, cameras of the Guinier type constructed at this institute were used. The cameras incorporated either a bent quartz monochromator or an aluminium monochromator with double curvature.

Besides other ternary oxides, a series of (Me', Me")O_x phases were obtained with the metal atoms randomly distributed in a hexagonal close-packed arrangement and the Me'/Me" ratio close to unity. The phase analysis indicated that the value of x can vary (to some degree) within the approximate limits $0.2 < x < 0.5$. The value of x never exceeded 0.5. By analogy with the carbides and nitrides of the L'3 type the ideal formula may be written Me'Me"O. The following combinations of transition metals were found to exist:

Me' = Mo — Me" = Fe, Co
Me' = W — Me" = Fe, Co
Me' = Re — Me" = Ti, V, Cr, Mn, Fe, Co

For Me' = Re some of the phases probably exist with $x = 0$, *i.e.* without any oxygen in the lattice. This is definitely not the case for the phases containing Mo or W. In the four ternary systems Mo—Fe—O, Mo—Co—O, W—Fe—O, and W—Co—O, phases of the "high speed steel carbide" (η-carbide) structure with the formula $Me_3'Me_3"O$ have been prepared, *i.e.* with an oxygen content of about 14 atom-%. These oxides are isomorphous with the compounds Me_3Ti_3O, where Me = Mn, Fe, Co, Ni, Cu earlier reported by the present author [4]. A cautious oxidation of the oxides of the η-carbide type containing Mo or W caused the formation of oxides of the L'3 type. This proves that the last-mentioned phases only exist with a higher oxygen content than 14 atom-%. An increase of the unit cell volume per metal atom further accentuates this fact.

In a few cases weak extra reflections appeared in the powder photographs of the Me'Me"O phases. These extra reflections do not necessitate an enlargement of the unit cell. They indicate a low degree of order in the metal lattice.

The calculation of $p|F|^2$ values for ordered oxygen atoms shows namely, that the resulting extra lines in this case should be hardly visible in the photographs for Me″ = Mo and undetectable for Me″ = W and Re.

The unit cell dimensions and interatomic distances are listed in Table 1. As indicated by the variation of the lattice constants — commonly of the order 0.1 % — the homogeneity ranges are comparatively small. The axial lengths reported are mean values. The observed mean interatomic distances are compared with those given by Pauling for 12-coordination of atoms [5]. The last column in Table 1 gives the calculated atomic radius for the oxygen atom. It is seen that the deviations from $r_0 = 0.60$ Å is very small. The data obtained for the α-Ti and α-Zr phases with interstitially dissolved oxygen in equilibrium with the TiO and ZrO phases respectively have also been included in the table.

SUMMARY

Metallic double-oxides including two transition elements (Me′, Me″) have been prepared in various ways and investigated by X-ray methods.

Me′Me″O_x phases with the metal atoms in hexagonal close-packing, where $0.4 < x < 1$, have been found to exist for the following combination of atoms:

Me′ = Mo — Me″ = Fe, Co
Me′ = W — Me″ = Fe, Co
Me′ = Re — Me″ = Ti, V, Cr, Mn, Fe, Co

For the Me′ = Re some of the phases probably exist with $x = 0$, *i.e.* without any oxygen in the lattice. This is definitely not the case for the phases containing Mo or W.

The author wishes to thank Professor G. Hägg for his kind interest in this investigation. A grant from *Statens Tekniska Forskningsråd* is gratefully acknowledged. I also wish to thank Mr. S. Hedling for his valuable assistance in the experimental work.

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Adsorption of Testicular Hyaluronidase upon Glass

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It has been observed quite accidentally by the author that bovine testicular hyaluronidase (h.dase) is adsorbed upon filterpaper, glass and quartz sand. Adsorption also takes place on cotton wadding and permutit. Up to now attempts to elute from filterpaper and wadding have been unsuccessful.

EXPERIMENTAL

The h.dase preparation used in the investigations described below was the commercial "Penetrase Leo", but different preparations have given essentially the same results.

The activities were measured in proportion to "Penetrase" as a standard, using the turbidimetric method of Dorfman and Ott [1] with slight modifications.

The adsorption upon glass and quartz sand is influenced by pH, ionic strength and the presence of other substances. Fig. 1 shows the effect of pH. Two ml portions of "penetrase", 50 mg/100 ml, in phosphate buffers of different pH were put in centrifuge tubes with 200 mg of pulverized quartz sand (particle size unknown). In one investigation (curve B), the buffers were 0.1 *M* and therefore the ionic strength increased with pH, in another (curve A) the ionic strength was kept constant (0.1) by appropriate dilution of the buffers. Adsorption is accomplished almost at once. After stirring 2 or 3 times with a glass spatula, the tubes were centrifuged and the supernatants analysed for activity.

The influence of the ionic strength is seen from Fig. 2. Two ml "penetrase", 50 mg/100 ml, in pH 7.2 phosphate buffers of different ionic strength was adsorbed on 50 mg of pulverized quartz sand.

Many attempts to elute the h.dase from quartz and glass have been made. Several monohexoses, fructose, glucose, galactose and glucosamine and also glycerol, all in concentrations of about 1—10 %, were able to hinder, or almost so, the adsorption of h.dase on quartz sand; moreover at an acid pH (3.8) these substances were able to elute some of the adsorbed h.dase. Urine has been found capable of eluting 70 % of the adsorbed h.dase. The best elution has been obtained with 1 *M* phosphate buffer, pH 5.6 (pH of minor importance).

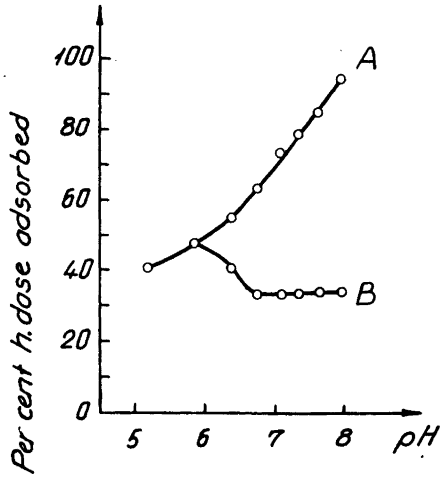

Fig. 1. Effect of pH on adsorption of testicular hyaluronidase upon pulverized quartz sand. In curve A a constant ionic strength of 0.1 is used. In curve B 0.1 M phosphate buffer is used.

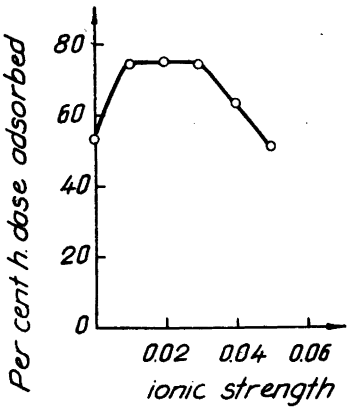

Fig. 2. Effect of ionic strength on adsorption of hyaluronidase upon pulverized quartz sand. Phosphate buffers, pH 7.2.

H.dase may be purified by adsorption upon a column of glass beads followed by elution with 1 *M* phosphate, pH 5.6 (see Fig. 3).

A 33 × 1 cm column of small glass beads ("Ballotini", diameter about 0.1 mm) was washed with phosphate buffer, pH 7.7, ionic strength 0.1, until the pH of the effluent was 7.7. The flow rate was 0.5 ml per minute. "Penetrase", 50 mg/100 ml, in this buffer was run through the column until considerable activity was obtained in the effluent, the column was thus "saturated" (76 ml, Fig. 3). After washing with nearly 20 ml solvent, 1 *M* phosphate, pH 5.6 was put on the column to elute the enzyme (beginning at 95 ml on the abscissa of Fig. 3). The effluent was collected as before in *ca.* 3 ml fractions. As a measure of protein concentration the absorption of the clear effluent fractions at 280 mμ is given in per cent of that of the original 50 mg/100 ml "penetrase" (for this solution the extinction was 0.465 in a 1 cm cell).

From Fig. 3 it is seen that a purification of h.dase is obtained in the third and fourth elution fraction (about 10 and 4.5 times respectively).

With a taller column containing twice the amount of glass beads, one of the elution fractions had a h.dase activity 15 times greater than that of the solution put on the column. Nevertheless, the purification was only of the same order of magnitude (10 times) as in the first experiment because the absorption at 280 mμ was also elevated (about 1.5 times).

The ability of glass to adsorb h.dase is especially important to keep in mind when working with purified h.dase. It has been found that h.dase is more easily adsorbed the purer it is. Consequently, it is also then more difficult to elute. A diluted (2 mg/100 ml) solution of Wyeth h.dase (declaration:

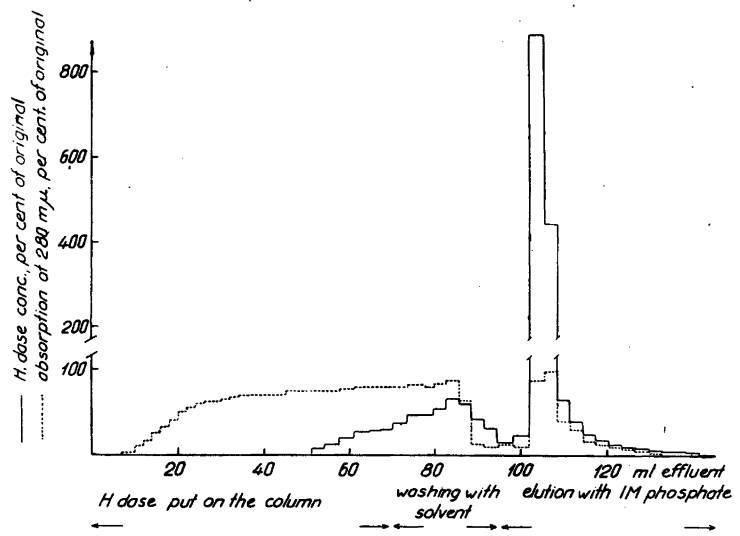

Fig. 3. Purification of hyaluronidase by adsorption upon a 1 × 33 cm column of glass beads followed by elution with 1 M phosphate, pH 5.6. Diameter of beads about 0.1 mm. 76 ml h.dase was run through the column, followed by washing with buffer. After collecting 95 ml effluent, elution with 1 M phosphate, pH 5.6 was begun.

1 400 T.R.U. per mg) in borate buffer, pH 7.3, 0.1 *M* NaCl, will lose more than 50 % activity if kept in a test tube half an hour, 3°. With this preparation (about 3 times more pure than "penetrase") it could be proved that much of the loss was due to adsorption, *i. e.* much of the lost activity could be eluted. Solutions of h.dase at least 50 times more pure than "penetrase", about the same purity as the best hitherto reported, very easily lose activity from contact with glass and other surfaces. Elution, however, has not been effected. Such purified h.dase preparations are said to be very unstable [2]. The author thinks that some of the "instability" of purified h.dase is due to adsorption upon glass, but investigations not yet completed seems to show that this "instability" is more complex.

These observations concerning the adsorption of h.dase upon negatively charged substances suggested that h.dase was possibly a basic protein. H.dase was therefore tried on a column of Amberlite IRC 50 in the same way as Moore, Stein and co-workers have done with ACTH [3], ribonuclease [4], and lysozyme [5]. Indeed the Wyeth preparation has now been purified 30-fold by this method. Details will be published later.

SUMMARY

Testis-hyaluronidase can be adsorbed upon glass, quartz sand, permutit, filterpaper and cotton wadding. Attempts to elute from filterpaper and wadding have not been successful.

Adsorption upon pulverized quartz sand is influenced by pH and ionic strength.

Purification of testicular hyaluronidase was obtained by adsorption upon a column of fine glass beads, followed by elution with 1 *M* phosphate, pH 5.6.

The author is indebted to Løvens Kemiske Fabrik, Copenhagen, for the "Penetrase Leo" used in this investigation.

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The Oxidation of Uric Acid with Horse Radish Peroxidase

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During the last decade the interest in the biological effects of the oxidation products of uric acid has been rapidly growing. Ascoli and Izar [1] described in 1909 an enzyme system in dog and calf liver which decomposed uric acid under aerobic conditions and resynthesized it from dialuric acid. The addition of uric acid to histamin (0.001 *M*) and impure hog kidney diamine oxidase increased the oxygen uptake [2]. The effect was attributed to a peroxidatic oxidation of the uric acid, the peroxide thus being unaccessible for catalatic decomposition. Dunn, Sheehan, and McLetchie [3] developed diabetes in rabbits by injecting alloxan, and discussed the possibility that alloxan formed *in vivo* from uric acid, might be involved in the pathogenesis of diabetes mellitus. Tipson and Ruben [4] found a substance in trichloroacetic acid extracts from a number of normal tissues which gave alloxan reactions. Alloxan was found in the liver of fasting white rat, rabbit, dog and man [5]. The injection of uric acid [6] or ribonucleic acid [7] developed hyperglycaemia in rabbits which were maintained on a diet low in cystine and methionine.

Alloxan reacts with amino and thiol groups of proteins and amino acids [8,9]. It inhibits hexosediphosphatase [10]. The structurally related ninhydrine inactivates tetanus toxin [11].

Agner [12] found that impure specimens of diphteria and tetanus toxins were inactivated by verdo-peroxidase (= myeloperoxidase) or horse radish peroxidase + hydrogen peroxide. Later on[13] he showed that pure toxins were not destroyed unless uric acid or casein hydrolysate were added. He also demonstrated that uric acid was oxidized by peroxidase + peroxide. Tuttle and Cohen [14] oxidized C^{14}-labelled uric acid with milk peroxidase and suggested tentatively that alloxan might be formed in connection with this oxidation of uric acid.

The purpose of the present investigation was to study the oxidation of uric acid with horse radish peroxidase and the conditions leading to the various possible oxidation products.

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MATERIAL AND METHODS

Crystalline horse radish peroxidase (HRP) was taken from the same preparation as previously used [15]. Commercial preparations (B.D.H.) of uric acid, xanthin, heteroxanthin, hypoxanthin, theobromine, adenine, uracil and alloxan were used. 1-methyl, 1,3-dimethyl, 1,9-dimethyl, 3,9-dimethyl, and 1,7-dimethyl-8-acetyl uric acid were kindly supplied by Dr. U. Lagerkvist. They had been purified by him by chromatography on starch (*n*-propanol : 0.5 *M* HCl 2 : 1, v/v).

Spectrophotometric analyses were made with a Beckman DU spectrophotometer. The optical densities were corrected for absorption due to HRP and solvents and for dilutions.

The experiments were made at room temperature (20°).

1-methyl uric acid was used for most experiments because of its greater solubility than uric acid.

RESULTS

The changes in light absorption, which occur at different wavelengths during the oxidation of 1-methyl uric acid are seen in Fig. 1. At least one intermediate substance is involved in the transformation of uric acid to the final product(s); it gives a low light absorption at 285 mμ and a higher one at 230 mμ. If the reaction was interrupted after five minutes by the addition of strong HCl practically no light absorption remained above 255 mμ, *i. e.* no uric acid was left. Thus the changes in density at 285 mμ, occurring after this time, reflect only reactions concerning the intermediate(s).

The following symbols are used in the sequel to facilitate the description:

$$\text{uric acid} \xrightarrow{k_1} \text{B} \xrightarrow{k_2} \text{C}$$

where B is the intermediate and C the final compound.

Reaction 1 (0—2 min., Fig. 1) was accelerated by the add tion of another portion of HRP + H_2O_2, whereas the same addition during reaction 2 (>4 min) had no influence. Thus peroxidase catalyses only the conversion of uric acid to B.

No change in pH was noticed in experiments at pH 3.5—4 in which only dilute HCl had been used to adjust the pH.

Curves 3—5 in Fig. 2 show that the oxidation of one mole of uric acid consumes one mole of hydrogen peroxide.

Spectrophotometric experiments

Reaction 1. The initial, rapid decrease in light absorption at 285 mμ fitted in most experiments well to curves for first order reactions. However, when the oxidation of uric acid was followed in terms of remaining amounts of uric acid, agreement with neither first nor zero order reactions was found (Fig. 3). The concentrations of peroxidase and peroxide, a possible inhibition of HRP by the products B or C, and the instability of B will all influence the light absorption changes. No significance can be attributed to the abovementioned finding of an apparent first order reaction.

Some information on the influence of pH on the velocity of reaction 1 was obtained by plotting $1/t_{30\,\%}$ against pH, where $t_{30\,\%}$ is the time during

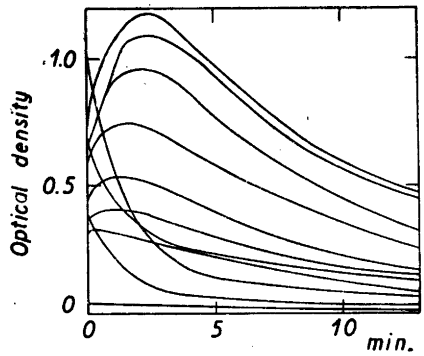


Fig. 1. Spectral changes during the oxidation of 1-methyl uric acid. The curves represent from above (at 10 min) the wave-lengths 230, 235, 240, 245, 250, 257, 270, 285, 300, and 310 mμ. HRP = 0.35 μM, 1-methyl u.a. = 82 μM, H_2O_2 = 97 μM. pH 3.55 (adjusted with dilute HCl, no buffer).

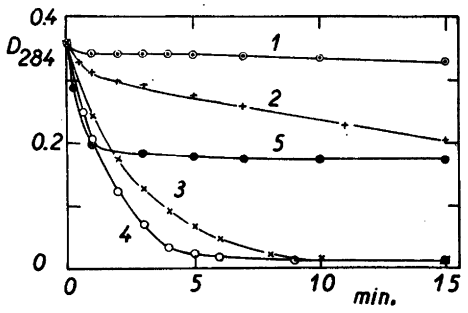


Fig. 2. Influence of $[H_2O_2]$. 1-methyl u.a. = 33 μM, HRP 0.40 μM, H_2O_2 for curves 1—5 32, 3.2 mM, 320, 32, and 16 μM. pH 3.70 (0.1 M acetate).

which the initial density at 285 mμ decreased by 30 % (Fig. 4). A few experiments were made at alkaline reaction. $t_{30\,\%}$ was roughly the same at pH 8.5 as at pH 7, while at pH 10.5 only a very slow reduction of the light absorption at 293 mμ of 1-methyl uric acid was seen.

Reaction 2. As mentioned no uric acid was left after a few minutes. Plots of

$$\log\,[(D_{5\text{min}}-D_{\text{final}})\,/\,(D_{5+t_{\text{min}}}-D_{\text{final}})]$$

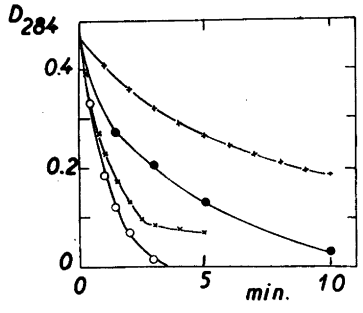


Fig. 3. Reaction 1 in terms of remaining amounts of 1-methyl u.a. + and × denote optical densities (without acidification) at pH 5.53 and 4.00. Same experiment repeated but 0.10 ml 8 M HCl added at various times to reaction mixtures of pH 5.53 (●) and 4.00 (○). HRP 0.87 μM, H_2O_2 81 μM, 1-methyl u.a. 46 μM. 0.01 M acetate buffer, volume 3.0 ml.

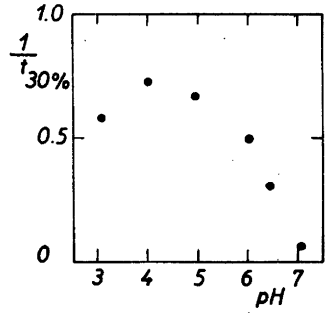


Fig. 4. Velocity of reaction 1 in terms of $1/t_{30\,\%}$ at various pH-values. HRP 0.12 μM, H_2O_2 82 μM, 1-methyl u.a. 45 μM, 0.05 M acetate.

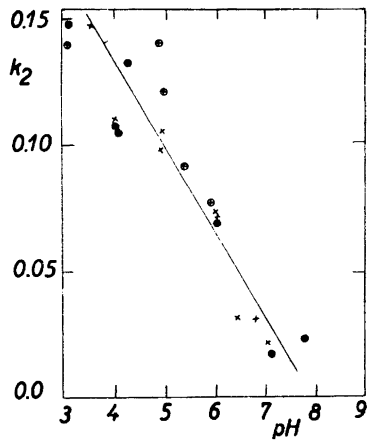


Fig. 5. Velocity constant of reaction 2.

● *235 mμ*	*HRP = 0.35 μM*	H_2O_2 *82 μM*	*1-me. u.a.*	*45—52 μM*
× *285* »	» *0.12* »	» *82* »	»	*45—52* »
⊕ *285* »	» *0.12* »	» *33* »	»	*32* »
+ *305* »	» *0.35* »	» *82* »	»	*52* »

(D = optical density) against time gave straight lines of constant and practically the same slope for all wavelengths in Fig. 1, indicating that only one compound was decomposing at this pH. Further determinations of k_2 were made at 235, 285 and, in a few cases, 305 mμ (Fig. 5). It was found that the velocity constant k_2 of the reaction B → C was independent of wavelength, ratio of H_2O_2 : uric acid and [HRP] during reaction 1 but varied with pH.

Spectrum of the intermediate B. The light absorption curve obtained by plotting the densities after 5 min. in Fig. 1 against wavelength gives the relative spectrum of B at pH 3.55 with an unknown but small contribution from C. The same experiment was then repeated, but phosphate buffer was added at 5 min. to give pH 7.60. (Fig. 6 : 1.) Compound B can obviously appear in one form ("B_1") at slightly acid reaction (pH 3.5—5) without the band at 307 mμ, and one in neutral or faintly alkaline solution ("B_2"). The latter has a lower light absorption at 230 mμ but gives a band at 307 mμ (*cf.* diagram, p. 643). The existence of the two forms was further confirmed by the following results.

1. In a series of experiments at pH 4.28 the reaction was followed at 305 mμ. After 5 min, when the density was stable and low pH was brought to values between 4.5 and 9 by means of phosphate buffers and the light absorption determined as rapidly as possible. It was then followed for a time, sufficiently long to permit the extrapolation back to $t = 5$ min. The curve obtained by plotting the density after the addition of the buffer against the resulting pH is given in Fig. 7. Thus the change from B_1 to B_2 is rapid (< 10 sec.), and its dependence upon pH seems to follow a dissociation curve with $n = 1$.

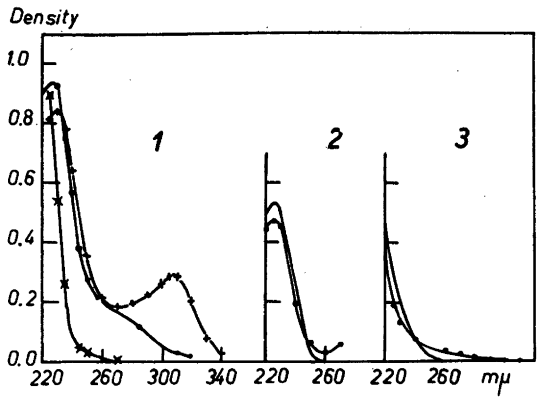


Fig. 6. 1. Relative spectra of compounds B_1 (●), B_2 (+), and B_3 (×). Spectrum of B_1 obtained from Fig. 1 (5 min.). Solutions of B_3 (pH<1) and B_2 (pH 7.60) obtained by the addition of HCl and sec. phosphate at 5 min to the same reaction mixture as used for B_1 (values corr. for dilution).
2. Spectrum of compound C_3 from solution, acidified at 2.5 min (cf. Fig. 7. Some unreacted 1-methyl uric acid but practically no C_1 in the solution). Reference curve: 79 μM 1-methyl alloxan in 0.35 M HCl.
3. Spectrum of C_1 at pH 4.05 (from 82 μM 1-methyl u.a.). Reference curve: 80 μM allantoin, pH 4.05.

2. The oxidation of uric acid was initiated as usually (pH 3.85). After 4 min pH was brought to 6.08 by means of phosphate buffer and the decreasing light absorption was followed alternatingly at 235 and 307 mμ. At both wavelengths the decrease followed a first order reaction curve with

$$k_{235\ m\mu} = 0.069\ \text{min}^{-1} \text{ and } k_{307\ m\mu} = 0.072\ \text{min}^{-1}.$$

3. In four different experiments (H_2O_2 = 81 μM; HRP = 0.53 μM; 1-methyl uric acid = 51 μM; pH 4.10, 0.05 M acetate) the changes in optical

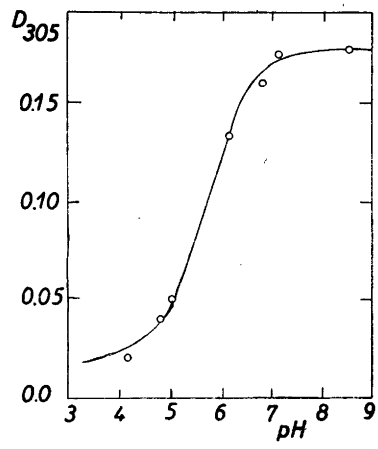


Fig. 7. Light absorption at 305 mμ after alkalinization from pH 4.18 to the values in the figure. Full drawn curve calculated for half conversion of B_1 to B_2 at pH 5.75 and n = 1. HRP 0.35 μM, H_2O_2 82 μM, 1-methyl u.a. 52 μM. Alkaline phosphate buffer added at 5 min.

Table 1. Correspondence between intermediates B_1 *and* B_2. *Reaction conditions, see text.*

	Addition of sec. phosphate min.			
	5	10	15	20
Density at 235 mμ above final level before addition of phosphate	0.342	0.200	0.110	0.060
Increase in density at 305 mμ upon the addition of phosphate	0.155	0.090	0.052	0.027
Ratio	2.23	2.22	2.12	2.22

density were registered at 235 and 305 mμ alternatingly. At 5, 10, 15, and 20 min. secondary phosphate was added to give pH 7.35, and the densities at 305 mμ determined immediately after the additions. The final levels (2 h) at 305 and 235 mμ at pH 4.10 were determined in a separate experiment. The results collected in Table 1, show that the increase in density at 305 mμ, *i. e.* formation of compound B_2 upon neutralization, is proportional to the optical density at 235 mμ at the moment of neutralization, *i. e.* to the concentration of compound B_1.

At low pH a third spectral form of the intermediate was found. 1-methyl uric acid was oxidized in the usual way to compound B, and the solutions were acidified after 3 min. with various amounts of hydrochloric acid (Fig. 8 A). At pH 4.28 and 2.33 continuously decreasing curves were found. At even lower pH the density decreased rapidly to a minimum and increased then slowly ($t_{1/2} \approx 4.5$ min). The more acid added at 3 min., the higher was the final (30 min.) level. Fig. 8 B shows that this level is proportional to the amount of compound B at the moment of acidification.

These observations are interpreted as follows. At pH 3—5 the intermediate B, at this acidity in the form B_1, yields a compound with low absorption at 235 mμ ("C_1"). The acidification of a solution of B_1 to pH 1—2 brings about two consecutive reactions. Within a very short time B_1 is converted to a form with a low light absorption at 235 mμ ("B_3"). A curve giving the inverse of $t_{1/2}$ for the rapid decrease in density at 235 mμ upon acidification *versus* pH approaches asymptotically the ordinate at pH 1. The rapid, initial decrease in density is completely reversed by an immediate (< 10 sec.) addition of an equivalent amount of alkali. In the succeeding, slower ($t_{1/2} \approx 4.5$ min.) reaction B_3 is converted to the final substance ("C_3"), which has a higher molar absorption at 235 mμ than C_1. At an intermediate pH both ways (leading to C_1 and C_3) for the decomposition of B are used.

The spectrum of compound B_3 (Fig. 6 : 1) was obtained in the same way as the spectrum of B_1. Consequently small amounts of compounds C_1 and C_3 may have been present.

The relative spectra of the final compounds C_1 and C_3 are given in Fig. 6 : 2 and 6 : 3. C_3 apparently gives the same spectrum as 1-methyl alloxan, while the spectrum of C_1 is less characteristic.

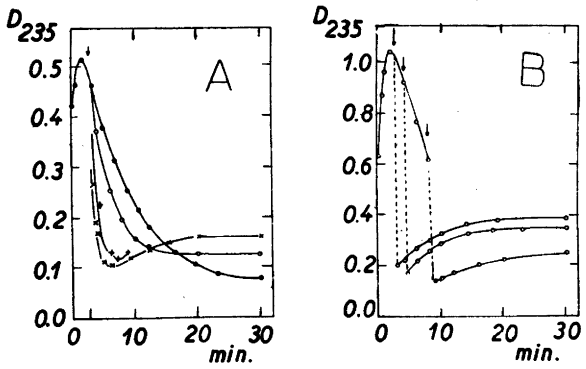
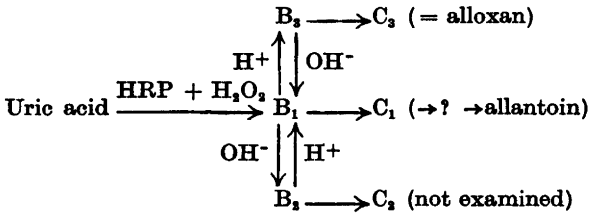


Fig. 8. Strong acidification of intermediate reaction mixture. A: HRP = 0.12 μM, H_2O_2 82 μM, 1-methyl u.a. 30 μM. pH 4.28 (●). At 3 min. HCl was added to give pH 2.33 (○) 1.56 (+) and 1.08 (×). B: HRP 0.35 μM, H_2O_2 100 μM, 1-methyl u.a. 85 μM. pH 3.55. Initial volume 3.09 ml. In three separate experiments 0.15 ml 6 M HCl were added at 2.5, 3.5 and 8 min. 235 mμ.

When 0.2 ml 6 *M* HCl was added to a Beckman cuvette, containing C_1 (pH 4.28, 0.01 *M* acetate, total volume 3.05 ml), no change in light absorption occurred except for that which was accounted for by the dilution and the effect of acidification on the acetate buffer.

The results of the spectrophotometric experiments are summarized in the diagram:



At those acidities where reaction 1 proceeds with its highest velocity, form B_1 of the intermediate is formed. B_1 is reversibly converted into forms B_2 and B_3 by basification or acidification respectively. The three forms of the intermediate B decompose non-enzymatically to the products C_1, C_2, and C_3. The two first-mentioned are not mutually convertible by changing the pH; C_2 was not studied.

In this pattern it has been assumed that B is the only intermediate between A and C. Occasionally we have seen traces of another compound at pH 4—5 when the decomposition of B_1 was followed at 285 mμ. It appeared as a plateau for 1—2 min immediately after the rapid decrease in optical density, *i. e.* 3—5 min after the addition of peroxidase. We did not, however, always trace it and it is doubtful if the plateau was significant. The difference between the spectrum of C_1 and that of allantoin (Fig. 6 : 3) is more obvious. Even if C_1 and allantoin are not identical, the changes in C_1 occur so slowly that they hardly influence the kinetic results given above.

Identification of C_1 and C_3. One mmole (168 mg) of uric acid, dissolved in an equivalent amount of lithium hydroxide (6 ml), was added in 20 portions during 3 1/2 h to 500 ml water, previously adjusted with hydrochloric acid to pH 4.1 and containing 0.27 μmoles of HRP (1.2 mg). After the tenth addition more HRP (0.13 μmoles) was added. Simultaneously to each addition of urate an equivalent amount of hydrogen peroxide was added, in all 1.01 mM. It was frequently checked spectrophotometrically that no unreacted uric acid accumulated, and pH was consistently found to be 4.1—4.2. Next day the solution was evaporated to 10 ml; a crystalline precipitate soon appeared. It was washed three times with ice-cold water and dried. Weight 70 mg. After recrystallization three times from hot water it melted at 230°—236° (Kofler-Block). The melting point of allantoin in a capillary tube has been given as 228—230° (slow heating) or 233—234° (bath preheated to 228°). Still higher melting points have been noticed on copper block [16]. (Found: C 30.63, H 3.86, N 35.40. Calc. for $C_4H_6O_3N_4$ (allantoin) (158.1): C 30.38, H 3.83, N 35.45.) C_1, or the substance deriving from it by a slow reaction (overnight) is thus allantoin. The yield of crude crystals was 44 %.

When a solution of C_3 was adjusted to pH 3.5, mixed with *o*-phenylene diamine, and illuminated with a Wood's lamp it gave a green fluorescence [17, 18], whereas compounds B_1, B_3 or C_1 gave no fluorescence under the same conditions. The increase in fluorescence during the conversion of B_3 to C_3 was followed by withdrawing samples from a solution of B_3 and matching them against a standard of 1-methyl alloxan. The final value amounted to about 50 % (Pulfrich photometer) of the value, calculated from the used amount of 1-methyl uric acid. Substance C_3 gave the same R_F-value as 1-methyl alloxan when chromatographed on starch (*n*-propanol : 0.5 *M* HCl 2 : 1 v/v) *. Moreover, since the relative spectrum of C_3 was practically the same as that of 1-methyl alloxan (Fig. 6 : 2) it seems to be clear that compound C_3 is 1-methyl alloxan. It was not possible to prepare a large amount of C_3 because of the necessary acidification. The yields of alloxan, calculated from Fig. 8 B, were found to be 78 (corr. for remaining uric acid), 80 and 56 %.

Action of HRP on other purine derivatives and uracil

Table 2 gives $t_{30\,\%}$ for uric acid and some of its methyl derivatives. The values $t_{30\,\%} = 0.3$—0.5 are not significantly different. The table confirms that the oxidizable compounds and hydrogen peroxide react in the molar ratio of 1 : 1.

No reaction could be detected spectrophotometrically at the wavelengths of maximal absorption within the range 240—270 mμ when HRP + peroxide were added, at pH 3.6 and 7, to solutions of xanthin, hypoxanthin, heteroxanthin, adenin, theobromine, and uracil.

* Dr. U. Lagerkvist.

Table 2. Oxidation of uric acid and some of its methyl derivatives. HRP 0.35 μM. H_2O_2 16.5 μM. Uric acid or derivative 29—31 μM. The reactions were followed at the wavelengths for maximal absorption (mμ). pH 3.78.

Substance	mμ	$t_{30\%}$ min.	Moles oxidized per mole of H_2O_2
Uric acid (u.a.)	284	0.4	1.1
1-methyl u.a.	284	0.5	1.0
1,3-dimethyl u.a.	286	2.5	1.0
1,9-dimethyl u.a.	285	0.5	1.0
3,9-dimethyl u.a.	292	13.5	—
1.7-dimethyl-8-acetyl u.a.	286	0.3	1.1

DISCUSSION

The highest velocity of reaction 1, as judged from the values for $t_{30\%}$, was found at pH 4—5 (Fig. 4). With increasing pH $1/t_{30\%}$ decreased, by half at pH 6.3. This value is higher than pK_1 for uric acid (5.4 [19]) and probably also for 1-methyl uric acid [20], but if it is taken into consideration that $t_{30\%}$ may only remotely reflect the velocity of reaction 1 the results of Fig. 4 may well be interpreted as showing that unionized uric acid rather than the monovalent anion is the actual subject of oxidation. Chance [21] found the decomposition of HRP-H_2O_2 Complex II by ascorbic acid (pK 4.21) to be slower above than below pH 5.3, and attributed the decrease to a change in the ascorbic acid molecule, possibly ionization. Neither of the cases is conclusive, but they indicate that (HRP + H_2O_2) reacts more easily with the uncharged donor molecule than with its ionized form. The action of peroxidases has been described [22] as a dehydrogenation, *i. e.* hydrogen atoms are removed.

Margules and Griffiths [23] have shown that uric acid can reduce ferricytochrome c without the cooperation of a dehydrogenase. Although the reaction was very slow an optimum could be found at a pH where the acid exists as the monovalent anion. The optimal pH for the action of uricase is 9.2—9.4 [24]. The sole function of this enzyme is, according to Bentley and Neuberger [25], to transfer two electrons from the mono- or divalent urate anion to oxygen.

The oxidation of cysteine by heart muscle preparation + cytochrome c has been found [26] to proceed at highest velocity at pH 8.0, whereas the maximal velocity of the reaction between ferricytochrome c and cysteine was found at pH 8.8. In the latter case the increase in velocity between pH 7 and 9 coincided with the titration curve of the sulphhydryl group of cysteine. Experiments on the oxidation of cysteine with HRP + added peroxide have shown that cysteine is more rapidly oxidized at faintly acid than at alkaline pH.

Peroxidases are, with a few exceptions, found in plants, where pH generally is lower than in tissues from at least higher animals. An acid substance AH_2 would then be oxidized in plants according to

$$AH_2 + H_2O_2 = A + 2H_2O$$

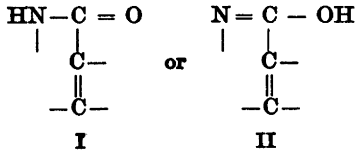
In tissues from animals the substance would be oxidized by electron transporting enzymes after protolytical release of one or several protons

$$AH_2 = A^{2-} + 2H^+$$
$$A^{2-} = A + 2\ e^-$$

As a consequence of this comparison between the modes of action of peroxidases and the exclusively electron transporting systems it must be assumed that the oxidation products, obtained when HRP and uricase act upon uric acid, at their pH optima, should become identical when brought to the same pH. Uricase oxidizes uric acid to allantoin, which also was the final product deriving from compound B_2. Moreover, B_2 and that of Praetorius' [27] intermediates, which possessed an absorption band at 325 mμ show similar spectra. His figures do not seem to exclude the possibility that the maximum could have been situated at a slightly shorter wavelength than 325 mμ.

In unsubstituted uric acid the hydrogen atoms at the positions 3 and 9 [20] (or possibly in the order 9 and 3[28]) are first dissociated. Methylation at these positions exerts the most pronounced influence on the velocity of reaction 1 (Table 2). The velocity of reaction 1 of the oxidation of a purin derivative cannot, however, be related only to the dissociations of the nuclear hydrogen atoms, since the presence of the carbonyl group at position 8 is a prerequisite for the oxidation.

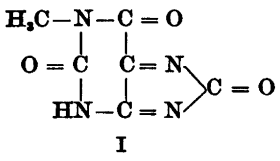
The most apparent spectral effect during the oxidation of uric acid to compound B is the vanishing of the strong band at around 285 mμ. This band is mainly caused by the chromophore [29]



I II

It is therefore probable that the 4,5-double bond vanishes simultaneously with the loss of two hydrogen atoms. Several structures for the intermediate B will then be possible, a common feature being a number of conjugated double bonds. The structures differ by the lack of one negative charge from the one attributed by Bentley and Neuberger to the primary product from uricase action on the monovalent urate anion. The rapid transformations between the three forms of B can be accounted for by tautometric shifts or ionisations.

The following way for the decomposition of B may be worth considering. One of the possible structures of B is (I). By the uptake of water it acquires a glycol form (II).



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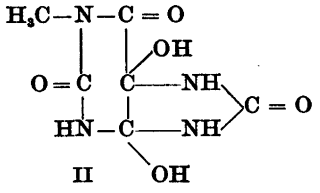

II

Biltz and Max [30] suggested that (II) could be an intermediate in the oxidation of uric acid with nitric acid to alloxan and with alkaline permanganate to allantoin. The dimethyl ether of (II) has been isolated [31] after oxidation of uric acid with chlorine in methanol.

Biltz [31] has described a procedure, supposed to give (II). It gave in our hands a preparation which was spectrophotometrically stable at pH 4 (235 mμ). The addition of 6 *M* HCl (final conc. 0.2 *M* HCl) caused no immediate decrease in optical density at 235 mμ, nor did the light absorption increase slowly. At pH 9.3 (235 mμ) the initial light absorption was higher than at pH 4 and decreased by one fourth in 3 min. and was then stable. Thus our compound B cannot be identical with the substance obtained according to Biltz. Archibald [18] found that Biltz' method gave a product, from which ammonia could be liberated with urease, and which gave alloxan reactions. He concluded that Biltz' compound was not the true glycol (II) but rather some adduct of alloxan and urea, a conclusion well compatible with our spectrophotometric observations.

The rapid transformation $B_1 \rightarrow B_3$ occurs at the pH where the first proton is dissociated from hydrated alloxan [32]. A negative charge acquired by the dissociation of the hydroxylic proton at the position 5 in (II) tends to split off by decarboxylation the 6-carbon atom, corresponding to reaction 2 within the range pH 2—5. Bentley and Neuberger showed that this carbon is lost during the conversion of uric acid to allantoin by uricase. A high hydrogen ion concentration would promote this decarboxylation, *i. e.* $k_2(B_1 \rightarrow C_1)$ should increase when pH is decreased (Fig. 5). This reaction leads to allantoin. At even lower pH the dissociation of the proton is suppressed, and no decarboxylation takes place. The spectral differences between B_1 and B_3 can possibly be accounted for by this dissociation. The slow reaction $B_3 \rightarrow C_3$ then corresponds to the hydrolysis of the (7—9) urea residue.

Thus it appears unlikely that alloxan is formed in the living organism by peroxidase or uricase action. The results of Tipson and Ruben can nevertheless be explained since they extracted the tissues with 10 % trichloroacetic acid. Since neither allantoin nor uroxanic acid explain the biological observations mentioned at the beginning, it is possible that the parent substance of alloxan, *i. e.* compound B, is responsible for the effects.

SUMMARY

1. The oxidation of 1-methyl uric acid with horse radish peroxidase and hydrogen peroxide has been studied.

2. The uncharged molecule was oxidized more rapidly than the monovalent anion. The divalent anion was apparently not oxidized at all.

3. The primary oxidation product, obtained at pH 3—5, could reversibly be transformed into two other, spectrophotometrically different forms by changing the pH to > 6 ($pK = 5.75$) or ≤ 1.

4. The primary oxidation product undergoes non-enzymatic decomposition to allantoin (pH 3—6) or alloxan (pH ≤ 1). The decomposition of the alkaline form of the primary oxidation product has not been studied.

5. Experiments on uric acid, some N-methyl substituted uric acid preparations and purins, lacking the carbonyl group in position 8, showed that the presence of this carbonyl group is a prerequisite for the oxidation.

6. It is likely that the primary oxidation products of uric acid, obtained by the actions of uricase and peroxidase, should become identical when brought to the same pH. Available results seem to support this idea.

Dr. Ulf Lagerkvist has kindly supplied some methyl derivatives of uric acid. Technical assistance was given by Miss Berit Nilsson. The investigation was financially supported by *Statens naturvetenskapliga forskningsråd.*

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The Assay of Peroxidase with Mesidine as the Hydrogen Donor

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A number of procedures have been suggested for the assay of peroxidases [1–3], but none of them is quite satisfactory. Disadvantages are lack of specificity as regards the catalyzing enzyme (*e. g.* in the oxidation of ascorbic acid by crude peroxidase preparations) and the formation of byproducts which influence the result. The purpurogallin test [4–7], which perhaps is most commonly used, may function quite well on one occasion and give duplicates of good agreement, but later the same operator may obtain quite different values with the same specimen of peroxidase. Thus Ettori [7] during the course of seven weeks found variations from 1 160 to 1 780 in the purpurogallin number of one horse-radish peroxidase (HRP) preparation. Such irregularities have been attributed to impurities in the pyrogallol [8] or to metal ions in the water [9]. Consequently the comparison of actual results with data from the literature or from other laboratories may give rise to misleading conclusions. There are also other disadvantages: a considerable blank value, the necessity of several ether extractions, and the occasional formation of byproducts, noticeable as a reddish colour in the aqueous residues.

Chapman and Saunders [10] examined the peroxidative oxidation of mesidine (1-amino-2,4,6-trimethyl-benzene), catalyzed by HRP. Only one condensation product was formed (2,6-dimethylbenzoquinone-4-(2,4,6-trimethyl benzene) anil, "purple compound"). The absence of other condensation products was attributed to the fact that mesidine contains only two free nuclear hydrogen atoms. We have attempted to use mesidine for the assay of HRP and some other peroxidases **.

MATERIAL AND METHODS

Horse-radish peroxidase was crystallized as described by Theorell [11]. Although the material appeared to be homogenous in solubility tests *ad modum* Cohn [12], the first batch of crystals contained 1.33 % haemin and the second, from the mother liquor of the first,

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** Dr. C. B. Saunders suggested mesidine for this purpose upon a question from Professor H. Theorell at the Biochemical Congress in Paris, 1952.

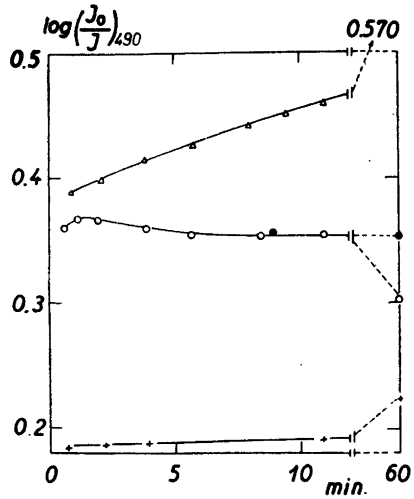

Fig. 1. Interruption of the enzymatic reaction by one volume of ethanol of various acidities. Five min. after the addition of HRP were added 2 ml of ethanol without acid (+), with 6 (△) or 20 (○) ml 3.5 M H_2SO_4 per 1 000 ml ethanol. (●) same as (○) but after further 7 min. were added 0.32 ml 1 M NaOH. Density values recalculated to 4 ml.

1.26 %. Material from the second batch was used for the experiments in this paper (P.N. 1 230, $D_{402}/D_{280} = 2.74$). Haemin was determined as pyridine haemochromogen, the molar absorption coefficient $\beta_{\alpha\text{-band}}$ being 8.0×10^7 cm² × mole $^{-1}$ [13]. [HRP] of the stock solutions were determined in the same way. Haemin contents for HRP earlier reported from this institute (1.36—1.48 %), had been based on the previously accepted value $\beta_{\alpha\text{-band}} = 7.3 \times 10^7$ cm² × mole^{-1} and should thus be multiplied with 0.915 to become correct.

A HRP preparation from the Molteno Institute, Cambridge, contained, according to the submitted analyses, 1.61 % haemin, P.N. 1 220 (1948), 983 (1953). The preparation had been kept at −15° since 1948. We found the haemin content of the Molteno preparation to be 1.36 % (calculated on $\beta = 8.0 \times 10^7$ cm² × mole^{-1}), a value close to those of our preparations. D_{402}/D_{280} of the Cambridge preparation was 2.79.

A third HRP preparation, obtained from Philadelphia, gave $D_{402}/D_{280} = 2.9$.

Hydrogen peroxide 30 % ("Perhydrol" Merck) was titrated with $KMnO_4$ and suitably diluted with water. The dilute solution was kept in icewater and made up twice daily.

Mesidine. Nitromesitylene [14] was refluxed with tin and 4 *M* HCl (molar ratios 1 : 10 : 10) for two hours on a sandbath, and the metal ions subsequently removed by H_2S. Mesidine hydrochloride was crystallized twice from water and kept in the solid state. An 0.2 *M* solution in water is stable for one week in the icebox.

Water. Ordinary distilled water (tin pipes) was generally used. No difference was noticed between this and glassdistilled water or, in a few experiments, tap water.

Spectrophotometric determinations were made with a Beckman DU spectrophotometer, 1-cm cells being consistently used.

EXPERIMENTS

General procedure. Buffer, mesidine, and peroxide are pipetted into a test tube. HRP, in a volume of 0.02—0.06 ml is added as a droplet on a hook-shaped glass rod, and simultaneously a stop-watch is started. The reaction is interrupted after a suitable time by blowing down one volume of acid ethanol, which immediately develops a blue colour, within a few minutes changing to orange-red. This colour in acid is not quite stable (Fig. 1) and therefore alkali sufficient to give a slight excess is added 6—9 min after the alcohol. The colour

then becomes purple red and stable for at least 18 h. It can also be seen from Fig. 1 that alcohol alone does not cause any colour change, and that one volume of neutral or insufficiently acid alcohol does not stop the enzymatic reaction. The optical density of the purple red solution is determined at 490 mμ. A solution of the anil in neutral or faintly alkaline aqueous alcohol follows Beer's law to an optical density of at least 1.2. The molar absorption coefficient β_{490} is 2.92×10^6 cm^2 $\times$ mole^{-1}.

The purpose of the alcohol is to keep the purple compound in solution; it is not necessary in itself for the reactions. It is sometimes preferable to substitute the acid ethanol by acid butanol, *e. g.* when turbid tissue extracts are examined.

The influences of some factors on the formation of the purple compound were studied.

pH. Aliquots of an 0.2 *M* solution of mesidine hydrochloride in 0.1 *M* acetate buffer were adjusted with 4 *M* NaOH or acetic acid to desired pH-values. At pH $>$ 6 mesidine separated to give an emulsion and therefore no higher pH than 6 was tried. The other reagents and HRP were added as described above. The largest amount of purple compound was formed when pH was kept at 4.2 during the enzymatic reaction. Since, however, only slightly slower formation took place at pH 4.9 this value was preferred because of the instability of the anil towards acids. At pH 6 no purple compound was formed; at pH 3 less than half of the quantity at pH 4.2.

Temperature. The formation of the purple compound was twice as rapid at 18° as at ice-water temperature. At 28° the rate was the same as for 18°. The latter fact depends probably upon the cancelling of a higher velocity at 28° by an increased rate of inactivation of the enzyme by the peroxide (Table 1). The determinations should therefore be made at a low temperature. It was found to be essential that the temperature was kept constant and at an easily reproducible level. The numerical values of the constants given below were obtained with finely crushed ice as cooling medium. With ice cubes significantly different numerical values and larger dispersion were noted.

[H_2O_2]. The colour density of the solution, after the addition of acid alcohol and alkali, is proportional to the available amount of peroxide, provided that an excess of mesidine and a sufficiently long reaction time have been employed. An initial peroxide concentration of 5 m*M* was found to give D_{490} around 1 under these conditions. The linear part of a curve, giving D_{490} *versus* time (*cf.* below) will then range from $D_{490} = 0.15$ to 0.8, which is suitable for spectrophotometric determinations without further manipulations.

Table 1. Effect of heat. Reactants denoted by (+), and buffer were pipetted into the tubes and warmed for 15 min. at 59°. After rapid cooling lacking reactants (−) were added, and after further 5 min. acid ethanol. Reactions of tubes 1 and 2 were made at 18° and 59°. Initial concentrations: Mesidine 13 mM, H_2O_2 7.5 mM, HRP 2.9 mμM.

Mesidine	H_2O_2	HRP	D_{490}	% of tube 1
+	+	+	0.370	—
+	+	+	0.105	28
+	—	—	0.370	100
—	+	—	0.340	92
—	—	+	0.105	28
+	+	—	0.380	103
+	—	+	0.140	38
—	+	+	0.000	0

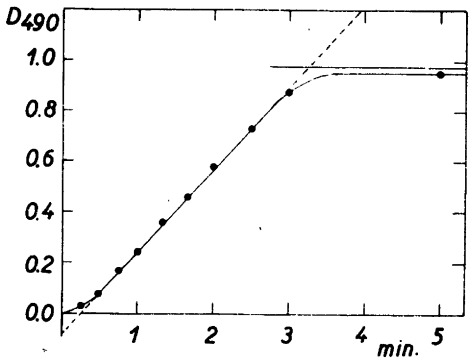

Fig. 2. Increase in amount of purple compound with time. [HRP] = 30.5 mμM. HRP added at t = 0. D_{490} = 0.98 calculated for 3 H_2O_2/1 purple compound. Dashed line: $D_{490} = D_{t_0} + k \times t$; 0°, H_2O_2 5 mM, mesidine 15 mM.

[*Mesidine*]. The overall formation of the purple compound per unit time was found to be proportional to the mesidine concentration to a value of around 13 mM; 20 mM gave only 35 % instead of 54 % higher overall velocity than 13 mM. Since the peroxide concentration should be 5 mM and two moles of mesidine correspond to three moles of peroxide in the net formula, nothing can be gained stoichiometrically by using [mesidine] > 13 mM, when a pure peroxidase is being examined. On the other hand a high mesidine concentration will counteract undesired side-reactions between the oxidation product of mesidine and amino groups in impure peroxidase preparations. The appearrance of a turbidity due to unreacted mesidine will eventually be seen upon the addition of alcohol if the initial mesidine concentration has been 20 mM or higher. We have used 15 mM mesidine.

Reaction time and [*HRP*]. In a number of experiments at one peroxide concentration the addition of acid ethanol *etc.* was made at different times. Plots of D_{490} of the final solution against time showed that for a certain time the enzymatic reaction proceeds as a zero order reaction (Fig. 2). The rectilinear part is limited upwards by the exhaustion of the peroxide, downwards by a "lag" phase. The duration of the latter varies slightly with the concentration of HRP, but for all tested concentrations (4.0—96.6 mμM HRP) the linear part extended at least from 0.150 to 0.750 density units. The results are collected in Table 2. The slope of the linear part (k in Table 2) is proportional to [HRP]. Thus it should be possible to calculate [HRP] from two determinations (D_1, t_1 and D_2, t_2) where $0.150 < D_1 < D_2 < 0.750$. Because of the lag phase at least two points are necessary. However, it seems to be more accurate to calculate [HRP] from the two experimental points in the following way. Let $t_{0.450}$ be the time which, at a certain HRP concentration, is needed to give $D_{490} = 0.450$ = mean of 0.150 and 0.750) of the final, alkaline aqueous solution. Table 2 shows that $1/t_{0.450}$ is proportional to [HRP]; the relation is

$$[\text{HRP}] = (49.8 \pm 1.00) \times \frac{1}{t_{0.450}} - 0.09 \qquad (1)$$

where HRP is obtained in millimicromolarity ($M \times 10^{-9}$) in the reaction mixture when t is given in min. The term — 0.09 can be neglected when $t_{0.450} >$ 5.3 min. (see below). The intensity of the violet colour, which develops during the reaction prior to the addition of alcohol, can be estimated by the eye.

Table 2. Formation of the purple compound at various peroxidase concentrations. The expression $D_{490} = D_{t_0} + k \times t$ *is used for the rectilinear part of a plot of density against time. For every [HRP] the coefficients* D_{t_0} *and k were calculated by means of the method of least squares.* $t_{0.450}$ *was obtained for every [HRP] by putting* $D_{490} = 0.450$.

HRP points mμM	Number of $0.150 < D_{490} < 0.750$	$-D_{t_0}$ density	k density × min^{-1}	$1/t_{0.450}$ min^{-1}
Our HRP				
4.0	5	0.062	0.035	0.068
8.7	7	0.005	0.072	0.158
15.1	9	0.056	0.148	0.292
20.2	4	0.055	0.207	0.410
30.5	6	0.079	0.326	0.616
40.5	8	0.095	0.465	0.853
50.7	7	0.026	0.529	1.111
60.7	5	0.123	0.676	1.180
72.8	5	0.125	0.840	1.461
86.7	6	0.164	1.057	1.721
96.6	6	0.129	1.111	1.919
Cambridge HRP				
18.9	4	0.068	0.215	0.415
37.9	8	0.090	0.422	0.781
68.1	10	0.170	0.798	1.287
94.0	7	0.150	1.065	1.775
Philadelphia HRP				
52.7	8	0.140	0.644	1.092

With a little practice it is easily possible to stop the reaction at such a time that the final solutions give $0.150<D_1<450<D_2<0.750$. From equiform triangles in the density-time diagram it follows that

$$t_{0.450} = \frac{\Delta t(0.450 - D_1)}{\Delta D} + t_1 \qquad (2)$$

[HRP] is then obtained from (1) and (2).

$$[\text{HRP}] = (49.8 \pm 1.00) \times \frac{\Delta D}{\Delta t(0.450 - D_1) + \Delta D \times t_1} \qquad (3)$$

Table 2 also gives the results with two other HRP preparations. Although a statistical comparison is not possible on these few points, there does not seem to be any appreciable difference in activity per haemin between the three preparations.

The values in Table 2 cannot be used to determine the error of the method, since the error in the dilutions of the stock solutions is included. A number of determinations were therefore made with one HRP concentration (adjusted to 20.2 mμM in the reaction mixture). The experimental values were divided into two groups ($0.150<D_1<0.450$ and $0.450<D_2<0.750$). Two values, one

from each group, were randomly combined, and [HRP] was calculated from every pair according to (3). The concentration was found to be 20.5 ± 0.11 * mμM (19.9—21.1, $n = = 9, \sigma_{\%} \pm 1.6$).

The upper limit for the peroxidase concentrations which can be assayed is probably dependent only upon the minimum time, necessary for the manipulations. Our highest concentrations, [HRP] = 96.6 mμM, corresponds to Δt = 31 sec. for $\Delta D = 0.60$. This concentration is close to the highest one which can practically be assayed, since we have found the error in the determination to be roughly proportional to $1/\Delta t$ (or $1/\Delta D$). Our lowest value is [HRP] = 4.0 mμM. It is, however, doubtful if the departure of that point from the rectilinear regression line is random. We think that [HRP] in the reaction mixture should be > 10 mμM, corresponding to $t_{0.450}$<5.3 min.

The "*Standard procedure*" is summarized as follows. Into each of two test tubes are pipetted 1.75 ml 0.1 *M* acetate buffer pH 4.9 + 0.15 ml 0.2 *M* mesidine hydrochloride solution in water + 0.10 ml 0.10 *M* hydrogen peroxide. The tubes are carefully cooled in a water bath containing crushed ice, and 0.02—0.06 (= *a*) ml of the HRP solution to be examined is added to one of them. When the violet colour has reached an intensity which can be estimated as corresponding to a density value of the final solution between 0.150 and 0.450, 2.0 ml acid ethanol (20 ml 3.5 *M* H_2SO_4/1 000 ml alcohol) are added. After further 6—9 min in air at room temperature 0.32 ml 1 *M* NaOH are added. A similar operation is performed in the second tube but here the final density should reach between 0.450 and 0.750. The solutions are measured at 490 mμ, $d = 1$ cm. [HRP] in the reaction mixture is then calculated according to (3). If the time is given in minutes [HRP] in the reaction mixture (1.75 + 0.15 + 0.10 + *a* ml) will be obtained in mμmolarity. [HRP] of the unknown solution is $(2 + a)/a$ times higher.

Other catalysts. A number of other possible catalysts were examined (Tables 3, 4 and 5). In the calculations of moles of purple compound per mole of haemin per min. it was assumed that the reaction was of zero order from the beginning.

Reaction formula. Chapman and Saunders established the structure of the purple compound. They also found that two moles of mesidine produced one mole of ammonia. As a hypothesis for the reaction mechanism they suggested that 2,6-dimethyl-4-methylene-quinone imine was formed as the primary oxidation product, the labile imino group being spontaneously liberated as ammonia by hydrolysis. The methylene group, they suggested, could be liberated as formaldehyde or possibly methane, giving 2,6-dimethyl benzoquinone. We have been able to confirm their hypothesis and to determine the formaldehyde as methylene dimedon.

HRP and peroxide, in slight excess, were added in five portions to 0.69 mg (4 μmoles) of mesidine hydrochloride in 3 ml acetate buffer of pH 4.9. The red precipitate was removed by centrifugation after every addition. The supernatant liquid was decanted, and 0.05 ml of a 10 % dimedon solution in alcohol was added. A white turbidity appeared

* $\bar{X} \pm \sqrt{\frac{\Sigma(X-\bar{X})^2}{n(n-1)}}$

Table 3. Catalytic effects of some haem enzymes. Standard conditions.

Enzyme	[Enzyme]	Reaction time min.	D_{490}
Cytochrome oxidase (as heart muscle prep.)	1 mg/ml	5 *	0
Cytochrome oxidase + cytochrome c	1 mg/ml 4.3 μM	5 *	0
Ditto	Ditto	5	0.148
Cytochrome c	4.3 μM	5	0.143
HRP	3.8 mμM	10	0.678
HRP + + catalase	3.8 mμM 3.8 mμM	10	0.662
HRP + + catalase	3.8 mμM 68.0 mμM	10	0.238
Catalase	68.0 mμM		
After 5 min. HRP	3.8 mμM	5 + 10	0.028
Catalase	1.2 μM	10	0

* No peroxide added.

Table 4. Catalytic effect of some haem-proteids. Standard conditions. Haemin determined as pyridine haemochromogen.

Catalyst	[Catalyst] in terms of haemin	Reaction time min.	D_{490}	Moles of purple compound per mole of haemin per min.
HRP	20 mμM	2	0.485	21 000
Methaemoglobin	38 μM	4	0.645	7
Metmyoglobin	22 »	4	0.500	10
Haemalbumin	18 »	4	0.205	4
Cytochrome c	4.5 »	5	0.143	11

Table 5. Effect of cupric and ferric ions. Standard conditions. Reaction time 4 min. The tubes were centrifuged 6 h after the addition of alkali. A very slight turbidity remained in the tube with only $CuSO_4$.

Catalyst	[Catalyst]	D
HRP	13.2 mμM	0.506
HRP $CuSO_4$	13.2 mμM 925 »	0.540
$CuSO_4$	15.4 mM	0.030
HRP $FeCl_3$	13.2 muM 0.9 mM	0.508
$FeCl_3$	2.5 mM	0.005
Blank		0

in thirty seconds and grew to a crystalline precipitate in three min. The rapid appearance of the turbidity indicated that formaldehyde was present [15]. It was recrystallized three times from ethanol-water and dried over calcium chloride. Yield 4.36 mg (3.2 μmoles of methylene dimedone), m.p. 181—190° (189° [16]). In another experiment the same amounts of reactants were used, but the formaldehyde was separated by steam distillation, and dimedon added to the distillate. Yield 3.70 mg (2.8 μmoles), m.p. 189—190°.

From Fig. 2 (and similar experiments with varying amounts of peroxide) it is obvious that three moles of peroxide are consumed to give one mole of purple compound. The over-all reaction formula is thus

2 mesidine + $3H_2O_2$ = 1 "purple compound" + 1 HCHO + 1 NH_3 + 3 H_2O.

When the reaction is carried out under the standard conditions and at a moderate HRP concentration, the solution remains colourless for about five seconds. This supports the assumption that 2,6-dimethyl-4-methylene quinone imine is the primary oxidation product, since quinone imine is colourless [17]. Then a yellow colour appears, which after another five-ten seconds changes to violet. The yellow substance, probably dimethyl benzoquinone, could not be isolated in pure form from the reaction mixture, although it accumulated as a precipitate when the reaction was made at pH 7 and 20°.

COMMENTS

It is essential for a determination method, specific for peroxidases, that the hydrogen donor should not be oxidizable by oxygen (eventually *via* an enzyme) but only by hydrogen peroxide. This criterion, examplified by a comparison between (dimethyl methylene quinone imine/mesidine) and (benzoquinone/hydroquinone), means that substances like ascorbic acid [18], cysteine, leucodyes and ferrocytochrome *c* cannot be used. The assay should also be designed to minimize sidereactions of the primary oxidation product, achieved in the present case by a high mesidine concentration. Moreover it is necessary that the structure of the substance to be oxidized (AH_2) should be such that only one compound (A-AH_2) is formed. In these respects the present method is satisfactory.

Since HRP and, to an even higher extent, some other peroxidases, are inactivated or destroyed by high peroxide concentrations, the ratio of three moles of hydrogen peroxide / one mole of coloured compound is unfavourable. The same ratio is found in the purpurogallin reaction. The initial peroxide concentration 5 *mM* is rather high, about three times higher than the one suggested by Ettori for the purpurogallin test. A reduction of the peroxide concentration to 1.6 *mM* reduces the above-mentioned linear part (Fig. 2) by more than three quarters. The disadvantage is strengthened by the relatively low molar absorption of the purple compound, $\beta_{490} = 2.9 \times 10^6$ cm^2 × × mole^{-1}. The corresponding value for purpurogallin is $\beta_{430} = 5.8 \times 10^6$. Both values are low, however, as compared to the absorption coefficients of some of the commonly used redox-dyes ($\beta_{596,\ \text{thionine}} = 13.1 \times 10^7$, $\beta_{665,\ \text{methylene blue}} = 19.6 \times 10^7$ cm^2 × mole^{-1}).

Until now the procedure described here has been applied mainly to HRP. With the purpurogallin method "yields" of up to 150 % have occasionally been noticed in the initial steps of HRP isolation. The new method gives good reproducibility with the crude extracts from horse-radish roots when the amount of extract and reaction time were varied in the determinations. After the initial ammonium salt fractionations [11] agreement was also found between

activity and haemin content of the preparations. Unpasteurized milk, as well as a ten years old preparation of crystalline milk peroxidase gave no colour, whereas orange peel extract gave a strongly positive reaction.

SUMMARY

1. A procedure has been designed for the assay of horseradish peroxidase, in which mesidine (sym. trimethylaniline) is used as hydrogen donor.

2. The peroxidase concentration of an unknown solution is obtained directly in molarity.

3. No significant differences in activity, referred to haemin, have been found between three horse-radish peroxidase preparations, obtained by different methods in three laboratories.

4. Catalase, the cytochrome system, haemoglobin, and myoglobin are inactive as catalysts, if a weak, unspecific haemin effect is disregarded. Cupric and ferric ions have no influence. The blank value (peroxide + mesidine only) is zero.

5. The method employs a rather high peroxide concentration. Therefore it cannot be used as a general test for peroxidases in biological material.

6. The net reaction formula is given.

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Studies on the Turnover Rate of ATP during Oxidative Phosphorylation *

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Investigations by Sanadi and Littlefield [1] and by Kaufman *et al.*[2,3] have demonstrated that the transfer of energy connected with the oxidation of α-ketoglutarate to succinate takes place according to the following reaction sequence:

α-ketoglutarate + DPN + CoA → succinyl ~ CoA + $DPNH_2$ + CO_2 (1)
succinyl ~ CoA + ADP + orthophosphate ⇌ succinate + CoA + ATP (2)

α-ketoglutarate + DPN + ADP + orthophosphate → succinate + $DPNH_2$ + ATP + CO_2

This reaction sequence is one of the pathways by which isolated mitochondria form ATP during respiration.

Earlier it was believed that the incorporation of orthophosphate into ATP was intrinsicly linked with oxidative energy generation and thus, that a stoichiometric relation existed between the turnover rate of ATP and the rate of oxidation. The situation becomes different, however, when, as in the case of the above mechanism, the orthophosphate enters into a preformed energy-rich bond by way of a reversible reaction.

A constant rate of oxidation and phosphorylation (by the above reaction sequence) can evidently be maintained only when ATP is removed from the system by an irreversible reaction. Under these conditions a steady state prevails; the rates of reaction (1) and of the irreversible trapping of ATP are equal. In such a system a constant level of the intermediates involved in reaction (2) is established. None of these levels could be zero, for were that the case the reaction chain would be interrupted.

Thus, in a steady state, reaction (2) must proceed in both directions. Accordingly, the rate from left to right should exceed the net rate of oxidative phosphorylation. In other words, in a steady state system the incorporation of orthophosphate into ATP must take place at a greater rate than does the net phosphate uptake.

Experiments supporting this reasoning are presented below. Some data concerning the renewal of ADP during oxidative phosphorylation are also given.

* This work has been presented at the Meeting of Swedish Biochemists, Stockholm, June 5th, 1953.

MATERIALS

Mitochondria from rat liver and rabbit heart were prepared by centrifugal fractionation of homogenates made in 0.25 *M* sucrose [4] containing 0.01 *M* versene [5]. The pH of the medium was adjusted to 7.8. The mitochondria were washed once with this solution.

Yeast hexokinase was prepared according to the method of Berger *et al.*[6], except for the cytolysis in toluene which was performed as described by Bailey and Webb [7]. The preparation obtained at step 5 was employed. This preparation contains no trace of myokinase, ATPase and phosphofructokinase, and is practically free from pyrophosphatase. It contains hexose-6-phosphate isomerase.

The reagents used were commercial products. $H_3P^{32}O_4$ was furnished by AERE Harwell, England.

EXPERIMENTAL

The mitochondria were suspended in an isotonic buffer solution (pH 7.8) containing glycyl-glycine and KCl, and brought to a final volume of 30 ml, in the case of liver, and 10 ml in the case of heart. The solution also contained α-ketoglutarate or glutamate, orthophosphate, adenylic acid, glucose, hexokinase and Mg^{++} in amounts specified under the respective experiments.

Samples of 1.5—2.0 ml of this suspension were incubated at 30° C, either in Warburg vessels or in open tubes under aeration. For chromatographic analyses, samples of 0.1 ml were removed from the open tubes at suitable intervals and fixed with 0.1 ml 10 % trichloroacetic acid containing 40 m*M* ATP.

Analytical methods

Three kinds of determinations were performed throughout the experiments: 1) oxygen consumption, 2) concentration of orthophosphate and phosphorylated intermediates, 3) turnover rate of the individual phosphate groups of ATP.

For this purpose each sample was incubated in triplicate. The first sample was incubated in a Warburg vessel where oxygen uptake was measured in the usual way. The respiration for the period of thermo-equilibration (7 minutes) was calculated by extrapolation. The second and the third samples were incubated in open vessels. Radioactive orthophosphate was added to one of these at the start. In this sample the activity contained in each intermediate formed during the experiment is a direct measure of the amount of the respective phosphate compound. In the middle of the experiment, when steady state had been established in the system, a drop of carrier-free radioactive orthophosphate was added to the second vessel, in order to compute the turnover rates of the intermediates. That identical conditions exist in the triplicates could be confirmed by determining orthopshophate colorimetrically [8] in all three samples after a given time of incubation.

The radioactivity of orthophosphate and phosphorus derivatives formed during incubation was determined after chromatographic separation of these compounds. For this purpose the samples fixed in TCA—ATP were centrifuged and the clear solutions neutralized by adding 0.2 ml of a solution containing 0.6 *M* $NaHCO_3$, 0.1 *M* glucose and 0.015 *M* $MgCl_2$. Each sample was thereafter divided into two equal aliquots. About 10 microliters of hexokinase solution was added to one aliquot. After 15 minutes at room temperature, 0.05 ml *iso*butyric acid was added to both aliquots. From each aliquot about 5 microliters was spotted onto a sheet of filterpaper. Chromatographic separation was performed with *iso*butyric acid-water-ammonia (100 : 36 : 1.36 by volume) as the solvent [9]. Blue spots, originating from the ATP added to the fixing solution, could be detected on the dried chromatograms under UV illumination. The spots from the two aliquots appeared at different heighths on the strips. The spots from aliquots containing hexokinase appeared at a level corresponding to the R_F value of ADP, whereas those from the untreated aliquots appeared at the level of ATP. This confirmed the conversion of all ATP to ADP in the aliquots containing hexokinase.

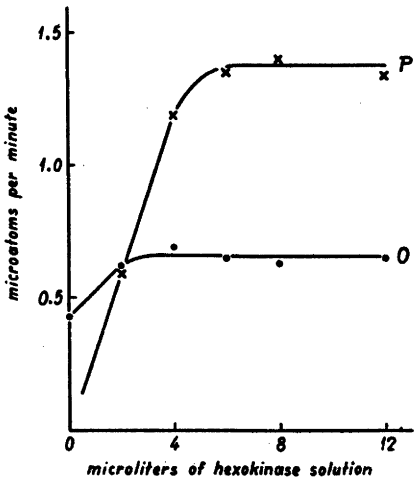

Fig. 1. Rate of oxidation and phosphorylation with varying amounts of hexokinase.

Determination of O_2 consumption: Each Warburg vessel contained: mitochondria (1/18 rat liver), glycyl-glycine (167 μmoles), KCl (130 μmoles), $MgCl_2$ (10 μmoles), K_2HPO_4 (91 μmoles), AMP (6.7 μmoles), potassium α-ketoglutarate (22.2 μmoles), glucose (185 μmoles) pH, 7.8. Hexokinase, a stock solution in 1 % glucose of about 25 ml, obtained as step 5 in a preparation starting from 11 kg yeast, added to each vessel in amounts as indicated in the figure. Final volume of each sample, 2.1 ml. Temp. 30° C. Gas phase: air.

Determination of rate of phosphorylation: Specimens of the same composition as above were incubated in open vessels from which samples were removed at different interval of time, for estimation of hexose-6-phosphate and phosphorylated intermediates.

Rates of oxidation and phosphorylation were determined by averaging the values obtained for the individual intervals from the above two series of incubation.

Also a third specimen for each hexokinase concentration was run, from which, by adding P^{32}-orthophosphate during incubation, the rate of turnover of the phosphorylated intermediates could be computed (see Fig. 2).

The strips corresponding to each aliquot were cut out and the radioactivity was measured along the entire length of the strip. This was accomplished by an automatic counter consisting of three units: a feeding device, an amplifier and a counting machine. The strips (fastened together in a long band) were wound up on the feeder-wheel and fed in half-centimeter portions per half-minute under a GM tube. The amplified impulses are registered by a decadic electron-tube system and recorded on the counting machine tape *.

Thus, for each chromatographic strip fed into the counter, a tape-record was obtained. From this record the successive peaks, corresponding to the different compounds separated by chromatography could be assessed. In this way, the activities of hexose phosphate, of orthophosphate plus ATP, and of ADP were determined. The activities of the individual labile phosphate groups of ATP were obtained from the increase in activity of hexose phosphate and ADP, respectively, in the aliquots treated with hexokinase. This method has already been described in detail [10].

* The automatic counting device was constructed and furnished by LKB-Produkter, Stockholm 12, Sweden.

Table 1. Concentration of phosphorus derivatives in three of the experiments included in Fig. 1.

	2 μl hexokinase			4 μl hexokinase			6 μl hexokinase		
Time, min.	Hexose-6-phosphate, μmoles/ml	ATP, μmoles/ml	ADP, μmoles/ml	Hexose-6-phosphate, μmoles/ml	ATP, μmoles/ml	ADP, μmoles/ml	Hexose-6-phosphate, μmoles/ml	ATP, μmoles/ml	ADP, μmoles/ml
5	1.24	1.47	0.92	1.85	1.03	1.00	1.96	0.75	0.98
10	2.32	2.57	0.38	3.66	1.63	0.92	4.49	1.04	0.98
15	3.73	2.94	0.12	5.75	2.27	0.60	7.20	1.29	1.00
20	4.98	2.93	0.13	8.22	2.56	0.35	10.16	1.44	0.93
25	6.10	2.68	0.11	10.83	2.64	0.24	12.81	1.55	0.86
30	7.40	2.95	0.13	12.79	2.80	0.17	15.40	1.66	0.77
35	8.61	2.90	0.11	15.26	2.64	0.18			
40	9.69	2.89	0.12	17.33	2.71	0.15			
45	10.71	2.84	0.11						
50	11.58	2.91	0.11						
Mean velocity of HMPformation μmoles/min	0.23			0.43			0.51		
Mean contration at steady state (N) μmoles/min		2.88	0.12		2.67	0.17		*	0.90

* The curve tends towards a level of about 1.70. In the calculation of the turnover rate for the interval 20—25 min (Fig. 2), the values between 1.44 and 1.55 were interpolated.

RESULTS

The results described here concern experiments on rat liver. Those performed on rabbit heart have given essentially the same results and will therefore only be tabulated in the next section.

When rat liver mitochondria are incubated in the presence of varying amounts of hexokinase, the rates of respiration and phosphorylation (both constant within a given experiment) vary in the way shown in Fig. 1. When no hexokinase is present all respiration is presumably dependent upon the hydrolytic breakdown of formed energy-rich phosphate, and this can be regarded as the limiting factor of oxidation. As hexokinase is introduced into the system, part of the ATP formed during respiration is converted into glucose phosphate with liberation of ADP. At hexokinase levels inducing a maximum rate of net phosphate uptake practically all the esterified phosphate is conserved as glucose phosphate, nothing being left over to be trapped by ATPase.

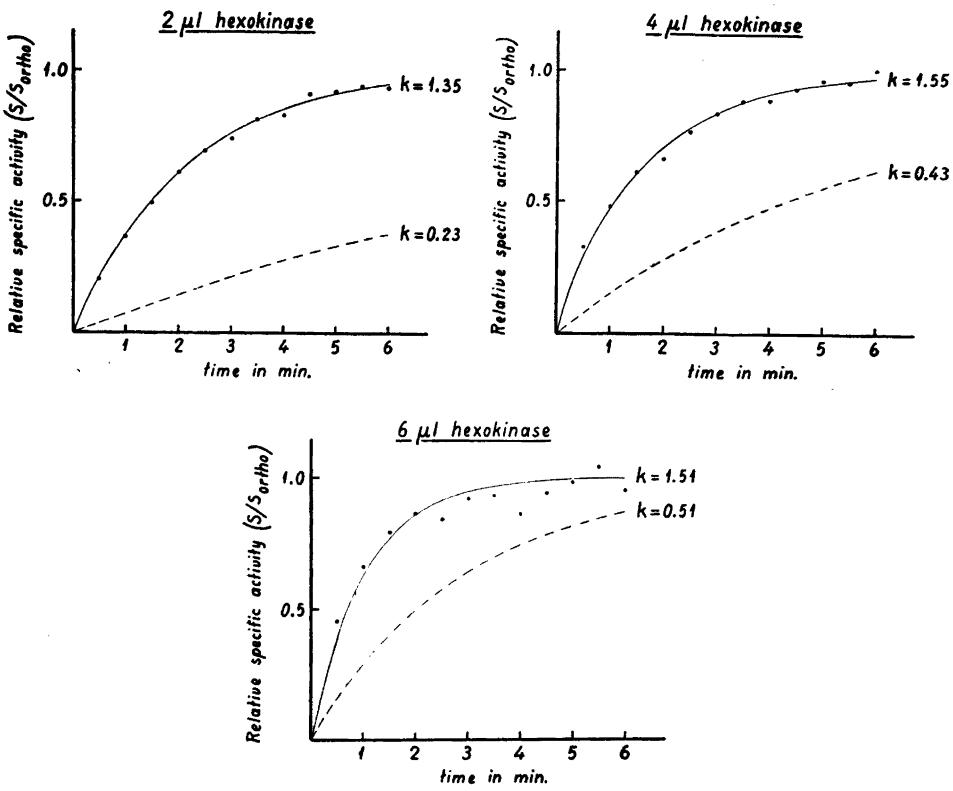

Fig. 2. Calculation of the turnover rate of the terminal phosphate group of ATP.

Points: relative spec. activity of terminal phosphate group of ATP, on the basis of values computed in Table 2.

Solid lines: theoretical curves covering the experimental points, assuming them to be fitted by equation (4) on p. 000. The values of k for these curves are the slopes of the lines obtained by plotting $-N\ln(1-S/S_{ortho})$ against t.

Dotted lines: theoretical curves corresponding to the above equation when putting k equal to the rate of hexose-6-phosphate formation in the resp. experiments (*cf.* Fig. 1).

Each point on the curve in Fig. 1 represents an individual steady-state experiment in which, as mentioned above, oxidation and phosphorylation proceed at a constant rate. In this steady state the phosphorylated intermediates, ADP and ATP, are maintained at constant concentrations, as shown in Table 1.

After these constant levels of intermediates had been attained in the three experiments shown in Table 1 (after 40, 30 and 20 minutes, respectively) radioactive orthophosphate was added to the incubation mixtures. Samples were now removed at half-minute intervals for a period of 6 minutes. In these samples the number of counts of the individual phosphate groups of ATP was determined (Table 2). The specific activity of orthophosphate during this

6-minutes period could be regarded as virtually constant. On this basis the relative specific activity of the terminal phosphate group of ATP was calculated and plotted *versus* time (Fig. 2). By taking orthophosphate as the immediate precursor of this phosphate group, the turnover rate expressed in micromoles / minute was computed from the equation:

$$N\frac{dS}{dt} = k\ (S_{ortho} - S) \tag{3}$$

which upon intergration over the period 0 to t gives,

$$S/S_{ortho} = 1 - e^{-\frac{k}{N}t} \tag{4}$$

where S designates the specific activity of the terminal phosphate group of ATP at a time t after the introduction of P^{32} into the incubation mixture (*cf.* Table 2); S_{ortho}, the specific activity of orthophosphate, taken as constant during the period concerned; N, the concentration of ATP in the steady-state system (*cf.* Table 1); and k, the turnover rate (μmoles. $ml^{-1} \cdot min^{-1}$) of the terminal phosphate group of ATP.

Table 2. Calculation of the turnover rate of the terminal phospha te group of ATP in the steady-state system.

The table refers to the same experiments as in Table 1. To each incubation, P^{32}-orthophosphate was added when steady state had well been established in the system. In the experiment with 2 μl hexokinase this was done after 40 min, with 4 μl hexokinase after 30 min, and with 6 μl after 20 min.

a. 2 μl hexokinase.

Time in seconds after addition of P^{32}	Activity (R) *Counts.* min^{-1} . 10^{-5}			Specific Activity ($S = R/N$) *Counts.* min^{-1} . $\mu M^{-1}.10^{-5}$ (N taken from Table 1)			Relative Specific Activity (S/S_{ortho}) ($S_{ortho} = 3.53$)		
	ATP terminal group	ATP middle group	ADP	ATP terminal group	ATP middle group	ADP	ATP terminal group	ATP middle group	ADP
33	2.05	0.04	0.00	0.71	0.01	...	0.20	...	...
64	3.73	0.12	0.03	1.30	0.04	...	0.37	0.01	...
94	5.00	0.16	0.03	1.74	0.06	...	0.49	0.02	...
126	6.18	0.32	0.03	2.15	0.11	...	0.61	0.03	...
154	6.99	0.40	0.05	2.43	0.14	...	0.69	0.04	...
185	7.50	0.49	0.05	2.61	0.17	...	0.74	0.05	...
215	8.20	0.61	0.07	2.86	0.21	...	0.81	0.06	...
245	8.41	0.75	0.07	2.94	0.26	...	0.83	0.07	...
280	9.19	0.85	0.08	3.20	0.30	...	0.90	0.09	...
306	9.29	0.93	0.08	3.23	0.32	...	0.91	0.09	...
334	9.51	1.06	0.08	3.31	0.37	...	0.94	0.10	...
365	9.57	1.17	0.09	3.30	0.40	...	0.93	0.11	...

b. 4 µl hexokinase.

Time in seconds after addition of P^{32}	Activity (R) *Counts. min^{-1} . 10^{-5}*			Specific Activity ($S = R/N$) *Counts. min^{-1} . µM.$^{-1}$10^{-5}* (N taken from Table 1)			Relative Specific Activity (S/S_{ortho}) ($S_{ortho} = 4.00$)		
	ATP terminal group	ATP middle group	ADP	ATP terminal group	ATP middle group	ADP	ATP terminal group	ATP middle group	ADP
36	3.46	0.23	0.06	1.30	0.09	...	0.33	0.02	...
67	5.10	0.31	0.04	1.91	0.12	...	0.48	0.03	...
97	6.47	0.44	0.05	2.43	0.17	...	0.61	0.04	...
126	7.04	0.56	0.04	2.65	0.21	...	0.66	0.05	...
156	8.13	0.85	0.10	3.05	0.32	...	0.76	0.08	...
186	8.89	1.23	0.08	3.33	0.43	...	0.83	0.11	...
216	9.38	1.26	0.11	3.51	0.48	...	0.88	0.12	...
246	9.43	1.43	0.11	3.54	0.54	...	0.88	0.14	...
277	9.88	1.72	0.08	3.70	0.65	...	0.93	0.16	...
306	10.24	1.56	0.11	3.85	0.59	...	0.96	0.15	...
336	10.19	2.27	0.14	3.82	0.86	...	0.96	0.22	...
369	10.68	2.43	0.11	4.01	0.92	...	1.00	0.23	...

c. 6 µl hexokinase.

Time in seconds after addition of P^{32}	Activity (R) *Counts. min^{-1} . 10^{-5}*			Specific Activity ($S = R/N$) *Counts. min^{-1} . µM.$^{-1}$10^{-5}* (N taken from Table 1) *			Relative Specific Activity (S/S_{ortho}) ($S_{ortho} = 3.22$)		
	ATP terminal group	ATP middle group	ADP	ATP terminal group	ATP middle group	ADP	ATP terminal group	ATP middle group	ADP
30	2.08	0.14	0.16	1.45	0.10	0.17	0.45	0.03	...
61	2.94	0.50	0.43	2.02	0.35	0.47	0.66	0.11	...
89	3.72	1.05	0.58	2.55	0.72	0.63	0.79	0.22	...
119	4.09	1.52	0.81	2.78	1.04	0.89	0.86	0.32	...
150	3.98	2.11	1.05	2.69	1.43	1.17	0.84	0.45	...
181	4.43	2.42	1.28	2.97	1.63	1.43	0.92	0.51	...
210	4.47	2.61	1.43	2.98	1.74	1.61	0.93	0.54	...
239	4.19	2.98	1.54	2.76	1.98	1.74	0.86	0.62	...
270	4.59	3.43	1.63	3.01	2.26	1.86	0.94	0.70	...
300	4.80	3.93	1.65	3.15	2.57	1.89	0.98	0.80	...
330	5.13	3.98	1.80	3.35	2.60	2.08	1.04	0.81	...
360	4.90	3.94	1.94	3.06	2.54	2.25	0.95	0.79	...

* Values of N intrapolated between 1.44 and 1.55.

The experimental points fit fairly well for $k = 1.35$, 1.55 and 1.51 μmoles $ml^{-1} \cdot min^{-1}$, respectively, in the three experiments. On the other hand, these values of k greatly exceed the actual rate at which glucose phosphate was formed in the three respective experiments. This becomes clearly evident when the experimental points are compared with the theoretical lines drawn on the basis of k-values equated to the rate of glucose phosphate formation. Thus, the incorporation of orthophosphate into the terminal group of ATP takes place at a rate considerably higher than that of the oxidative phosphate uptake. Moreover, the former rate seems to be rather independent of the latter.

Equation (3) is valid only under the condition that there is no flow of unlabeled phosphate from ADP or from the middle group of ATP to the terminal group. To take the extreme case, if there were an instantaneous equilibrium between these three phosphate groups (*i. e.*, an infinitely high myokinase activity), the value of N would be much greater than when only the terminal group is involved. The maximum error is, thus, $(N_{terminal} + N_{middle} + N_{ADP}) / N_{terminal}$, *i. e.* about 2 to 2.5 times the value obtained in the present experiments. The relative specific activities of the middle group of ATP and the labile group of ADP are, however, relatively low (Table 2). Thus, the error in the calculation of k (caused by the myokinase reaction) is insignificant.

DISCUSSION

These experimental data are in accord with the principles we have advanced in the introduction. According to these, the reversible transfer-reactions leading from the primary energy-carrier to the formation of ATP, in a respiring mitochondrial system in a steady state, proceed at a greater rate than does the oxidative formation of the primary high-energy bonds. The disparity between the velocities of the two reactions is indeed significant, as summarized in Table 3. In these systems the rate of incorporation of orthophosphate into the terminal group of ATP seems to be rather independent of the rate of oxidative phosphorylation.

Table 3. Rate of net phosphate uptake and turnover rate of the terminal group of ATP in respiring mitochondria supplemented with yeast hexokinase.

A relative amount of hexokinase = 0.5 designates the amount necessary to give half maximal net phosphate uptake.

Experimental conditions: *rat liver.* see Fig. 1; *rabbit heart,* mitochondria from one heart suspended to a final volume of 10 ml in a solution of the same composition as in the case of rat liver. As a substrate glutamate, rather than α-ketoglutarate, was used in these experiments.

RAT LIVER			RABBIT HEART		
Relative amount of hexokinase	Rate of phosphate uptake μmoles / min. per ml suspension	Rate of turnover of the terminal group of ATP μmoles/min. per ml suspension	Relative amount of hexokinase	Rate of phosphate uptake μmoles/min. per ml suspension	Rate of turnover of the terminal group of ATP μmoles/min per ml suspension
0.44	0.23	1.35	0.33	1.30	5.6
0.87	0.43	1.55	0.66	2.25	7.0
1.31	0.51	1.51			

A reasonable explanation for the fact that the turnover rate of the terminal group of ATP is higher than the rate of the oxidative phosphorylation might be that only part of the ATP is trapped by hexokinase while another part is hydrolyzed by ATPase to orthophosphate and ADP. This explanation may certainly be valid for the case when addition of more hexokinase can further increase the rate of net phosphate uptake. This situation prevailed in the first two cases recorded in Table 2. In the third case, however, hexokinase was present in an amount such that further addition of it could no longer increase the rate of net phosphate uptake. In such a system it is hardly probable that any significant leakage of ATP effected by ATPase can occur. Furthermore, in the first two cases the discrepancy between rate of phosphorylation and of ATP turnover is too great to be explained by this mechanism.

From Fig. 1 it can be seen that the maximum number of phosphate molecules esterified per atom of oxygen consumed (P/O ratio) was about 2.1 for those cases where hexokinase was present in excess. The rate of turnover of the terminal group of ATP exceeded the rate of phosphate uptake in the presence of an excess of hexokinase by a factor of about 2.5. Thus, a P/O ratio of about five would have been obtained if the ATP turnover rate had been taken as a basis for the P/O calculation. Recently Krebs *et al.*[11] used this method to determine the P/O ratio in a similar system. However, direct comparision of the present experiments with those by Krebs *et al.* is difficult, for the experimental conditions differ. The experiments by Krebs *et al.* were performed on homogenates rather than mitochondria, without the addition of hexokinase, and in the presence of about equivalent amounts of orthophosphate and adenine nucleotides. Nevertheless, these differences from the conditions of our experiments do not appear to be such as to reduce the risk of error involved in calculating the P/O ratio on the basis of ATP-turnover.

In the experiments by Krebs *et al.* no incorporation of P^{32}-orthophosphate into ATP occurred under anaerobic conditions. This circumstance is regarded by the authors as a proof that a stoichiometric relation exists between oxygen consumption and renewal of the terminal phosphate group of ATP. It is, however, questionable whether a true steady state with respect to the intermediates occurring along with the reversible energy-transferring reactions prevails in an anaerobic system. Moreover, the disparity between the rate of oxidative phosphorylation and the rate of renewal of the terminal phosphate group of ATP (as found in the present experiments) need not be restricted to the step, α-ketoglutarate → succinate; a similar disparity might also occur in phosphorylating reactions connected with the respiratory chain.

Recently it has been frequently debated whether the P/O ratio obtained for the step α-ketoglutarate → succinate is 3 or 4. Slater and Holton [12] obtained in agreement with earlier data [13–17] P/O values below or equal to 3 while Judah [18] and recently Copenhaver and Lardy [19] using the same system but apparently at a greater efficiency, obtained values indicating a P/O ratio of 4. The values calculated by Krebs *et al.*[11] would support Copenhaver and Lardy's [19] value if the turnover rate of ATP could really be regarded as an index of phosphorylation rate. Our own values might seem to support the low ratios obtained by Slater and Holton [12]. There is, however, an important aspect to be discussed in this connection.

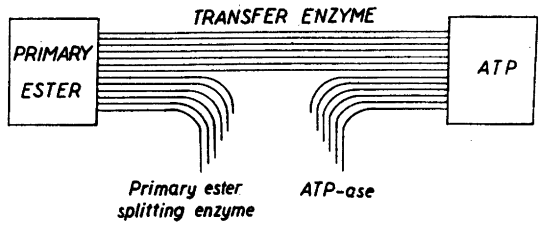

Fig. 3. Schematic illustration to a hypothesis, discussed in the text, about the way in which mitochondrial ATPase may influence P/O ratio.

In the experiment described here (see Fig. 1) we obtained a maximal P/O ratio of 2.1. This value is rather low, even when compared with those obtained by Slater and Holton [12]. In an attempt to explain this discrepancy we have carried out some preliminary experiments which we will not describe in detail here. These experiments show that the maximum P/O ratio obtainable with a sufficient excess of hexokinase is higher, the lower the capacity of the system to oxidize substrate without the addition of hexokinase. If we assume that this capacity is a direct function of the ATPase activity of the mitochondrial preparation, we can conclude that the presence of ATPase activity somehow implies a decrease of net phosphate uptake, even when a large excess of hexokinase is introduced.

It is a known fact that mitochondrial ATPase may show properties of a latent enzyme [15]. In view of this we have suggested [20] that the appearance of ATPase activity in a mitochondrial preparation during ageing or as a result of hypotonic treatment or addition of dinitrophenol, might be due to a splitting of transphosphorylation enzymes which mediate the transfer of phosphate between primary esters and ATP. It was proposed that such a splitting might result in the formation of a pair of hydrolytic enzymes, one splitting ATP and the other dephosphorylating the primary ester. If this were true, the presence of ATPase activity in a native mitochondrial preparation would imply the presence of an "equivalent" amount of primary ester-splitting enzyme. This enzyme would cause a loss of esterified phosphate, which could never be prevented by addition of hexokinase. This situation is visualized schematically in Fig. 3.

It can be readily deduced from the scheme, that in a system where the ATPase activity of the mitochondrial preparation is high, no considerable increase in the oxidation rate can be obtained by the addition of hexokinase, and the maximal P/O ratio attainable in this way is relatively low. This seems to be the case in the present experiments and perhaps also in those by Slater and Holton [12]. When the ATPase activity is low in the native preparation, a high stimulation of the oxidative rate and a high P/O ratio will be obtained by the addition of an excess of hexokinase. This situation apparently prevailed in the experiments of Copenhaver and Lardy [19]. According to this reasoning, a true value of the P/O ratio, *i. e.*, one with which leakage of phosphate does not interfere, can only be obtained in an ideal mitochondrial preparation in which ATPase activity is entirely absent.

Ever since isotopes became available for the study of metabolic processes, a great deal of work has been devoted to the estimation of the metabolic activity of different tissues by injecting P^{32}-orthophosphate into an animal and determining the renewal rate of the ATP phosphorus. This rate has been regarded as a measure of metabolic activity, *i. e.*, of energy generation in the tissue in question. From the present work it is clear that the rate of renewal of ATP in a steady-state system is largely independent of the rate of oxidation. Thus, the conclusions regarding metabolic activity which have been drawn from isotopic *in vivo* experiments of ATP labeling have to be revised. A high turnover rate of the ATP phosphorus need not indicate a corresponding high rate of oxidative energy generation in a given tissue; it merely means that the reversible reaction catalyzing the introduction of phosphate into a primary energy carrier, and its transfer to ATP is fast.

In some instances [21–24] it has been found that the renewal rate of ATP *in vivo* could be altered by the introduction of certain hormones. In connection with these experiments the question has often been raised: is it the rate of oxidation which is influenced by hormones, or is it the permeability of the cell membranes? In view of the above discussion we incline to favour the second alternative. It must be kept in mind, however, that an alteration of active permeability mechanisms implies, at least secondarily, an altered metabolic activity.

SUMMARY

Isolated mitochondria from rat liver were incubated to oxidize α-ketoglutarate in the presence of glucose and varying amounts of yeast hexokinase. In the steady state system, oxidation and phosphorylation proceed at constant rates, and constant levels of ATP and ADP become established. P^{32}-orthophosphate was added to the steady state system and the turnover rate of the terminal phosphate group of ATP determined. This was found to exceed, by a factor of about 2.5, the rate of net phosphate uptake, even when the latter was not limited by hexokinase. The middle phosphate group of ATP had a specific activity considerably lower than the terminal one throughout the experiments. Similar results have been obtained with rabbit heart mitochondria.

The disparity between the rate of net phosphate uptake and the turnover rate of the terminal ATP group is interpreted as resulting from a reversible phosphoroclastic cleavage of a primary energy carrier.

It is concluded that, in the system studied, no stoichiometric relation prevails between turnover of ATP and oxidative generation of energy. The implications of this finding as to the possibilities of estimating energy yield by determination of ATP turnover, *in vivo* or *in vitro*, are discussed.

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The Isolation of Nebularine and the Determination of its Structure

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Nebularine, an antibiotic active against *Mycobacteria* and present in the mushroom *Agaricus (Clitocybe) nebularis* Batsch. has been studied since 1945 [1–4]. In 1952 we isolated nebularine and in a preliminary report [4] we were able to give information on its composition, the structure being formulated as 9-(D-ribosyl)purine. Since then we have demonstrated the compound to be a furanoside. The configuration of the glycosidic centre has not been investigated by us but was demonstrated through the synthesis of nebularine by G. Brown and Weliky [5] at the Sloan-Kettering Institute for Cancer Research in New York. This synthesis shows definitely the correctness of our previous conclusions and in addition that the glycosidic centre has the β-configuration. — We will now describe in detail the isolation of nebularine and the determination of its structure.

The isolation of nebularine from the mushroom may be considered as difficult. The content of nebularine in the fresh mushroom is very low, *i. e.* about 0.002 % *. As we had no chemical test for the identification of the compound, we had to check every fraction against *tubercle bacilli* **. Other difficulties were the high water solubility of nebularine as compared with its low solubility in lipoid solvents, and the impossibility of finding a selective precipitation reaction. — The first steps in the isolation procedure have been described previously [3]. These steps include an aqueous extraction of the mushroom, precipitation of the proteins with acetone, the removal of lipoid matter by extraction with chloroform and with ether, adsorption of nebularine on active carbon followed by elution, and chromatography on aluminum oxide. The fraction thus obtained showed a high biological activity but nevertheless we had the problem of separating nebularine from at least ten amino acids, eight "nucleo" derivatives (purines, pyrimidines, and nucleosides), and a number of other compounds, highly soluble in water. We tried to separate nebularine

* Calcd. not only from the yield but also from the bacteriological activity.

** For the current experiments we used *Mycobacterium avium*, strain Bang, in Dorset medium.

from the amino acids by a simple extraction procedure of the aqueous solution with a suitable organic solvent. But it appeared *a priori* impossible according to the fact that nebularine was extracted together with the amino acids. Finally, however, the problem was solved by shaking out the water solution fifteen times with a solvent mixture chloroform-ethanol 2 : 1 (v/v) *. All the amino acids remained in the water phase whereas all the nebularine had been transferred to the chloroform-ethanol mixture. By using the paper chromatography technique developed by Löfgren [7] for the analysis of purines, pyrimidines, and nucleosides we could show that the chloroform-ethanol extract contained nine nucleo derivatives, *viz.* adenine, adenosine, guanosine, hypoxanthine, cytosine, cytidine, uracil, uridine, and one unknown. In order to find a R_F value of nebularine we ran a one-dimensional paper chromatogram of a special type, the starting line being totally covered with spots. The paper strip was then cut into many parts, each of which was eluted with a 0.2 % solution of 2-octanol in 30 % ethanol. Each fraction was then tested for tuberculostatic activity. Only the region corresponding to the unknown compound gave an active extract. Therefore we were able to conclude with a high degree of probability that our nebularine belonged to the purine or the pyrimidine group.

Thus it seemed possible for us to divide the nucleo derivatives into fractions by means of chromatography on a suitable ion exchanger. The use of Dowex 50 in the hydrogen form seemed to give a good result. The active principle appeared together with adenosine and cytosine on elution with dilute ammonia. On the paper chromatogram, however, the fraction showed a much higher spot intensity than was to be anticipated from the measured tuberculostatic activity. Because of this somewhat confusing result we avoided the ion exchange technique. Later, when we had isolated nebularine and had found purine to be one of its components we could explain the phenomenon. We were then able to demonstrate that nebularine was partly hydrolyzed in contact with the ion exchanger. In the effluent from the column, purine appeared together with nebularine, both of which had the same R_F value in the resolving liquid used.

From the chloroform-ethanol solution the solvents were removed and the residue was distributed between *n*-butanol and water. After separation of the two layers the butanolic solution was extracted three times with water. From the combined four aqueous solutions the water was evaporated and replaced by dry *n*-propanol. Addition of much ether resulted in a solid precipitate which consisted mainly of nucleo derivatives including a relatively small part of the nebularine. The filtrate was found to contain adenine, adenosine, uracil, uridine, most of the nebularine, and some other compounds not belonging to the purine or the pyrimidine group. Redissolving and reprecipitating of the solid fraction gave another filtrate containing some more nebularine. By evaporation of the combined filtrates, a solid crystalline mass was obtained. — In order to get further separation we tried a number of procedures. However, as the usual routine methods failed we applied the countercurrent distribution technique and found it to be successful. The distribution was carried out between *n*-butanol and water. At first we determined the partition coefficient

* Stoll, Renz, and Kreis [6] have used this mixture succesfully in shaking out highly water-soluble glycosides.



Fig. 1. Photomicrograph of crystalline nebularine (8 ×).

of nebularine. For this purpose a distribution was made in a Craig apparatus, the number of transfers being 49. The relative concentrations of nebularine in the tubes were determined by bacteriological experiments and also by estimating spot intensities on paper chromatograms. Both methods gave the same value of the partition coefficient. From the same run, by analysing spot intensities, we also obtained the partition coefficients of adenosine, uracil, and uridine. (As mentioned above, these compounds, and also adenine, were present together with nebularine in the propanol-ether soluble product.) We found the same values as recorded previously by Tinker and Brown [8] who determined the partition coefficients for a number of nucleo derivatives in distributing the compounds between *n*-butanol and a phosphate buffer of pH 6.5. Because of this agreement between their experiments and ours, we took their value for adenine (*cf.* above) without any further experimental checking. With the aid of these values of the partition coefficients we could show by calculation that a distribution carried to 120 transfers should give a fraction containing all the nebularine and uracil (both have the same partition coefficient) and a relatively small amount of the adenosine (for details, see the experimental part). After this distribution had been carried out, the desired fraction was found within the anticipated tube interval. By washing the dry product with ice-cold absolute ethanol most of the impurities could be removed without considerable loss of nebularine. The product was then crystallized from absolute ethanol. The crystals, as shown by paper chromatography, contained no adenosine but a small amount of uracil. A second recrystallization from the solvent mixture ethyl methyl ketone-methanol 11 : 3 (v/v) increased the melting point by 1—2 degrees. Only traces of uracil could be found. Another recrystallization from the same solvent mixture resulted in a product with a sharp melting point and free from uracil. Further recrystallizations did not alter the product as to its melting point and elementary composition.

From the solvent mixture ethyl methyl ketone-methanol (*cf.* above), nebularine is obtained as small rhombohedral crystals (see Fig. 1). The

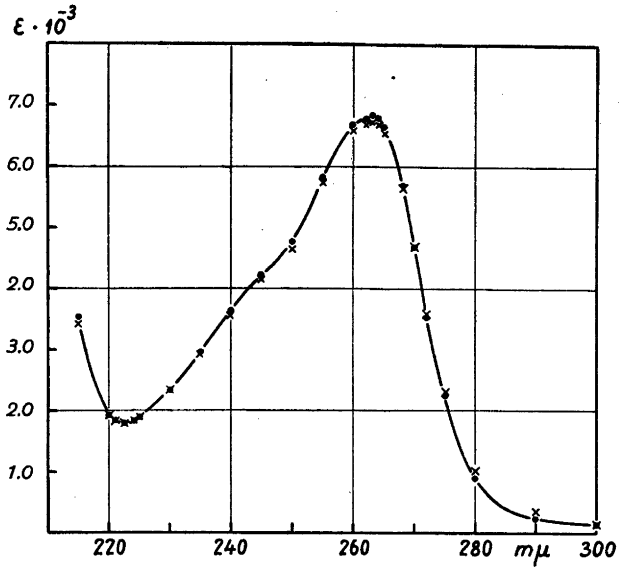


Fig. 2. Ultraviolet absorption of water-solutions of:
—●—●—● *Nebularine from Agaricus (Clitocybe) nebularis Batsch.*
× × × *Synthetic 9-(β-D-ribofuranosyl)purine.*

melting point of the pure nebularine is 181—182° (corr.). Nebularine is considerably soluble in cold water (about 10 %), slightly soluble in cold ethanol, very slightly soluble in acetone and ether, and almost insoluble in chloroform. The specific rotation, $[\alpha]_D^{20}$, in aqueous solution is $-47.5°$ at $c = 2$, and $-47.3°$ at $c = 1$; the difference between the two values might possibly fall within the limits of experimental error. In the ultraviolet, nebularine has a maximum at 263 mμ and a minimum at 222.5 mμ; see Fig. 2, where the absorption curve of the natural product is compared with that of the synthetic compound *. The partition coefficient $C_{n\text{-butanol}} / C_{\text{water}}$ is 0.42. In the solvent mixtures given by Löfgren[7], nebularine has the following R_F values on Whatman No. 4 filter paper at 22° ± 0.5° : 0.73 ("*iso*butyric acid liquid"), 0.52 ("butanol-*iso*butyric acid liquid"), 0.82 ("morpholine liquid"), and 0.66 ("piperidine liquid"). In water-solution, nebularine is markedly thermostable. Thus it endures three hours' heating in aqueous solution at 100° without decomposition (a longer heating time has not been tried). In dilute ammonia solutions the stability may be considered as fairly high (no decomposition in 0.1 *N* ammonia at room temperature after 8 days), but it is less pronounced in aqueous solutions of diethylamine, piperidine, and mineral acids. Nebularine gives a positive Molisch reaction.

The tuberculostatic activity ** on *Mycobacterium avium*, strain Bang, in Dorset medium is 1 : 500 000 but pronounced activity has been observed down

* A sample of the synthetic product was kindly sent to us by Dr. G. Brown.
** The minimal concn. at which growth still can be traced.

to a concentration of 1 : 3 000 000. This effect is noticed during at least 30 days. The effect is about the same for *Mycobacteria* of human and bovine types and for *Mycobacterium phlei*. The compound has no antibiotic effect on the following bacteria: *Sarcina lutea*, *Staphylococcus aureus*, *Escherichia coli*, *Aerobacter aerogenes*, *Proteus vulgaris*, and *Bacillus subtilis*. Its bacterial spectrum may therefore be stated as very selective. When tested in tissue culture, nebularine strongly retards the cell growth of both normal mouse embryo skin and sarcoma 180[5]. In considerable dilutions there is some preferential activity against the cancer cells. In experiments with barley seedlings we found nebularine to inhibit the growth almost completely at a dilution of 1 : 3 000. On root tips of *Allium cepa*, nebularine shows inhibition of the cell division, disturbances of the spindle function, and a remarkable chromosome contraction. These effects were demonstrated in the concentration range 0.01—0.001 % *. When tested subcutaneously in white mice, nebularine shows a surprisingly high toxicity, the LD_{50} value being approximately 100 mg / kg. Nebularine has haemolytic activity only at a relatively high concentration (0.5 %).

Elementary analysis of nebularine gave the formula $C_{10}H_{12}N_4O_4$. On hydrolysis two cleavage products were found.

One of them was shown to be identical with purine, the actual compound and synthetic purine having the same melting points, R_F values, and spectra in the ultraviolet. Furthermore the hydrolysis product gave a picrate of the same composition and melting point as purine picrate. The mixed melting point of the two picrates showed no depression. — This is the first time purine has been demonstrated to be a component of a natural product.

The other cleavage product was found to be identical with D(−)ribose. The identity of the two sugars was established by means of paper chromatography, from measurements of optical rotation, and further by comparing the two α,α-benzylphenylhydrazones as to their compositions and melting points, and by determining the mixed melting point.

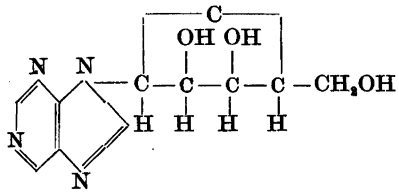
The uptake of one mole of periodate without production of formic acid showed that nebularine has the furanoside structure.

A study of ultraviolet spectra indicated that the ribose is attached at position 9 of the purine nucleus. We planned to make further investigations on this point by comparing the ultraviolet spectra of nebularine, 7-methylpurine and 9-methylpurine, and eventually to synthesize 9-(D-ribofuranosyl)purine, but in the meantime we got the information from Dr. G. Brown at the Sloan-Kettering Institute in New York that he was engaged in synthesizing this nucleoside. We therefore awaited the results from the Sloan-Kettering Institute. Recently Brown and Weliky [5] succeeded with the synthesis of the nucleoside which was shown to have the β-configuration. We sent a sample of nebularine to this institute and by comparing the crystalline forms, melting points, spectra, and biological activity, and by examining the mixed melting point, Brown and Weliky could demonstrate our nebularine to be identical with their synthetic product [5]. The $[\alpha]_D$ value of the synthetic product at $t = 25°$ and at $c = 1$ in aqueous solution was found by Brown and Weliky to be $-48.6°$.

* Experiments performed by B. Kihlman.

Under the same conditions but at $t = 20°$, our measurements on the natural product gave a specific rotation $= -47.3°$.

Thus, it is made clear that nebularine is 9-(β-D-ribofuranosyl)purine:



Nebularine = 9-(β-D-ribofuranosyl)purine

EXPERIMENTAL

*The isolation of nebularine from Agaricus (Clitocybe) nebularis Batsch.**. By means of a mincing machine, 1 130 kg of fresh mushroom were ground and from the slurry thus obtained, the juice was pressed off hydraulically at 150 kg/sq.cm. The resulting firm mass was then suspended in its own weight of water. After some time the liquid was separated as before by expressing. The two solutions were combined and mixed with 2 920 kg of acetone. A protein precipitate formed and the mixture was kept at 0° over-night. Most of the supernatant solution was siphoned off and the remaining mixture filtered with suction. The precipitate was suspended in 75 % acetone and after some time the mixture was filtered at the pump. The filtrate was combined with the former solutions. The acetone and part of the water were distilled off under reduced pressure and at a liquid temperature of 25° (thermometer immersed in the liquid). The distillation was stopped when 778 l of solution remained. This solution which had a dry content of 2.25 % was now extracted five times with 260-l portions of chloroform and three times with 130-l portions of ether. From the aqueous solution the dissolved ether was removed by distillation under reduced pressure. The dry content was adjusted to 2 %. — Under vigorous stirring, this solution was treated with 3.05 kg of active carbon (Merck's "Carbo Activatus" — washed several times with hot water). Stirring was continued for half an hour and the mixture was then kept at 0° over-night. The carbon was sucked off and eluted with 3 × 763 kg of a 0.2 % solution of 2-octanol in 30 % ethanol. During each elution the mixture was stirred mechanically for five hours and then allowed to stand over-night at 0°. The supernatant solution was every time siphoned off from the carbon sediment and then, without further separation, the next portion of eluant was added. After the third and last elution, the separation was completed by centrifugation. The combined eluates were concentrated to a volume of 120 l by distillation under reduced pressure and at a maximum liquid temperature of 30°. — To this solution, 20 kg of aluminium oxide ("Brockmann") ** were added under vigorous stirring which was continued for one hour. The aluminium oxide was allowed to settle for some time and most of the supernatant solution was siphoned off. The remaining mixture was filtered at the pump and the aluminium oxide was washed with 40 l of water. The combined solutions were sucked through a filter covered with pure wet aluminium oxide, the dry weight of which was 3 kg. The oxide layer was then washed with 5 l of water and the filtrates were combined. All these filtrations through aluminium oxide were made in a refrigeration room of approximately 0°. The main part of the water was now distilled off under diminished pressure and at a maximum liquid temperature of 20°. The distillation was stopped when a volume of 40 l remained. — This solution was extracted 15 times with 40-l portions of a solvent mixture chloroform-ethanol 2 : 1 (v/v).

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* Each step in the isolation procedure means an increase in the tuberculostatic activity.

** Previously [3] the purifying step with Al_2O_3 was carried out as a true chromatography. Here the procedure has been simplified.

From a smaller portion of the combined extracts an aqueous solution was now prepared. In analysing this solution for nucleo derivatives by means of paper chromatography and using Löfgren's technique [7], the presence of adenine, adenosine, guanosine, hypoxanthine, cytosine, cytidine, uracil, uridine, and one unknown compound could be demonstrated. Much attention was paid to the latter compound. In the "piperidine liquid" (*cf.* Löfgren [7]) on Whatman No. 4 filter paper at 22°, the unknown compound gave a spot with the R_F value 0.66. Experiments were now made in order to find the R_F value of nebularine. For this purpose sections from paper chromatograms were eluted and the resulting extracts tested for tuberculostatic activity. To each of two filter-paper strips six spots were applied, each containing a relatively high content of substance. The chromatograms were developed by means of the "piperidine liquid". After drying, each of the two papers was cut into six sections, corresponding to the following R_F intervals: 1) 0.00—0.30 2) 0.30—0.57 3) 0.57—0.63 4) 0.63—0.69 5) 0.69—0.75 6) 0.75—1.00. The cuttings with the same numbers were matched and each pair was eluted with a 0.2 % solution of 2-octanol in 30 % ethanol. Only section No. 4 corresponding to the R_F value of the unknown compound gave an active extract when tested for tuberculostatic activity. It was therefore concluded, with a high degree of probability, that the spot with the R_F value 0.66 belonged to nebularine. (The subsequent experiments gave full evidence for this assumption.)

The combined chloroform-ethanol extracts were concentrated by distillation under reduced pressure and at a bath temperature of 40—45° to a volume of 3.6 l. — The residue which consisted of an aqueous solution containing some solid matter was shaken with 3.6 l of *n*-butanol. Most of the solids dissolved in the butanolic phase. The whole mixture was filtered with suction. The butanolic layer, separated from the aqueous solution, was then extracted with its own volume of water and this procedure repeated twice. The combined *four* aqueous solutions were evaporated to dryness under reduced pressure and at a maximum *liquid* temperature of 25°. At the end of the distillation the *bath* temperature was kept at 40°. The residue, finally dried over silica gel in a vacuum desiccator, consisted of 86 g of a light brown mass. — The finely powdered and carefully dried product was divided into four parts. Each portion, weighing 21.5 g, was mixed with 700 ml of dry *n*-propanol in a round-bottomed flask equipped with a condenser and a separatory funnel, both fitted with a calcium chloride tube, and a mercury-sealed Hershberg stirrer. The powder was dissolved by heating the flask in a paraffin oil bath which was maintained for some time at 70°. When the material had gone into solution, heating was discontinued without removing the oil bath. As soon as the temperature of the oil bath had fallen to 35—40°, 3.5 l of absolute ether were introduced under vigorous stirring and during a period of four hours. A yellow precipitate formed. The flask was carefully closed and kept at 0° over-night. The precipitate was sucked off and the filtrate evaporated to dryness under reduced pressure and at a maximum bath temperature of 40°. The residue consisted of a yellowish syrup which after further drying over silica gel in a vacuum desiccator soon solidified to a crystalline mass. The amount deposited from the four runs was 57.2 g. From the combined four precipitates an additional 7.8 g was obtained by redissolving in propanol and reprecipitating with ether — the yield from the whole procedure thus being 65 g. By analysis of this highly active product for nucleo derivatives it was found to contain nebularine contaminated with adenine, adenosine, uracil, and uridine.

The propanol-ether soluble product was then purified by means of countercurrent distribution between *n*-butanol and water. At first the partition coefficient was determined by distributing a small amount of the product in a Craig apparatus, made up of small compartments. The number of transfers was 49. When the distribution was completed all fifty water-phases were tested for tuberculostatic activity. The maximum activity was found in the tubes 13—16. The position of maximum concentration was also investigated by analysis of spot intensities on paper chromatograms (visual estimation of fluorescence intensity). The same result was obtained. The tube number of maximum concentration was therefore set to 14.5 corresponding to a partition coefficient $C_{\text{butanol}}/C_{\text{water}} = 0.42$. Estimation of spot intensities also gave the partition coefficients of adenosine, uracil, and uridine. The corresponding value for adenine was taken from Tinker and Brown [8] who distributed a number of nucleo derivatives between *n*-butanol and a phosphate buffer of pH 6.5 (*cf.* above). Thus all necessary figures were ascertained in order to calculate the possibility of separating nebularine from the other nucleo derivat-

ives having the following partition coefficients: adenine 2.77, adenosine 0.76, uracil 0.40, uridine 0.12. Calculations on a countercurrent distribution * carried to 120 transfers showed that nebularine should occur in the tubes 22—48. In this calculation the loss in yield was set to 1 %. Furthermore it was calculated that in this interval no adenine would interfere, whereas a little of the uridine, some of the adenosine and all the uracil would contaminate nebularine. (Nebularine and uracil have approximately the same partition coefficients; *cf.* above.) However, it could also be anticipated that a purer product might be obtained from the tubes 27—44, since in this case all uridine and more of the adenosine should be avoided. In other words: in carrying out 120 transfers, the selection of tubes 27—44 might result in a fraction containing almost all of the nebularine and uracil, little of the adenosine (about 8 % of its starting amount), and none of the other compounds. This scheme was followed in the procedure. A big Craig apparatus ** in which each compartment had a capacity of 2 × 250 ml was used. Of the available starting material (65 g) one third was used in each of three distribution procedures. In agreement with the calculations the maximum concentration of nebularine occurred in the compartments 35—36. The contents of compartments 27—44 were mixed together and evaporated to dryness under reduced pressure and at a maximum *liquid* temperature of 25°. At the end of the distillation the *bath* temperature was kept at 40°. The residue consisted of a pale yellow syrup which after further drying over sulphuric acid in a vacuum desiccator soon solidified to a crystalline mass. From the 65 g of starting material, 29.9 g were obtained. This product was analyzed for nucleo derivatives by means of paper chromatography and proved to contain the expected compounds, *i. e.* nebularine, uracil, and adenosine. As to the proportions, nebularine predominated over the two others — the content of adenosine being small.

To the finely divided and carefully dried product (29.9 g), 120 ml of ice-cold absolute ethanol were added and the mixture was kept at 0° for 18 hours. The powder was sucked off and once more treated with ethanol in the same way. The remaining, almost white crystalline powder weighed 23.4 g. An analysis of this product as well as of the combined light yellow alcoholic solutions showed that almost all of the nebularine had been retained in the powder, whereas the main part of the uracil together with some of the adenosine had gone into solution. The powder thus contained nebularine contaminated by small amounts of uracil and adenosine. The product was now recrystallized from absolute ethanol. From this operation 17.6 g of small prisms melting at 178—180° (corr.) were obtained. In this product no adenosine could be detected but a small amount of uracil. For further purification, 3.00 g were recrystallized from a solvent mixture ethyl methyl ketone-methanol 11 : 3 (v/v). The yield was 2.13 g of a product melting 1—2 degrees higher. Only traces of uracil could be found. Another recrystallization from the same solvent mixture resulted in a product melting at 181—182° (corr.) and free from uracil. Further recrystallizations did not alter the product as to its melting point and elementary composition. From the solvent mixture ethyl methyl ketone-methanol the pure nebularine appeared in the form of small rhombohedral crystals (Fig. 1). (Found: C 47.7, H 4.83, N 22.0, O 25.4 ***.) Calc. for $C_{10}H_{12}N_4O_4$ (252.2): C 47.6, H 4.80, N 22.2, O 25.4.)

Optical rotation of nebularine. A solution of $c = 2$ was prepared by dissolving 400.0 mg of nebularine in water to a total volume of 20.00 ml. The rotation for the D line at $l = 2.00$ dm and $t = 20.0°$ was found to be $-1.90°$. After dilution of this solution to $c = 1$ the rotation was again determined and found to be $-0.945°$. Thus for $c = 2$, $[\alpha]_D^{20} = -47.5°$ and for $c = 1$, $[\alpha]_D^{20} = -47.3°$.

Spectrum of nebularine in the ultraviolet. The measurements were made in aqueous solution by using a Beckman quartz spectrophotometer. Fig. 2 shows the absorption curves of natural nebularine and of the synthetic product (the latter sent to us by Dr. G. Brown). The coinciding curves have a maximum at 263 mμ and a minimum at 222.5 mμ.

Purine obtained from nebularine by hydrolysis. Nebularine, 19.0 mg, was dissolved in one ml of 3 % hydrochloric and the mixture kept at 80° for one hour in a water bath. The solution was then neutralized with ammonia and poured on a column of Dowex 50

* Concerning such calculations, see for instance Nichols [9].

** The type of the glass vessels was similar to that given by Craig, Gregory, and Barry [10].

*** By means of the Unterzaucher method.

in the hydrogen form (particle size, 70—100 mesh U.S. series; diameter and height of the column, 7 and 40 mm respectively). Frequent microchemical tests for sugars (Molisch) and for nucleo derivatives (fluorescence of spots on filter paper) were carried out on the effluent. Very soon a sugar fraction appeared. The column was washed with somewhat more water than was necessary to obtain a negative Molisch reaction. Then the column was eluted with 0.2 *N* ammonia. After some time and within a short period, a fraction appeared which was positive in the spot test on filter paper (*cf.* above). The eluate was evaporated to dryness. The brownish crystalline residue contained no nebularine and melted at about 204° (corr.). Yield 8.7 mg (97 % assuming the product to be purine formed from ribosyl purine). The product was now extracted with 0.5 ml of boiling toluene. The resulting solution was decanted from the solid material and yielded crystals on cooling. The extraction procedure was repeated five times, each time using the mother liquor. The insoluble residue consisted of a black deposit. The total yield of fine colourless needles melting at 216—217° (corr.) was 4.8 mg. The melting point is in agreement with that of purine given by Fischer [11].

The substance gave the same R_F values as synthetic purine. Thus, in the solvent mixtures given by Löfgren [7], both purine and the hydrolysis product were found to have the following R_F values on Whatman No. 4 filter paper at 22.0° ± 0.5° : 0.82 ("*iso*butyric acid liquid"), 0.68 ("butanol-*iso*butyric acid liquid"), 0.82 ("morpholine liquid"), and 0.66 ("piperidine liquid").

Another evidence for the identity of the cleavage product with purine was established in comparing their maxima and minima in different media in the ultraviolet. Maxima and minima were measured in neutral, acidic, and alkaline solutions, using a Beckman instrument. Synthetic purine and the hydrolysis product gave the same values as shown in Table 1. (No spectral data of purine are available from the literature.)

Table 1. Maxima and minima in the ultraviolet of synthetic purine and of the cleavage product from nebularine. Measurements in neutral, acidic, and alkaline media; λ in mμ.

Medium	λ_{max}	λ_{min}
Water	262.5	221
1 *N* HCl	260	229
1 *N* NaOH	271	236

For further characterization the picrate was prepared: In one drop of water, 3 mg of substance were dissolved and then 0.5 ml of a saturated aqueous solution of picric acid was added. The separated needles were recrystallized from water. The melting point was found to be 205—208° (corr.), in agreement with the recorded melting point of purine picrate [11]. When mixing synthetic purine picrate with the actual picrate, no depression of the melting point occurred. (Found: C 37.8, H 1.84, N 28.3. Calc. for $C_{11}H_7N_7O_7$ (349.2): C 37.8, H 2.02, N 28.1.)

D(—)*Ribose obtained from nebularine by hydrolysis.* In 5 ml of 0.1 *N* sulphuric acid, 49.9 mg of nebularine were dissolved and the solution was kept at 100° for four hours. After cooling, the solution was neutralized to pH 7 with dilute ammonia and then poured on a column of Dowex 50 in the hydrogen form (particle size, 70—100 mesh U.S. series; diameter and height of the column, 7 and 40 mm respectively). Frequent microchemical tests for sugars (Molisch reaction) and for nucleo derivatives (*cf.* above) were carried out on the effluent. Purine and unchanged nebularine were adsorbed on the resin, whereas a sugar fraction passed through. This fraction was taken up in a mixture of water and freshly prepared barium carbonate. The column was washed with somewhat more water than was necessary to obtain a negative Molisch reaction. The solid material ($BaSO_4$ + $BaCO_3$) was then filtered off with suction and washed with water. The combined filtrate and washings were evaporated to dryness under reduced pressure. A syrup containing some barium carbonate was obtained. This mixture was extracted with a small amount of hot absolute ethanol. The barium carbonate was filtered from the solution and washed with a little hot alcohol. From the combined filtrate and washings the alcohol was evaporated over silica gel in a vacuum desiccator. The residue consisted of a crystalline mass. Yield 22.6 mg (76 % assuming the product to be ribose formed from ribosyl purine).

When Bial's test was carried out, the product gave a positive reaction on pentoses and of exactly the same type as found in a parallell test made with commercial ribose. Spot tests on filter paper using Partridge's naphtoresorcinol reagent [12] gave a negative reaction for ketoses.

For further characterization of the sugar, paper chromatography was applied. A technique developed by Löfgren * was used.

The irrigating liquid used in this technique is made up of 60 ml of ethyl acetate, 17 ml of glacial acetic acid, and 17.5 ml of water. This mixture has a high resolving power on sugars. In order to enhance the resolution the mobile phase is allowed to run off the lower edge of the strip (Whatman's No. 4 filter paper; length 57 cm). Thus the chromatogram is run over a rather long period (usually 21 h 45 min.). Under these conditions it is, for instance, possible to separate a mixture of the four aldopentoses into individual components. Rhamnose which travels rapidly on the chromatogram has been used as a standard. Thus a sugar is characterized by its R_r value, *i. e.* the ratio between the distance the sugar travels and the distance through which the rhamnose has moved. The R_r values for the aldopentoses at a temperature of 20.0° ± 0.3° are the following: arabinose 0.62, xylose 0.70, lyxose 0.79, and ribose 0.87.

Using chromatograms of this type, the sugar originating from nebularine was compared with the four aldopentoses. After developement of the chromatograms the spots were revealed by means of Horrocks' benzidine reagent [13]. Each of the four aldopentoses occurred at different positions (*cf.* the R_r values given above). The hydrolysis product from nebularine gave only one spot and at the same position as ribose.

Measurement of the optical rotation in aqueous solution gave $[\alpha]_D^{20} = -19.4° \pm 1.5°$ ($c = 2.8$). For D(−)ribose in water $[\alpha]_D^t$ values corresponding to somewhat higher temperatures than 20° are available from Kuhn *etal.*[14] By extrapolation from these values **: $[\alpha]_D^{20} = -19.9° \pm 0.1°$; *cf.* the recorded value [16] for L(+)ribose: $[\alpha]_D^{20} = +20.7°$ ($c = 4$).

Furthermore, the *α,α*-benzylphenylhydrazone was prepared. In a test tube 16.2 mg of the hydrolysis sugar were dissolved in one ml of ethanol and then a solution of 80 mg of *α,α*-benzylphenylhydrazine in one ml of ethanol was added. The mixture was kept in a boiling water bath for 35 minutes. During this time almost all of the ethanol evaporated. On cooling the syrup crystallized. The crystals were washed carefully three times with 2.5−ml portions of a mixture ether-petroleum ether 1 : 1 (v/v). The yield was 32.6 mg (91 %, when the calculation is based on ribose) of a product melting at 121−124°. Recrystallization from *iso*butanol gave a product (21 mg) with a melting point 128−129° (corr.). This melting point is approximately the same as recorded for the corresponding hydrazones of D(−)ribose *** and D(−)lyxose ****, both of which were prepared. When admixing D(−)ribose benzylphenylhydrazone with the actual hydrazone, no depression in the melting point was found. Admixture with the lyxose derivative gave a large depression of about 30°, when equal parts were mixed. (Found: C 65.0, H 6.74. Calc. for $C_{18}H_{22}N_2O_4$ (330.4): C 65.4, H 6.71.)

Periodate oxidation of nebularine *****. In a 250−ml volumetric flask 100.9 mg of nebularine (0.4000 millimole) were dissolved in 150 ml of water. Then 5 ml of 0.2500 *M* $NaIO_4$ were added and the solution diluted to the mark. After 24 and 45 hours, 50−ml aliquots were taken out with a pipette and the unchanged metaperiodate was estimated iodometrically by the method of Barneby [20] using 0.1000 *N* sodium arsenite. In both cases 3.28 ml of arsenite solution, corresponding to a consumption of 1.07 moles of periodate per mole of nebularine, were needed. The remainder in the flask was investigated as to its content of formic acid (*cf.* Jackson and Hudson [21]). No formic acid could be found.

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* A thorough description of this method and its possibilities of separating sugars will be published later.

** From Kuhn *et al.*[14]: $[\alpha]_D^{24} = -25°$ ($c = 0.9$), $[\alpha]_D^{23} = -23.7°$, $[\alpha]_D^{22} = -22.5°$ ($c = 0.9$). By extrapolation from these values: $[\alpha]_D^{20} = -19.9° \pm 0.1°$. Levene and Jacobs [15] give a value $[\alpha]_D = -19.5°$ but they do not state any temperature.

*** 129° (uncorr.) according to Haiser and Wenzel [17].

**** 128° (corr.) according to Ruff and Ollendorff [18].

***** The general method given by Lythgoe and Todd [19] was followed.

SUMMARY

Nebularine, *i.e.* 9-(β-D-ribofuranosyl)purine, is a naturally occurring compound with strong inhibitory action on cell growth and on *Mycobacteria*. The isolation of nebularine from a mushroom, *Agaricus (Clitocybe) nebularis* Batsch., and the determination of its structure have been described.

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The Definition and Affinity of the Deterging Process

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A system in which a deterging process takes place has so many variables that it is not a priori clear how to define the deterging process and its affinity. So the problem of how to evaluate detergents leads to a thermodynamical analysis of systems of the above kind with due regard to the practical purpose. Certain results of this analysis may be generalized for use in other evaluation problems. For the dish washing process, the writer has previously [1] suggested as affinity measure the adhesion work W_A in isothermal reversible separation of soil and detergency object when both phases are surrounded by the detergent analogous with Dupré's definition concerning the separation of two phases surrounded by air. The significance and reasons are explained quantitatively for applying this, the simplest possible, thermodynamically justifiable, convention. The expression for W_A comprises interfacial tensions between solid-liquid phases, but may be rewritten in various ways to make it accessible to experimental determination.

1. EVALUATION OF DETERGENTS

The evaluation of a detergent is most often done in one of the following ways:

A. by practical washing tests, or
B. by measuring its surface tension γ_{DG} against air at varying concentrations.

The latter method was, at any rate, in common use during the early stages of synthetic detergents. The former method takes long time and presents certain practical difficulties. The latter method — according to which the detergent showing lowest surface tension at lowest concentration is claimed to be best — is based on the determination of quantities easily accessible to accurate measurement; but the method is lacking a well-grounded experimental and theoretical foundation.

Now the question arises whether it is possible either to establish method B or to substitute for it another, more well-founded method with similar favourable properties.

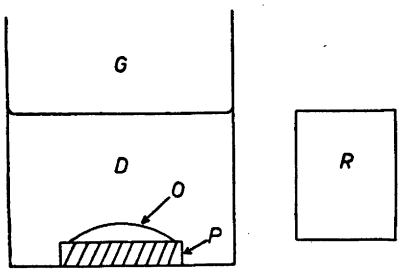

Fig. 1.

A pre-condition for solving this problem — or altogether to attach any meaning to it — is above all the *postulate* that method A can lead to an unambiguous evaluation of the efficacy of various detergents (referred to in the following as D-agents), *i. e.* that in practice the variations in the washing method are insignificant so as not to influence the evaluation. To this postulate the writer has [1], as far as dish washing agents are concerned, added that the quantity:

$$W_A = -\gamma_{PO} + \gamma_{PD} + \gamma_{OD} \qquad (1)$$

shows accordance with this evaluation and that γ_{DG} does not, if:

γ_{XY} = interfacial tension between X and Y
P = the detergency object (porcelain)
O = the soil (oil)
D = D-agent
G = air

The thermodynamical significance of the quantity W_A will appear from the following. Now the question is whether the above statement can be established.

2. CONVENTIONS AND FACTS

As a model to which the considerations may be appropriately attached, we will consider a system as shown in Fig. 1.
It consists of a detergent system composed of a vessel with a P-plate, an O-drop and a large D-phase. The detergent system is surrounded by the atmosphere G. Furthermore, there is a reservoir for mechanical energy R (R represents the washing machine or the person that does the dish washing).

In order to evaluate D as a dish washing agent, it is necessary to define what to understand by a deterging process in the detergent system by fixing its initial and final state. However, this is not quite so simple as it may seem to be. What is usually called a dish washing consists of a series of more or less well-defined operations during which a detergent system passes through a succession of state changes $a_1 \rightarrow a_2 \rightarrow a_3 \ldots$ Examples of such links $a_i \rightarrow a_{i+1}$ are: the detergent's solution in water, the placing of P (with O) in the D-phase, overcoming the adhesion O—P. If the link $a_i \rightarrow a_{i+1}$ is spontaneous, it would mean that $a_i \rightarrow a_{i+1}$ occurs in the detergent system even if R is not coupled in. In these circumstances the process naturally entails an entropy increase ΔS

in the detergent system plus surrounding G-phase, with which the detergent system is in thermal contact. By introducing R, an amount of energy $\Delta E_R = T\Delta S$ could be gained if the process $a_i \rightarrow a_{i+1}$ takes place at maximum utilization, T being the temperature (in °K).

If we consider two detergents during the course of $a_i \rightarrow a_{i+1}$ in two detergent systems, one would be apt to suppose that the detergent with the highest value of ΔE_R had functioned best during the operation $a_i \rightarrow a_{i+1}$ because it is useful energy (in R) we want to save. There is, however, the *essential qualification* to add in this *practical* example that it must be possible to couple R to the detergent system. If *e. g.* we consider the operation of the pure detergent's solution in water, we have clearly a spontaneous link which theoretically might be utilized. It is, however, quite irrelevant to the practical evaluation of the two detergents whether one detergent might *theoretically* give greater gain of energy in R than the other at the above dissolution, since there is no possibility of coupling. If only the link is spontaneous, we are satisfied. What is desired is to save useful energy in those links which in practice correspond to coupling. In order to obtain comparable values for these links, it is necessary to use maximum utilization (reversible coupling), because the possibilities of utilization are not known in detail. Broadly it can be said that it is of no use *e. g.* to be able theoretically to gain much mixing work by the detergent's solution in water, if later on O adheres very strongly to P. *Thus there exist a number of links which must not be included in the definition of the deterging process for our purpose*, since the only requirement is that they are spontaneous. It might be expedient to include this requirement in the definition of a detergent. As such links the writer has also grouped the wetting and de-wetting processes in the immersion and removal of P into and from the D-phase. *But possibly this assumption is too rough as it would certainly be for textiles.*

In accordance with what is said above, it is now the task to try to define the initial state a and the final state b for the deterging process in the system illustrated in Fig. 1. It will soon appear that the fixing of a and b involves uncertainties owing to lack of knowledge of the kinetics of practical dish washing. The only approach is, therefore, — at any rate for the time being — to remove the uncertainties through conventions. After selecting conventions for the fixing of a and b, the efficacy measure for D will be the reversible isothermal work W that might be gained by the introduction of R if the process $a \rightarrow b$ takes place in the detergent system.* The experimental proof that the states a and b are properly chosen must be obtained from a large-scale examination of the connection between this value and evaluations by the above method A. The theoretical establishment lies in the fact that W denotes ΔE_R for a (idealized, it is true) deterging process, whereas γ_{DG} measured alone (method B) does not refer to anything at all with relation to what causes trouble and expenses in a deterging process. As conventions we would of course in the first instance select the simplest that are not incompatible with what is said above.

Let us consider first the final state b. In this state O must be removed from P, and be surrounded by D (or perhaps D and G). In traditional thermodynamics states of equilibrium are widely operated with, but for our purpose

* *i. e.* the work done by R with inverted sign.

it must be noted that after overcoming the adhesion O—P, O may assume any shape as shown in Fig. 2 where only the sphere at extreme right could represent mechanical equilibrium.

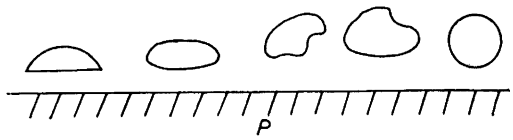

Fig. 2.

Broadly it can be said that the removal of O in unchanged shape (extreme left, Fig. 2) corresponds to an instantaneous "cutting off', and the sphere to a slow "rolling off" of O. The higher O's viscosity is, the easier it is to imagine the removal in unchanged shape. If b is defined as corresponding to the latter, and imagined realized, it would if the detergent system is left to itself be followed by a process $b \rightarrow b_0$ (Fig. 3), which might have done useful work.

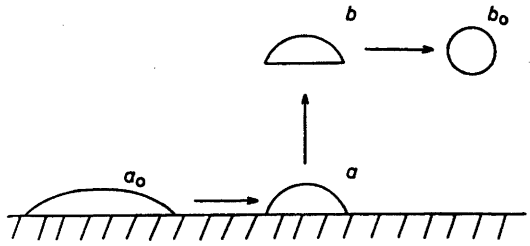

Fig. 3.

This theoretically obtainable work would be of no use in practice if practice corresponds to a "cutting off". If so, D should have "assisted in" the "cutting off". In practice it would most likely be somewhere between the said possibilities. Quite similar uncertainties are encountered when trying to fix the initial state a.

If a represents the state when O's removal is started, it would be possible to include in the definition certain preceding links $a_0 \rightarrow a$ (Fig. 3). When comparing two detergents one must consider the same O-amount adhering to P before immersion into the D-phases, and if a represents the state immediately after the immersion, O has at state a the same area σ_{PO} in common with P as in the air before the immersion. If the detergent system is left to itself before the removal, the area σ_{PO} will change through a spontaneous process, partly because of an interchange of matter between O and D during mutual dissolution, and partly because γ_{OG} and γ_{PG} are replaced by γ_{OD} and γ_{PD}. Also here we are lacking in kinetic knowledge. Are *e. g.* the processes of interchange of matter allowed time to occur in practice and "to lend a hand", or do they not attain any importance until state b_0 (Fig. 3) is reached?

This important point will be touched later; here we shall only point out that processes of the type $a_0 \rightarrow a$ do not necessarily always "lend a hand". If the detergent is pure water, more water will be dissolved *e. g.* in oleic acid than the other way round. O's volume is not always reduced and the equilibrium tensions may have spread O over P. Perhaps it would not be quite reasonable to consider pure water a detergent for dish washing. If we assume, however, that processes of the kind $a_0 \rightarrow a$ and $b \rightarrow b_0$ "lend a hand", we would (by leaving them out of the definition of the deterging process) *never obtain too optimistic an evaluation* of the detergent through ΔE_R for the process $a \rightarrow b$. This means some safety in an evaluation where it is impossible to state with certainty what is the "right" set of conventions. To the above considerations may be added that some of them are of quite a general nature and apply to all evaluations where it is a question of carrying through certain processes by means of certain agents *.

3. QUANTITATIVE THERMODYNAMICAL TREATMENT

a. the Energy differential

We can now make a stringent summary of the foregoing and at the same time study the properties of W_A through a quantitative thermodynamical treatment.

if:

E	represents	energy
S	»	entropy
v	»	volume
σ	»	interfacial area
n_i	»	number of moles of substance i
T	»	temperature
p	»	pressure
μ_i^f	»	chemical potential for substance i in phase f
γ	»	interfacial tension
Index K	»	interfacial layers (referred to in the following as capillary phases)
Σ_i	»	summation over the substances
Σ_f	»	» » » phases

we have for states in the detergent system with homogeneous bulk phases:

$$dE = \underset{f}{\Sigma} T dS - \underset{f}{\Sigma} p dv + \underset{f}{\Sigma}\underset{i}{\Sigma} \mu_i^f dn_i^f + \underset{K}{\Sigma} \gamma d\sigma$$

from which is obtained the maximum isothermal work dE_R which by a differential process can be gained in R by leaving out $\Sigma T dS$ and $p_D dv'$ as $-dv' = dv_0 + dv_D$ is introduced and the sign inverted.

* Examples of quite another kind of evaluations are *e. g.* organoleptic tests.

Thus we find:

$$dE_R = (p_0 - p_D)dv_0 - \gamma_{PO}d\sigma_{PO} - \gamma_{PD}d\sigma_{PD} - \gamma_{OD}d\sigma_{OD} - \sum_f \sum_i \mu_i^f dn_i^f \quad * \qquad (2)$$

We now find that on certain assumptions (2) is converted to:

$$dE_R = (\gamma_{PO} - \gamma_{PD} - \gamma_{OD})d\sigma_{PD} = -W_A d\sigma_{PD} \qquad (3)$$

Above all, *we have simplified the problem by leaving out of consideration gravity, buoyancy and electric charges in the phases*, since (2) does not contain variables representing these phenomena. Furthermore, we have put $dv_0 = 0$ and $-d\sigma_{PO} = d\sigma_{PD} = d\sigma_{OP}$ which corresponds to a removal of the drop in unchanged shape. Finally we have put:

$$\Sigma\Sigma\mu dn = 0 \qquad (4)$$

which causes the greatest difficulties.

b. $W_A^{initial}$ and W_A^{final}

A safe way of fulfilling (4) is to operate with material equilibrium in the detergent system during the process. In that case no chemical processes will occur (hydrolysis etc.) and the phases are mutually saturated throughout the process.

The detergent system has, besides the degrees of freedom of a simple thermodynamic system (pressure (p) — temperature (T) — and concentrations (c) in all phases) as many — f_k — further degrees of freedom as there are capillary phases, but accordingly also f_k further phases which are to be taken into consideration with their concentrations. If f_0 represents the number of bulk phases, x the number of degrees of freedom, and $\varkappa$ the number of components, the phase rule will accordingly maintain its form:

$$f_0 + x = \varkappa + 2 \qquad (5)$$

Actually, this is not quite correct, because the bulk phases are usually of different pressure owing to curved capillary phases, but as we are considering condensed systems, we can with approximation disregard the effect of the pressure on the potentials provided the curvature of the interfaces is not very pronounced (as in very small drops). As furthermore the external pressure and the temperature are fixed in our system, the phase rule is expressed by:

$$f_0 + x = \varkappa \qquad (5\ a)$$

Here x is the same whether or not we group porcelain under f_0 and $\varkappa$. Irrespective of the "additional" degrees of freedom it can accordingly be laid down that in a 2-component-system (*e. g.* oil-water) (or in a 3-component-system, if we include the P-phase) $x = 0$, that is to say we have a fixed composition of the

* Here we treat the K-phases as mathematical surfaces without volumes according to Gibbs. Alternatively, they may be considered of arbitrary volumes. The result is with good approximation the same. Regarding this problem reference is made *e. g.* to Guggenheim's Thermodynamics [2].

phases and fixed interfacial tensions throughout the process. In a 3-component-system (*e. g.* oil-water-synthetic detergent) (or in a 4-component-system, when including P) $x = 1$, *i. e.* the same as above holds if we fix D's concentration C_D.

By the above considerations we have, however, accounted only for the intensive states of the phases when $a \rightarrow b$ is imagined taking place at material equilibrium, but the detergent system, being materially isolated, is subject also to constraints of extensive nature, namely stoichiometric relations, since the amounts of the capillary phases vary.

However, we assume that the D-phase is large in relation to O, and accordingly that C_D = constant = initial value during the process $a \rightarrow b$. According to common thermodynamical parlance this simplifying assumption can be so expressed that the dish-washing process under consideration is regarded as a differential process. Accordingly, (3) can be immediately integrated assuming constant tensions. We will designate W_A by the above described process (at material equilibrium) W_A^{final} and accordingly we obtain (Δ representing finite differences and γ^{final} the γ's corresponding to the process):

$$\frac{dE_R}{d\sigma_{PD}} = \frac{\Delta E_R}{\Delta\sigma_{PD}} = -W_A^{\text{final}} = \gamma_{PO}^{\text{final}} - \gamma_{PD}^{\text{final}} - \gamma_{OD}^{\text{final}}$$

There is, however, quite another way of satisfying (4): The material changes may be insignificant, *i. e.* it may with approximation be possible to put $dn_i^f = 0$. This cannot be attained completely throughout the process, because the variations in the interfacial areas entail transferring of matter between bulk phases and capillary phases. If we disregard this, it is possible in certain cases to leave out of consideration irreversible transferring of matter caused by material disequilibrium. Such transferring may be so slow, or the duration of the process so short, as to make it insignificant. They may also be imagined blocked. If we regard the detergent system at a state immediately after new D—O and P-surfaces having been brought in contact, the tensions in the interfaces would have certain more or less well-defined values γ^{initial}. Such tensions are in literature often called "dynamical tensions". The quantity W_A (1) computed with these tension values, we will call W_A^{initial}. This quantity corresponds to $\Delta E_R = \int dE_R$ by a reversible removal with blocked material processes, provided we disregard the work $\Sigma\Sigma\mu dn$ corresponding to the area-work in connection with transferring of matter owing to chemical potential differences between bulk- and capillary phases. This assumption will not be dealt with in this paper. We would only point out that the introduction of W_A^{initial} and W_A^{final} corresponds to the — in literature [3] well-known — definition of initial and final values of the so-called spreading coefficients, and that we can, of course, in the same manner as introducing a semi-initial value for these, imagine $W_A^{\text{semiinitial}}$ or $W_A^{\text{semifinal}}$ defined. W_A^{final} or $W_A^{\text{semifinal}}$ corresponds in practice to the final states of a dish washing comprising a large number of washing objects in the vessel where the D-phase has not been renewed.

c. Properties of W_A.

Now it is also easily seen that the quantity W_A has a relation to spreading phenomena. This fact is immediately realised if we let O and D (Fig. 1) exchange roles. We imagine in other words a D-drop adhering to P and a large surrounding O-phase. Further, we imagine the D-drop widely spread — possibly through external influence — so that the contact angle is practically $= 0$. The concentration of the drop must be $= C_D$ in the removal test from which is obtained W_A for an O-drop on P surrounded by D. For further spreading of the D-drop we approximately have:

$$d\sigma = d\sigma_{PD} = -d\sigma_{PO} = d\sigma_{DO} \text{ and } p_D = p_O$$

and hence (2) for $\Sigma\Sigma\mu dn = 0$ making

$$dE_R = (\gamma_{PO} - \gamma_{PD} - \gamma_{DO})\, d\sigma = Pd\sigma$$

or

$$dE_R = -W_A d\sigma$$

where $d\sigma > 0$ for spreading, and W_A refers to the originally considered process, *i. e.* removal of the O-drop. Indicating the drop with index and as above the spreading coefficient with P, we have with easily understood designations:

$$P^D = -W_A^O \text{ and specially also}$$
$$P^{D,\,final} = -W_A^{O,\,final} \tag{6}$$

However P^{final} must be < 0 (*cf.*[3])

Thus it follows from (6) that we always will have:

$$W_A^{O,\,final} > 0 \tag{6 a}$$

while nothing prevents

$$W_A^{O,\,initial} \text{ from being } \gtrless 0$$

Now it will be natural to examine the significance of W_A's sign. Let us assume that the detergent system is in stable equilibrium with an O-drop adhering to P and with contact angle Θ. It is now easily derived that

$$W_A^{final} = \gamma_{OD}^{final}\, (1 + \cos\Theta)^{final} \tag{7}$$

whence — leaving out index 'final' — follows:

$$0 \leqq W_A \leqq 2\gamma_{OD} \tag{7 a}$$

which is accordingly a condition for stable drop on P. It is, however, clearly seen that this condition is not sufficient, for an evaluation of stability concerns ΔE_R by the process $a \to b_0$ (Fig. 3) where b_0 is the state of equilibrium upon removal and not ΔE_R by the process $a \to b$. We have now with easily understood symbols:

$$\Delta E_R\ (a \to b_0) = \Delta E_R\ (a \to b) + \Delta E_R\ (b \to b_0)$$

or per unit area of the surface PO according to (2)

$$\Delta E_R'\ (a \to b_0) = -W_A - \gamma_{OD}\, \Delta\sigma_{OD}\ (b \to b_0) + (p_O - p_D)\, \Delta v_0\ (b \to b_0) \tag{8}$$

or when expressing the latter part by W':

$$\Delta E_R\ (a \rightarrow b_0) = -W_A + W' \qquad (8\ a)$$

where, owing to $b \rightarrow b_0$'s spontaneousness $W' > 0$

if $-W_A + W' > 0$, the drop is not stable;
if $-W_A + W' < 0$, the drop is stable.

Here it must, however, be remembered that gravity, buoyancy and electric charges are left out of consideration. It is, of course, possible to compute W' on certain simple assumptions. If once again we presuppose that the change in pressure by variation in curvature through the change spherical cap → sphere, is of no consequence to the solubility equilibrium, so that the above process takes place without movement of matter between the phases, and if further — more we disregard the change in volume through the increase in pressure — which is, of course, a good approximation — we have in (8): $\Delta v_0 = 0$, and $W' = -\gamma_{OD}\ \Delta\sigma$.

By simple, but somewhat lengthy geometrical computations we find on these assumptions:

$$W' = \gamma_{OD}\ (2 + t^2 - (t^3 + 3\ t)^{\frac{2}{3}})$$

where t = tangent of the spherical cap's half contact angle.
As corresponding values can *e. g.* be given:

$t = 0$, $W' = 2\gamma_{OD}$
$t = \frac{1}{2}$, $W' = 0.87\gamma_{OD}$
$t = 1$, $W' = 0.5\gamma_{OD}$
$t = 2$, $W' = 0.2\gamma_{OD}$

The term W' in (8 a) accounts for processes of the kind $b \rightarrow b_0$ (Fig. 3). *But as pointed out above, it is by no means certain that these links or the links $a_0 \rightarrow a$ should be taken into consideration when fixing a practical evaluation measure for D-agents.* As regards processes of the kind $a_0 \rightarrow a$, they will not be discussed in detail in this paper. We would only point out that on certain assumptions we learn something also about such processes from the quantities W_A *and* γ_{OD}. Let us assume that we have a stable O-drop on the P-plate for two D-agents I and II. From (7) is obtained, leaving out indices "final":

$$\cos\Theta_I - \cos\Theta_{II} = \frac{W_A^I}{\gamma_{OD}^I} - \frac{W_A^{II}}{\gamma_{OD}^{II}}$$

which determines whether

$$\Theta_I \gtreqless \Theta_{II}$$

If, therefore, the dissolving processes do not change the conditions, it will be known which final area is the larger when the drops were equal at the outset.

4. EXPERIMENTAL INVESTIGATIONS AND SOME KINETIC CONSIDERATIONS

Designating with Θ^{Z}_{XY} the contact angle between phases X and Y when X and Y are surrounded by phase Z, the equation (7) will be:

$$W_A = \gamma_{OD}\,(1 + \cos\Theta^{D}_{PO}) \qquad (7)$$

This equation can be used for the determination of W_A through measuring γ_{OD} and Θ^{D}_{PO}; always provided, of course, that the drop is stable. Thus it will *e. g.* not be applicable to the determination of negative W_A-values (*cf.* 7 a). In a previous paper [1] the writer suggested for various reasons the application of another equation, namely:

$$W_A = \gamma_{OG}\cos\Theta^{G}_{PO} - \gamma_{DG}\cos\Theta^{G}_{PD} + \gamma_{OD} \qquad (9)$$

According to (9) the contact angles for O- and D-drops are measured against air (G). Its validity is, however — as can be shown by a detailed analysis — subject to a number of pre-conditions which in practice can only with difficulty be fulfilled; and if they are not fulfilled, the results arrived at would be quite wrong. Consequently the writer now considers this equation of merely theoretical interest. A series of experiments have been carried out with D-agents, peanut oil and glass in accordance with (9), which were described in the original manuscript, but as the writer has changed his technique *, he thinks it more reasonable to mention some comments received from several researchers during the exchange of experiences. This exchange has also directed the writer's attention to various available works in the detergent literature. Of interest are *e.g.* works by Kling [4,5].

Strange enough, there have been no objections to the practical application of (9), but the chief objection seems to be that the velocity has been ignored in the considerations advanced in [1]. The velocity is by some researchers considered of even primary importance. To this objection should above all be said that the velocity for the washing process cannot be unambiguously connected with the detergent system as such, for a dish-washing is not done by placing the washing objects in a vessel with the D-agent and then just sit back and wait, *i.e.* to leave the system alone. The velocity is a factor that is influenced by the operator, provided it is not — for certain processes $a_i \rightarrow a_{i+1}$ — so extremely high that they occur before the operator interferes.

Another objection is that the drop can practically always come loose by itself — quickly or slowly — so that there will be no contact angle. ($\cos\Theta^{D}_{PO}$ in (7) is undefined).

So the drop is said to be unstable and consequently it is possible for the system to do the washing by itself releasing free energy. If this is so for two D-agents, no energy will *in principle* be required to hasten the attainment of the final state if it is possible reversibly to recover the kinetic energy. *The two D-agents might then be considered equally good* quite irrespective of which of them releases most free energy when left to themselves. Now such reversible

* With the kind assistance of the Danish technical University, dpt. of technical physics, a very simple device has been designed for measurements in accordance with (7).

recovery will not be possible in practice. If we, however, consider two systems I and II in which the spontaneous dish-washing takes place, quickly and slowly respectively, the question arises: What causes the retardation of one drop in relation to the other?

The velocities are determined by 1) the pressure gradients 2) the weight of the drops 3) the friction (viscosity) 4) the charges in the interfaces (and the phases) 5) the adhesion as expressed *e. g.* in W_A and γ_{OD} (disregarding any dissolving processes).

1) and 2) must be about equal for I and II — (the specific gravity is about 1 for both D-solutions and we are considering the same O-amount and O-kind).

As the systems are liquid, we can with approximation disregard 3) as the cause of retardation in II, for the friction is very small when the process is slow. Then 4) and 5) remain. We have in these preliminary considerations ignored 4). Thus W_A will be highly co-responsible for the velocity of the spontaneous washing process. Roughly, it can be said, u representing the velocity and f a function

$$u_I - u_{II} = f(W_A^I, \gamma_{OD}^I, \Delta E_R^I, W_A^{II}, \gamma_{OD}^{II}, \Delta E_R^{II})$$

assuming that we now influence the systems from outside. (The consideration is of course very *rough*, particularly because it is not a question of a single value of velocity but of a velocity field. The writer is striving to establish the considerations in a quantitative form). Accordingly, if we want to bring about the same velocity (velocity field) in I and II, and if we know the W_A's and the γ_{OD}'s, the difference between the two required values of ΔE_R is determined by that knowledge.

Finally should be said: It will often be found that the drop is removed *almost* by itself when the P-plate is placed so that the buoyancy assists in removing it. According to the writers' experiences this is strongly dependent on the pre-treatment of the plate. As pure water gives a defined contact angle D's concentration must also be taken into consideration.

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Short Communications

o- and *m*- *Iso*propylbenzoic Acids

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During the course of work, which will be described in a separate communication, it became necessary to prepare pure samples of the three isomeric *iso*propylbenzoic acids. The *p*-isomer, cumic acid, is well known and adequately characterised, but the methods which have been employed for the preparation of the *o*- and *m*-isomers are not entirely satisfactory, and no convenient derivatives of these acids have been described. Thus, *o-iso*propylbenzoic acid has been obtained by the reduction of 3,3-dimethylphthalide with hydrogen iodide and red phosphorus [1], and as a by-product in preparations giving predominantly the *p*-isomer [2]. Likewise, *m-iso*propylbenzoic acid has been obtained by the alkylation of benzoic acid with di-*iso*propyl ether using hydrogen fluoride as a catalyst [3]. It has been stated that the acid melts at about 20°, or is an oil [4,5].

When the present work was nearing completion a paper by Crawford and Stewart [6] appeared, in which syntheses of the two acids in question were described. However, as the melting points of the acids we obtained are significantly higher than those recorded by Crawford and Stewart, and, furthermore, two derivatives of each acid were prepared, it seems desirable to record our own findings.

*o-Iso*propylbenzoic acid was prepared by hydrolysis of *o-iso*propylbenzonitrile obtained from *o*-cumidine by the method recommended for the preparation of *o*-tolunitrile [7]. The pure acid melted at 63—63.5°, and on oxidation with dilute nitric acid in a sealed tube at 180° gave phthalic acid.

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Substance	m. p. * °C	Crystal Form and solvent	Analysis ** Found, %			Required, %		
			C	H	N	C	H	N
*o-Iso*propylbenzoic acid	63—63.5	needles, petrol ether	73.1	7.38	—	73.1	7.37	—
Anilide	137—8	plates, EtOH/H_2O	80.5	7.04	5.89	80.3	7.16	5.85
p-Bromophenacyl ester	54—5	needles, EtOH/H_2O	60.0	4.69	—	59.8	4.74	—
*m-Iso*propylbenzoic acid	51—2	prisms, petrol ether	73.1	7.32	—	73.1	7.37	—
Anilide	117—8	needles, EtOH/H_2O	79.8	7.28	5.94	80.3	7.16	5.85
p-Bromophenacyl ester	96	plates, EtOH	60.5	4.81	—	59.8	4.74	—

* All melting points uncorrected.
** Analyses by W. Kirsten, Uppsala, and A. D. Campbell, University of Otago.

*m-Iso*propylbenzoic acid was prepared by the carbonation of *m-iso*propylphenyl magnesium bromide [8] either with gaseous carbon dioxide at 0°, or, more conveniently, with solid carbon dioxide. Crawford and Stewart prepared the acid by the same method. The pure acid melted at 51–52° and on oxidation with dilute nitric acid at 180° gave *iso*phthalic acid.

The anilides and *p*-bromophenacyl esters were prepared by standard methods.

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A Preliminary X-Ray Investigation of Re_2O_7

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In connexion with work on chromium oxides, dichromates, and polychromates, the author has preliminarily investigated the crystal structure of Re_2O_7. This compound was first prepared by W. Noddack in 1928 by direct oxidation of Re-metal by oxygen [1,2], who in this way obtained it as extremely deliquescent, canary-yellow, "hexagonal" plates and prisms. The compound begins to sublime at 250° C at atmospheric pressure. Vapour density measurements (at 520° C) indicated the existence of Re_2O_7-molecules in the gas phase.

In this preliminary investigation the oxide was prepared according to the method given in *Inorganic Syntheses* [3], starting from Re-metal of 99.5 % purity (Mackay). After the reaction and subsequent sublimation of the Re_2O_7, the sample was preserved by sealing off the reaction tube using a torch. All manipulations of the substance had to be carried out in a carefully dried atmosphere.

The single crystal photographs were taken with the crystals sealed up in thin tubes of lithium borate glass, using Cr*K* and Cu*K* radiation. Numerous crystals were tried, all of which, however, consisted of two or more individuals with the *c*-axis (the prism axis) in common. The powder photograph was taken in a 19 cm Bradley camera with Cu*K* radiation using a sample obtained directly from the reaction vessel after drawing out one end of the sealed-off Pyrex tube into a capillary.

The Laue symmetry, derived from Laue and Weissenberg diagrams (*h*0*l*, *h*1*l*) was D_{2h}/mmm. The unit cell dimensions obtained from the powder photograph were found to be

$$a = 15.25 \pm 0.1 \text{ Å}; \; b = 5.48 \pm 0.02 \text{ Å};$$

$$c = 12.5 \pm 0.1 \text{ Å}; \; V = 1044.6 \text{ Å}^3.$$

The density of the compound as determined by Biltz and Lehrer [4] ($d = 6.103$) evidently indicates a unit cell content of 8 (7.92) formula units of Re_2O_7.

The only reflexions systematically missing were *hk*0 with *k* odd, which suggests the symmetry to be $D_{2h}^5/Pmmc$.

Further studies on the crystal structure are planned.

This preliminary report forms part of an investigation on the structure of metal oxides, financially supported by the *Swedish Natural Science Research Council*.

The author wishes to thank Dr. Arne Magnéli for valuable discussions.

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The only reflexions systematically missing were *hk*0 with *k* odd, which suggests the symmetry to be $D_{2h}^5/Pmmc$.

Further studies on the crystal structure are planned.

This preliminary report forms part of an investigation on the structure of metal oxides, financially supported by the *Swedish Natural Science Research Council*.

The author wishes to thank Dr. Arne Magnéli for valuable discussions.

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β-Mercaptopyruvate as a Substrate for Rhodanese

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Wood and Fiedler[1] recently reported that liver extract catalyzed the formation of thiocyanate from β-mercaptopyruvate and cyanide. This was taken as evidence for β-mercaptopyruvate being a substrate for rhodanese, an enzyme catalyzing the formation of thiocyanate from thiosulfate and cyanide[2]. As this enzyme recently has been purified and crystallized[3], it was thought desirable to investigate the effect of a purified enzyme preparation on a mixture of β-mercaptopyruvate and cyanide. When this experiment was carried out no formation of thiocyanate was observed and β-mercaptopyruvate is consequently not a substrate for rhodanese. A rhodanese preparation, about 50 % pure, was used in the previously described[3] test system for rhodanese assay, but sodium thiosulfate was replaced by ammonium β-mercaptopyruvate. When instead a rat liver homogenate was used as the enzyme source, a small but significant formation of thiocyanate was obtained, which could be increased 3-fold by preincubating the homogenate with β-mercaptopyruvate before the cyanide was added. From these results (shown in Table 1) it is evident that liver contains an enzyme system, which catalyzes the formation of thiocyanate from β-mercaptopyruvate and cyanide, but which is different from rhodanese. When the same liver homogenate was examined under the conditions of the test systems used by Wood and Fiedler (Table 2), the yield of thiocyanate obtained from β-mercaptopyruvate (as compared with the yield from thiosulfate) was considerably increased. A further increase was observed when the liver homogenate was replaced by an extract of acetone dried liver, which was the enzyme source used by Wood and Fiedler. Even in this case less thiocyanate was obtained from β-mercaptopyruvate than from thiosulfate, although Wood and Fiedler reported that β-mercaptopyruvate and thiosulfate were equally active as sulfur donors for the formation of thiocyanate. This discrepancy can easily be explained since different liver extracts can be expected to differ in their relative content of rhodanese and the enzyme system responsible for the formation of thiocyanate from β-mercaptopyruvate. This enzyme system is probably identical with a recently described[4] enzyme, which catalyzes the formation of free sulfur from β-mercaptopyruvate. The reaction between free sulfur and cyanide is then catalyzed by sulfhydryl and disulfide groups[5], abundantly present in the liver proteins. The claim[2] that this reaction is catalyzed by rhodanese has previously[6] been disproved.

Table 1. Thiocyanate formation of rhodanese preparations.

The test system contained 0.05 *M* sulfur donor and cyanide in a final volume of 2.5 ml. The pH was 8.6 and the system was incubated 5 min. at 20° C.

Enzyme	Sulfur donor	CNS^- formed
		μ-eq.
Purified rhodanese	Thiosulfate	4.30
» »	β-Mercaptopyruvate	0
Liver homogenate	Thiosulfate	5.53
» »	β-Mercaptopyruvate	0.23
* » »	»	0.84

* The homogenate was preincubated 30 min at 38° C with the β-mercaptopyruvate.

Table 2. Enzymatic formation of thiocyanate.

The test system contained 0.017 *M* sulfur donor and 0.0056 *M* cyanide in a final volume of 2.5 ml. The pH was 9.1 and the system was incubated 30 min. at 38° C.

Enzyme	Sulfur donor	CNS^- formed
		μ-eq.
Liver homogenate	Thiosulfate	14.8
» »	β-Mercaptopyruvate	6.75
Acetone liver extract	Thiosulfate	14.9
» »	» β-Mercaptopyruvate	9.32

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Use of Thiophene in Lengthening Carbon Chains of Aliphatic Acids by five Carbon Atoms

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In connection with other works, lengthening of aliphatic acids with several carbon atoms by a simple method was of interest. Since thiophene compounds are readily available and easily desulphurated to saturated aliphatic compounds, a method using thiophene as a starting material seems to be convenient.

Starting from an aliphatic acid RCOOH, the corresponding acylthiophene $RCOC_4H_3S$ can be prepared by one of the general methods given by Hartough [1-3]. This acylthiophene is reduced to the corresponding alkylthiophene $RCH_2C_4H_3S$ by a modified Wolff-Kishner method [4]. From this alkylthiophene the 5-alkyl-2-acetylthiophene $RCH_2C_4H_2SCOCH_3$ is prepared and oxidized to the 5-alkyl-2-thiophenecarboxylic acid $RCH_2C_4H_2SCOOH$ [2]. From thiophenecarboxylic acids, sulphur has been removed by means of Raney nickel. This operation requires great amounts of Raney nickel in order to give the hydrogen required. Since much hydrogen is produced in the preparation of Raney nickel from the alloy, it might be advantageous to use the Raney alloy directly. In this way the amount of the alloy can be greatly reduced compared with the amount necessary if the Raney nickel is first prepared. Another advantage is that neither the alloy nor the nickel residue obtained by the reduction is pyrophoric, if the alloy is used.

The method is exemplified by the preparation of *n*-heptanoic acid from acetic acid.

Experimental. 2-Acetothienone was prepared according to *Organic Syntheses* [5], *cf.* Hartough and Conley [2].

2-Ethylthiophene. In a 1-l round-bottomed flask fitted with a condensor set for distillation, 79.5 g of potassium hydroxide were dissolved in 560 ml of diethylene glycol, 200 ml of hydrazine hydrate (85 %) and 103 g of 2-acetothienone were added and the mixture heated until no more 2-ethylthiophene distilled over. After washing with water, drying over sodium sulphate and distilling, the yield was 78 g, b.p. 135—140°.

5-Ethyl-2-acetothienone was prepared by the same method as 2-acetothienone.

5-Ethyl-2-thiophenecarboxylic acid was prepared by a modified procedure of that given for the preparation of 2-napthoic acid from 2-acetylnaphtalene in *Organic Syntheses* [6], *cf.* Hartough and Conley [2].

Removal of sulphur from 5-ethyl-2-thiophenecarboxylic acid. 75 g of the finely ground nickel-aluminium alloy (the minimum quantity necessary) was added slowly to a solution of 75 g of sodium hydroxide in 300 ml of distilled water and 8.8 g of 5-ethyl-2-thiophenecarboxylic acid contained in a 1-l three-necked round-bottomed flask fitted with a mechanical stirrer, a reflux condensor and a stopper, surrounded by ice. The mixture was refluxed for 5 hours, filtered while warm and the catalyst washed with a small quantity of water. The combined filtrate was acidified with concentrated hydrochloric acid and extracted with benzene. The benzene layer was washed with water, dried and distilled. The yield was 5.8 g of *n*-heptanoic acid, b.p. 122—124°/14 mm. A quantity of the acid was converted into the amide, m.p. 97 (from water) and this showed no melting point depression when mixed with an authentic sample of *n*-heptamid.

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Reindarstellung und Bestimmung von Nikotinsäurebenzylamid

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Bei der Herstellung von Nikotinsäurebenzylamid nach der Methode von Billman und Rendall [1] erhält man eine Substanz, welche in Übereinstimmung mit der Literatur bei 72—73° schmilzt und bei der Gesamtstickstoffbestimmung einen Gehalt von 13,08 % Stickstoff ergibt.

Auf der Suche nach einer mehr spezifischen analytischen Methode griffen wir zur acidimetrischen Bestimmung im wasserfreien Medium nach Kahane [2] und Kahane und Sackur [3]. Diese ergibt, berechnet auf das Molekulargewicht für Nikotinsäurebenzylamid (212,25), einen Gehalt von 107,1 % bei der laut Literatur hergestellten Substanz. Da die Substanz in wässriger Lösung neutral reagiert, kann die Verunreinigung nur aus unumgesetzten nikotinsaurem Benzylamin bestehen, welches noch nicht Wasser abgespalten hatte.

$$C_5H_4NCOOH + NH_2CH_2C_6H_5 = C_5H_4NCOOH \cdot NH_2CH_2C_6H_5$$

$$C_5H_4NCOOH \cdot NH_2CH_2C_6H_5 \rightarrow C_5H_4NCONHC_6H_5 + H_2O$$

Während sich Nikotinsäurebenzylamid wie eine einbasische Verbindung bei Titration mit Perchlorsäure im wasserfreien Medium verhält, da die Amidogruppe neutral reagiert, wird die Nikotinsäure von der starken Perchlorsäure aus dem Salz verdrängt und reagiert das Salz daher zweibasisch. Eine Substanz, die nach der Perchlorsäuretitration einen Gehalt vom 107,1 % zeigt, muss also noch 13,7 % unverändertes Salz enthalten. Berechnen wir für eine solche Mischung den Stickstoffgehalt, so finden wir, dass er einem Wert von 13,05 % entspricht. Beide Werte, 13,05 % und 13,08 %, liegen aber innerhalb der Fehlergrenze von dem für das reine Amid berechneten Werte: 13,20 %. Daraus geht aber hervor, dass eine Bestimmungsmethode, welche den Gesamtstickstoff sowohl beim Amid als auch beim Salze erfasst, kein Kriterium für die Reinheit der Substanz bietet. Die Titration mit Perchlorsäure hingegen bestimmt beim Amid bloss den basischen Stickstoff im Pyridinkern, während sie beim Salze sowohl den Kernstickstoff als auch den Aminstickstoff erfasst. Dies lässt in diesem Falle eine grosse Genauigkeit zu.

Dass die Substanz wirklich verunreinigt war, geht daraus hervor, dass sie beim Umkristallisieren aus Toluol und Ausfällen mit abs. Äther eine reinere Substanz liefert, welche bei 78—79° schmilzt und einen scheinbaren Amidgehalt von 104,5 % bei der Titration aufweist. Wird die gleiche Substanz in Chloroform gelöst, die Lösung mit verdünnter Natronlauge ausgeschüttelt und die Substanz aus der eingeengten Chloroformlösung mit abs. Äther gefällt, so weist sie einen Schmelzpunkt von 83—83,5° und einen Gehalt von 99,3 % Amid auf.

Experimenteller Teil: Herstellung von reinem Nikotinsäurebenzylamid. Zwei Mole Benzylamin und ein Mol Äthylnikotinat werden unter Rückflusskühlung im Ölbad bei einer Badtemperatur von 160—170° 25 Stunden erhitzt. Nach Abkühlen der Mischung auf Zimmertemperatur wird abs. Äther hinzugefügt. Man lässt darauf die Mischung zwei Tage bei 6° stehen und saugt ab. Die Kristalle werden auf dem Filter gründlich mit abs. Äther gewaschen. Es wird bei Zimmertemperatur getrocknet. Schmp. 83—83,5° Gehalt an Amid 100,1 %.

Reinigung von Benzylnikotinamid. 20 g Benzylnikotinamid, die man nach der Methode von Billman und Rendall erhalten hat, werden in 50 ml Chloroform gelöst. Diese Lösung wird 3 mal mit 20 ml 2 *N* Natronlauge und 3 mal mit 20 ml dest. Wasser gewaschen. Darauf wird das Lösungsmittel unter vermindertem Druck entfernt. Der Rückstand wird mit 50 ml abs. Äther versetzt und nach zweitägigem Stehen bei 6° wird abgesaugt. Die auf dem Filter befindlichen Kristalle werden mit abs. Äther gewaschen und bei Zimmertemperatur getrocknet. Ausbeute 17 g Schmp. 83—83,5°. Gehalt Amid 99,3 %.

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* Die Analysen wurden vom Chef des analytischen Laboratoriums der A.B. Hässle, Ing. Arne Hagsund, durchgeführt.

Determination of Some Inorganic Substances in the Labyrinthine Fluids

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As a part of a general investigation of the chemical composition of the labyrinthine fluids we have made an attempt to determine some inorganic substances in the endo- and perilymph of sharks *(Acanthias vulgaris)*.

The contents of sulphur was determined by the method of Bürger [1], modified by Zimmermann [2]. We found that the endo- and perilymph contained 75.47 mg, respectively 51.75 mg of sulphur per 100 ml of the fluids.

On account of the scantiness of material, and the complete ignorance as to the contents of inorganic substances contained in the labyrinthine fluids we examined these fluids by means of spectrographic analysis.

After incineration in a platinum crucible the sample to be analysed was placed in a small core in the lower graphite electrode, the upper one being another graphite electrode ground to a blunt point. The sample was now vaporised in the course of 45 seconds during which time one spectrum was recorded within the wavelengths of about 2 200 – 3 200 Å. The intensity of the emission lines was registered on a photografic film. The result is in Fig. 1, which gives the spectra of the endo- and perilymph, and the spectra of iron and sodium chloride for qualitative identification. The more important lines of analysis are denoted by their chemical symbols and their wavelengths in Å. The spectrum of the perilymph shows several lines which are not found in the other spectra. These lines arise from platinum, the perilymph being, it is supposed, contaminated with platinum as a result of its treatment in the platinum crucible.

Besides, sodium and potassium were determined quantitatively by means of a Beckman flame spectrophotometer. The result is in Table 1.

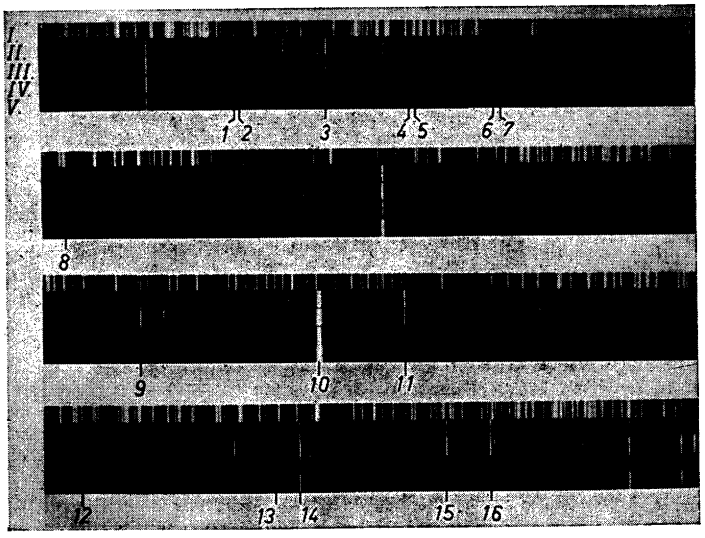

Fig. 1. The spectrogrammes of endo- and perilymph. I Pure Fe. II Endolymph. III Perilymph. IV and V NaCl. 1 B 2496.8, 2 B 2497.7, 3 Si 2516.1, 4 P 2534.0, 5 P 2535.7, 6 P 2553.3, 7 P 2554.9, 8 Fe 2599.6, 9 Mg 2795.5, 10 Na 2852.8, 11 Si 2881.6, 12 Ni 3002.5, 13 Al 3082.2, 14 Al 3092.7, 15 Ca 3158.9, 16 Ca 3179.3. Explanation in the text.

Table 1. The contents of sodium and potassium in the endo- and perilymph.

	Na mmoles	K mmoles
Endolymph	2.80	0.60
Perilymph	2.47	0.37

The authors wish to thank Laboratoriet for Metalundersøgelser, A/S Nordiske Kabel- og traadfabriker, Copenhagen S, for the courtesy of placing the spectrograph at our disposal, and professor J. A. Christiansen for his interest in this investigation. Financial support from *Det teknisk-videnskabelige forskningsraad* and *Carlsbergfondet* is gratefully acknowledged by C. E. Jensen.

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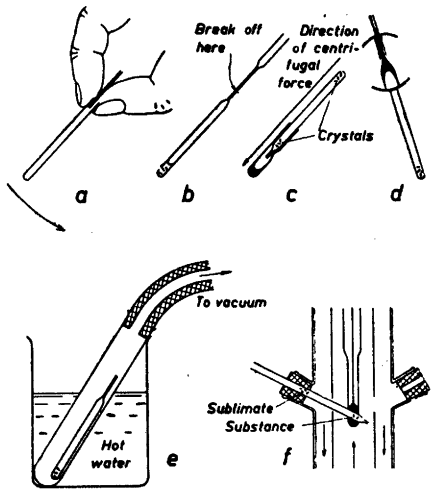

Fig. 1. Explanation in the text.

Syntheses in Capillary Tubes

NIILO KAJOLA

Institute of Chemistry, University of Helsinki, Finland

For several years the students at the Institute of Chemistry, Helsinki University, have carried out several syntheses on a micro scale, using as textbook A. A. Morton's "Laboratory Technique in Organic Chemistry". Experiment on a micro scale included in this book have chiefly been devised by A. Fuchs [1]. The reactions and the purification of substances are carried out in a capillary tube about 2 mm in diameter. Fuchs freed the crystals from the mother liquor by centrifuging the contents of the reaction tube to the bottom and sucking off the liquid with a capillary pipette. However, it is not possible to remove all the liquid in this way. This is a serious drawback, since it is known that complete removal of the mother liquor is a necessary condition for the purification of substances by crystallization.

The present author has devised some improvements to Fuchs' technique, that make the micromanipulation easier and more effective. The most noteworthy of them is the manner of removing the mother liquor from the crystals.

This is a general course of a synthesis with modifications:

A capillary tube, 2 mm in diameter is drawn out from 10 mm glass tubing. 6 cm long pieces of this capillary tube are sealed at one end. The small "test tubes" so made are here called reaction tubes.

Liquid substances are best introduced by means of 3 cm long pieces of 1 mm capillary tube (the same kind of capillary tubing as is used in melting-point determinations). Each piece of tube is used for filling one substance only, a new piece being taken for each substance. When a liquid is touched with one end of a capillary tube, it rises spontaneously in the tube. The height of the liquid column in the capillary tube is a measure of the quantity of the liquid. Any excess of liquid is removed by touching a piece of filter paper with the lower end of the capillary tube. To transfer the substance from the filling capillary to the reaction tube, the former is placed half-way into the latter (Fig. 1 a). The liquid can be shaken to the bottom of the reaction tube by jerking the tubes by hand. When all the necessary substances are in the reaction tube, it is sealed and heated in a metal block for the time required for the reaction. When the reaction is complete, the tube is opened. At this stage the reaction mixture can be stirred with a capillary stirring rod to promote the

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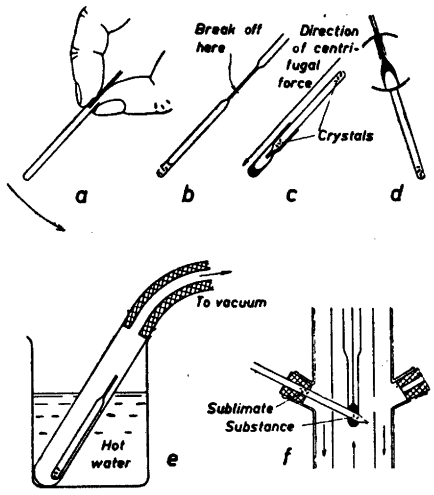

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crystallization. The reaction tube is then drawn to a fine capillary near its open end (Fig. 1 b). The capillary is cut off at the place marked in Fig. 1 b. A 3 cm long piece of 3 mm tubing is sealed at one end and is heated at one point in a micro flame to produce a small recess. The reaction tube is then placed in this filtering tube, the capillary end inwards; the filtering is achieved by centrifuging the liquid away from the reaction tube (Fig. 1 c). On centrifuging, the crystals usually remain at the closed end of the reaction tube; if they come loose, they are held back by the capillary end, which functions as a filter. If the precipitate is very fine, it is better to use an asbestos filter (Morton, p. 166). If some crystals reach the capillary end of the reaction tube, they can be brought to the bottom by dropping the reaction tube through a long glass tube against some hard object.

For recrystallization of a substance, a little solvent is taken up in the filling capillary. The reaction tube is gently heated, and its capillary end is put into the filling capillary (Fig. 1 d). On cooling, the reaction tube sucks the solvent in. Washing liquids and precipitating agents can be introduced in the same way. After the solvent has been centrifuged to the bottom, the crystals are dissolved by heating the tube in a metal block (not over a naked flame!). It is best to let part of the substance remain undissolved; it then initiates crystallization on subsequent cooling. If a supersaturated solution does not begin to crystallize, a minute amount of liquid is allowed to drain out of the capillary end. On the outer surface of the capillary the solution can be brought to crystallize by rubbing with a capillary glass rod. The crystallization then propagates through the capillary part into the reaction tube.

The crystals are then freed from the liquid by centrifuging as above. The drying of the crystals is best accomplished by heating the reaction tube in an evacuated test tube (Fig. 1 c). The vacuum is released a couple of times during drying. After drying the melting-point of the substance is determined in the same tube. The crystallization can then be repeated if necessary.

For sublimations, the capillary tube is heated in the same way as in melting-point determinations (Kajola's apparatus [2]). The sublimation is greatly accelerated if a minute hole is left at the bottom of the capillary tube. Since the air in the melting-point apparatus is in rapid motion, a slow current of air is produced through the capillary tube; the air current conveys the vapours of the substance to the colder part of the tube, where they are condenced (Fig. 1 f).

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*iso*Thiocyanates IX. The Occurrence of Ethyl *iso*Thiocyanate in Nature

ANDERS KJÆR and IVAN LARSEN

Chemical Laboratory, University of Copenhagen, Denmark

The naturally occurring *iso*thiocyanate-containing glycosides can, for practical reasons, be classified in two groups according to whether their *iso*thiocyanates are volatile with steam or not. Within the former group the occurrence of allyl, (+)-*sec*-butyl, benzyl and β-phenylethyl *iso*thiocyanate was convincingly established before these studies were initiated two years ago. Since then, we have proved 3-butenyl *iso*thiocyanate to be derivable from a glycoside of, a.o. *Brassica napus* L.[1], and demonstrated by isolation the wide-spread occurrence of *iso*propyl *iso*thiocyanate in the family *Cruciferae* [2]. To these findings we now wish to adduce experimental evidence for the presence in nature of a glycoside containing ethyl *iso*thiocyanate, the occurrence of which has not been suggested previously.

In the course of a paperchromatographic scanning of a large number of seed-samples for their contents of volatile *iso*thiocyanates, the results of which have been partly published [3], a rather unique spot with an R_{Ph}-value [4] of 0.15 was noticed from the seeds of *Lepidium Menziesii* DC. This value is in accord with that of authentic N-ethylthiourea, suggesting the presence of ethyl *iso*thiocyanate in the glycoside of the seed in question. Unfortunately, the small amount of seed available at that time did not allow substantiation of this assumption by isolation. Through the courtesy of

crystallization. The reaction tube is then drawn to a fine capillary near its open end (Fig. 1 b). The capillary is cut off at the place marked in Fig. 1 b. A 3 cm long piece of 3 mm tubing is sealed at one end and is heated at one point in a micro flame to produce a small recess. The reaction tube is then placed in this filtering tube, the capillary end inwards; the filtering is achieved by centrifuging the liquid away from the reaction tube (Fig. 1 c). On centrifuging, the crystals usually remain at the closed end of the reaction tube; if they come loose, they are held back by the capillary end, which functions as a filter. If the precipitate is very fine, it is better to use an asbestos filter (Morton, p. 166). If some crystals reach the capillary end of the reaction tube, they can be brought to the bottom by dropping the reaction tube through a long glass tube against some hard object.

For recrystallization of a substance, a little solvent is taken up in the filling capillary. The reaction tube is gently heated, and its capillary end is put into the filling capillary (Fig. 1 d). On cooling, the reaction tube sucks the solvent in. Washing liquids and precipitating agents can be introduced in the same way. After the solvent has been centrifuged to the bottom, the crystals are dissolved by heating the tube in a metal block (not over a naked flame!). It is best to let part of the substance remain undissolved; it then initiates crystallization on subsequent cooling. If a supersaturated solution does not begin to crystallize, a minute amount of liquid is allowed to drain out of the capillary end. On the outer surface of the capillary the solution can be brought to crystallize by rubbing with a capillary glass rod. The crystallization then propagates through the capillary part into the reaction tube.

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the Botanical Garden of the University of Copenhagen, however, a larger seed sample was provided after the plant had been cultivated in the Garden on a larger scale during the summer of 1953.

The seeds (*ca.* 60 g) were defatted and cleaved enzymatically in the usual way [3], whereupon a strong smell of *iso*thiocyanate was noticed. The ethereal oil was removed by steam distillation, collected in aqueous ammonia and the resulting crude thiourea isolated upon evaporation. After unsuccessful attempts to provide a homogeneous specimen by numerous recrystallizations and sublimations, the tenaciously adhering impurities were eventually removed by partition chromatography (*cf.* experimental part). Repeated recrystallizations resulted in an analytically pure sample (31 mg) of N-ethylthiourea, the identity of which was further established by mixed melting point, paper chromatography and infrared spectroscopy.

It appears very remarkable that the seed of *Lepidium Menziesii* DC. is the only specimen among approximately one hundred investigated so far, which contains ethyl *iso*thiocyanate. Other species belonging to the genus *Lepidium* have consistently been rich in benzyl *iso*thiocyanate. From the initial routine investigation it seemed as if *Lepidium Menziesii* DC. contains solely ethyl *iso*thiocyanate. However, paper chromatography of the mother liquors from the above crystallizations disclosed the presence also of very small amounts of *iso*propyl, *sec*-butyl and benzyl *iso*thiocyanate which had been sufficiently enriched for detection by the use of a larger seed-sample.

Experimental. A seed-sample (56.8 g) of *Lepidium Menziesii* DC. (source: *Hortus Botanicus Hauniensis*), which by our spectrophotometrical assay [3] was estimated to contain *ca.* 135 mg of ethyl *iso*thiocyanate, was pulverized and thoroughly defatted by repeated hot extractions with a mixture of ligroin (230 ml) and ethanol (115 ml). The fat contents were about 29 %. The finely divided material was then submitted to enzymatic cleavage by suspension in 400 ml of water and addition of 5 ml of a cell-free myrosinase preparation. After having been kept for 18 hours at room temperature in a tightly stoppered flask the liberated volatile *iso*thiocyanate was removed in a stream of steam and the distillate collected in 200 ml of concentrated ammonia. After standing for 24 hours at ordinary temperature the solution was evaporated to dryness *in vacuo* at a bath temperature not exceeding 50°. The residue consisted of crystalline material embedded in an oil, which was removed by careful treatment with cold chloroform. There remained 199 mg of a crystalline product which, however, melted over a very wide range. The crystals were treated with ethanol (4 ml) and the insoluble residue (23 mg) was discarded. Extraction with ethyl acetate removed another 7 mg of insoluble material. Two recrystallizations from ethyl acetate and one from ethylene chloride afforded a sample with a considerably sharper melting point (103—109°). After repeated sublimations *in vacuo* had failed to remove the final impurities, recourse was taken to partition chromatography.

A column of kieselguhr (Hyflo-Supercel), 250 × 18 mm, made up from 40 g of kieselguhr with water as the stationary phase and ethyl acetate as the moving phase, was loaded with 108 mg of the above thiourea. The chromatography was performed in a constant temperature room (23.4°) and 50 fractions of each 3.0 ml were taken by means of an automatic fraction collector. The contents of the tubes Nos. 13—29 gave a positive Grote-reaction (*cf.* Ref.[4]) and were pooled and evaporated to dryness *in vacuo*, leaving 95 mg of a crystalline, slightly yellow material. This product was carefully recrystallized three times from ethyl acetate and an analytical specimen (31 mg) was thus obtained as beautiful, rhombic plates, m.p. 110—111°, alone, or in admixture with an authentic sample of N-ethylthiourea (m.p. 110—111°). Calc. for $C_3H_8N_2S$ (104.2): C 34.58; H 7.74; N 26.89; S 30.77. Found: C 34.68; H 7.76; N 26.96; S 30.83. Paper chromatography of the sample in chloroform-water, ethyl acetate-water and 2-butanone-water gave spots which were undiscernible from those given by authentic ethylthiourea. Furthermore, the infrared absorption spectra determined in Nujol mulls of the two samples to be compared coincided completely. When the mother liquors from the ethyl acetate recrystallizations above were pooled and submitted to paper chromatography in chloroform-water, four spots appeared with R_{PK}-values of 0.15, 0.40, 0.73 and 0.90 (*cf.* Ref.[4]). From previous experience these can be assigned with reasonable safety to ethyl, *iso*propyl, *sec*-butyl and benzyl *iso*thiocyanate, respectively.

The authors are much indebted to *The Botanical Garden of the University of Copenhagen* for its assistance which made the present investigation possible. The gift of an automatic fraction collector to one of us (A.K.) from *Statens*

Almindelige Videnskabsfond (The National Science Foundation) is greatly acknowledged. Microanalyses were performed in this laboratory by Mr. W. Egger. This work is part of investigations supported by *Carlsbergfondet (The Carlsberg Foundation)*.

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Conversion of Orotic Acid to Uridine Phosphates by Soluble Enzymes of Liver

ROBERT B. HURLBERT* and PETER REICHARD

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In rat liver *in vivo* radioactive orotic acid is converted to a group of uridine nucleotides before the label is incorporated into the pyrimidines of the ribonucleic acid [1]. These nucleotides, which are uridine-5-phosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP) and three compounds consisting of UDP linked to a carbohydrate, have recently been found in rat liver as well as in other sources (*cf.* Ref.[1,2]). Since it has furthermore been established [3,4] that orotic acid can be formed *in vitro* in rat liver from the essential metabolites aspartic acid, CO_2, and NH_3, it is apparent that the compound is an ideal precursor for the study of some of the enzymatic mechanisms in the normal biological synthesis of nucleic acids. The objective of the present project is to examine *in vitro* the nature of the ribosidation stage in the conversion of orotic acid to the uridine-5-phosphates.

The soluble enzymes of rat and pigeon liver are found to be capable of converting orotic acid-2-C^{14} to the uridine nucleotides

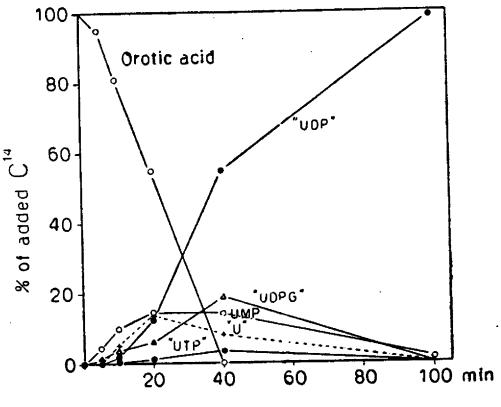

Fig. 1. The dialyzed supernatant fraction [5] from 400 mg of liver is incubated aerobically at 37° with 0.5 µmoles of orotic acid-2-C^{14} (500 000 c/min), 20 µmoles of hexose diphosphate, 3 µmoles of DPN and 120 µmoles of nicotinamide in a volume of 5.0 ml. The reaction is stopped by chilling the flask and adding perchloric acid to 0.4 N. The proteinfree extract is neutralized with KOH and chromatographed directly. ⊙ = *orotic acid;* + = *"U";* ○ = *UMP;* ● = *"UDP";* △ = *"UDPG";* ◐ = *"UTP".*

in vitro. The enzyme preparation is the dialyzed supernatant fraction of the liver after homogenization and high speed centrifugation (*cf.* Ref.[5]). The conversion of the orotic acid is obtained when fructose-1,6-diphosphate or ribose-5-phosphate with the cofactors DPN, ATP and Mg ion are added. A number of assisting and competing enzymes appear to be involved and the relative effectiveness of various combinations of these added components is different in preparations of pigeon liver as compared with rat liver.

The reaction products are separated by chromatography on 6 × 1 cm Dowex-2 (formate) columns by gradient elution with formic acid and formic acid-ammonium formate (*cf.* Ref.[6]). In addition to the unchanged orotic acid, five other radioactive peaks are found. One of these has been identified as UMP and three of them contain UMP which can be liberated by acid hydrolysis. They correspond in chromatographic behaviour to the components identified in rat liver *in vivo* [6] and have been designated here as "UDPG", "UDP" and "UTP" pending complete identification. The other radioactive fraction, "U", is found in the effluent from

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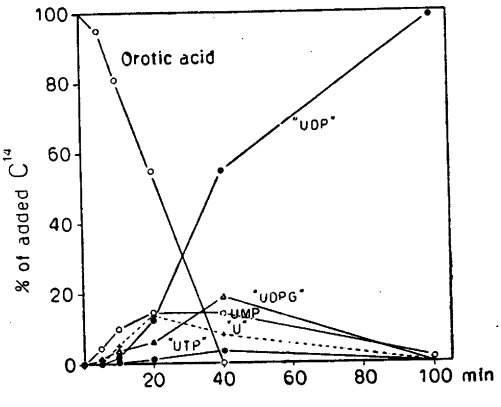

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In these experiments the UMP appears to be the first detectable product of the reaction. Fig. 1 shows the results of a time study of the reaction in which the isotope first appeared in the UMP and subsequently was distributed among the other uridine fractions and the "U" fraction.

In another time study similar to that of fig. 1, 4 μmoles of non radioactive UMP was added with the radioactive orotic acid. The incorporation of the label from the orotic acid in the "UDPG", "UDP" and "UTP" peaks was greatly diminished even though about 1 μmole of the added UMP was converted into the latter compounds. At the 40 minute time point of this experiment a large part of the radioactivity appeared in the "U" fraction in the form of uracil and uridine which were identified by chromatography on starch columns [7].

In a third time study it was found that 0.25 μmoles of added radioactive UMP, in the absence of orotic acid, was converted to the other uridine phosphate derivatives within 10 minutes.

The presence of 2 μmoles of non labeled uridine did not influence the incorporation of C^{14}-orotic acid into the UMP derivatives and thus uridine is excluded as an intermediate in the reaction.

The fixation into uridine phosphates of ribose formed from hexose metabolism may provide a useful system for evaluating the current concepts of ribose synthesis. The possibility is being examined that the ribotide formation from hexosediphosphate or ribosephosphate involves ribose-1,5-diphosphate as does the conversion of adenine to the adenine nucleotides [5]. The enzyme preparation is seen to be capable of degrading the UMP as well as of phosphorylating it, and the enzymes interconverting the "UDPG", "UDP" and „UTP" are also present.

The further investigation of these reactions by substrate limitation, inhibition and enzyme fractionation is continuing.

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The Structure of Selenium Diselenocyanate

OLA AKSNES and OLAV FOSS*

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The crystal structure of selenium diselenocyanate [1,2], $Se(SeCN)_2$, has been determined from X-ray data, by two-dimensional Patterson and Fourier methods.

The unit cell dimensions, from oscillation and Weissenberg photographs (Cu$K\alpha$ radiation, $\lambda = 1.542$ Å) are: $a = 10.07 \pm 0.03$ Å, $b = 13.35 \pm 0.04$ Å, $c = 4.48 \pm 0.02$ Å. There are four molecules per unit cell. Absent reflections, $0kl$ when $k + l$ is odd, $hk0$ when h is odd. The space group is the centrosymmetric one, D_{2h}^{16}—$Pnma$.

Patterson projections along the a, b and c axes revealed the positions of the selenium atoms. In the subsequent Fourier syntheses, signs of the reflections were initially based on the selenium contributions alone. The final atomic coordinates, in fractions of corresponding cell edges, are:

	x	y	z
Se_1	0.540	0.250	0.492
Se_2	0.442	0.115	0.249
C	0.295	0.112	0.488
N	0.203	0.095	0.586

The reliability factor, $R = \Sigma\|F_{obs}| - |F_{calc}\|/\Sigma|F_{obs}|$, is 0.15, 0.12 and 0.13, respectively, for the $hk0$, $0kl$ and $h0l$ reflections, with an over-all value of 0.14 for the three zones.

The selenium diselenocyanate molecule possesses, by space group requirements, a

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the sample placed onto the column. None of the fractions have as yet been found to contain a component resembling orotic acid.

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mirror plane of symmetry. The three selenium atoms and the cyano groups form an unbranched and non-planar chain, with a Se-Se bond length of 2.33 Å and a Se-Se-Se bond angle of 101°. The values, 94° and 95°, respectively, were found for the SeSeSe/SeSeC dihedral angle and the Se-Se-C bond angle.

The crystals are isomorphous with those of selenium dithiocyanate [3,4], $Se(SCN)_2$.

Details of the structure of selenium diselenocyanate will be published later.

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A Note on the Occurrence of Dimethyl Sulphone in *Cladonia deformis* Hoffm.

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NILS ANDREAS SØRENSEN

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In the course of an investigation on the presence of triterpenoids in lichens a sample of *Cladonia deformis* Hoffm., collected in a peat-bog, through which the railway runs, some 100 miles north of Trondheim, was investigated. The material was extracted with ether. The neutral fraction left after acidic substances had been removed by alkali was subjected to chromatography on alumina. The fraction eluted with ether-methanol (49 : 1) contained a substance that sublimed on the water-bath in a vacuum. It was sublimed twice and then melted at 98—105° (15 mg). Its IR-spectrum contained bands which could be due to the presence of the sulphone grouping. The simplest sulphone, dimethyl sulphone, has m.p. 109°, and, indeed, its IR-spectrum showed almost complete identity with that of the isolated substance. A mixture of the two compounds melted at 105—108°; the melting points of the two substances and the mixture were taken at the same time. Recrystallisation of the isolated sulphone from methanol-ether raised the m.p. to 107—108°, no depression on admixture with the authentic dimethyl sulphone.

During an investigation of sulphate turpentine (unpublished results with E. Hafnor) we isolated dimethyl sulphone, which we regard as arisen by aerial oxidation of dimethyl sulphide. This prompted us to consider the possibility of the presence of dimethyl sulphone, presumably originated in a similar way, in the large amount (3—4 litres) of solvent used. We think it unlikely that the sulphone should have been present as an impurity in the petroleum b.r. 40/70° and the methanol used during the chromatography, and have checked that it was not present in the benzene or the ether by filtering 12 litres of each solvent through 200 g of alumina with subsequent elution of any adsorbed substance with methanol *etc.*, as for the isolated substance. No crystalline substance could be detected. Similarly, we have also checked that no dimethyl sulphide was present in the benzene. None was detected.

Dimethyl sulphone has previously been isolated from cattle blood [1], from the adrenal gland [2] and from the horse-tail, *Equisetum palustre* [3], and other *Equisetum* species [4].

One of us (T.B.) thanks *Norges Almenvitenskapelige Forskningsråd* for a Research Fellowship. The work was supported by a grant from *A/S M.H. Lundgreen's Enke's Fund.*

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4. Karrer, P., Eugster, C. H. and Patel, D. K. *Helv. Chim. Acta* **32** (1949) 2397.

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mirror plane of symmetry. The three selenium atoms and the cyano groups form an unbranched and non-planar chain, with a Se-Se bond length of 2.33 Å and a Se-Se-Se bond angle of 101°. The values, 94° and 95°, respectively, were found for the SeSeSe/SeSeC dihedral angle and the Se-Se-C bond angle.

The crystals are isomorphous with those of selenium dithiocyanate [3,4], $Se(SCN)_2$.

Details of the structure of selenium diselenocyanate will be published later.

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A Note on the Occurrence of Dimethyl Sulphone in *Cladonia deformis* Hoffm.

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In the course of an investigation on the presence of triterpenoids in lichens a sample of *Cladonia deformis* Hoffm., collected in a peat-bog, through which the railway runs, some 100 miles north of Trondheim, was investigated. The material was extracted with ether. The neutral fraction left after acidic substances had been removed by alkali was subjected to chromatography on alumina. The fraction eluted with ether-methanol (49 : 1) contained a substance that sublimed on the water-bath in a vacuum. It was sublimed twice and then melted at 98—105° (15 mg). Its IR-spectrum contained bands which could be due to the presence of the sulphone grouping. The simplest sulphone, dimethyl sulphone, has m.p. 109°, and, indeed, its IR-spectrum showed almost complete identity with that of the isolated substance. A mixture of the two compounds melted at 105—108°; the melting points of the two substances and the mixture were taken at the same time. Recrystallisation of the isolated sulphone from methanol-ether raised the m.p. to 107—108°, no depression on admixture with the authentic dimethyl sulphone.

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Über den papierchromatographischen Nachweis von Saccharinsäuren

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Die Trennung der beim Erwärmen von Zuckern mit Alkali neben anderen Produkten entstehenden Saccharinsäuren durch fraktionierte Kristallisation ihrer Alkaloidsalze ist, wie die Arbeiten von Kiliani [1] und Nef [2] gezeigt haben, äusserst mühsam. Im Zusammenhang mit Untersuchungen über den alkalischen Abbau von Polysacchariden [3] schien eine papierchromatographische Methode zum Nachweis der verschiedenen Säuren wünschenswert.

Auf Whatman-Papier Nr. 1 gelingt eine einigermassen befriedigende Trennung bei Anwendung eines Lösungsmittelgemisches Äthylacetat — Wasser — Essigsäure im Verhältnis 10 : 1 : 1,3. Die Lage der einzelnen Flecken wird durch Besprühen mit ammoniakalischer Silberlösung bestimmt, wobei Mengen von 1 γ noch erkennbar sind. Folgende R_F-Werte wurden gefunden: α-Dextrometasaccharin 0,41, β-Dextrometasaccharin 0,36, α-Galaktometasaccharin 0,30, β-Galaktometasaccharin 0,35, α-Isosaccharin 0,43, 1,3-Dioxybuttersäurelacton 0,69, 1,3-Dioxybuttersäure 0,41, Milchsäure 0,67 und Glykolsäure 0,52. Die den genannten C_6-Lactonen entsprechenden Säuren haben ziemlich übereinstimmende R_F-Werte von 0,14—0,15. Die entsprechenden C_5-Säuren aus Pentosen haben R_F-Werte von 0,22. Die Chromatogramme der Säuregemische, welche beim Erhitzen von Glukose und Galaktose mit 8 *N* Natronlauge entstehen, zeigen neben Flecken der C_6-Saccharinsäuren u.a. auch Flecken, die auf die Anwesenheit von C_5-Saccharinsäuren hindeuten. Die Chromatogramme der analog aus Xylose und Arabinose erhaltenen Säuregemische zeigen u.a. Flecken, die auf die Anwesenheit von C_6-Saccharinsäuren hinweisen.

Bei der Untersuchung der Säure- bzw. Lactongemische, welche beim Alkaliabbau von Polysacchariden oder auch beim Sulfataufschluss des Holzes entstehen, erwies es sich zweckmässig, das Rohgemisch zunächst zu acetylieren und die Acetate mittels fraktionierter Destillation einer groben Trennung zu unterwerfen. Die entacetylierten Fraktionen werden chromatographiert. In vereinzelten Fällen konnten nach der Entacetylierung kristallisierte Anteile erhalten werden.

Die Ausarbeitung einer Methode zur zweidimensionalen Papierchromatographie der Saccharinsäuren ist im Gang.

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On the Growth Stimulating Activity and Utilization of Phosphopeptone and Phosphorylated Amino Acids on some *Lactobacilli*

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In recent experiments from this laboratory [1] it was found that phosphopeptone prepared from casein was a very active growth stimulator for *L. casei* and *L. delbrückii* cultivated in a complete synthetic medium. In the light of current information on the impermeability of cell membranes to most phosphorylated intermediates [2] we were interested in a more detailed investigation of the utilization and growth stimulating activity of phosphopeptone and some phosphorylated amino acids.

EXPERIMENTS AND RESULTS

The microbiological procedures have previously been described in detail [3,4]. The total volume of medium was 5 ml per tube. The original medium (OM) of Steele *et al.*[5] was also used in the modified form (MM) recently described [6]. The microorganisms were *L. casei* (7469), *L. mesenteroides* P—60 (8042), and *L. delbrückii* (9595). Phosphopeptone as a rather complicated mixture of phosphopeptides [7] was obtained through the courtesy of professor O. Mellander, prepared according to the method outlined by him [8]. Phosphoserine and phosphothreonine were crystallized samples prepared in this laboratory from casein [9-11]. A synthetic sample of phosphothreonine [12] was also used. Phosphoglycine was prepared according to Winnick and Scott [13] as the magnesium salt.

In Table 1 a comparison is made of the growth effects of phosphopeptone on the three microorganisms cultivated in the medium of Steele *et al.*[5]. When compared with the corresponding control series where phosphopeptone was not present in the medium it is quite evident that *L. casei* and *L. delbrückii* were stimulated to a conspicous growth and acid production even by the addition of 0.1 mg of phosphopeptone per ml of medium. *L. mesenteroides* was not influenced at all by these additions.

Table 1. The growth stimulating effect of phosphopeptone on some lactobacilli. Addition of 1 mg and 0.1 mg of phosphopeptone per ml of medium.

Microorganism	Growth after							
	12 hours		24 hours		48 hours		72 hours	
	1 mg	0.1 mg	1 mg	0.1 mg	1 mg	0.1 mg	1 mg	0.1 mg
L. delbrückii	67*	25*	335*	118*	415*	280*	415*; 10.5**	315*; 7.6**
Control	7		29		83		129 ; 2.1	
L. casei	92	15	265	125	350	275	350 ; 10.0	300 ; 8.1
Control	7		20		85		170 ; 3.0	
L. mesenteroides P-60	230	224	290	283	350	350	365 ; 7.7	360 ; 7.7
Control	223		270		330		340 ; 7.6	

* Given as scale readings on the Klett-Summerson photoelectric colorimeter. The readings 0 corresponds to 100 % transmission. The values are mean values from three tubes and have been corrected for the blank values.

** Given as ml of 0.1 *N* NaOH to titrate 5 ml of final solution. The values are mean values from three tubes and have been corrected for the blank titrations.

Experiments were next made to find out if the esterified phosphate of the peptide mixture could be utilized when the inorganic phosphate, which normally must be present in the medium, was excluded. The results from a typical series are given in Table 2. There seemed to be a slight effect with 1 mg samples of phosphopeptone on the growth of *L. casei*. Calculation shows that the original medium contains 12.5 mg of potassium phosphate per 5 ml of

Table 2. The growth stimulating effect of phosphopeptone on L. casei and L. mesenteroides P-60 when inorganic phosphate was excluded from the medium. Addition of 1 mg and 0.1 mg per ml of medium.

Microorganism	Growth after							
	12 hours		24 hours		48 hours		72 hours	
	1 mg	0.1 mg	1 mg	0.1 mg	1 mg	0.1 mg	1 mg	0.1 mg
L. casei	1*	1*	1*	1*	11*	7*	24*; 0.6**	8*; 0**
Control without any phosphate at all	0		1		6		9 ; 0	
L. mesenteroides P-60	2	2	2	3	5	5	7 ; 0	7 ; 0
Control without any phosphate at all	2		3		3		3 ; 0	

* Given as scale readings on the Klett-Summerson photoelectric colorimeter. The readings 0 corresponds to 100 % transmission. The values are mean values from three tubes and have been corrected for the blank values.

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medium which corresponds to 2.4 mg of phosphorus. From 5 mg of calcium phosphopeptone only 0.2 mg of phosphorus will be available. This inadequate supply may be one explanation to the low effect observed, another may be the absence of potassium in the medium. Both possibilities will be further investigated.

The growth stimulating effects of some phosphorylated amino acids when added to the original medium of Steele *et al.*[5] or to the corresponding improved medium [6] is demonstrated by the figures of Table 3. When compared with the corresponding controls phosphoserine seemed to have a slight growth stimulating effect. This medium probably contains too little serine since the growth effect disappeared in the improved medium. Here both phosphoserine and phosphothreonine had a slight growth-inhibiting activity. If this is a parallel to the recent find of azaserine as a growth inhibitor of tumours and microorganisms [14] can at present not be decided. Of interest is that phosphoglycine which contains nitrogen-bound phosphorus had a slight stimulating effect on the growth and lactic acid production after 24 hours of incubation. With regard to the instability of this compound [13] it was aseptically added to the sterilized medium. Phosphoglycine had no growth stimulating effect after 4 or 8 hours of incubation. From these experiments the conclusion can be drawn that phosphopeptone does not stimulate the growth of lactobacilli through a preliminary digestion in the medium with the formation of the free phosphorylated amino acids.

Since these amino acids did not stimulate growth it was of interest to test their ability to replace the corresponding unphosphorylated amino acids. *L. mesenteroides* P-60 was used as test organism. In Fig. 1 some typical results with phosphoglycine are given. The samples of phosphorylated amino

Table 3. The effects of some phosphorylated amino acids on the growth of L. c a s e i. Additions of 1 mg and 0.1 mg of amino acid per ml of medium.

Amino acid	Growth after							
	24 hours		48 hours		72 hours			
	1 mg	0.1 mg	1 mg	0.1 mg	1 mg		0.1 mg	
L-Phosphoserine [1]	14*	14*	74*	48*	159*;	2.8**	116*;	1.9**
DL-Phosphothreonine [1]	15	16	57	58	117 ;	1.9	118 ;	1.8
Phosphoglycine [1]	59	22	—	—	—	—	—	—
Control [1]	16		17		124 ;	2.1		
L-Phosphoserine [2]	20	14	152	138	252 ;	5.5	224 ;	5.1
DL-Phosphothreonine [2]	21	16	171	128	240 ;	5.4	220 ;	4.6
Control [2]	25		203		284 ;	5.9		

[1] Medium of Steele *et al.* (4).

[2] Improved medium for *L. casei* (5).

* Given as scale readings on the Klett-Summerson photoelectric colorimeter. The readings 0 corresponds to 100 % transmission. The values are mean values from three tubes and have been corrected for the blank values.

** Given as ml of 0.1 *N* NaOH to titrate 5 ml of final solution. The values are mean values from three tubes and have been corrected for the blank titrations.

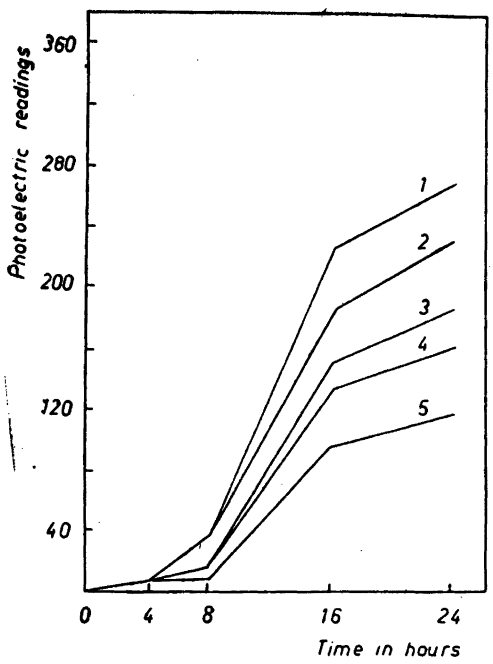

Fig. 1. Capacity of phosphorylated glycine to replace free glycine in the growth of L. mesenteroides P-60. 1. 20 μg of glycine. 2. 65 ug of Mg-phosphoglycine. 3. 5 μg of glycine. 4. 17 μg of Mg-phosphoglycine. 5. Control.

acids in this and the following experiments were calculated to yield the same amounts of unphosphorylated amino acids as the corresponding levels of free amino acids. From Fig. 1 it is evident that phosphoglycine is not utilized to the same degree as free glycine. The results with phosphoserine and phosphothreonine are given in Table 4. There is a striking difference between the utilization of phosphoserine and phosphothreonine. The first compound can

Table 4. The utilization of phosphoserine, phosphothreonine and the corresponding free amino acids by L. mesenteroides P-60. Incubation time 72 hours.

Amino acid	Lactic acid production by adding to the medium in μg			
	20	10	5	2.5
L-serine	11.5*	10.4*	9.5*	8.2
DL-serine (added in double amounts)	13.7	12.5	11.3	10.4
L-phosphoserine	11.8	10.7	10.0	8.8
L-threonine	9.4	5.2	2.8	0.9
DL-threonine (added in double amounts)	9.4	5.2	2.8	1.0
L-phosphothreonine	1.1	0.4	0.2	0.1

* Given as ml of 0.1 *N* NaOH to titrate 5 ml of final medium. The values are mean values from three tubes and have been corrected for the blank titrations.

replace serine as an essential amino acid for the growing microorganism while phosphothreonine can not be substituted for threonine. At present time it can not be decided if this dissimilarity originates in differences in cell permeability or in initial dephosphorylation of the two compounds in the medium. The series with racemic amino acids show as could be anticipated that *L. mesenteroides* can utilize D-serine but not D-threonine.

SUMMARY

Phosphopeptone is a very active growth stimulator for *L. casei* and *L. delbrückii* but not for *L. mesenteroides* P-60. This growth stimulating effect is not produced by the free phosphorylated amino acids of phosphopeptone. Phosphopeptone can to a certain degree be used in place of inorganic phosphate by *L. casei* but not by *L. mesenteroides*. When serine or threonine is excluded from the medium, the last mentioned microorganism can completely utilize phosphoserine for growth but only to a very slight degree phosphothreonine. Phosphoglycine, also, can not completely replace glycine for growth.

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Amine Catalysis in the Decarboxylation of Oxaloacetic Acid

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It is known that the decarboxylation of β-keto carboxylic acids is catalysed by primary amines, but a satisfactory explanation of the reaction has not been given. The aim of this paper is to give a contribution to the solution of this problem by studying the amine catalysis in the decarboxylation of oxaloacetic acid. Three catalysts, ammonium ions, ethylammonium ions, and anilinium ions were examined in solvents of different acidity: solutions of hydrogen chloride and acetate buffer solutions. By an analysis of the results, separate rate constants were determined for each of the three simultaneous reactions: decomposition of undissociated oxaloacetic acid, of univalent hydrogenoxaloacetate ion, and of divalent oxaloacetate ion.

It was found that the uncatalysed decomposition of the univalent ion is accelerated by acetate ions. Aminium ions catalyse the decomposition of both the univalent and the divalent oxaloacetate ion. The former (and perhaps also the latter) catalysis is further accelerated by acetate ions. The anilinium ion is a much more effective catalyst than the two other ions, especially in the decarboxylation of the divalent oxaloacetate ion. It has not been possible to explain an apparent disagreement between the results on anilinium-ion catalysis in acetate buffers and in solutions of hydrogen chloride.

It is concluded from the experiments that aminium ions form reactive intermediates with the di- and uni-valent oxaloacetate ion (and possibly also with the undissociated acid), *e. g.*, by reactions of the type $>CO + NRH_3^+ \rightleftharpoons >CNRH^+ + H_2O$. It has in all computations been assumed that the concentrations of such complexes are negligible. Results of glass-electrode measurements reported in the last part of the paper are in agreement with this assumption.

The decomposition of oxaloacetic acid in aqueous solution into pyruvic acid and carbon dioxide is catalysed by certain metal ions and by primary amines. The uncatalysed reaction and the metal-ion catalysis were examined in an earlier paper [1], and a study of the amine catalysis is the object of the present paper. It is a general rule that the decarboxylation of β-keto carboxylic acids is catalysed, within certain pH-limits, by primary amines. This catalysis has been most thoroughly examined on acetoacetic acid (Widmark and Jeppsson [2], Ljunggren [3], Beniya [4]) and on α,α-dimethyl acetoacetic acid (Pedersen [5], Westheimer and Jones [6]). The catalysis of various amines and amino acids

in the decarboxylation of oxaloacetic acid has been studied by Kaneko [7] and by Bessman and Layne [8]. A satisfactory explanation of the amine catalysis has not been given. The author of the present paper has shown [9] that the decarboxylation is not preceded by an isomerisation of the keto-acid into an enol-form, and it has been suggested that a reactive intermediate is formed by a fast reaction of the amine with the keto-group, *e. g.*,

$$CH_3COCH_2COO^- + NRH_3^+ \rightleftharpoons CH_3C(NRH^+)CH_2COO^- + H_2O$$

This suggestion has been examined and modified by Westheimer [6,10].

In the present paper, three catalysts, ammonium ions, ethylammonium ions, and anilinium ions, were examined in solutions of various acidities. Since oxaloacetic acid is present in the solutions partly in the form of undissociated acid H_2Ox and partly in the form of the ions HOx^- and Ox^{--} (degree of dissociation α_1 and α_2, respectively), the rate of decarboxylation may be expressed as

$$-\frac{dx}{dt} = k_0\,[H_2Ox] + k_1\,[HOx^-] + k_2\,[Ox^{--}] = [k_0(1-\alpha_1-\alpha_2) + k_1\alpha_1 + k_2\alpha_2]x$$

where at least one of the coefficients depends on the nature and concentration of the catalyst. When the hydrogen ion concentration is constant, the reaction will be of first order, and the rate constant will be

$$k = k_0(1-\alpha_1-\alpha_2) + k_1\alpha_1 + k_2\alpha_2 \qquad (1)$$

The solutions examined may be divided into two groups: highly acid solutions (where $\alpha_2 < 0.013$) and weakly acid solutions (where $\alpha_2 > 0.5$). The solvents to which oxaloacetic acid was added were in the former group water or solutions of hydrogen chloride, in the latter group buffer solutions of acetic acid and sodium acetate. The solutions contained, in addition, the chloride of the catalyst and potassium chloride up to an ionic strength of approximately 0.3. The temperature was 37.0° C, and the experimental method the same as in the earlier paper [1]. The first-order law was always found to hold for the highly acid solutions. In agreement with this, glass-electrode measurements [11] had shown that pH of a solution of oxaloacetic acid in pure water remains practically unchanged during the complete reaction. In the experiments on weakly acid solutions, small deviations from first-order law were observed. This is explained by a gradual change in pH, and consequently also in α_1 and α_2, taking place when oxaloacetic acid decomposes. Here k_∞, the rate constant corresponding to the final composition of the solution, was computed by means of a formula deduced in the earlier paper [1]. This formula could, however, not be applied to the experiments on anilinium ion catalysis since, here, not only α_1 and α_2 but, owing to displacement of the equilibrium: $AnH^+ \rightleftharpoons An + H^+$, also the coefficients k_0, k_1, and k_2 change during the reaction.

In accordance with practice in previous papers, the rate constants marked * are defined in terms of decadic logarithms ($k^* = 0.4343\ k$), and the time unit is the minute. The degrees of dissociation α_1 and α_2 of oxaloacetic acid were calculated by means of the two dissociation constants of the acid obtained from

glass-electrode measurements [11]. In all calculations in this paper, it has been assumed that the concentrations of possible complexes of the amines with oxaloacetic or pyruvic acid are negligible. This assumption is supported by electrometric measurements on solutions of aniline and the acids (reported in the last part of this paper).

Materials. The oxaloacetic and pyruvic acid were of the same purity as the preparations used in earlier papers [1,11]. The other chemicals were purified either by distillation or by recrystallisation from redistilled water. Standard solutions of ethylammonium chloride and anilinium chloride were prepared from the pure bases and standard hydrochloric acid. The anilinium chloride solution was tested for excess or deficiency of hydrochloric acid by measurements with the glass electrode (see later). All solutions were made with water redistilled in an all-glass still.

EXPERIMENTAL RESULTS ON HIGHLY ACID SOLUTIONS

Uncatalysed reaction (Table 1, first 4 lines). When equation 1 is applied, and the last (small) term is calculated by means of $k_2^* \cdot 10^3 = 1.81$ (taken from experiments on weakly acid solutions), it is found that the following values of the two other coefficients

$$k_0^* \cdot 10^3 = 0.176 \text{ and } k_1^* \cdot 10^3 = 6.47 \qquad (2)$$

give the best agreement with k^* found by experiment. When k^* is calculated from equation 1 and the values of the coefficients given here, the results shown in the next to the last column of Table 1 are obtained.

Table 1. Decomposition of oxaloacetic acid (initially 0.0150 molar) at 37° C in the solvent: h molar hydrogen chloride, c molar ammonium chloride, (0.300 − h − c) molar potassium chloride. The data in the last column refer to solutions containing in addition 0.418 molar acetic acid.

h	c	a_1	a_2	$k^* \cdot 10^3$	$k^* \cdot 10^3$ calc.	$k^* \cdot 10^3$ acet. acid
0.0000	0.000	0.4841	0.0126	3.243	3.244	
0.01995	0.000	0.2352	0.0020	1.665	1.660	1.673
0.0499	0.000	0.1229	0.0005	0.942	0.950	0.963
0.0999	0.000	0.0670	0.0001	0.600	0.599	
0.0000	0.100	0.4841	0.0126	3.359	3.355	
0.0000	0.200	0.4841	0.0126	3.467	3.467	
0.01995	0.200	0.2352	0.0020	1.740	1.742	1.759
0.0499	0.200	0.1229	0.0005	0.987	0.990	1.009
0.0999	0.200	0.0670	0.0001	0.620	0.619	
0.0000	0.300	0.4841	0.0126	3.578	3.578	

Ammonium-ion catalysis (Table 1, last 6 lines). When the first term of equation 1 is calculated by means of $k_0^* \cdot 10^3 = 0.176$, and the last term by means of the values of k_2^* obtained from experiments on ammonium-ion catalysis in acetate buffers (equation 10), we get agreement with the experimental results when we set

$$k_1^* \cdot 10^3 = 6.47 + 1.47\ [NH_4^+] \qquad (3)$$

This is seen from the next to the last column where k^* calculated by means of the equation is given.

Effect of acetic acid. Addition of acetic acid has only a small effect on k^* as shown in the last column of Table 1.

Ethylammonium-ion catalysis (Table 2). When $k_0{}^*10^3 = 0.176$ and values of $k_2{}^*$ obtained from experiments on ethylammonium-ion catalysis in acetate

Table 2. Decomposition of oxaloacetic acid (initially 0.0150 molar) at 37° C in the solvent: h molar hydrogen chloride, c molar ethylammonium chloride, (0.300 − h − c) molar potassium chloride.

h	c	a_1	a_2	k^*10^3	$k_1{}^*10^3$	$k_1{}^*10^3$ equ. 4
0.0000	0.100	0.4841	0.0126	3.605	7.10	7.10
0.01995	0.100	0.2352	0.0020	1.816	7.10	7.10
0.0000	0.200	0.4841	0.0126	3.994	7.79	7.73
0.01995	0.200	0.2352	0.0020	1.966	7.70	7.73

buffers (equation 11) are introduced into equation 1, we calculate the values of $k_1{}^*$ given in the next to the last column of Table 2. They may be expressed by the following linear relation

$$k_1{}^*10^3 = 6.47 + 6.3\ [C_2H_5NH_3{}^+] \qquad (4)$$

Values calculated by means of this equation are given in the last column.

Anilinium-ion catalysis. The composition of the solutions examined and the rate constants found are given in Table 3. An analysis of the data shows that the catalytic effect of the anilinium ion on the decomposition of the divalent oxaloacetate ion is so enormous that its contribution to the total rate is large, although $a_2 < 0.013$. The experiments fall in four groups. Within each group, the anilinium ion concentration is constant while the hydrogen ion concentration varies. For each group the coefficients $k_0{}^*$, $k_1{}^*$, and $k_2{}^*$ of equation 1 are determined by the method of least squares. The results are given in Table 3a. They may be expressed by the formulae

$$k_0{}^*10^3 = 0.176 + 1.19\ [C_6H_5NH_3{}^+] \qquad (5)$$

$$k_1{}^*10^3 = 6.47 + 404\ [C_6H_5NH_3{}^+] + 260\ [C_6H_5NH_3{}^+]^2 \qquad (6)$$

$$k_2{}^* = 0.00181 + 38.4\ [C_6H_5NH_3{}^+] + 55\ [C_6H_5NH_3{}^+]^2 \qquad (7)$$

The increase of $k_0{}^*$ has, however, so small an influence on the total rate that it is uncertain whether it is significant. When the coefficients are calculated by means of equations 5, 6, and 7, the values given in the 3rd, 5th, and 7th column of Table 3a are obtained. In the last column of Table 3 are shown rate constants calculated by means of equation 1 and the values of the coefficients computed from equations 5, 6, and 7.

EXPERIMENTAL RESULTS ON WEAKLY ACID SOLUTIONS

In this section are reported results of measurements on solutions containing an acetate buffer system. Since, here, only a very small fraction (less than 1.2 %) of the oxaloacetic acid is undissociated and, further, k_0 is always much smaller than k_1 and k_2, we may neglect the first term of equation 1 and write

Table 3. Decomposition of oxaloacetic acid (initially 0.0150 molar) at 37° C in the solvent: h molar hydrogen chloride, c molar anilinium chloride, (0.300 − h − c) molar potassium chloride.

h	c	a_1	$a_1 10^3$	$k^* 10^3$	$k^* 10^3$ calc.
0.00000	0.0100	0.4808	12.41	10.06	10.05
0.00993	0.0100	0.3247	4.28	5.213	5.227
0.02043	0.0100	0.2316	1.90	3.329	3.329
0.02990	0.0100	0.1813	1.09	2.490	2.490
0.04985	0.0100	0.1228	0.47	1.640	1.637
0.09985	0.0100	0.0670	0.13	0.926	0.927
0.00988	0.0200	0.3250	4.29	8.242	8.265
0.02038	0.0200	0.2318	1.91	5.076	5.049
0.0498	0.0200	0.1228	0.47	2.334	2.342
0.0998	0.0200	0.0670	0.13	1.272	1.270
0.00474	0.0500	0.3940	7.05	25.36	25.47
0.00973	0.0500	0.3260	4.32	18.06	17.97
0.02023	0.0500	0.2325	1.92	10.49	10.48
0.04965	0.0500	0.1231	0.47	4.519	4.524
0.0996	0.0500	0.0671	0.13	2.314	2.316
0.1994	0.0500	0.0349	0.03	1.253	1.252
0.00948	0.1000	0.3276	4.37	35.35	35.55
0.01444	0.1000	0.2765	2.89	26.75	26.57
0.01998	0.1000	0.2336	1.94	20.40	20.34
0.02945	0.1000	0.1827	1.11	14.20	14.20
0.0494	0.1000	0.1236	0.47	8.48	8.50
0.0994	0.1000	0.0672	0.13	4.205	4.208
0.1490	0.1000	0.0461	0.06	2.851	2.851
0.1992	0.1000	0.0350	0.03	2.186	2.186

Table 3a.

c	$k_0^* 10^3$	$k_0^* 10^3$ calc.	$k_1^* 10^3$	$k_1^* 10^3$ calc.	k_2^*	k_2^* calc.
0.0100	0.182	0.188	10.53	10.54	0.394	0.392
0.0200	0.198	0.200	14.67	14.65	0.785	0.792
0.0500	0.243	0.236	27.08	27.3	2.079	2.06
0.1000	0.295	0.295	50.0	49.5	4.34	4.39

Table 4. Decomposition of oxaloacetic acid (initially 0.0150 molar) at 37° C in the solvent. a molar acetic acid, b molar sodium acetate, (0.300 − b) molar potassium chloride.

a	b	$a_1\infty$	$a_2\infty$	$k^*\infty 10^3$	$k^*\infty 10^3$ calc.	
0.1043	0.2991	0.064	0.936	2.16	2.16	
0.2087	0.2991	0.113	0.887	2.42	2.43	$k_1^* = 7.40 \times 10^{-3}$
0.3125	0.2991	0.157	0.842	2.70	2.68	$k_2^* = 1.80 \times 10^{-3}$
0.4174	0.2991	0.198	0.801	2.90	2.90	
0.1045	0.1993	0.095	0.905	2.37	2.35	
0.2090	0.1993	0.164	0.835	2.70	2.73	$k_1^* = 7.33 \times 10^{-3}$
0.4181	0.1993	0.275	0.722	3.36	3.34	$k_2^* = 1.83 \times 10^{-3}$
0.6275	0.1993	0.359	0.636	3.79	3.79	
0.0522	0.0997	0.114	0.886	2.42	2.40	
0.1045	0.0997	0.186	0.813	2.77	2.79	$k_1^* = 7.12 \times 10^{-3}$
0.2090	0.0997	0.298	0.698	3.36	3.38	$k_2^* = 1.80 \times 10^{-3}$
0.4181	0.0997	0.448	0.542	4.20	4.16	

$$k = k_1\alpha_1 + k_2\alpha_2 \quad (8)$$

Since α_1 and α_2 change a little during the reaction, we shall, where it is possible, compute k_∞, $\alpha_{1\infty}$, and $\alpha_{2\infty}$, the values for solutions where (practically) all oxaloacetic acid has been transformed into pyruvic acid. Inserting them into equation 8, we get

$$k_\infty = k_1\alpha_{1\infty} + k_2\alpha_{2\infty} \quad (9)$$

In order to calculate α_1 and α_2, it is necessary to know with good accuracy the ratio K_{HAc}/K_2, where K_{HAc} is the dissociation constant of acetic acid and K_2 the second dissociation constant of oxaloacetic acid. In the earlier paper [1], k_2^* for the uncatalysed reaction was computed without using this ratio. Rate constants were determined for a series of solutions which differed only with respect to the concentration of free acetic acid. The reasonable assumption was made that k_1^* has the same value here as in solutions of hydrochloric acid. On the basis of equation 9, a formula was deduced which made it possible to determine k_2^* by extrapolation and, in addition, obtain a value of K_{HAc}/K_2. The latter did, however, not agree well with that found from electrometric measurements. For 0.3 molar sodium chloride at 37° C, it was estimated from measurements by Harned and Hickey [12] that $K_{HAc} = 3.20 \times 10^{-5}$, and glass-electrode measurements [11] have shown that $K_2 = 1.97 \times 10^{-4}$. Hence, $K_{HAc}/K_2 = 0.162$, while the much higher value 0.197 was found from the kinetic measurements for 0.3 molar sodium acetate. The discrepancy was confirmed in this paper, and experiments on amine catalysis have decisively shown that it is not permissible to assume that k_1^* has the same value for acetate buffer solutions as for solutions acidified with hydrochloric acid. In the following, we shall therefore, for the solutions examined, which have all an ionic strength of approximately 0.3, adopt the value 0.162 for the ratio K_{HAc}/K_2. By means of this value, we shall calculate $\alpha_{1\infty}$ and $\alpha_{2\infty}$, and then, by means of equation 9, determine k_1^* and k_2^*. Fortunately, practically the same value of k_2^* for the uncatalysed reaction was obtained by the former and the latter method (1.80×10^{-3} and 1.81×10^{-3}, respectively).

Uncatalysed reaction. In the three groups of solutions examined (Table 4), the concentration of sodium acetate (before addition of oxaloacetic acid) was 0.3, 0.2, and 0.1 molar, respectively. The rate constants found from the measurements are given in column 5. When equation 9 is applied to each group separately, the values of k_1^* and k_2^* shown in the last column are obtained. Introducing them into equation 9, we calculate the values of k^*_∞ presented in column 6. As seen from the last column, k_2^* is the same for all three groups (mean 1.81×10^{-3}), while k_1^* increases with increasing acetate ion concentration and is considerably greater than the value 6.47×10^{-3} found for the highly acid solutions. If the latter value of k_1^* were adopted, a recalculation of the data in Table 4 by the old method [1] would give $K_{HAc}/K_2 = 0.206$, a value which is much higher than that derived from electrometric measurements (0.162).

Ammonium-ion catalysis. Within each of the four groups of experiments (see Table 5), both b and c are constant. When equation 9 is applied to each group separately, we find the values of k_1^* and k_2^* shown in the third and

Table 5. Decomposition of oxaloacetic acid (initial concentration 0.0150 molar, except in the experiments marked x and y where it was 0.0100 and 0.0200 molar, respectively) at 37° C in the solvent: a molar acetic acid, b molar sodium acetate, c molar ammonium chloride, (0.300 − b − c) molar potassium chloride.

	a	b	c	$a_{1\infty}$	$a_{2\infty}$	$k^*_\infty 10^3$	$k^*_\infty 10^3$ calc.
	0.1045	0.1993	0.050	0.095	0.905	3.86	3.83
	0.4181	0.1993	0.050	0.275	0.722	4.59	4.66
	0.6275	0.1993	0.050	0.359	0.636	5.07	5.04
	0.1045	0.1993	0.100	0.095	0.905	5.33	5.32
x	0.2090	0.1993	0.100	0.158	0.841	5.49	5.52
	0.2090	0.1993	0.100	0.164	0.835	5.62	5.54
y	0.2090	0.1993	0.100	0.171	0.828	5.65	5.57
	0.4181	0.1993	0.100	0.275	0.722	5.80	5.89
	0.6275	0.1993	0.100	0.359	0.636	6.15	6.15
	0.1045	0.0997	0.100	0.186	0.813	5.49	5.48
	0.2090	0.0997	0.100	0.298	0.698	5.73	5.75
	0.0522	0.0997	0.200	0.114	0.886	8.25	8.25
	0.1045	0.0997	0.200	0.186	0.813	8.21	8.21
	0.2090	0.0997	0.200	0.299	0.698	8.03	8.14
	0.4181	0.0997	0.200	0.448	0.542	8.14	8.03

Table 5a.

b	c	$k_1^*10^3$	$k_2^*10^3$	$k_1^*10^3$ equ. 3	$k_2^*10^3$ equ. 10
0.1993	0.050	8.03	3.39	6.54	3.42
0.1993	0.100	8.24	5.02	6.62	5.02
0.0997	0.100	7.52	5.02	6.62	5.02
0.0997	0.200	7.87	8.30	6.78	8.23

fourth column of Table 5a. Introducing them into equation 9, we calculate the values of k_2^* presented in the last column of Table 5. The coefficient k_2^* is independent of the acetate ion concentration and may be represented by the following linear function of the ammonium ion concentration

$$k_2^*10^3 = 1.81 + 32.1\ [NH_4^+] \qquad (10)$$

k_2^* calculated by means of this equation is given in the last column of Table 5a. Values of k_1^* calculated from equation 3, which was found to hold for acetate-free solutions, are shown in the next to the last column. They are much lower than those found for acetate buffers (column 3). The discrepancy cannot be explained away by an error in the value of the ratio K_{HAc}/K_2. If we, instead of 0.162, apply 0.206 (see above), the values computed for $\alpha_{1\infty}$ and $\alpha_{2\infty}$ will change considerably, but those for k_1^* only little, and they will still be much higher than those calculated from equation 3.

Ethylammonium-ion catalysis. When equation 9 is applied to each of the four groups of measurements presented in Table 6, the values of k_1^* and k_2^* shown in the fourth and fifth column of Table 6a are found. Introducing them into equation 9, we calculate the values of k^*_∞ given in the last column of Table 6. The coefficient k_2^* is nearly a linear function of the ethylammonium

Table 6. Decomposition of oxaloacetic acid (initially 0.0150 molar) at 37° C in the solvent: a molar acetic acid, b molar sodium acetate, c molar ethylammonium chloride, (0.300 − b − c) molar potassium chloride.

a	b	c	$a_{1\infty}$	$a_{2\infty}$	$k^*_\infty 10^3$	$k^*_\infty 10^3$ calc.
0.1043	0.1993	0.0500	0.095	0.905	4.67	4.68
0.2085	0.1993	0.0500	0.164	0.835	4.96	4.95
0.6260	0.1993	0.0500	0.358	0.636	5.72	5.72
0.1045	0.1993	0.1000	0.095	0.905	7.04	7.11
0.2090	0.1993	0.1000	0.164	0.835	7.36	7.30
0.4181	0.1993	0.1000	0.275	0.722	7.60	7.59
0.6275	0.1993	0.1000	0.359	0.636	7.79	7.81
0.1042	0.0997	0.1000	0.185	0.814	6.94	6.95
0.2087	0.0997	0.1000	0.298	0.698	7.14	7.14
0.4175	0.0997	0.1000	0.448	0.542	7.38	7.39
0.0421	0.0997	0.2002	0.098	0.901	11.13	11.17
0.1042	0.0997	0.2002	0.185	0.814	11.16	11.10
0.2089	0.0997	0.2002	0.298	0.698	11.04	10.98
0.4176	0.0997	0.2002	0.447	0.543	10.74	10.79

Table 6a.

b	c	$[Ac^-]_\infty$	$k_1^* 10^3$	$k_2^* 10^3$	$k_1^* 10^3$ equ. 4	$k_2^* 10^3$ equ. 11
0.1993	0.0500	0.184	8.35	4.29	6.78	4.32
0.1993	0.1000	0.184	9.61	6.85	7.10	6.83
0.0997	0.1000	0.085	8.51	6.60	7.10	6.56
0.0997	0.2002	0.085	10.47	11.26	7.72	11.32

ion concentration but it seems to increase a little also with increasing acetate ion concentration. The latter effect is, however, so small that it is uncertain whether it is significant. The values found for k_2^* may be expressed by the following equation

$$k_2^* \ 10^3 = 1.81 + (45.2 + 27 \ [Ac^-]) \ [C_2H_5NH_3^+] \qquad (11)$$

k_2^* calculated by means of this equation is given in the last column of Table 6a. The acetate buffer has a large influence on k_1^*. If k_1^* is calculated from equation 4, which holds for acetate-free solutions, the values given in the next to the last column are obtained. They are much lower than those found for acetate buffers (column 4). It has, however, not been possible to express the effect of acetate on k_1^* by means of a formula.

Anilinium-ion catalysis. As already mentioned, it was here too difficult to correct for the change in pH due to the reaction, but when the kinetic data were plotted in the usual way, only small deviations from the first-order law were found. The rate constants given in the next to the last column of Table 7, therefore, correspond to an average composition of the reacting solution. The values given for the concentrations of acetate ion $[Ac^-]$ and anilinium ion $[AnH^+]$, and for α_1 and α_2, are the averages of their initial and final values. For the dissociation constant of the anilinium ion we have used the value

Table 7. Decomposition of oxaloacetic acid (initially 0.0150 molar) at 37° C in the solvent: a molar acetic acid, b molar sodium acetate, (0.300 − b) molar potassium chloride, 1.000 × 10⁻³ molar anilinium chloride. In the experiments marked x and y, the concentration of anilinium chloride was 0.500 × 10⁻³ and 2.000 × 10⁻³, respectively.

	a	b	$[Ac^-]$	$[AnH^+]10^3$	a_1	a_2	k^*10^3	k^*10^3 calc.
	0.1043	0.2991	0.276	0.294	0.069	0.931	7.08	7.07
x	0.2087	0.2991	0.277	0.215	0.119	0.881	5.80	5.83
	0.2087	0.2991	0.277	0.432	0.119	0.881	9.29	9.34
y	0.2087	0.2991	0.276	0.864	0.119	0.880	16.44	16.29
	0.3125	0.2991	0.277	0.523	0.163	0.836	10.65	10.70
	0.4174	0.2991	0.278	0.589	0.204	0.795	11.62	11.59
	0.1043	0.1998	0.178	0.393	0.104	0.896	8.54	8.55
	0.2087	0.1998	0.178	0.540	0.173	0.826	10.71	10.72
	0.4174	0.1998	0.179	0.690	0.283	0.714	12.54	12.49
	0.1043	0.0999	0.079	0.592	0.206	0.793	11.18	11.18
	0.2087	0.0999	0.080	0.723	0.317	0.679	12.45	12.53
	0.4174	0.0999	0.081	0.830	0.461	0.528	12.96	12.98

3.52×10^{-5} which, according to glass-electrode measurements [13], holds for 0.3 molar sodium chloride at 37° C. An analysis of the data given in Table 7 has shown that the rate constants may be expressed by equation 8 when the coefficients have values given by the formulae

$$k_1^*10^3 = 6.47 + 4.4 \times 10^3\ [AnH^+] \qquad (12)$$

$$k_2^*10^3 = 1.81 + (16.4 + 4.8\ [Ac^-])\ 10^3\ [AnH^+] \qquad (13)$$

where k_1^* and k_2^* for the uncatalysed reaction have, in advance, been given values found earlier in this paper. Rate constants calculated by means of these equations are presented in the last column of Table 7.

There is a serious discrepancy between the results of the anilinium-ion catalysis obtained here and those found for solutions containing hydrochloric acid (compare equations 12 and 13 with equations 6 and 7). When rate constants for the solutions presented in Table 7 are calculated by means of equations 6 and 7, the values obtained are from 64 to 93 % too high. An attempt was made to explain this disagreement in the following way. It has hitherto in all the computations been assumed that the concentrations of reactive intermediates is negligible, but this may not be true in all cases. If a considerable fraction of the anilinium salt added to the acetate buffers were bound to oxaloacetate ions, the concentrations of free anilinium ion would be lower than shown in Table 7, and this might possibly explain the unexpectedly low rate found here. An examination showed, however, that it was not possible to explain the results of experiments on both acetate buffers and solutions of hydrochloric acid in this way. It has further been shown by electrometric measurements on solutions of oxaloacetic acid and aniline (reported in the last part of this paper) that the concentrations of possible complexes of this type are too small to explain the discrepancy.

CONCLUSIONS OF KINETIC EXPERIMENTS

The rate constants for the uncatalysed decarboxylation of the undissociated oxaloacetic acid, the univalent ion, and the divalent ion were found to be $k_0^* = 0.176 \times 10^{-3}$, $k_1^* = 6.47 \times 10^{-3}$, and $k_2^* = 1.81 \times 10^{-3}$, respectively. Higher values of k_1^* were found when the solvent contained an acetate buffer system. It seems as if acetate ions (or perhaps bases in general) catalyse the decomposition of the univalent oxaloacetate ion, but the experimental material is not large and accurate enough to draw a definite conclusion.

Table 8 gives a summary of the catalytic effects of the amines examined. It is seen that they have all a greater effect on the divalent ion than on the

Table 8. Catalytic coefficients.

Catalyst, A^+	Solvent	$\frac{(k_1^* - 6.47)10^3}{[A^+]}$	$\frac{(k_2^* - 1.81)10^3}{[A^+]}$
NH_4^+	Hydrochloric acid	1.47	(32.1)
	Acetate buffer	7.0 to 31.2	32.1
$C_2H_5NH_3^+$	Hydrochloric acid	6.3	(45.2)
	Acetate buffer	20.0 to 37.6	45.2 + 27 [Ac^-]
$C_6H_5NH_3^+$	Hydrochloric acid	404	38 400
	Acetate buffer	4 400	16 400 + 4 800 [Ac^-]

univalent ion, and that the anilinium ion is a much more effective catalyst than the ammonium and the ethylammonium ions. The reaction of the univalent oxaloacetate ion is undoubtedly catalysed more strongly by amines in acetate buffers than in solutions of hydrochloric acid. In the amine catalysis of the divalent ion, the effect of acetate ions is less certain, and, with the anilinium ion as catalyst, much lower values were found for acetate buffers than for solutions of hydrochloric acid, a result to which it has not been possible to give any explanation. It may be concluded from the experiments on amine catalysis that the aminium ions form reactive intermediates with both the univalent and the divalent oxaloacetate ion, and that the decarboxylation of (at least) the former is accelerated by acetate ions.

GLASS-ELECTRODE MEASUREMENTS

The measurements were carried out at the temperature 25° C and by the method described in a previous paper [11]. The cells had the composition

Pt | S, quinhydrone | glass | X | 3.5 *M* KCl | S, quinhydrone | Pt

where S denotes 0.00999 *M* HCl, 0.0900 *M* NaCl and X the solution under investigation. When E volts is the e.m.f. of the cell (after subtraction of a small asymmetry potential) and $[H^+]$ the hydrogen ion concentration of solution X, we have

$$-\log [H^+] = A + 16.90\ E \qquad (14)$$

Table 9. Determination of A (equ. 14) for solutions of the composition: c molar anilinium chloride, (0.300 — c) molar sodium chloride, h molar hydrogen chloride at 25° C.

c	h	$-\log [H^+]$	E	A	A (mean)
0.1000	0.01921	1.714	−0.0182	2.021	
0.1000	0.00922	2.024	0.00005	2.023	2.023
0.1000	0.004215	2.327	0.0179	2.024	
0.0500	0.00961	2.012	−0.00055	2.022	2.021
0.0500	0.004600	2.316	0.0175	2.020	

By means of this equation we may find $[H^+]$ when A is known or A when $[H^+]$ is known.

First A was determined for solutions containing anilinium chloride and small concentrations of hydrogen chloride (Table 9). In the calculation of $[H^+]$ from the composition of the solutions, it is sufficient to know an approximate value of the dissociation constant of the anilinium ion. The values applied were 2.33×10^{-5} when c was 0.1 and 2.26×10^{-5} when c was 0.05. A calculated by means of equation 14 is shown in the two last columns of the table.

The measurements presented in Table 10 were carried out in order to determine the hydrochloric acid deficiency in a standard 1.000 molar anilinium

Table 10. Electrometric determination of the hydrochloric acid deficiency in a standard solution of anilinium chloride.

$h\ 10^3$	E	$[H^+]10^5$	$y\ 10^5$	$y\ 10^5$ (calc.)
−1.006	0.0617	86.0	1.648	1.648
0.000	0.0537	117.4	1.405	1.406
1.024	0.0449	165.3	1.054	1.037
2.047	0.03365	227.8	0.532	0.556

chloride prepared from pure aniline and hydrochloric acid. By dilution of the standard solution were prepared a 0.1000 molar "anilinium chloride", 0.200 molar sodium chloride and solutions containing in addition small concentrations of hydrogen chloride (h molar) or sodium hydroxide ($-h$ molar). If a fraction ε of the aniline in the standard solution is not neutralised, we have for the diluted solutions, when K is the dissociation constant of the anilinium ion,

$$[H^+]\ ([H^+] + 0.1000\varepsilon - h) = K\ (0.1000(1-\varepsilon) - [H^+] + h)$$

which may also be written in the form

$$y = K - \varepsilon([H^+] + K) \tag{15}$$

where

$$y \equiv ([H^+] + K)\ ([H^+] - h) / 0.1000 \tag{16}$$

The hydrogen ion concentrations given in the third column of Table 10 are calculated from E by means of equation 14 and $A = 2.023$ found in Table 9. Introducing them into equation 16, we calculate y given in the next to the last column. We get the best agreement with equation 15 when $K = 2.33 \times 10^{-5}$ and $\varepsilon = 0.0077$. When y is calculated by means of equation 15 and these

Table 11. Determination of the dissociation constant K of the anilinium ion at 25° C in solutions of the composition: d molar aniline, 0.0500 molar hydrogen chloride or pyruvic acid, 0.250 molar sodium chloride.

	Hydrogen chloride			Pyruvic acid		
d	E	$-\log [H^+]$	$-\log K$	E	$-\log [H^+]$	$-\log K$
0.0750	0.1382	4.357	4.657	0.1391	4.372	4.663
0.1000	0.1557	4.652	4.652	0.15625	4.662	4.658
0.1500	0.17355	4.954	4.653	0.17385	4.959	4.657
		Mean:	4.654		Mean:	4.659

values of K and ε, we obtain the results shown in the last column. As a result of the examination we have found that the standard solution had a hydrochloric acid deficiency of 0.77 % and that $K = 2.33 \times 10^{-5}$ (compare $K = 2.16 \times 10^{-5}$ found for 0.300 molar sodium chloride in a previous paper [13]).

In order to examine whether aniline or the anilinium ion form complexes with the pyruvate ion in dilute solution, measurements were carried out on solutions of aniline partly neutralised with either hydrogen chloride or pyruvic acid. The composition of the solutions and the results obtained are shown in Table 11. The dissociation constant of the anilinium ion was calculated from the measurements on the assumption that no complexes are formed. A correction has been made for the concentration of free pyruvic acid by means of the dissociation constant 6.37×10^{-3} for pyruvic acid [11]. Since practically the same values of K are found for solutions without and with pyruvate, we conclude that complexes are not formed to a measurable extent.

Measurements of the same kind with oxaloacetic acid instead of pyruvic acid were much more difficult and less accurate owing to the rapid decomposition of oxaloacetate in the presence of aniline. An attempt was, however, made (Table 12). E_0 given in the third column of the table was found by

Table 12. Determination of the dissociation constant K of the anilinium ion at 25° C in solutions of the composition: d molar aniline, x_0 molar oxaloacetic acid, 0.300 molar sodium chloride.

d	x_0	E_0	$\left(\frac{dE}{dt}\right)_0$	$-\log[H^+]_0$	$[HOx^-]_0$	$[Ox^{--}]_0$	$-\log K$
0.00999	0.00297	0.1513	0.0014	4.571	0.00038	0.00259	4.67
0.01499	0.00299	0.1691	0.0012	4.872	0.00020	0.00279	4.67
0.01499	0.00600	0.1348	0.0031	4.293	0.00128	0.00471	4.68
0.02000	0.00600	0.1518	0.0026	4.580	0.00074	0.00526	4.69
						Mean:	4.68

extrapolation of the observed E values to the moment when the oxaloacetic acid was dissolved. The fourth column shows the increase of E in volts per minute at the beginning of the reaction. The next column gives $-\log[H^+]_0$ calculated from E_0 and values of A for 0.3 molar sodium chloride found earlier [11]. The initial concentrations of $[HOx^-]$ and $[Ox^{--}]$ were computed by means of the values $K_1 = 6.0 \times 10^{-3}$ and $K_2 = 1.87 \times 10^{-4}$ for the

Table 13. Determination of the dissociation constants of pyruvic and oxaloacetic acid at 25° C from glass electrode measurements on solutions of the composition: x_0 molar pyruvic or oxaloacetic acid, b molar sodium hydroxide, c molar anilinium chloride, (0.300 − c) molar sodium chloride.

		−log K for pyruvic acid			−log K_1 for oxaloacetic acid		
x_0	b	c = 0.0000	c = 0.0500	c = 0.1000	c = 0.0000	c = 0.0250	c = 0.0500
0.0100	0.0000	2.174	2.193	2.191	2.225	2.230	2.234
0.0100	0.0050	2.186	2.199	2.187	2.217	2.224	
0.0200	0.0000	2.175	2.182	2.183			
0.0200	0.0100	2.180	2.191	2.192			
	Mean:	2.179	2.191	2.188	2.221	2.227	2.234

first and second dissociation constant of oxaloacetic acid [11], and the initial concentration of anilinium ion by means of the equation $[AnH^+]_0 = [HOx^-]_0 + 2[Ox^{--}]_0 - [H^+]_0$. Finally, the values of −log K given in the last column of the table were found. They are so close to those found in Table 11 that we conclude, considering the lower accuracy, that the concentrations of the complexes are too small to be estimated by this method.

Table 13 gives a summary of a determination of the dissociation constant of pyruvic acid and the first dissociation constant of oxaloacetic acid in 0.300 molar sodium chloride and in solutions where a part of the sodium chloride had been replaced by anilinium chloride. In the computation it was assumed that the concentration of the complexes was negligible, and the measurements do not disprove this assumption, since the dissociation constants found are nearly independent of the concentration of anilinium ion.

As a result of the electrometric measurements we conclude that possible complexes of aniline or anilinium ion with either pyruvate or oxaloacetate ion are present in such small concentrations that they are undetectable by the method applied here. They were therefore neglected in all the computations in this paper.

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The Biosynthesis of Riboflavin in *Eremothecium ashbyii*

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The ascomycete *Eremothecium ashbyii* is well known for its capability of synthesizing riboflavin. Under controlled conditions remarkably high yields are obtained. (Robinson [1], Hickey [2]).

The organism is usually grown under aerobic conditions (submerged) in a liquid medium containing some sources of nitrogen and carbohydrate. Synthetic media have been described, some of which support the growth exellently, and give high yields of riboflavin. (Schopfer and Guilloud [3], Yaw [4], Hickey [2]).

According to MacLaren [5], there is no direct correlation between the growth of the organism and its ability of synthesizing riboflavin. It was therefore found of importance to investigate the dependence of riboflavin synthesis upon growth, and experiments have been performed where washed mycelium of *Eremothecium ashbyii* was incubated in a solution of inorganic salts.

As acetic acid enters a great many metabolic processes, the possible role of this compound in the riboflavin synthesis has been investigated.

EXPERIMENTAL AND RESULTS

Eremothecium ashbyii (American Type Culture Collection, 6 747) was kept on agar slants of the composition: 4.0 % glucose, 0.5 % peptone (Difco), 0.5 % yeast extract (Difco) and 1.8 % agar. The cultures were incubated at 28° C for 24 hours, and stored at room temperature. Cultures used in the experiments were transferred daily.

The medium for the liquid cultures had the same composition, except that the agar was omitted. The flasks were shaken in a water-bath at 28° C, at a rate of approximately 100 strokes per minute.

In the experiments with washed mycelium the following buffer was used: 100 parts 0.9 % sodium chloride, 5 parts 1.15 % potassium chloride, 5 parts 3.83 % magnesium sulfate (epsomite) and 15 parts 0.1 *M* di-sodium hydrogen phosphate buffer of pH 7.4. The pH of the mixture was adjusted to 6.9 before use.

Growth and riboflavin production. Preliminary experiments were made by measuring the oxygen consumption of mycelium suspended in fresh medium, and in phosphate buffer, by the direct method of Warburg. Mycelium was collected by centrifugation from a 24 hours old shaking culture, washed twice with buffer and resuspended in fresh medium. The oxygen consumption per unit time increased at a rate of 6—8 % per 10 minutes, indicating abundant growth. When washed mycelium was incubated in phosphate buffer no growth took place, the volume oxygen taken up being linear with time.

Table 1. Riboflavin production in Eremothecium Ashbyii. Washed mycelium.

Experiment No.	pH	Riboflavin, μg/ml	
		Content	Net increase
I			
Zero time	6.9	6	—
Incubated 72 h	7.0	47	41
II			
Zero time	6.9	7	—
Incubated 45 h	—	60	53

40 ml liquid medium, inoculated from agar slant, was incubated at 28° C for 48 h. Mycelium was collected by centrifugation, washed twice and resuspended in 4 × 20 ml buffer. The numbers are averages from 3 incubated flasks. The riboflavin was determined fluorometrically.

Experiments were then made to determine the formation of riboflavin when washed mycelium was incubated in phosphate buffer. Washed mycelium from a 48 hours old shaking culture was re-suspended in buffer of twice the original volume of the medium. The suspension was measured into 100 ml flasks in portions of 20 ml, and shaken at 28° C for 45 to 72 hours. The riboflavin was determined fluorometrically, and compared to a non incubated sample of the suspension (Table 1).

The data given in Table 1, clearly demonstrate that *E. ashbyii* is able to synthesize riboflavin, even when deprived of organic materials except those residing in the cells themselves, and under conditions when no growth takes place.

Experiments with 1-C ^{14} *acetic acid.* Liquid medium (50 ml) was inoculated from a 24 hours old shaking culture. After 24 hours shaking at 28° C 10 μcurie 1-C ^{14} acetate was added and the shaking continued for another 72 hours. The yield of riboflavin was 220 μg/ml as determined fluorometrically.

Most of the riboflavin was isolated by extraction with butanol and precipitation with petroleum-ether [1], 40 mg carrier riboflavin was added and the mixture was crystallized from water/alcohol. The riboflavin was then counted by direct plating by means of an ordinary end window G.M. counter. The activity recovered in riboflavin was very low, amounting to less then 0.01 % of the added C ^{14}.

Degradation experiments. A small portion of the C^{14} containing riboflavin was degraded without addition of carrier. An aqueous solution was made alkaline with sodium hydroxide and irradiated with a 100 watt lamp for 2 hours. The impure lumiflavine was extracted by shaking the acidified solution several times with chloroform. After removal of the chloroform by distillation the residue was dissolved in 1 *N* sodium hydroxide and heated on a boiling water-bath for 30 minutes, made acid with acetic acid and again extracted with chloroform [6,7]. After removal of the chloroform the residue should consist of *the hydrolysis product* of lumiflavin, and *lumicrome*, and of some unchanged *lumiflavin* [8,9].

The difficulties in obtaining small quantities of these substances in the pure state led to an attempt to separate them by means of paper chromatography. Water saturated *n*-butanol was used as the solvent, and the paper was Whatman No. 1. Standard reference substances were prepared according to Kuhn *et al.*[7], and Karrer *et al.*[8]. The three substances gave well defined spots, visible by their fluorescence in ultraviolet light (Fig. 1).

For estimation of the distribution of C^{14} along the paperchromatogram, a strip 3.5 cm broad and holding all the spots was cut out of the paper. This strip was fitted into a double aluminum screen, the upper plate of which was supplied with a slit, 1.0 cm broad and 3.0 cm long. The slit was centered relative to the window of the G. M. tube, and the paper strip was moved past the slit, one cm at the time, and counted.

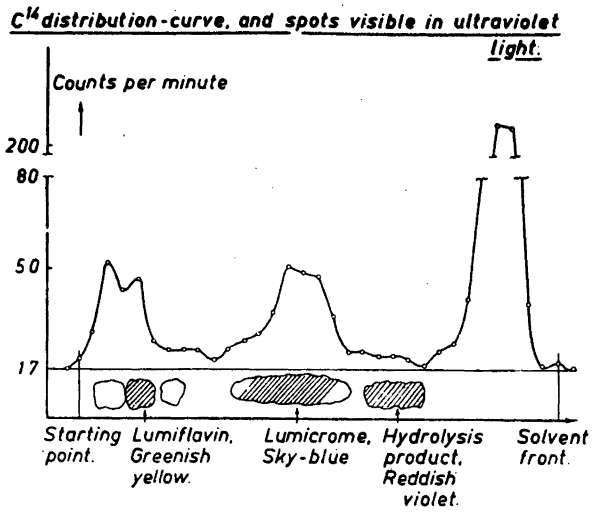


Fig. 1. C^{14} activity in degradation products of riboflavin, separated by means of paper chromatography. Counted along the intact paper strip, one cm at the time. Riboflavin formed by Eremothecium ashbyii incubated in a medium containing 1-C^{14} acetate.

In Fig. 1 the obtained C^{14} distribution curve is drawn, and the positions of the spots are indicated. As will be seen from the curve the C^{14} content of the hydrolysis product of lumiflavin is low compared to that of lumiflavin and lumicrome. The high activity on the right hand side of the paper is probably due to lower fatty acids.

The experiment was repeated with washed mycelium. Ten ml suspension was incubated with 50 μcurie 1-C^{14} acetate for 72 hours. The riboflavin was degraded directly in the centrifuged medium, and the degradation products were separated by paper chromatography as before. The results are given in Fig. 2.

DISCUSSION

The demonstration of riboflavin synthesis in washed mycelium of *Eremothecium ashbyii* incubated in a solution of inorganic salts (Table 1), proves that riboflavin is a product of the metabolism in the already formed cells. Thus, growth and riboflavin synthesis may be regarded as independent phenomena. However, growth factors will obviously stimulate the riboflavin formation, as more cells are formed when such substances are present in the medium.

MacLaren [5] reported that xanthine and some other purines stimulated riboflavin synthesis in *Eremothecium ashbyii*, while the growth was little affected. Hickey [2] and Yaw [4] found no effect of xanthine on growth and riboflavin formation in *E. ashbyii*. It is apparent from the papers of Hickey and Yaw that they tested the effect of xanthine as a growth factor in a sub-optimal medium. In accordance with MacLaren's data they found no such effect. In MacLaren's experiments xanthine was added to a medium where the organism grew well, and stimulated under such conditions the formation of riboflavin.

These aspects are discussed here because the results from the experiments with 1-C^{14} acetate indirectly support MacLaren's hypothesis of some purine-like substance as a precursor in riboflavin synthesis.

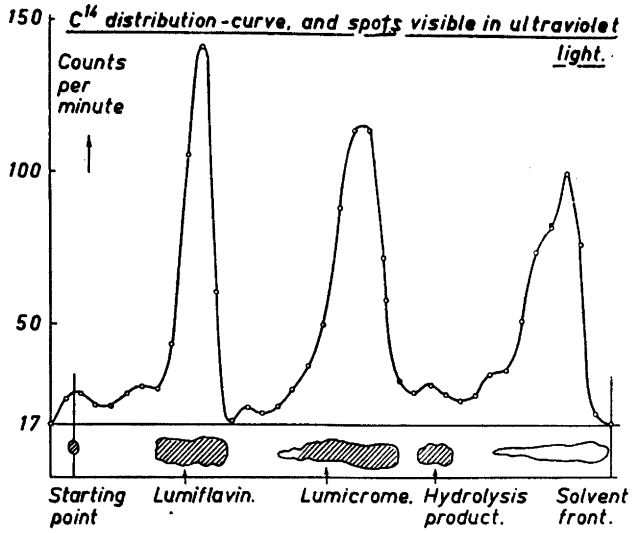
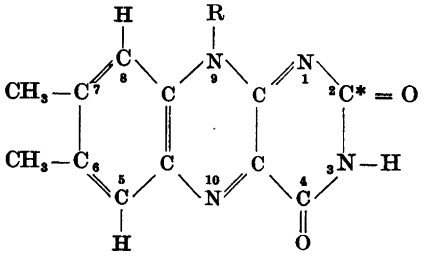
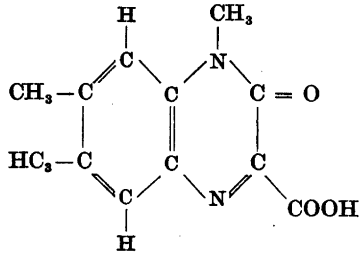


Fig. 2. C^{14} activity in degradation products of riboflavin, separated by means of paper chromatography. Counted along the intact paper strip, one cm at the time. Riboflavin formed by Eremothecium ashbyii incubated in a phosphate buffer containing 1-C^{14} acetate.

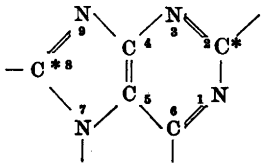
The C^{14} present in lumiflavin is split off by hydrolysis, yielding a practically inactive hydrolysis product (Figs. 1 and 2). Thus, C^{14} from 1-C^{14} acetic acid is present in carbon No. 2 in lumiflavin.



Riboflavin: R = ribityl-chain.
Lumiflavin: R = CH_3-group.
Lumicrome: R = H.



Hydrolysis product of lumiflavin.



The purine nucleus.

According to Schulman, Sonne and Buchanan [10] the precursor for carbons 2 and 8 in the purine nucleus is formic acid, while acetic acid is not incorporated into the purines in pigeon liver extract. According to Krebs, Gurin and Eggleston [11] the tricarboxylic acid cycle is not the mayor pathway of acetate oxidation in yeast and other microorganisms. The following pathway is tentatively suggested: Acetyl-coenzyme A, glycollyl-, glyoxalyl-, oxalyl- and formyl-coenzyme A. Thus, carboxyl labelled acetate would give rise to purines labelled in the positions 2 and 8. If a purine nucleus is formed as a precursor of riboflavin the carbon 8 will probably be split off, leaving the riboflavin nucleus labelled in position 2, in accordance with the reported data. It is therefore probable that a substance with purine structure is utilized in the synthesis of the riboflavin molecule.

SUMMARY

1. When incubated in a buffer containing only inorganic constituents *Eremothecium ashbyii* produced significant amounts of riboflavin.

2. Separation of the degradation products of riboflavin by means of paper chromatography is described.

3. C^{14} from carboxyl-labelled acetic acid is found in carbon No. 2 in the riboflavin nucleus, thus giving indirect evidence of a purine precursor in riboflavin synthesis in *Eremothecium ashbyii*.

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The Reaction between Carbon Dioxide and Dimethoxyphenylmagnesium Bromides

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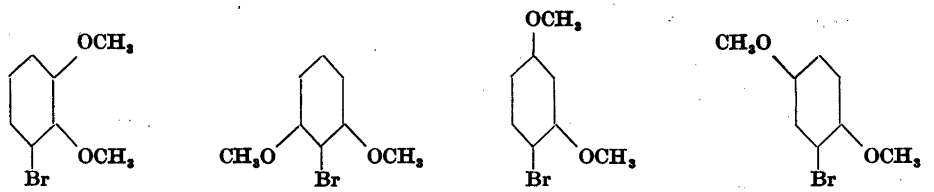
Some time ago the author published a report on the reaction between carbon dioxide and methoxy substituted phenylmagnesium and tolylmagnesium bromides [1]. It was then stated that a methoxy group in ortho position relative to the magnesium atom leads to the formation of the corresponding ketone in addition to the normal reaction product, the carboxylic acid. However, a methyl group in ortho position relative to the magnesium atom or relative to the methoxy group prevented the ketone formation.

The investigations have now been extended to the dimethoxyphenylmagnesium bromides. Some of the reactions have previously been carried out. Grignard [2] isolated veratric acid from the reaction products of carbon dioxide and 3,4-dimethoxyphenylmagnesium bromide. 2,4-Dimethoxybenzoic acid has been prepared from 2,4-dimethoxyphenylmagnesium bromide and carbon dioxide by Rice [3] and from the corresponding iodide and carbon dioxide by Kauffmann and Kieser [4]. However, since in none of these investigations attention has been paid to the formation of ketones, it was considered necessary to repeat the reactions with 3,4- and 2,4-dimethoxyphenylmagnesium bromides.

Indeed, Kauffmann and Fritz [5] isolated 2,5,2′,5′-tetramethoxybenzophenone and 2,5-dimethoxybenzoic acid from the reaction products of 2,5-dimethoxyphenylmagnesium bromide and carbon dioxide, but to be sure that the same conditions prevailed in all experiments also this reaction was repeated.

During the course of the work, results very similar to those obtained in the investigations with the methoxyphenylmagnesium and methoxytolylmagnesium bromides were obtained. It was found that if neither of the methoxy groups was in ortho position relative to the magnesium atom, only the corresponding carboxylic acids were formed. Two of the four compounds in which a methoxy group and the magnesium atom were in ortho position relative each other, *viz.* those prepared, from 2,3-dimethoxybromobenzene (3-bromoveratrole; I) and 2,6-dimethoxybromobenzene (II), reacted to give only the acids, whereas the Grignard reagents prepared from 2,4-dimethoxybromobenzene (III) and 2,5-dimethoxybromobenzene (IV) gave both ketones and acids.



I II III IV

If, thus, as in the preceding report, the ketone formation is considered a type of ortho effect, this effect is cancelled by a second methoxy group in ortho position relative to either the magnesium atom or the first methoxy group, *i. e.* the first ortho effect is again cancelled by a second ortho effect.

These results are in good accordance with those of Wittig and Pockels [6]. In addition to other reactions between carbon dioxide and organic lithium compounds, they investigated the reactions between carbon dioxide on one hand and 2,4-dimethoxyphenyllithium and 2,6-dimethoxyphenyllithium on the other, and established that the corresponding acids, ketones and triphenylcarbinols are formed in both cases. It is not surprising that 2,6-dimethoxyphenyllithium gives the above-mentioned products while the corresponding Grignard reagent gives only the carboxylic acid, since the organolithium compounds are more reactive than the organomagnesium compounds.

For the sake of completeness, it should be mentioned that Gilman, Swiss and Cheney [7] have isolated *o*-veratric acid from the reaction mixture of 2,3-dimethoxyphenyllithium and carbon dioxide. However, attention has not been paid to the formation of the corresponding ketone.

EXPERIMENTAL

2,3-Dimethoxybromobenzene (3-Bromoveratrole). Guajacol (124 g), dissolved in an aqueous solution (560 ml) of potassium hydroxide (56 g), was methylated with dimethyl sulphate (126 g) by shaking for 15 minutes. Any unchanged sulphate was then destroyed by heating the mixture for 30 minutes on a boiling water bath. The oil that had formed was separated and the aqueous layer was extracted with ether. The combined organic phases were washed with water and dried with calcium chloride. After the solvent had been evaporated, veratrole was distilled under reduced pressure. The yield of material boiling at 88—90°/10 mm was 72.5 % (100 g).

The veratrole was nitrated to yield 4-nitroveratrole according to Cardwell and Robinson [8]. This substance could have been converted into 3-bromo-5-nitroveratrole by bromination according to Jones and Robinson [9], but as the method is time-consuming and a considerable portion of the desired compound is hydrolyzed to 6-bromo-4-nitroguaiacol during the performance, another method was chosen. 4-Nitroveratrole was hydrolyzed with aqueous potassium hydroxide to give 4-nitroguaiacol according to Pollecoff and Robinson [10]. It was, however, found that the period of heating in this synthesis could be shortened from 36 to 12 hours. The 4-nitroguaiacol obtained was brominated according to Robinson [11] to yield 6-bromo-4-nitroguaiacol. As no experimental details are to be found in the literature, the procedure followed in the bromination is given here.

4-Nitroguaiacol (66 g) was dissolved in acetic acid (330 ml) and a solution of bromine (21 ml) in acetic acid (130 ml) was added. After a few minutes, crystals of the 6-bromo compound began to separate. Forty-five minutes after the bromine addition, water (1.5 l) was added. The precipitated 6-bromo-4-nitroguaiacol was filtered and dried. The yield of material melting at 150—151° was 93.5 % (90.5 g).

Methylation of 6-bromo-4-nitroguaiacol with dimethyl sulphate in aqueous potassium hydroxide is not satisfactory, because the yield is only 35 % owing to the low solubility of the potassium salt of the bromo compound. The methylation is more conveniently performed by allowing an excess of undistilled diazomethane in ether solution to react with 6-bromo-4-nitroguaiacol (35 g) dissolved in ether (1 l). After the addition of the diazomethane solution, the mixture was allowed to stand for 15 minutes. The excess of diazomethane was then destroyed by adding acetic acid in ether. In order to remove the acid and any unreacted 6-bromo-4-nitroguaiacol, the mixture was shaken with an aqueous solution (200 ml) of potassium hydroxide (30 g). After the ether solution had been washed with water, it was dried with anhydrous calcium chloride. Evaporation of the solvent yielded a solid residue from which 3-bromo-5-nitroveratrole (34.2 g; m.p. 112—113°) was isolated after boiling with methanol (50 ml) and allowing the mixture to cool. A small amount (1.1 g) of the same compound was isolated after addition of water to the filtrate. The total yield was thus 95.5 %.

This 3-bromo-5-nitroveratrole was reduced to the corresponding amino compound in the following way. A solution of stannous chloride was prepared by dissolving tin (178 g) in concentrated hydrochloric acid (720 ml) in a three-necked flask, fitted with a reflux condenser and a stirrer. Ethyl alcohol (250 ml) was added, the stirrer started, and the temperature raised to 45°. A boiling solution of 3-bromo-5-nitroveratrole (10 g) in ethyl alcohol (70 ml) was added through the free neck of the flask. The bromonitro compound precipitated at first, but the precipitate disappeared in a few minutes. The addition of the bromonitro compound was then continued in portions (15 g, 25 g, 25 g, 25 g, and 26.5 g) dissolved in ethyl alcohol (105 ml, 175 ml, 175 ml, 175 ml, and 185 ml, respectively). Before each addition the temperature was adjusted to 45°, and the precipitated bromonitro compound was allowed to disappear before the next portion was added. Half an hour after the last precipitate of 3-bromo-5-nitroveratrole had disappeared, the stirrer was stopped. On the next day, the alcohol was removed by distillation under reduced pressure. After cooling, the precipitate that had formed during the distillation was filtered and treated with a mixture of ether (1 l) and technical sodium hydroxide (500 g) dissolved in water (3 l). The mixture was thoroughly shaken and the layers were separated. The water phase was extracted once more with ether and the combined ether solutions were dried with potassium hydroxide. After the ether had been evaporated, a yellowish white solid substance remained, which yielded 5-amino-3-bromoveratrole upon crystallization from benzene (250 ml). This substance (85.9 g) was isolated by filtration. The filtrate was concentrated to half its volume, and after addition of ligroin to the hot solution until a turbidity appeared, a small amount of impure amino compound was isolated. After recrystallization from benzene the purified substance (11.8 g) was combined with the main portion. The total yield of 5-amino-3-bromoveratrole, m.p. 100—100.5°, was 87 %.

Kimoto, Kimura, and Sakai [12] who performed the reduction with iron and hydrochloric acid, have reported the melting point 95—97° for this compound.

A small amount of the amine was converted into its acetyl derivative. After recrystallization from aqueous ethyl alcohol, 5-acetamino-3-bromoveratrole melting at 159—160° was obtained. (Found: N 5.38; Calc. for $C_{10}H_{12}O_3NBr$: N 5.43).

5-Amino-3-bromoveratrole has previously been deaminated in good yields by Kimoto, Kimura, and Sakai [12] by treating the corresponding diazonium salt with 40 % hypophosphorous acid. As the last-mentioned reagent could not be obtained, the deamination had to be performed in another way. Reduction of the diazonium salt with ethyl alcohol did not seem suitable, because only a small amount of acetaldehyde was detected when the reaction was performed on a test-tube scale. As a consequence of this fact, the diazonium salt was reduced with alkaline sodium stannite in the following way.

The amine (79 g) was dissolved in a hot mixture of concentrated sulphuric acid (31.6 ml) and water (475 ml). Crushed ice was added until the temperature had dropped to 0°, after which a solution of sodium nitrite (24 g) in water (200 ml) was gradually added. As the amine reacts slowly, the rate of addition was held very low. When the addition was completed, the mixture was allowed to stand for some time, and then added in small portions to an ice-cold mixture of stannous chloride (127 g) in water (650 ml), and sodium hydroxide (160 g) in water (190 ml). During this addition, nitrogen was evolved, and a tarry semi-liquid lump was formed. After the mixture had stood for an hour, crude 3-bromoveratrole was distilled over with steam. The oil in the distillate was dissolved in

ether, and the ether solution was shaken with a dilute solution of sodium hydroxide. After washing with water, the ether solution was dried with anhydrous sodium sulphate. Evaporation of the solvent followed by distillation under reduced pressure yielded pure 3-bromoveratrole, b.p. 128—129°/17 mm. The yield was 20 % (14.9 g).

2,4-Dimethoxybromobenzene. Resorcinol (30 g) dissolved in an aqueous solution (200 ml) of sodium hydroxide (22 g) was methylated with dimethyl sulphate (69 g) by shaking for an hour. Any unchanged sulphate was then destroyed by heating the mixture for 30 minutes on a boiling water bath. The methoxy compound was distilled with steam from the reaction mixture. The distillate was extracted with ether and the ether phase dried with anhydrous sodium sulphate. Evaporation of the solvent and distillation of the residue under reduced pressure yielded 1,3-dimethoxybenzene, b.p. 92—95°/16 mm. The yield was 85 % (32 g).

2,4-Dimethoxybromobenzene was prepared from this dimethoxybenzene according to the procedure described by Noelting and Werner [13] for the corresponding 2,5-dimethoxy compound.

2,5-Dimethoxybromobenzene. Hydroquinone was converted into 1,4-dimethoxybenzene by the method given for 1,3-dimetoxybenzene under the sub-heading "2,4-Dimethoxybromobenzene". The yield of material boiling at 109—110°/20 mm was 70 %.

The preparation of 2,5-dimethoxybromobenzene from 1,4-dimethoxybenzene was performed according to Noelting and Werner [13].

2,6-Dimethoxybromobenzene. Resorcinol was nitrated according to Kauffmann and de Pay [14] to give 2-nitroresorcinol. This compound was methylated according to Baeyer [15] and the 2,6-dimethoxynitrobenzene obtained was reduced according to Kauffmann and Franck [16] to 2,6-dimethoxyaniline.

This amine (20 g) was dissolved in 48 % hydrobromic acid (39.5 ml). The solution was cooled to 0° and a solution of sodium nitrite (9 g) in water (64 ml) was added gradually. In the meantime, a mixture of cuprous bromide (10.5 g) and 48 % hydrobromic acid (10.5 ml) was prepared in a flask provided with a dropping funnel, a steam inlet tube, and a condenser set for distillation. While steam was led through the mixture of cuprous bromide and hydrobromic acid the solution of the diazotized amine was allowed to run through the dropping funnel into the flask rather rapidly. During this operation, some crude 2,6-dimethoxybromobenzene distilled with steam. When the addition had been completed, the steam distillation was continued until no more organic material passed over. The solid in the distillate was filtered, broken up, and washed with dilute sodium hydroxide and water. After recrystallization from ethyl alcohol (50 ml), pure 2,6-dimethoxybromobenzene, m. p. 93—94°, was obtained. The yield was 53 % (15 g). (Found: Br 36.90; Calc. for $C_8H_9O_2Br$: Br 36.82).

3,4-Dimethoxybromobenzene. (4-Bromoveratrole) was prepared according to Freudenberg, Fikentscher, and Marden [17] by bromination of guaiacol followed by methylation with dimethyl sulphate in alkaline solution. The boiling point of the 4-bromoveratrole was 127—128°/13 mm.

3,5-Dimethoxybromobenzene. 3,5-Dinitroanisole (54.5 g), prepared according to Organic Syntheses [18], was dissolved in ethyl alcohol (100 ml). To this solution 15 % ammonium hydrogen sulphide solution (210 ml) was added in portions. After each addition, the mixture was thoroughly shaken. When all the sulphide solution had been added, the mixture was heated for 15 minutes on a boiling water bath. After cooling, the solid substance was filtered, washed with water, and extracted with hot dilute hydrochloric acid. The extraction was repeated several times or until no precipitate was formed when the extracts were made alkaline. Ammonia was then added to the combined extracts until they were alkaline. The precipitated 3-amino-5-nitroanisole was filtered off and recrystallized from ethyl alcohol. The yield of the compound, melting at 119—120° was 85 % (39 g).

This amino compound was converted into 3-bromo-5-nitro-anisole according to Blanksma [19]. When the synthesis was continued to 5-bromo-3-aminoanisole, the description for the corresponding iodo compound given by Hodgson and Wignall [20] was followed. From the bromoamine, 5-bromo-3-methoxyphenol was prepared according to Hodgson and Wignall [21] and this compound was methylated to yield 3,5-dimethoxybromobenzene by the following method.

The phenol (20.3 g) was dissolved in an aqueous solution (63 ml) of potassium hydroxide (7 g) and to this solution dimethyl sulphate (15.8 g) was added. The mixture was

Table 1. Yields, melting points, and analyses.

Dimethoxybromo benzene	Carboxylic acids				Ketones			
	g	m. p.	*p*-Nitrobenzyl esters Calc. N 4.41		g	m. p.	2,4-Dinitrophenylhydrazones Calc. N 11.61	
			m. p. [a]	Found N			m. p. [a]	Found N
2,3—	1.13	121.5—122.5 [b]	97—98	4.53	—			
2,4—	0.50	106—108 [b]	128—130	4.63	1.05	132—133 [c]	146—147	11.43
2,5—	1.00	74—76 [d]	128—130	4.39	1.30	108—109 [a]	175—176	11.53
2,6—	0.78	172—174 [b]	154—155	4.52	—			
3,4—	0.71	177—179 [b]	143—144	e	—			
3,5—	0.57	180—181 [c]	121—122	4.50	—			

[a] Recrystallized from ethyl alcohol.
[b] Recrystallized from water.
[c] Recrystallized from a mixture of water and alcohol.
[d] Recrystallized from ligroin.
[e] Previously prepared.

shaken for one hour and after addition of dilute sodium hydroxide solution, 3,5-dimethoxybromobenzene was distilled with steam. It was collected from the distillate by filtration. After recrystallization from ethyl alcohol, it melted at 64—65°. (Found: Br 37.01; Calc. for $C_8H_9O_2Br$: Br 36.82).

The yield was 83 % (18 g).

The reactions between Grignard reagents and carbon dioxide were examined as has been described previously [1]. The results are collected in Table 1.

SUMMARY

1. Dimethoxyphenylmagnesium bromides in which neither methoxy group is in ortho position relative to the magnesium atom react with carbon dioxide to give the corresponding carboxylic acids only.

2. If one of the methoxy groups is in ortho position relative to the magnesium atom and the other in ortho position relative to the first-mentioned methoxy group or relative to the magnesium atom, the dimethoxyphenylmagnesium bromide gives only the corresponding carboxylic acid in the reaction with carbon dioxide.

3. The other dimethoxyphenylmagnesium bromides react with carbon dioxide to give both the corresponding carboxylic acids and the corresponding tetramethoxybenzophenones.

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Synthesis of Some 3-Benzalcoumaran-2-ones

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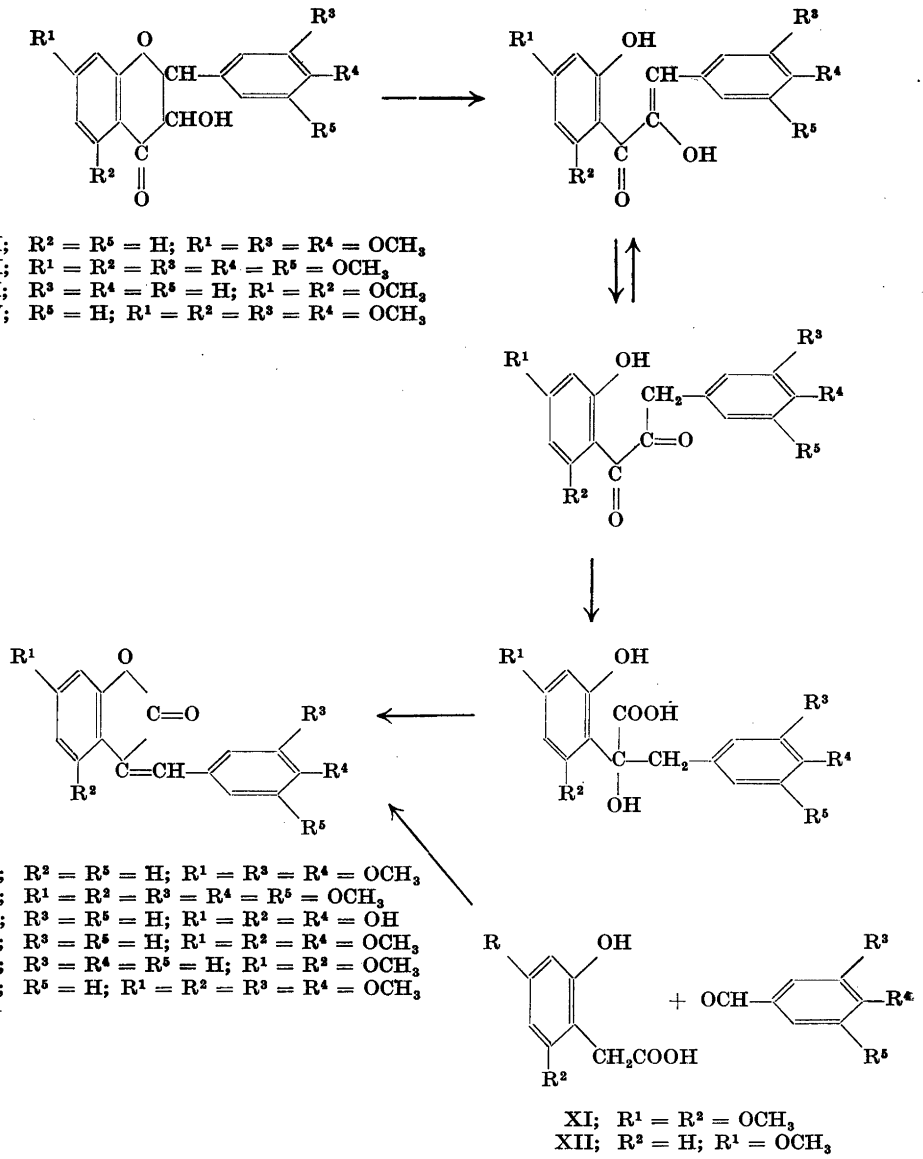
3-Hydroxyflavanones have been found to be very unstable towards alkali. They are partly dehydrogenated to 3-hydroxyflavones and partly rearranged. It has recently been shown [1] that this rearrangement in some cases gives 2-hydroxy-2-benzylcoumaran-3-ones, but it is reported in the literature that in other cases the reaction can go further, giving acids, which easily yield anhydrolactones. The formation of these acids has been assumed to take place through a benzilic acid rearrangement. Thus fustintrimethylether (3-hydroxy-3′,4′,7-trimethoxyflavanone) (I) and ampeloptinpentamethylether (3-hydroxy-3′,4′,5,5′,7-pentamethoxyflavanone) (II) give anhydrotrimethylhazeyl lactone [2] (V) and anhydropentamethylampeloptin lactone [3] (VI) respectively.

A third lactone is garcinol [4] (VII) obtained from fukugetin by treatment with alkali [5]. Because the structure of fukugetin is not known with certainty, it is impossible to say whether the formation of garcinol is quite analogous to the formation of the two other lactones.

It was at one time thought that the rearrangement of pinobanksindimethylether [6] (3-hydroxy-5,7-dimethoxyflavanone) (III) might be analogous to the above rearrangement and that the product obtained, which is now regarded as 2-hydroxy-2-benzyl-4,6-dimethoxycoumaran-3-one [1] was possibly the acid corresponding to (IX). It was therefore decided to synthesise this acid and the corresponding lactone (IX) and at the same time to synthesise the lactones (V) and (VI) and the trimethylether of (VII). The lactone (X), which should be produced by the benzilic acid rearrangement of taxifolintetramethylether (3-hydroxy-3′,4′,5,7-tetramethoxyflavanone) (IV) was also synthesised. None of these lactones, which can also be regarded as substituted 3-benzalcoumaran-2-ones, has previously been synthesised, although the methylester-methylether of the free acid corresponding to (VIII) has been synthesised [4].

The route followed in these syntheses was essentially that given by Czaplicki, v. Kostanecki and Lampe [7], *viz.* condensation of the appropriate 2-hydroxyphenylacetic acid with benzaldehyde or a substituted benzaldehyde. The 2-hydroxyphenylacetic acids (XI) and (XII) were prepared by the Willgerodt reaction, as modified by Schwenk and Bloch [8], from the corresponding 2-hydroxyacetophenones. According to Schwenk and Bloch their method is



I; $R^2 = R^5 = H$; $R^1 = R^3 = R^4 = OCH_3$
II; $R^1 = R^2 = R^3 = R^4 = R^5 = OCH_3$
III; $R^3 = R^4 = R^5 = H$; $R^1 = R^2 = OCH_3$
IV; $R^5 = H$; $R^1 = R^2 = R^3 = R^4 = OCH_3$

V; $R^2 = R^5 = H$; $R^1 = R^3 = R^4 = OCH_3$
VI; $R^1 = R^2 = R^3 = R^4 = R^5 = OCH_3$
VII; $R^3 = R^5 = H$; $R^1 = R^2 = R^4 = OH$
VIII; $R^3 = R^5 = H$; $R^1 = R^2 = R^4 = OCH_3$
IX; $R^3 = R^4 = R^5 = H$; $R^1 = R^2 = OCH_3$
X; $R^5 = H$; $R^1 = R^2 = R^3 = R^4 = OCH_3$

XI; $R^1 = R^2 = OCH_3$
XII; $R^2 = H$; $R^1 = OCH_3$

not adaptable to the preparation of acids with a free hydroxyl group, whereas such acids have been prepared by the usual Willgerodt-Kindler reaction [9]. We find however that the method of Schwenk and Bloch can be used, although the yields are low. As the starting materials were easily available the method was, in spite of the low yield, convenient for the preparation of the small amounts of acids necessary for our purposes.

The condensation with the aldehyde was carried out in the presence of acetic anhydride and triethylamine [10].

As can be seen from Table 1 the m.p:s of the synthetic 3-benzalcoumaran-2-ones are in good agreement with the values given for the compounds obtained through rearrangement from natural products. Although no mixed m.p. determinations could be carried out, owing to lack of authentic material, these syntheses are however additional evidence in support of the structures given to anhydrotrimethylhazeyl lactone, anhydropentamethylampeloptin lactone and garcinoltrimethylether respectively.

Table 1.

(V)	m.p. 183—184°	Anhydrotrimethylhazeyl lactone	m.p. 184—185° [2]
(VI)	m.p. 159—160°	Anhydropentamethylampeloptin lactone	m.p. 159—160° [3]
(VIII)	m.p. 167°	Garcinoltrimethylether	m.p. 167° [4]
(IX)	m.p. 169.5—170.5°		
(X)	m.p. 173—174°		

3-Benzal-4,6-dimethoxycoumaran-2-one (IX) was converted by hydrolysis into the free acid, the m.p. of which cannot be determined as upon heating it is converted back into the lactone thus exhibiting its m.p. Although this m.p. coincides with the m.p. of the rearrangement product of pinobanksindimethylether [6], mixed m.p. determination shows that they are definitely different compounds.

EXPERIMENTAL

2-Hydroxy-4,6-dimethoxyphenylacetic acid (XI). 2-Hydroxy-4,6-dimethoxyacetophenone (5 g) was heated with sulphur (2 g) and morpholine (4.35 g) in an oil-bath at 150—160° for 5 hours. The mixture was then dissolved in chloroform and the chloroform solution was washed with water and hydrochloric acid. The chloroform was removed in vacuum and the residue crystallised from alcohol. The thiomorpholide thus obtained was hydrolysed by boiling with 30 % potassium hydroxide (20 ml). The alkaline solution was diluted with water, extracted with ether and then acidified, whereupon 2-hydroxy-4,6-dimethoxyphenylacetic acid was precipitated as an oil. It was taken up in ether, the acid extracted in sodium hydrogen carbonate and precipitated once again. Extraction with ether and removal of the ether in vacuum gave a yellow oil which crystallised. Crystallisation from ether-light petroleum gave colourless crystals, m.p. 140—140.5°. Yield 0.8 g (15 %). (Found: C 56.7; H 5.6. $C_{10}H_{12}O_5$ requires: C 56.6; H 5.7.)

4,6-Dimethoxycoumaran-2-one. When the above mentioned acid is sublimed in vacuum it loses water and is converted into its lactone. M.p. after crystallisation from ether 154°. (Found: C 61.6; H 5.1. $C_{10}H_{10}O_4$ requires: C 61.8; H 5.2.)

2-Hydroxy-4-methoxyphenylacetic acid (XII) was prepared from 2-hydroxy-4-methoxyacetophenone by the same procedure as described above for the preparation of 2-hydroxy-4,6-dimethoxyacetophenone. In this case the thiomorpholide could not be obtained crystalline and was therefore hydrolysed in the crude state. Crystallisation from ether-light petroleum gave the acid, m.p. 132—133° (Lit. 133° [11]).

3-Benzal-4,6-dimethoxycoumaran-2-one (IX). 2-Hydroxy-4,6-dimethoxyphenylacetic acid (1 g), benzaldehyde (0.55 g), acetic anhydride (2 g) and triethylamine (0.5 g) were heated together on a water bath for 6 hours. Water (50 ml) was then added to the reaction mixture which was subsequently boiled for 15 minutes. The semicrystalline reaction product was separated and dried on a porous plate. It was then dissolved in acetone and filtered through aluminium oxide. Crystallisation from alcohol gave yellow plates, m.p. 169.5—170.5°. (Found: C 72.0; H 5.0. $C_{17}H_{14}O_4$ requires: C 72.3; H 5.0.)

2-Hydroxy-4,6-dimethoxystilbene-α-carboxylic acid. The 3-benzal-4,6-dimethoxycoumaran-2-one was hydrolysed by boiling with 10 % potassium hydroxide. Crystallisation from benzene, of the product obtained upon acidification of the alkaline solution gave pale yellow needles. Upon heating it turns dark yellow and melts at 169°. (Found: C 67.7; H 5.55. $C_{17}H_{16}O_5$ requires: C 68.0; H 5.4.)

3-(4-Methoxybenzal)-4,6-dimethoxycoumaran-2-one (VIII) was made as described above using anisaldehyde, except that the time of heating was 20 hours. The product was obtained as long yellow needles, m.p. 167°. (Found: C 69.1; H 5.1. $C_{18}H_{16}O_5$ requires: C 69.2; H 5.2.)

3-(3,4-Dimethoxybenzal)-4,6-dimethoxycoumaran-2-one (X) was prepared in a similar manner from veratraldehyde, giving small orange coloured needles, m.p. 173—174°. (Found: C 66.7; H 5.3. $C_{19}H_{18}O_6$ requires: C 66.5; H 5.4.)

3-(3,4,5-Trimethoxybenzal)-4,6-dimethoxycoumaran-2-one (VI) was prepared from gallaldehydetrimethylether, giving orange-red needles, m.p. 159—160° (Found: C 64.3; H 5.3. $C_{20}H_{20}O_7$ requires: C 64.5; H 5.4).

3-(3,4-Dimethoxybenzal)-6-methoxycoumaran-2-one (V) was prepared from 2-hydroxy-4-methoxyphenylacetic acid and veratraldehyde, giving bright yellow needles, m.p. 183—184°. (Found: C 68.8; H 5.3. $C_{18}H_{16}O_5$ requires: C 69.2; H 5.2.)

SUMMARY

A number of methoxysubstituted 3-benzalcoumaran-2-ones have been prepared by condensation of methoxysubstituted 2-hydroxyphenylacetic acids with benzaldehyde or methoxysubstituted benzaldehydes.

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Infra-Red Absorption Spectra of SiD_3Cl, SiH_3F, and SiD_3F

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The infra-red absorption spectra of SiD_3Cl, SiH_3F, and SiD_3F are recorded in the 667—3 000 cm^{-1} region on a rock-salt instrument. Observed and estimated values of the fundamental vibrational frequencies are (estimated values in parenthesis): for SiD_3Cl: $\nu_1 = 1\,619$; $\nu_2 = 848$; ($\nu_3 = 500-560$); $\nu_4 = 1\,584$; $\nu_5 = 696$; ($\nu_6 = 470-520$); for SiH_3F: $\nu_1 = 2\,215$; ($\nu_2 \sim 1\,350$); $\nu_3 = 874$; $\nu_4 = 2\,215$; $\nu_5 = 980$; $\nu_6 = 739$; for SiD_3F: $\nu_1 = 1\,610$; $\nu_2 = 959$; $\nu_3 = 885$; $\nu_4 = 1\,570$; $\nu_5 = 704$; ($\nu_6 \sim 506$). All frequencies are in cm^{-1}.

In connection with microwave work on the silyl halides [1,2] the infrared absorption spectra of SiD_3Cl, SiH_3F, and SiD_3F were recorded as a check of the purity of the compounds. Because of the small quantities prepared [2] the gas-pressures which could be obtained were rather small (see experimental part). The spectra are, therefore, by no means complete but in view of the fact that no other investigations of the vibrational spectra of these compounds have so far been published it was decided to give the observed frequencies together with an assignment of them.

FUNDAMENTAL VIBRATION FREQUENCIES OF SiD_3Cl

In this case the assignment may, with relative ease, be made by using the results obtained by Monfils for SiH_3Cl[3,4]. His assignment of the fundamental vibrational frequencies of SiH_3Cl is indisputable since it is based on studies of the fine-structure of the bands. Because practically no fine-structure features could be seen by us with our instrument (Beckmann I.R. II) in the case of SiD_3Cl, the assignment which follows (Table I) has been based on several kinds of calculations (1°, 2°, 3°).

1°. Valence-force calculations

With reference to Fig. 1 the potential function used was:

$$\Delta 2V = f_1\ (\Delta r_1)^2 + 3\ f_2\ (\Delta r_2)^2 + 3d_1\ (r_1\Delta\gamma)^2 + 3d_2\ (r_2\Delta\beta)^2 \qquad \text{(I)}$$

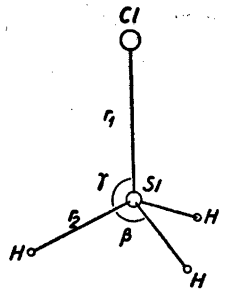

Fig. 1. Showing molecular geometrical parameters of SiH_3Cl.

The corresponding six equations which connect observed frequencies and the constants f_1, f_2, d_1, and d_2 have been given by Wagner [5]. Setting ν_1, ν_2, ν_3 (frequencies of totally symmetric vibrations) = 2 196, 1 097.5, 551 from the SiH_3Cl spectrum[4] we calculate $f_1 = 3.00 \cdot 10^5$, $f_2 = 2.82 \cdot 10^4$, and $D = 0.64 \cdot 10^5$ dynes / cm where D is given by

$$D = \frac{r_1}{r_2} d_1 + 9 \frac{\sin^2\gamma \cos^2\gamma}{\sin^2\beta} d_2^2 \qquad \text{(II)}$$

Using ν_4, ν_5, ν_6 (frequencies of twofold degenerate vibrations) = 2 147.4, 951.2, 666.2 (likewise from the SiH_3Cl spectrum) we get $f_2 = 2.54$, $d_1 = 0.32$, and $d_2 = 0.12$ in units of 10^5 dynes/cm. The deviation between the two values of f_2 does not exceed what must be expected in the valence-force approximation. If the calculated values of d_1 and d_2 are inserted in (II) using $r_1 = 2.048$, $r_2 = 1.48$ Å; $\beta = 110.18°$, $\gamma = 108.75°$ one calculates $D = 0.55 \cdot 10^5$ dynes/cm which, too, is sufficiently consistent with the value of D ($0.64 \cdot 10^5$) calculated above. Now, at the pre-calculation of ν_1, ν_2, ν_3 for SiD_3Cl we have used f_1, f_2, D = (3.00; 2.82; 0.64) 10^5 dynes/cm while f_2, d_1, d_2 = (2.54; 0.32; 0.12) 10^5 dynes/cm were used at the pre-calculation of ν_4, ν_5, ν_6 for SiD_3Cl. This might

Table 1. Observed and calculated normal vibration frequencies of SiD_3Cl.

Observed frequencies (cm^{-1})		Calculated normal vibration frequencies for SiD_3Cl (cm^{-1})			
SiH_3Cl[4]	SiD_3Cl	Valence-force calc.	Noether's rule[6] (A)	(B)	Teller's rule[7]
2 196	1 619	ν_1 = 1 564	1 595	1 600	
1 097.5	848	ν_2 = 831		834	
551	Not obs.	ν_3 = 523	557	523	495
2 147.5	1 584	ν_4 = 1 542	1 525	1 614	
951.2	696 *	ν_5 = 651	683	692	
666.2	Not obs.	ν_6 = 519		509	474

* Maximum of optical density. ⊥-band structure observed.

seem inconsistent but it must be remembered that no *precise* physical meaning can be ascribed to the constants in the abbreviated potential function which is used at 'valence-force' calculations. The results of the calculations are given in the third column of Table 1. It is consistent with the assignment suggested in the second column of the same table.

2°. Application of Noether's rule[6]

Noether's semi-empirical rule was originally applied succesfully by him in cases such as (III):

$$\frac{\nu_i\ (CH_3Cl)}{\nu_i\ (CD_3Cl)} = \frac{\nu_i\ (CH_3Br)}{\nu_i\ (CD_3Br)} \qquad \text{(III)}$$

Here, the rule was used in its original form (A) and in a slightly deviating form (B):

$$\frac{\nu_i\ (SiH_3F)}{\nu_i\ (SiD_3F)} = \frac{\nu_i\ (SiH_3Cl)}{\nu_i\ (SiD_3Cl)} \quad \text{(A)} \qquad \frac{\nu_i\ (CH_3Cl)}{\nu_i\ (CD_3Cl)} = \frac{\nu_i\ (SiH_3Cl)}{\nu_i\ (SiD_3Cl)} \quad \text{(B)}$$

The silyl fluoride frequencies necessary for the use of (A) are given in Table 2. The methyl halide frequencies were taken from Herzberg [8].

3°. Application of Teller's rule

Teller's theoretically well-established rule was used in the form

$$\frac{\text{Product of } \begin{Bmatrix}\text{totally symmetric}\\ \text{twofold degenerate}\end{Bmatrix} \text{ vibration frequencies of } SiD_3Cl}{\text{» » » » » » » } SiH_3Cl = \begin{Bmatrix} S \\ E \end{Bmatrix}$$

where $$S = \frac{m_H}{m_D}\sqrt{\frac{M(SiD_3Cl)}{M(SiH_3Cl)}} \quad \text{and} \quad E = \left(\frac{m_H}{m_D}\right)^{3/2}\sqrt{\frac{M(SiD_3Cl)B(SiH_3Cl)}{M(SiH_3Cl)B(SiD_3Cl)}}$$

Here, m_H is the mass of hydrogen, $M(SiH_3Cl)$ the mass of SiH_3Cl etc. while $B(SiH_3Cl)$ is the rotational constant of SiH_3Cl corresponding to rotation about an axis perpendicular to the threefold axis of symmetry. Inserting the values of the masses and $B(SiH_3Cl) = 6\ 674$ MHz, $B(SiD_3Cl) = 5\ 918$ MHz[1,2] we get $S = 0.512$ and $E = 0.384$. Taking three observed frequencies from the SiH_3Cl spectrum and two observed frequencies from the SiD_3Cl spectrum, ν_3 and ν_6 for SiD_3Cl were calculated. Since observed frequencies, rather than frequencies corrected for anharmonicity had to be used, the calculated values of ν_3 and ν_6 probably represent *lower* limits to the true values. It is seen from Table 1 that all calculated frequencies agree pretty well with the observed spectral lines.

FUNDAMENTAL VIBRATION FREQUENCIES OF SiH_3F AND SiD_3F

To the best of our knowledge no infra-red absorption spectrum of 'light' and 'heavy' silyl fluoride has hitherto been reported. Inspite of the small quantities of the silyl fluorides prepared by us we were able to localize five of

the six fundamentals in each of the spectra. Furthermore sufficient fine-structure features could here be seen to warrant an assignment independent of any calculations. The calculations carried out (following the same scheme as above) were undertaken in order to get a check on the assignment made and, too, to get a reasonable good prediction of the localization of the still unobserved fundamental vibrational frequencies.

1°. Valence-force calculations

The straight-forward application of Wagner's formulae to 'light' and 'heavy' silyl fluoride soon leads into trouble such as imaginary force-constants etc. This might perhaps most easily be recognized by observing that $\nu_3(SiD_3F)$ is *larger* than $\nu_3(SiH_3F)$ (see Table 2). The following procedure was, therefore, adopted: since evidently the effect of isotopic substitution is small on ν_3, we introduced $\nu_3(SiH_3F) = \nu_3(SiD_3F)$ in Wagner's formulae.

Table 2. Observed and calculated normal vibration frequencies of SiH_3F and SiD_3F (cm^{-1}).

Observed frequencies		Calculated frequencies		
SiH_3F	SiD_3F	Valence-force calc.	Noether's rule (A)	Teller's rule
2 215 ± 20	1 610 ± 10	ν_1 (SiD_3F) = 1 575	ν_1 (SiH_3F) = 2 186	
Not obs.	959 ≠	ν_2 (SiH_3F) = 1 350	ν_2 » = 1 240	ν_2 (SiH_3F) = 1 370
874 ≠	885 ≠	ν_3 (SiD_3F) = 874		
2 215 ± 20	1 570 ± 10	ν_4 (SiD_3F) = 1 575	ν_4 » = 2 130	
980 *	704 *	ν_5 (SiD_3F) = 762	ν_5 » = 961	
739	Not obs.	ν_6 (SiD_3F) = 506		ν_6 (SiD_3F) = 570

≠ P, Q, and R-branches observed. Calculated P−R separation for SiH_3F : 32.5 cm^{-1}, found for 874-band: 34.5. Calculated for SiD_3F: 33.4 cm^{-1}. Found for 885-band: 33.0, for 959-band about 30 cm^{-1} (weak band).

* Maximum of optical density. ⊥-band structure observed.

This simplification results in the formulae (V) and (VI) which are given together with (IV), an approximative formula introduced by Wagner himself:

$$\varkappa_1^2 = \frac{f_2}{m_2}\left(1 + \frac{3m_2}{M}\cos^2\alpha\right) \text{ (IV) } \varkappa_2^2 = \frac{D}{m_2}\left(1 + \frac{3m_2}{M}\right) \text{ (V) } \varkappa_3^2 = \frac{f_1}{m_1}\left(1 + \frac{m_1}{M}\right) \text{ (VI)}$$

(Wagner's notation)

From (IV)—(VI) we calculate $f_1 = 5.11 \cdot 10^5$, $f_2 = 2.87 \cdot 10^5$, and $D = 0.892 \cdot 10^5$ dynes/cm. Furthermore, since ν_1 and ν_4 practically coincide we also equalize $\varkappa_1$ and $\varkappa_4$ and introduce this simplification in Wagner's formulae for the two-fold degenerate vibrations together with $f_2 = 2.87 \cdot 10^5$ just calculated. Using 'light' frequencies and ignoring a small imaginary part of d_1 we find

$d_1 = 0.314 \cdot 10^5$ and $d_2 = 0.165 \cdot 10^5$ dynes/cm. These force-constants give rise to the precalculated, twofold degenerate 'heavy' frequencies of Table 2. The value of D corresponding to d_1 and d_2 is $0.494 \cdot 10^5$ dynes/cm.

2°, 3°. Application of Noether's and Teller's rules is self-explanatory in this case.

COMPILATION OF FORCE-CONSTANTS FOR METHANE AND SILANE AND MONO-CHLORO AND MONO-FLUORO DERIVATIVES

Table 3 shows that by at large consistent values of stretching and deformation constants are obtained for the group of molecules in question.

Table 3. Force-constants for CH_4, CH_3F, CH_3Cl, SiH_4, SiH_3F *and* SiH_3Cl *(in units of* 10^5 *dynes/cm).*

	Carbon/Silicon -Halogen stretching		Carbon/Silicon -Hydrogen stretching		Hydrogen-Carbon (or Silicon)-Halogen deformation	Hydrogen-Carbon (or Silicon)-Hydrogen deformation
	I	II	I	II		
CH_4			5.04	4.66		0.461
CH_3F	5.64 [b]	5.44	4.71 [b]	4.50	0.946 [b]	0.517 [b]
CH_3Cl	3.34 [a]	3.25	4.96 [a]	4.74	0.340 [a]	0.414 [a]
»	3.35 [b]	3.25	4.90 [b]	4.74	0.701 [b]	0.523 [b]
SiH_4			2.84 [a]	2.63		0.189 [a]
SiH_3F	5.11 [a]	5.32	2.87 [a]	2.86	0.314 [a]	0.165 [a]
SiH_3Cl	3.00 [a]	2.96	2.82 [a]	2.77	0.32 [a]	0.12 [a]
»	5.13 [c]	2.96	2.66 [c]	2.77	0.28 [c]	0.19 [c]

I: Quadratic potential function used; a: valence-force system; b: valence-force system improved by one constant of interaction; c: valence-force system improved by two constants of interaction. II: $\nu = \frac{1}{2\pi c}\sqrt{\frac{f}{\mu}}$, valid for diatomic molecules, used, considering *e. g.* CH_3F as consisting of F and a methyl'atom' (group).

It is seen that fairly independent of the approximative potential function assumed, the stretching constants come out almost identical. They represent, therefore, probably not only a mathematical but also a physical quantity (the force necessary to stretch the bonds 1 cm) in contrast to the deformation constants in which several effects seem to merge. The value $5.13 \cdot 10^5$ given by Monfils for the silicon-chlorine stretching constant may be due to a misprint (3.13?) or a computational error since all the other constants given by Monfils agree very well with our results.

Unfortunately, no theoretically derived rule exists connecting observed force-constants with interatomic distances, electron configuration *etc.* But the

force-constants found here are in agreement with the empirically very well-established rule by Badger [9]:

$$f = \frac{1.86}{(r - \Delta_{ij})^3} \, 10^5 \text{ dynes/cm.} \quad r \text{ in Å units.}$$

where f is the stretching constant, r the length of the bond considered and Δ_{ij} depends on the *periods* (rows) in the periodic table to which the two atoms involved belong. In the present case we get:

	Stretching constant	Δ_{ij}	Interatomic distance (Å) Calc. by Badger's rule	Observed
SiH_4	2.84 10^5	0.59	1.46	1.46
SiH_3F	5.11 »	0.90	1.62	1.59
SiH_3Cl	3.00 »	1.18	2.03	2.05

EXPERIMENTAL PART

The infrared spectra were taken on a Beckmann I.R. II spectrophotometer using NaCl optics. The length of the gas-cell was 10 cm. At the experiments with SiD_3Cl, SiH_3F, and SiD_3F the pressure was, respectively, 11, 6, and 10 mm of Hg. The temperature was 20° C. Details of the preparation of the samples used are given in reference 2.

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Hydrolysis and Alcoholysis of 2,3-Dichlorodioxan

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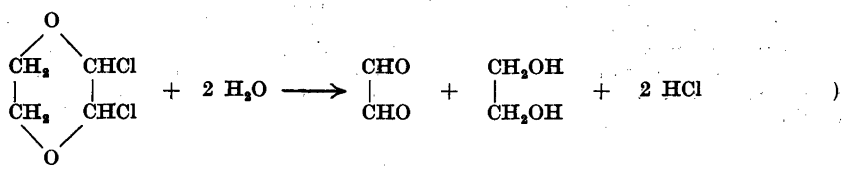
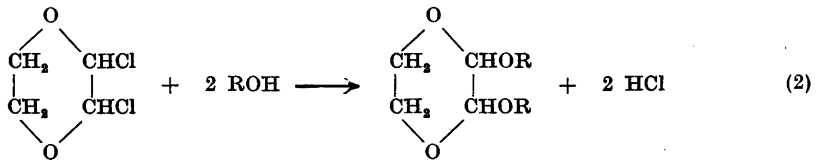
The chlorine atoms in 2,3-dichlorodioxan are known to be very reactive, and therefore the compound is widely used as a starting material in the preparation of several dioxan derivatives [1-3], and in the preparation of glyoxal and its derivatives [4]. The compound hydrolyzes readily according to the equation



Theoretically 2,3-dichlorodioxan is capable in existing in two modifications, the *cis* and *trans* forms, but the two isomers have never been isolated. Butler and Chretcher [4] have assumed that the compound is a mixture of the two isomers. It is to be noted, however, that in the displacement reactions of the chlorine atoms only one product has been isolated [3]. The only exception hitherto known is the reaction with ethylene glycol. This reaction yields two different products [1,2] which were formerly assumed to be the two stereoisomers of 1,4,5,8-naphtodioxan. Furberg and Hassel [5] have recently shown that the other reaction product is not an isomer of naphtodioxan, but bi-1,3-dioxa*cyclo*pentyl(2), and its formation thus involves further complications.

In a previous work [6] the author investigated the kinetics of the alcoholysis reaction of alkyl halogenomethyl ethers. Since 2,3-dichlorodioxan is also an 1-halogenoether, it seemed of interest to conduct a kinetic investigation of the hydrolysis (1) and alcoholysis (2)



(2)

reactions of the compound in order to elucidate the mechanism by which the chlorine atoms are displaced in the solvolytic reactions of the compound and its possible existence as a mixture of two isomers.

EXPERIMENTAL

Materials. 2,3-Dichlorodioxan was prepared by the chlorination of dioxan according to Böeseken, Tellegen and Cohen Henriquez [1] and Baker and Shannon [2]. The product obtained was distilled three times under reduced pressure. The purified product used in the kinetic experiments had a constant boiling point 82° C at 14 mm. (Found: Cl 44.94. Calc. for $C_4H_6O_2Cl_2$ (157): Cl 45.17.)

95.4 mg of the compound consumed after hydrolysis 23.50 ml of 0.0514 *N* sodium hydroxide, calculated 23.64 ml. The glyoxal formed in the hydrolysis of 56.1 mg of the compound was equivalent to 17.46 ml of 0.0813 *N* iodine, calculated 17.58 ml, determined by the modified bisulphite method described by the author for the determination of certain other aldehydes [6,7]. The analysis of the products of hydrolysis indicates that 2,3-dichlorodioxan hydrolyzes quantitatively according to eq. (1).

On standing a few weeks the 2,3-dichlorodioxan prepared solidified, *cf.* ref [2]. The melting point of the solid product was 31° C. Some kinetic experiments were also conducted with this form of 2,3-dichlorodioxan. Since the results obtained were identical with those obtained with the liquid 2,3-dichlorodioxan in the same solvents, the latter product was usually employed.

Acetone was a product of E. Merck (*pro analysi*) which was dried over anhydrous potassium carbonate and distilled. 1,4-Dioxan of technical grade (May & Baker Ltd.) was purified according to Hess and Frahm [8]. Ethanol was purified by the method described by Lund and Bjerrum [9]. The water used in the experiments was carbon dioxide-free distilled water. Lithium chloride and lithium perchlorate were purified as described earlier [6].

Kinetic measurements. In the kinetic measurements, the reactions were carried out in all-glass reaction vessels in an electrically heated water thermostat. A mercury contact regulator maintained the temperature constant within 0.02° C. A well stirred ice-water mixture was used as a thermostat at 0° C. When the reaction solution (water, water-acetone or water-dioxan mixture, or ethanol) had attained the temperature of the thermostat, 2,3-dichlorodioxan was added with vigorous shaking. The progress of the reaction was followed by taking 8—11 samples of the reaction mixture with a semiautomatic pipet during the course of the reaction and analysing both the hydrogen chloride formed and the unreacted 2,3-dichlorodioxan by the method described below. In most cases the initial concentration of 2,3-dichlorodioxan was about 0.05 *M* and the volume of the samples taken was 5 ml. In solvents containing 65 % or more water by weight, lower initial concentrations (0.03—0.01 *M*) were used, since 2,3-dichlorodioxan is less soluble in these solvents. In these experiments the volume of the samples taken from the reaction mixture was 10 or 20 ml. In all experiments the reaction mixture was homogeneous.

For the analysis of the samples taken from the reaction mixture, the following procedure based on preliminary experiments on the kinetics of the reaction was selected. The samples were run into Erlenmeyer flasks which were immersed in an ice-water mixture and which contained an amount of the standard sodium hydroxide solution used in the titrations which approximately neutralized the hydrogen chloride formed (or hydrochloric acid solution in the experiments in which the reaction solution was alkaline), and an amount of acetone or dioxan sufficient to correspond to at least 50 % of the total volume of the solution. By this latter addition, it was possible to stop the reaction efficiently also in such cases in which the reaction was very rapid, since preliminary experiments had indicated that the solvolysis rate is very low when the solvent contains at least 50 % acetone or dioxan, especially at low temperatures (the half-period of the reaction in such a mixture is more than four hours at 5° C). After the sample had been taken, the solution was neutralized with standard sodium hydroxide or hydrochloric acid solution, and the concentration of hydrochloric acid produced at the time of taking of the sample was calculated. An amount of water was then added to the neutralized solution which was sufficient to increase the water content to at least 90 % by volume, after which the

mixture was allowed to stand for half an hour at the room temperature. The hydrochloric acid formed by hydrolysis was then titrated to obtain the concentration of unreacted 2,3-dichlorodioxan at the time of taking of the sample. Preliminary experiments had indicated that the solvolysis rate of 2,3-dichlorodioxan is very high when the solvent contains 90 % or more water, the half-period of the reaction at room temperature being less than one minute. Thus the 2,3-dichlorodioxan present in the sample had time enough to hydrolyze quantitatively according to eq. (1). Approximately 0.05 *N* sodium hydroxide and hydrochloric acid solutions were used in the titrations.

Conducted kinetic measurements indicated that both procedures described above, the analysis of hydrogen chloride formed and the remaining 2,3-dichlorodioxan, gave conforming results and therefore only the results of the determination of the latter were used for the calculation of specific rates. In the hydrolysis reaction in alkaline solution, however, the rate of the decrease of the hydroxyl ion concentration was somewhat higher than the twofold rate of decrease of the 2,3-dichlorodioxan concentration. This is presumably to be attributed to a subsequent reaction, the Cannizzaro reaction of glyoxal: $(CHO)_2 + OH^- \rightarrow CH_2(OH)COO^-$. As will be shown, this complication does not, however, affect the results when the specific rates are calculated from the concentration of unreacted 2,3-dichlorodioxan in the reaction mixture.

RESULTS AND DISCUSSION

The order of the solvolytic reactions. The experiments conducted indicated that the hydrolysis and alcoholysis reactions of 2,3-dichlorodioxan are of the first order, the rate being proportional to the concentration of 2,3-dichlorodioxan in the reaction mixture. The specific rates (k) were calculated from the usual equation: $k = 1/t \ln a/(a-x)$, in which a denotes the concentration of 2,3-dichlorodioxan at the time of taking of the first sample and $a-x$ its concentration after time t. In all calculations, the second was used as the unit of time. To save space only two typical experimental runs are given in detail in Tables 1 and 2. The abbreviations "50.8 % dioxan", "25.3 % acetone" etc., used here and in the following, refer to dioxan-water or acetone-water mixtures which contain, respectively, 50.8 % dioxan and 25.3 % acetone by weight, etc.

Table 1. Hydrolysis of 2,3-dichlorodioxan in 50.8 % dioxan at 25° C. a = 0.0488

t (min.)	10	20	30	40	50	60	70	80	90
$a-x$	0.0385	0.0306	0.0242	0.0189	0.0148	0.0118	0.0093	0.0072	0.0055
$10^4 \cdot k$	3.95	3.89	3.90	3.95	3.98	3.94	3.95	3.99	4.04
							Average	3.95	

Table 2. Alcoholysis of 2,3-dichlorodioxan in ethanol at 35° C. a = 0.0482

t (hrs.)	6	12	24	30	36	48	56	72	80
$a-x$	0.0420	0.0366	0.0275	0.0240	0.0205	0.0157	0.0130	0.0092	0.0075
$10^4 \cdot k$	6.38	6.37	6.50	6.46	6.60	6.49	6.51	6.39	6.46
							Average	6.47	

The solvolytic reactions of 2,3-dichlorodioxan lead to the displacement of both of the chlorine atoms, the displacement of either of which may be the slower and thus rate-determining. The present results indicate that it is the displacement of the first chlorine atom that is the slower reaction, since otherwise the formation of hydrogen chloride would have been rapid at first, which was never observed in the experiments. The first-order equation was satis-

factorily obeyed in all cases independent of whether the decrease of 2,3-dichlorodioxan or the increase in the hydrogen chloride concentration was followed (see above).

Solvolysis in alkaline solution. Table 3 shows an example of experiments in which the hydrolysis of 2,3-dichlorodioxan was investigated in alkaline solution. It is seen that the addition of sodium hydroxide to the reaction solution does not alter the order of the reaction. Table 4 contains the average values of the first-order specific rates obtained with different alkali concentrations. The addition of sodium hydroxide does not influence the reaction rate significantly; at low sodium hydroxide concentrations the rate is practically unaffected, at relatively high concentrations, a slight decrease in the specific rate values is observed.

Table 3. Hydrolysis of 2,3-dichlorodioxan in 50.8 % dioxan at 35° C in the presence of sodium hydroxide. [NaOH] = 0.0624, a = 0.0381

t (min.)	2	4	6	8	13	16	20	25
$a-x$	0.0325	0.0277	0.0239	0.0204	0.0139	0.0116	0.0085	0.0058
$10^3 \cdot k$	1.32	1.33	1.30	1.30	1.29	1.24	1.25	1.26
							Average	1.29

Table 4. Influence of sodium hydroxide on the first-order specific rate of the hydrolysis of 2,3-dichlorodioxan in different solvents.

Solvent	° C	a	[NaOH]	$10^3 \cdot k$ sec.$^{-1}$
Water	15	0.0105	—	11.2
»	15	0.0139	0.0504	11.2
»	15	0.0123	0.0932	11.4
44.1 % acetone	35	0.0340	—	1.65
44.1 » »	35	0.0492	0.0972	1.68
44.1 » »	35	0.0510	0.1428	1.68
50.8 % dioxan	35	0.0553	—	1.26
50.8 » »	35	0.0381	0.0624	1.29
50.8 » »	35	0.0449	0.1475	1.23
50.8 » »	35	0.0414	0.3030	1.05
50.8 » »	35	0.0436	0.3620	0.97
50.8 » »	35	0.0441	0.5110	0.80

The fact that the addition of sodium hydroxide does not alter the order of the reaction and increase its rate indicates that the reaction takes place by the S_N1 mechanism [10], the rate-determining step being the unimolecular reaction involving the ionization of the first of the carbon-chlorine bonds. Similar results were also obtained in the alcoholysis reaction of alkyl halogenomethyl ethers [6], which reaction was shown to take place by the S_N1 mechanism. It is also interesting to note the recent results of Newth and Phillips [11]. The authors investigated the solvolytic reactions of *O*-acylglycosyl 1-halides, which contain lactol ring oxygen atom in the 1-position to the halogen atom, and found the reactions to take place by the S_N1 mechanism.

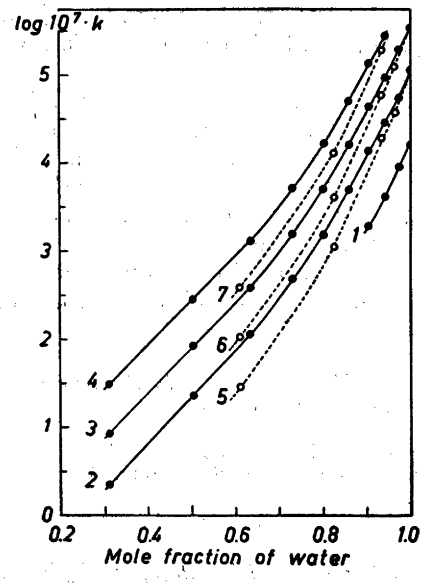


Fig. 1. Influence of solvent on the hydrolysis rate of 2,3-dichlorodioxan. Log k as a function of the mole fraction of water in different acetone-water and dioxan-water mixtures. 1, 2, 3 and 4 acetone-water mixtures at 0°, 15°, 25° and 35° C, 5, 6 and 7 dioxan-water mixtures at 15°, 25° and 35° C, respectively.

It is interesting that relatively high concentrations of sodium hydroxide lower the rate (Table 4). Similar effects have been also observed by Benfey, Hughes and Ingold [12] in the hydrolysis of diphenyl methyl bromide in aqueous dioxan, which reaction takes place by the S_N1 mechanism.

Small additions of sodium alcoholate do not materially influence the rate of the alcoholysis reaction of 2,3-dichlorodioxan. For instance, the value obtained for the first-order specific rate in ethanol solution at 15° C was $4.17 \cdot 10^{-7}$ sec.$^{-1}$ in the presence of 0.156 *M* sodium ethylate, whereas the value obtained without the alcoholate addition was $4.04 \cdot 10^{-7}$ sec.$^{-1}$.

Solvent effects. Fig. 1 shows the dependence of the logarithm of the first-order specific rate of hydrolysis of 2,3-dichlorodioxan on the solvent composition in different acetone-water and dioxan-water mixtures. In both solvent mixtures a very abrupt increase in the specific rate is observed with increasing water content of the solvent; in acetone-water mixtures, for example, the specific rate increases more than $5 \cdot 10^4$-fold when the mole fraction of water increases from 0.3 to 1.0. The results are in accord with the S_N1 mechanism [10]. Since the rate-determining step involves formation of ions, the rate may be expected to increase rapidly with the ionizing power of the solvent. Similar results have been also obtained by Tommila and Jutila [13] for the hydrolysis of *iso*propyl benzenesulphonate in the same binary mixtures, which reaction was also shown to take place by the S_N1 mechanism. Moreover, it is interesting to note that the inhibiting effect of dioxan is also in the present case somewhat greater than that of acetone for the same mole fractions of the organic components.

Salt effects. The investigation of salt effects furnishes a further criterion for the reaction mechanism. In a S_N1 reaction of an organic halide, two dif-

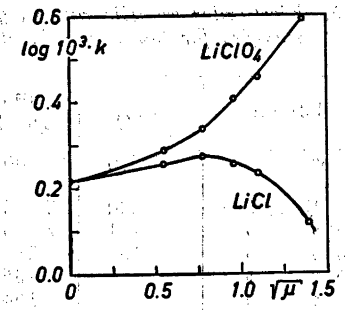


Fig. 2. Salt effects in the hydrolysis of 2,3-dichlorodioxan in 44.1 % acetone at 35° C in the presence of lithium chloride and lithium perchlorate. The dependence of the logarithm of the first-order specific rate on the square root of ionic strength.

ferent salt effects are possible [14]. An increase in the rate is to be expected following addition of electrolyte (ionic strength effect), since the rate-determining stage involves formation of ions. If, however, the anion of the added electrolyte is the same as that formed in the reaction, the situation may be reversed. This is because with increasing concentration of the common anion, the rate-determining ionization reaction may be partially reversed whereupon the rate is decreased (mass-law effect). Thus in the presence of non-common-ion salts the rate is always increased, whereas in the presence of common-ion salts the rate is increased or decreased depending on whether the ionic strength effect is strong enough to compensate the influence of the mass-law effect. Examples of both cases are to be found in the literature [6,11,14]. In an one-stage S_N2 reaction, on the contrary, the mass-law effect is not possible.

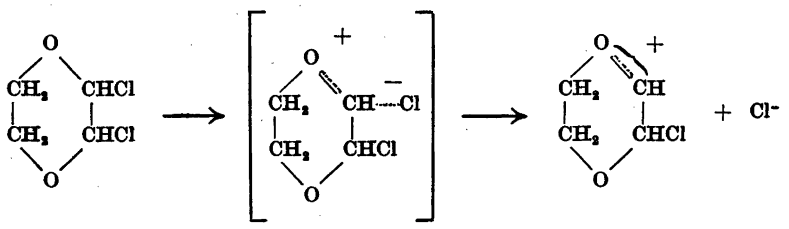
Fig. 2 shows the dependence of the logarithm of the specific rate of the hydrolysis on the square root of the ionic strength in the presence of lithium chloride and lithium perchlorate. As seen from the figure, lithium perchlorate causes the rate to increase continuously with increasing salt concentration, whereas in the case of lithium chloride the rate increases only slightly at lower concentrations. At higher lithium chloride concentrations the rate begins to decrease continuously. This is obviously because the mass-law effect becomes great enough at higher lithium chloride concentrations to overcome the ionic strength effect. The different influence of lithium perchlorate and lithium chloride on the rate is thus in good accord with the S_N1 mechanism.

Parameters of Arrhenius equation. In all solvents investigated the Arrhenius equation was obeyed within the limits of experimental error. Table 5 contains the logarithms of the frequency factors A and the activation energies E for the solvolysis of 2,3-dichlorodioxan in different media with the corresponding values of the first-order specific rates. The values of log A and E were calculated by the method of least squares. It is seen from the table that in the hydrolysis reaction the activation energy decreases and the frequency factor increases with water concentration. Both effects act in the same direction and thus the rate increases rapidly with increasing water concentration. The variation of log A and E with the water content of the solvent is similar to that observed by Tommila and Jutila [13] in the hydrolysis of *iso*-propyl benzenesulphonate in acetone-water mixtures.

Table 5. First-order specific rates, logarithms of the frequency factors and the activation energies for the solvolysis of 2,3-dichlorodioxan in different solvents.

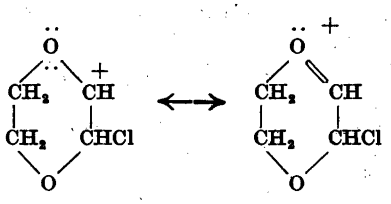
Solvent	$10^6 \cdot k$ sec.$^{-1}$ 0° C	15° C	25° C	35° C	log A	E cal.
Water	1 580	11 200	33 200		13.05	19 790
8.1 % acetone	890	5 940	18 900		12.79	19 800
16.5 » »	421	2 890	9 110	27 300	12.56	19 910
25.3 » »	191	1 360	4 320	13 400	12.50	20 260
34.5 » »		495	1 590	4 970	12.15	20 370
44.1 » »		154	504	1 650	12.07	20 940
54.2 » »		48.4	159	519	11.57	20 950
64.8 » »		11.4	39.3	131	11.41	21 560
75.9 » »		2.32	8.42	28.8	11.24	22 250
87.7 » »		0.224	0.854	3.20	11.16	23 480
15.4 % dioxan	581	3 780	12 400		12.60	19 800
25.6 » »		1 930	5 910	18 600	12.45	20 000
50.8 » »		113	395	1 260	12.22	21 310
75.6 » »		2.88	10.6	37.7	11.68	22 710
Ethanol		0.404	1.65	6.47	12.20	24 520

The activation energy for the alcoholysis of 2,3-dichlorodioxan in ethanol, 24 520 cal., is very high compared with the activation energy of methyl chloromethyl ether [6], 14 510 cal., in the same solvent. The latter reaction is much more rapid; *e. g.*, at 15° C about 160 000 times as rapid as the alcoholysis of 2,3-dichlorodioxan. The effect of structure is in accord with that observed in other S_N1 reactions [6,10], since an electron-attracting substituent near the site of the reaction, *e. g.* chlorine atom, may be expected to decrease the ionizability of the bond broken in the rate-determining ionization. The difference between the rate of solvolysis of methyl chloromethyl ether and 2,3-dichlorodioxan seems, however, to be too great to be accounted solely for by the inhibiting effect of the second chlorine atom on the ionization of the first. An additional explanation is suggested below when the reaction mechanism is discussed in more detail. The rate-determining step in the solvolysis of 2,3-dichlorodioxan may be presented as follows (*cf.* ref. [6]):



Transition state

That the reaction takes place by the S_N1 mechanism is to be attributed to the resonance of the nascent cation. The cation formed can be considered as a resonance hybrid of the following two valence bond structures:



In the hybridized structure one of the carbon-oxygen bonds has a partial double-bond character, which demands coplanarity of the adjacent carbon-oxygen and carbon-carbon bonds directly attached to the resonating system [15]. Thus the hybridized structure must be able to overcome steric strain, which is due to the cyclic structure of the molecule, and the resonance energy liberated in the formation of the carbonium cation is reduced and the activation energy is relatively high.

The rate-determining ionization in the solvolysis of 2,3-dichlorodioxan is then followed by a rapid reaction with the solvent. The substitution of one of the chlorine atoms is followed by a more rapid displacement of the second, apparently by the S_N1 mechanism. The fact that the displacement of the second chloride atom is more rapid than that of the first is in good accord with the S_N1 mechanism, since hydroxyl and alkoxyl groups are less electron-attracting substituents than chlorine (*cf.* ref. [6]), and thus their retarding effect on the ionization is smaller.

The present results give no support to the existence of 2,3-dichlorodioxan as a mixture of the two isomerides, the *cis* and *trans* forms; at least the concentration of the other isomer is very low. The different modifications would apparently have different rates of solvolysis and good first-order specific rates could not be obtained for their mixture. The fact that different products have never been isolated in the displacement reactions of the chlorine atoms is in accord with our results. The observations of Summerbell and Bauer [3] are interesting in this respect. The authors found that the reaction of chlorine with 2,3-dioxen yields 2,3-dichloro-dioxan, similar to that obtained by the direct chlorination of dioxan. Since normal double-bond addition reactions yield *trans* forms [16], 2,3-dichlorodioxan has presumably *trans* chlorine atoms.

SUMMARY

The kinetics of hydrolysis and alcoholysis of 2,3-dichlorodioxan have been investigated in different solvents and in the presence of electrolytes. The results indicate that the solvolytic reactions of the compound take place by S_N1 mechanism, the rate-determining reaction being the ionization of the first of the carbon-chlorine bonds.

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Enzymatic Hydrolysis of Organophosphorus Compounds

I. Occurrence of Enzymes Hydrolysing Dimethyl-amido-ethoxy-phosphoryl Cyanide (Tabun)

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Compared with the biochemical effects of the organophosphorus compounds as cholinesterase inhibitors, little is known about the degradation and detoxication mechanism of these agents in the living organism. The presence of an enzyme ("phosphofluorase" or "dialkylfluorophosphatase") capable of catalysing the hydrolysis of di*iso*propyl fluorophosphate (DFP) in the plasma and tissues of rabbit and man was first reported by Mazur [1]. The purification and properties of such an enzyme (DFP-ase) from kidney was recently described by Mounter *et al.*[2], who in a second paper [3] reported studies on the activation and inhibition by metals of this enzyme. In these three reports DFP was used as substrate and no serious attempts have as yet been made to elucidate the specificity of the enzyme or enzymes.

Aldridge [4,5] has demonstrated that an esterase, widely distributed in the tissues of rabbit and rat and present in the sera from many different species, hydrolyses diethyl *p*-nitrophenyl phosphate (mintacol, E 600). This esterase is identical with the so-called A-esterase which hydrolyses *p*-nitrophenylacetate. The enzymatic hydrolysis of mintacol by human plasma was confirmed in this laboratory [6]. The *in vivo* degradation of parathion (E 605), the sulphur analogue of mintacol, was studied by Pankaskie *et al.*[7] and by Lieben *et al.*[8], who showed that no parathion was excreted from the body.

It has been shown previously in this laboratory [9] that the organophosphorus compounds react spontaneously with dihydroxyphenyl derivatives, the reaction resulting in phosphorylation of one of the phenolic groups. Such a reaction could be of importance in the mechanism of cholinesterase inactivation or in the mechanism of an enzymatically controlled detoxication process. A series of experiments were carried out *in vitro* on the enzymatic degradation of organophosphorus compounds; a short communication of the results obtained was recently published [10]. The present report deals with some preliminary studies on the enzymatic hydrolysis of tabun or dimethylamido-ethoxy-phosphoryl cyanide.

METHODS AND MATERIAL

Enzyme activity was determined by the Warburg technique. Generally, 1.6 ml of the enzyme preparation was placed in the main compartment of a Warburg flask and 0.4 ml of the substrate solution in one of the side bulbs. All enzyme preparations were made up in a solution containing 0.034 *M* sodium bicarbonate and 0.12 *M* chloride. In the first of these experiments 0.0012 *M* magnesium chloride was also present, but was later omitted since it was found that magnesium ions do not influence the enzymatic hydrolysis of the compounds studied. The solutions were equilibrated with a mixture of 95 % N_2 + 5 % CO_2. The pH of the reaction mixture after mixing substrate and enzyme was 7.6. The liberation of CO_2 from the bicarbonate buffer was taken as evidence of the production of acid. All experiments were made at 25° C. Readings were taken at various intervals over 40 minutes or longer. Activities were calculated from the initial slope of the reaction curve (in a CO_2 (in μl) — time diagram). The extrapolated 30 minute value (a_{30}), minus the amount of CO_2 evolved during the same period of time by non-enzymatic hydrolysis, is generally taken as unit of enzyme activity, symbolised by b_{30}.

Substrates. A great variety of organophosphorus compounds have been tested as substrates. In the present paper, the experiments carried out mainly with tabun will be described; in some cases the results were compared with those obtained with DFP (di*iso*propyl fluorophosphate). In general, 0.4 % (by volume) solution of pure tabun in distilled water was prepared immediately before use; the concentration of tabun (mol. wt. = 162.13, d_{20} = 1.077) in the reaction mixture (2.00 ml) during activity determinations was 5.3×10^{-3} *M*. The time between the dilution procedure and mixing the substrate and enzyme in the Warburg flask was in most instances 60 minutes (for technical reasons). During this period the substrate solutions became somewhat acid due to spontaneous hydrolysis.

In the preliminary studies various solvents were tried for the dilution of the organophosphorus compounds. Highly purified propylene glycol (1,2-propanediol) is not suitable as it was found that CO_2 is evolved spontaneously when such a solution is mixed with the bicarbonate solution or with plasma diluted with that buffer. In addition, the initial rate of enzymatic hydrolysis of tabun is unfavorably influenced when the enzyme preparation is mixed with propylene glycol prior to the addition of substrate. The propylene glycol probably interferes with the enzyme in some way which has not yet been investigated in detail.

Peanut oil is not suitable when the enzymatic hydrolysis of these compounds are studied. When it is used as a solvent for the substrates, a lower initial enzyme activity is observed compared with controls. The actual substrate concentration in the aqueous phase is reduced for such an oil-water system. Due to the spontaneous hydrolysis of organophosphorus compounds in alkaline solutions, a bicarbonate solution is not suitable for the preparation of substrate solutions. It was decided therefore to use distilled water as a solvent and this was done throughout the investigations described.

Enzyme preparations. The catalytic effect of blood plasma from various animals on the hydrolysis of tabun has been investigated. Blood was taken with heparin and centrifugated free of blood cells. The enzyme activity of rabbit tissues was also studied. The organs were homogenised and extracts made up in bicarbonate solution. The hydrolysis capacity of kidney and adrenal glands from rabbit was compared with that of the same organs from pig and cow.

The various fractions of human serum (postpartum) studied were those obtained by a partly modified method No. 6 of Cohn *et al.*[11]. These fractions were prepared by A/B Kabi (Stockholm).

RESULTS

Introduction. The hydrolysis of tabun catalysed by blood plasma (*e. g.*, human plasma) is of an enzymatic nature. The rate of this reaction is much higher than the hydrolysis in aqueous solution without the plasma. The catalytic reaction is continuous and the rate of hydrolysis proportional to the amount of plasma added (*cf.* below and Fig. 3). The catalysis is completely

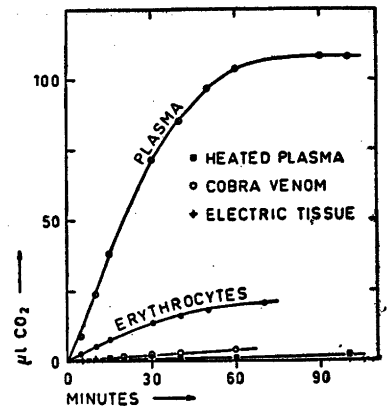

Fig. 1. Enzymatic hydrolysis of tabun (correction made for spontaneous hydrolysis) by various preparations known to contain cholinesterases. Plasma and erythrocytes from human blood. Electric tissue from Torpedo marmorata. Plasma heated for 15 minutes at 70° C.

prevented by heating for 10 minutes at 70° C (Fig. 1). After dialysis for 24 hours against running water, the catalytic activity of the plasma is reduced only a few per cent. It was concluded therefore that this hydrolysis of tabun by blood plasma is an enzymatic reaction. For the sake of convenience this enzyme will provisionally be referred to as "tabunase". It should be noted, however, that the enzyme activity demonstrated in various animal tissues is not specific for tabun, other organophosphorus compounds being hydrolysed as well; at least some of the enzymes studied split DFP and sarin. " Phosphorylphosphatase" is suggested by the authors as a more general name for enzymes hydrolysing organophosphorus compounds, characterised by the phosphoryl (PO) group and a variety of organic substituents. It should be noted also that these enzymes, as far as can be discussed, are not related to the ordinary phosphatases, *e. g.*, the kidney and plasma phosphatases.

The phosphorylphosphatases have no relation to the cholinesterases. However, the importance of the discovery of such enzymes is obvious when studying the organophosphorus compounds as anticholinesterase drugs. Preparations known to contain acetylcholinesterase as the only esterase, *e. g.*, those of electric tissue and cobra venom, do not hydrolyse tabun (Fig. 1) or other organophosphorus compounds studied. Haemolysate of human erythrocytes splits tabun at a comparatively low but detectable rate. It is known, however, that acid is produced when the compounds react with cholinesterases [9], but the concentrations necessary to inactivate cholinesterase completely are too low to give sufficiently large amounts to be detected by the technique used in the present study. Systems containing cholinesterases and phosphorylphosphatases as enzyme mixture, and acetylcholine and an organophosphorus compound as substrate-inhibitor mixture will be discussed in more detail in a subsequent paper.

Blood plasma as enzyme source. Typical hydrolysis curves for the enzymatic destruction of tabun are illustrated in Fig. 2 where horse plasma was used as the enzyme source. As will be described in a subsequent paper the production of CO_2 from a bicarbonate buffer when tabun is used as substrate, is due to the liberation of dimethyl-amido-ethoxy-hydroxy-phosphine oxide which

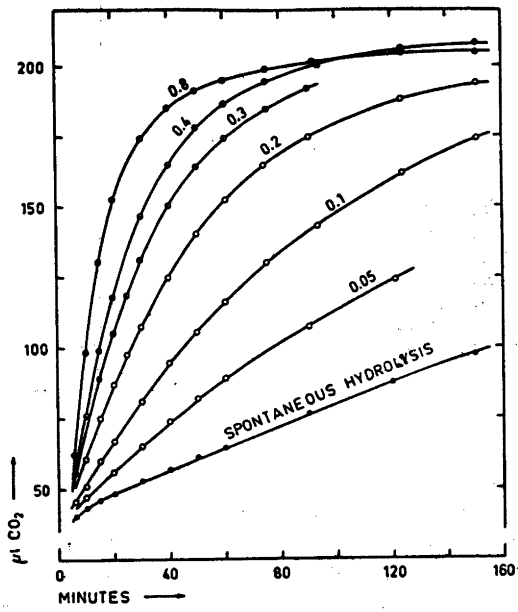

Fig. 2. Hydrolysis of tabun by the action of horse plasma. Numbers refer to ml plasma per reaction mixture (2.00 ml). Substrate, 5.3 × 10^{-3} M tabun.

is a strong acid [12]. Hydrocyanic acid is too weak an acid to be the cause of CO_2 production and therefore no more than one mole CO_2 per mole tabun is liberated. Generally we have found that 80 to 90 % of the theoretical amount of CO_2 (238 µl in Fig. 2) was evolved when crude enzyme preparations were used. In Fig. 3 the enzyme activity is recorded as a function of plasma concentration.

Blood plasma from other animal species, including man, have been tested. The results are demonstrated in Fig. 3 where enzyme activity is plotted against the amount of plasma present. For most preparations direct proportionality was found between hydrolysis rate and plasma concentration. Rabbit plasma has the highest activity and is about 10 times more active than human plasma. Amongst the eight plasma examined guina-pig plasma is least active. Bovine plasma, which is known to have no cholinesterase activity, has a moderate tabunase activity. The results obtained with a purified preparation (Fraction IV—1) from human serum are inserted in Fig. 3.

Separation of tabunase from cholinesterase. In order to purify tabunase of blood plasma and to separate it from the plasma cholinesterase, ammonium sulphate fractionation was tried without much success. Table I shows that the enzyme hydrolysing tabun cannot be separated by precipitation with this salt; even in 70 % saturated solutions appreciable amounts of active material were still detected in solution. It is known from earlier studies [13] that cholinesterase is completely precipitated at this concentration of ammonium sulphate. With

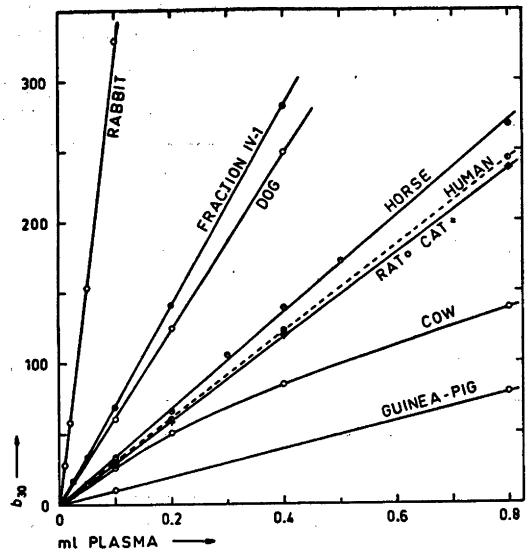

Fig. 3. Enzymatic hydrolysis of tabun by the plasma of various animals as a function of plasma concentration. Substrate, 5.3 × 10^{-3} M tabun. Fraction IV—1 was used as a 2.5 % solution.

a similar fractionation procedure, Aldridge [5] could not precipitate his A-esterase, which hydrolyses mintacol, successfully from rabbit serum.

Using the fractionation procedure of Cohn *et al.*[11] with human postpartum serum the tabunase came down almost completely in the globulin Fraction IV—1. Using this method the cholinesterase is found in Fraction IV—6 and no active material comes down in Fraction IV—1. The results obtained are recorded in Table 2. The direct proportionality between tabunase activity and concentration of Fraction IV—1 is shown in Fig. 3.

Tissues as enzyme source. The ability of various tissues of rabbit to hydrolyse tabun was then examined. The results are tabulated in Fig. 4 together

Table 1. Fractionation of human plasma with ammonium sulphate to separate the enzymes hydrolysing tabun and acetylcholine.

Fraction	b_{30}	
	Tabun	Acetylcholine
Original plasma	58.5	213
Precipitation, 50 % saturation with sulphate	47	35.5
Precipitation, 70 % saturation with sulphate	49	88.5
Centrifugate after 70 % saturation with sulphate	53	0

Table 2. Fractionation of human postpartum serum according to a partly modified (Kabi) method No. 6 of Cohn et al. [11] *to separate the enzymes hydrolysing tabun and acetylcholine.*

Fraction	b_{30}/10 mg proteine	
	Tabun	Acetylcholine
Original serum	40	111
I	8	14
II (γ-globulin)	2	0
III (β-globulin)	1	11
IV–1	135	14
IV–4	3	510
IV–5 +6	2	475
IV–6 + 9	0	880
IV–6	–	4 500
V (albumin)	2	0
Electric tissue *(Torpedo)*	0	5 000

with those obtained with acetylcholine as substrate. Adrenal glands are the most active; kidney and liver come next in activity amongst the tissues tested. Those tissues known to contain acetylcholinesterase (*e. g.*, brain, spinal cord) are weakly active. It will be shown in a subsequent paper that tabunase is not identical with the phosphatases known to be present in plasma, adrenal glands, kidney, and liver.

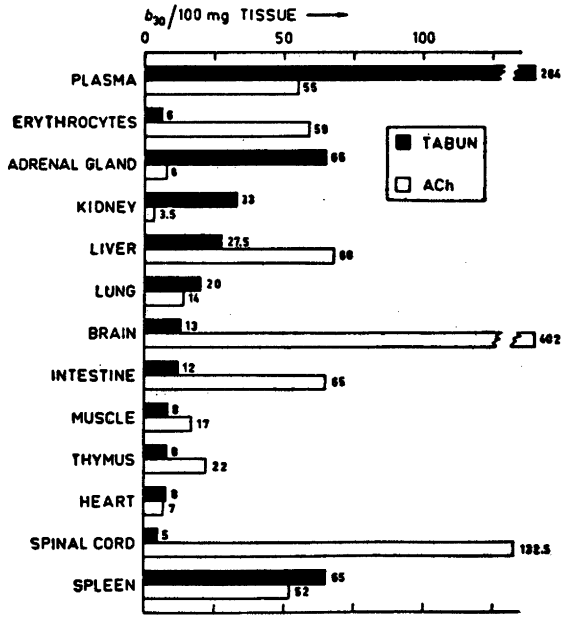

Fig. 4. The activity of various rabbit tissues in the hydrolysis of tabun and acetylcholine (ACh).

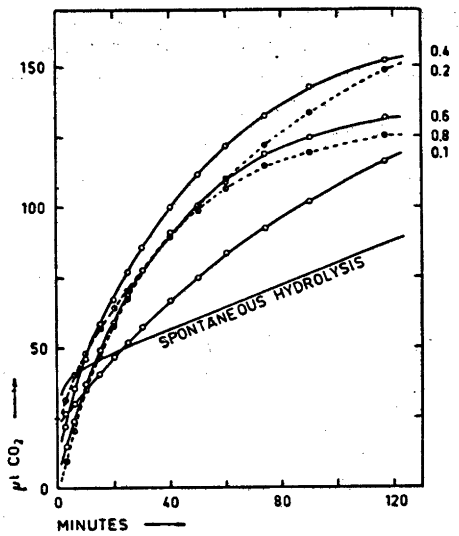

Fig. 5. Hydrolysis of tabun by the action of rabbit liver (not perfused). Numbers refer to grams of liver per reaction mixture (2.00 ml). Substrate, 5.3 × 10⁻³ M tabun.

An example of the hydrolysis of tabun by a tissue phosphorylphosphatase (rabbit liver) is shown in Fig. 5. Due to the relatively high spontaneous hydrolysis of tabun there is an immediate liberation of CO_2 when the enzyme (in bicarbonate buffer) and the substrate (in aqueous solution) are mixed. The

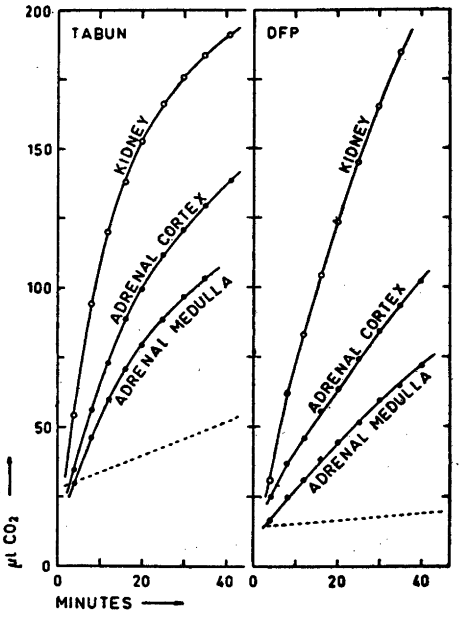

Fig. 6. Hydrolysis of tabun and DFP by kidney cortex and by the cortex and medulla of adrenal glands (pig). Substrate, 5.3 × 10⁻³ M; tissue, 200 mg per reaction mixture (2.00 ml). Dotted lines refer to spontaneous hydrolysis of substrates.

Table 3. The hydrolysis of tabun and DFP by kidney and adrenal glands of cow and pig. Enzyme activities are expressed in b_{30} *per 100 mg tissue. Cf. Fig. 6.*

Animal	Kidney cortex		Adrenal gland			
			Cortex		Medulla	
	Tabun	DFP	Tabun	DFP	Tabun	DFP
Cow	101.5	40	41	28	27	17
Pig	140	158	51	31	34.5	22.5

recorded amount of this spontaneously produced CO_2 is the less, the larger the amount of crude extract used.

Mazur [1] and Mounter *et al.*[2] have shown that kidney hydrolyses DFP. The hydrolysis of this substrate and that of tabun were compared using kidney and adrenal glands of pig and cow. The results obtained are shown in Fig. 6 and Table 3. In both animals the kidney is more active against the two substrates than are the adrenal glands. The adrenal cortex is more active than the adrenal medulla. The various tissue preparations show proportionate activities for each substrate and most probably the same enzyme in these tissues is responsible for the hydrolysis of tabun and DFP. Evidence will be presented later that the phosphorylphosphatase of plasma is not identical with that of kidney.

DISCUSSION

The presence of enzymes which hydrolyse the highly toxic organophosphorus compounds, is of great importance for our understanding of the pharmacological and biochemical effects of the latter on living organisms. Such enzymes in all probability interfere with the inactivation process of cholinesterases by such compounds. They will also be actively engaged in the detoxication mechanism of these anticholinesterase drugs, of which mechanism we know comparatively little.

From biochemical point of view a study of the enzymatic hydrolysis of organophosphorus compounds is likely to be of value for understanding of the mechanism of the inactivation of cholinesterases, which process underlies the toxicity of such compounds. In the opinion of the authors the hydrolysis of the compounds and cholinesterase inactivation by them involve principially the same reaction mechanism. In both these reactions the same linkage of the active molecule is broken, *i. e.*, the bond between the phosphorus atom and the acid group (halogen, cyanide, or an organic radical). In the hydrolysis process the phosphorus compound formed by the splitting of the toxic molecule is in itself not toxic. In the cholinesterase inactivation process that very part of the original molecule is more or less irreversibly bound to the esteratic site of a cholinesterase molecule. Further evidence for this working hypothesis is being presented later together with a more complete discussion of the results obtained.

SUMMARY

The presence of an enzyme, provisionally called tabunase (general name suggested, phosphorylphosphatase) and having the property of hydrolysing dimethylamido-ethoxy-phosphoryl cyanide (tabun), has been demonstrated in the blood plasma of eight various animals including man; the rabbit plasma is the most active. Erythrocytes are almost inactive. Amongst the tissues of rabbit the adrenal glands, kidney, and liver are particularly active. It appears probable from experiments with pig and bovine tissues that tabun and DFP are split by the same enzyme of kidney and adrenal glands.

The phosphorylphosphatase and cholinesterase of human serum were separated from each other by the method of Cohn.

Tabun was prepared at the Research Institute of National Defence, Department 1, Chief Professor Gustaf Ljunggren, to whom we are indebted for his continual interest. The authors wish to express their sincere thanks to Ing. Henrik Björling of A/B Kabi, Stockholm, for the preparation of the various serum fractions.

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Enzymatic Hydrolysis of Organophosphorus Compounds

II. Analysis of Reaction Products in Experiments with Tabun and Some Properties of Blood Plasma Tabunase

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In a previous paper [1] evidence has been put forward for the existence of an enzyme (phosphorylphosphatase, "tabunase") which catalyses the hydrolysis of dimethylamido-ethoxy-phosphoryl cyanide or tabun. The enzyme is present in blood plasma and various animal tissues. In the present paper the analysis of the products formed in such a reaction are described together with some properties of tabunase.

METHODS AND MATERIAL

The enzymatic hydrolysis of tabun was followed by the Warburg technique [1]. Enzyme activity is expressed in b_{30} values, *i. e.*, μl CO_2 evolved from a bicarbonate solution in 30 minutes, corrections being made for the spontaneous hydrolysis of the substrate.

Tabun was used as substrate exclusively and the plasma from rabbit and horse were used as enzyme preparations. In studies on the pH stability, fraction IV—1 of human serum (prepared as described by Cohn) [1] was employed.

Dimethylamine was determined by Dowden's [2] method which is based upon the reaction of dimethylamine with carbon disulphide and copper ion, forming cupric dimethyl-dithiocarbamate which is water-insoluble and imparts a deep yellow colour to chloroform, benzene, and other organic solvents in which it is soluble. The method was previously used by Larsson [3] to study the spontaneous hydrolysis of tabun and by Hall, Stohlman and Schechter [4] for the determination of octamethyl pyrophosphoramide (OMPA, Schradan).

The principle of Epstein's method [5] used to analyse the reaction mixture for cyanide involves the conversion of cyanide to cyanogen chloride with chloramine T solution; a blue colour, stable for at least 30 minutes, is obtained by adding pyridine, containing 0.1 % bis-pyrazolone and 1-phenyl-3-methyl-5-pyrazolone. With this method 0.2 to 1.2 μg cyanide can be determined. Recently Holmstedt [6] followed the spontaneous hydrolysis of tabun by this method.

The bicarbonate buffer, pH 7.6, used in the enzyme activity determinations contained 0.034 *M* sodium bicarbonate and 0.12 *M* sodium chloride. In the determination of the pH stability of human serum tabunase, *M*/15 phosphate buffers were used.

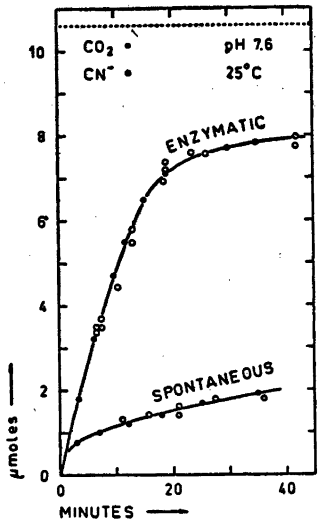


Fig. 1. Enzymatic hydrolysis of tabun by rabbit plasma followed by the CO_2 production in bicarbonate solution and the simultaneous production of cyanide. Original amount of tabun present, 10.6 μmoles.

RESULTS

Enzymatic hydrolysis of tabun by rabbit plasma and analysis of the reaction products at equilibrium and at intervals during the reaction were carried out. There was no release of inorganic phosphate during the reaction, nor was ethanol liberated.

Determination of dimethylamine. Compared with the spontaneous hydrolysis of tabun as control no extra dimethylamine was found to be split off by enzymatic degradation. When hydrolysis had reached equilibrium, the reaction mixture, containing 1.06×10^{-5} moles per ml of tabun originally, contained 11.5 μg or 1.64×10^{-6} moles of dimethylamine per ml. Based upon the amount of free dimethylamine, only 15 % of the tabun was destroyed. The production of dimethylamine was not due to an enzyme as the spontaneous hydrolysis of tabun over the same period (3.5 hours) and under the same experimental conditions (pH 7.6, temp. 25° C) yielded about the same amount, 11.0 μg of dimethylamine per ml.

Determination of cyanide. The liberation of CO_2 from a bicarbonate buffer during the enzymatic hydrolysis of tabun by rabbit plasma parallels the production of cyanide. This result is illustrated in Fig. 1. When equilibrium is reached about 80 % of tabun is hydrolysed, as determined by both the CO_2 and CN methods. After the same period (40 minutes) 16 % of the same initial amount of tabun was hydrolysed spontaneously. These experiments show that the enzymatic hydrolysis of tabun by rabbit plasma involves splitting of the P-CN linkage. The production of CO_2 is not due to hydrocyanic acid ($K_a = 7.2 \times 10^{-10}$), but to the other strong acid formed, dimethylamido-ethoxy-hydroxy-phosphine oxide.

Effect of the reaction products on the tabunase and acetylcholinesterase activities. When equilibrium is reached the reaction mixture has a weak inhibiting effect on the tabunase activity. Cyanide of the same concentration as that at equilib-

Table 1. The effect of cyanide, and the reaction products after "complete" hydrolysis of tabun by tabunase, on the enzymatic hydrolysis of tabun by a purified preparation of tabunase (Fraction IV—1). The heated preparation of the reaction mixture was prepared by incubation for 3 minutes in boiling water. Substrate, 5.3 × 10^{-3} M tabun, which was also the concentration of tabun in the original reaction mixture.

Inhibitor	b_{30}	Percentage inhibition
No inhibitor present (control)	78	—
NaCN, 10^{-3} *M*	67.5	13
Reaction mixture, heated	62.5	20
» » not heated	77.5	0

rium inhibits tabunase to about 10 %. The inhibiting effect of the reaction mixture is somewhat higher than that and it is therefore concluded that the other phosphorus containing part of the original tabun molecule also inhibits the tabunase activity. This inhibiting effect of the reaction products explains the decrease of reaction velocity during the enzymatic hydrolysis of tabun. The results obtained are illustrated in Table 1. In order to test the inhibiting effect of the reaction products the remaining tabunase activity of a mixture containing these products was destroyed by heating in boiling water. The reaction mixture before heating has still sufficient tabunase activity to split additional amounts of tabun and therefore the inhibiting effect of the reaction mixture in this case is masked.

It was suggested that the phosphorus containing part of the tabun molecule liberated during the enzymatic hydrolysis shows no cholinesterase inhibiting effect. This suggestion is a logical conclusion from previous studies on cholinesterase inhibiting activity and chemical structure of the organophosphorus compounds. The compound in question has not been available, but it has been found that the reaction products formed after "complete" enzymatic hydrolysis of tabun, using a purified enzyme preparation (Fraction IV—1), have no inhibiting effect on acetylcholinesterase in the concentrations used. Table 2 demonstrates the results in one of a series of experiments performed. The activating effect of the tabunase preparation on acetylcholinesterase (electric tissue), as seen in Table 2, will be discussed in a future paper.

pH stability of purified tabunase. The stability at various pH values of a purified preparation of tabunase (Fraction IV—1 of human serum) was deter-

Table 2. The effect of the reaction products after "complete" hydrolysis of tabun by tabunase on the acetylcholinesterase (electric tissue of T o r p e d o). Tabunase was a purified preparation (Fraction IV—1). Substrate, 3.3 × 10^{-3} M acetylcholine chloride. Concentration of tabun during esterase determination, 2.6 × 10^{-6} M.

Enzyme-inhibitor-system	b_{30}	
Acetylcholinesterase	168	153
Tabunase	14	14
Acetylcholinesterase + Tabunase	194	185.5
Acetylcholinesterase + Tabun	30.5	6
Acetylcholinesterase + Reaction mixture	195	189.5

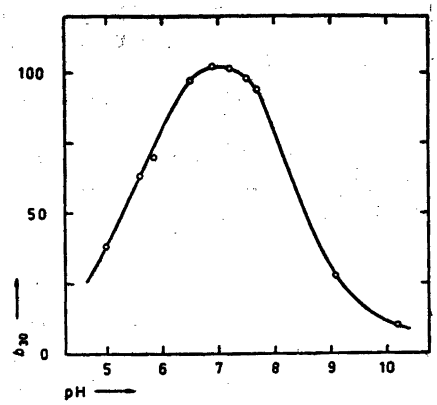


Fig. 2. The pH stability of a purified preparation (Fraction IV—1) of human serum tabunase. Incubation for 24 hours at 4.5° C.

mined in the following way. Solutions of the preparation in phosphate buffers, containing 25 mg tabunase per ml, were incubated at various pH and 4.5° C. After 24 hours 0.3 ml of the test solutions were diluted with 1.3 ml bicarbonate buffer to give pH 7.6. The enzyme activity was then determined using 5.3 $\times$ 10^{-3} *M* tabun. The results obtained are shown in Fig. 2 where enzyme activity is plotted against pH. The tabunase preparation was found to be most stable in neutral solutions.

Determination of the dissociation constant of the tabun-tabunase complex. The most convenient graphical method of evaluating the relationship between enzyme activity and substrate concentration is to plot the initial reaction velocity (v) of the enzyme reaction against v/[S] where [S] is the molar substrate concentration [7]. For simple enzyme-substrate systems this method gives, by extrapolation, the values of maximum velocity (V_{max}) and the dissociation constant (K_S) of the enzyme-substrate complex. This method was applied

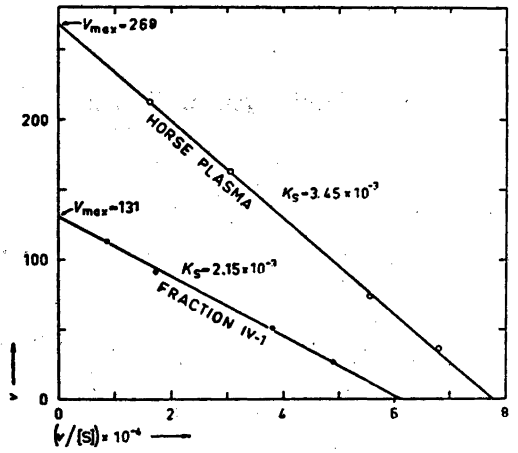
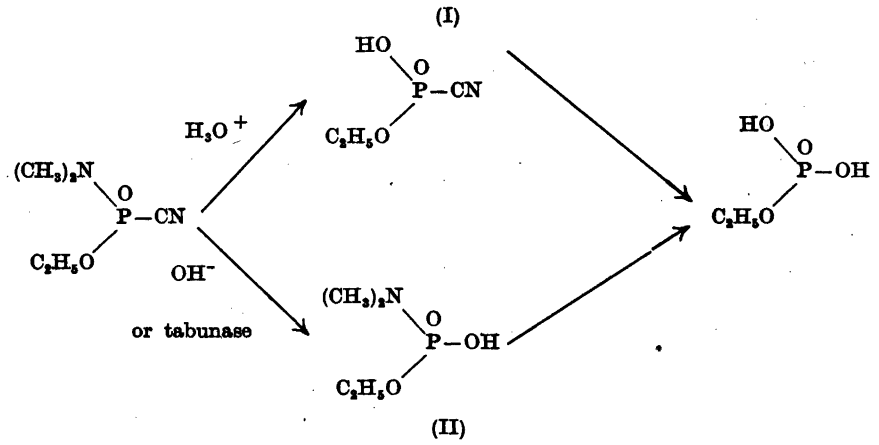


Fig. 3. Evaluation of K_S *for the enzymatic hydrolysis of tabun by horse plasma and Fraction IV—1.* v, *expressed in* b_{30} *values;* [S], *molarity of tabun in the reaction mixture.*

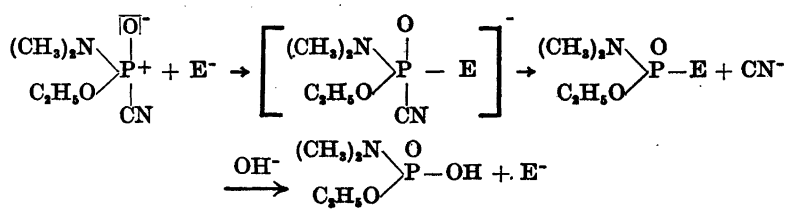
to the enzymatic hydrolysis of tabun by horse plasma and purified tabunase (Fraction IV—1 of human serum). The results are demonstrated in Fig. 3. Horse plasma, containing a variety of esterases including cholinesterase, gave a somewhat higher K_S value (3.45×10^{-3}) than did the preparation free from cholinesterase (2.15×10^{-3}). The difference obtained is significant. It is due to the presence of cholinesterase in horse plasma which in its turn necessitates a higher concentration of substrate (tabun) to saturate tabunase and thus giving maximum reaction velocity for this enzyme.

DISCUSSION

Larsson [3] showed that the decrease in toxicity of tabun during spontaneous hydrolysis parallels the decrease in cholinesterase inhibiting activity. From the fact that neither dimethylamine nor hydrocyanic acid alone is liberated at the same rate as the toxicity decreases, he concluded that the spontaneous hydrolysis must proceed by the two parallel reactions I and II:



The splitting off of the dimethylamido group (I) is catalysed by hydroxonium ions and that of the cyanide group (II) by hydroxyl ions. In the present investigation on the enzymatic hydrolysis of tabun it has been found that the splitting of the P—N linkage (I) is not influenced by enzymes, but that hydrocyanic acid is liberated (II). Hence the mechanism of the enzyme reaction is similar to the base-catalysed hydrolysis. This enzyme reaction probably takes place according to the following scheme:



The reaction is supposed to be controlled by the reaction of a nucleophilic group ("esteric site") of the enzyme molecule with the phosphorus atom, which, dependent on the electrophilic strength of the acid substituent (CN in the case of tabun), is more or less in an anionic state.

The phosphorus containing hydrolysis product does not inhibit cholinesterase in concentrations which are ten fold higher than that of tabun giving almost complete inhibition of that enzyme. Due to the inhibition of tabunase by the reaction products (cyanide and dimethylamido-ethoxy-hydroxy-phosphine oxide) it is concluded that about 15 % of the tabun originally present is not or is very slowly attacked by enzymatic hydrolysis. This amount of unreacted tabun may be responsible for the cholinesterase inhibition of the reaction mixture when used in higher concentrations.

SUMMARY

The enzymatic hydrolysis of tabun by rabbit plasma involves splitting off of the cyanide group. The production of a strong acid, dimethylamido-ethoxy-hydroxy-phosphine oxide, parallels that of hydrocyanic acid. No dimethylamine, ethanol, or inorganic phosphate are liberated by enzymatic degradation of tabun.

Cyanide and the phosphorus containing compound formed in the reaction have a weak inhibiting effect on the tabunase activity, but no such effect on acetylcholinesterase.

The reaction mechanism is discussed.

A purified tabunase preparation from human serum has its optimum stability at pH 7. The Michaelis' constant for the tabunase of horse plasma and that of a purified preparation of human serum were determined and found to differ due to the interaction of cholinesterase in the untreated plasma.

Tabun was prepared at the Research Institute of National Defence, Department 1, Chief Professor Gustaf Ljunggren, to whom we are indebted for his continual interest. The authors wish to express their sincere thanks to Ing. Henrik Björling of A/B Kabi, Stockholm, for the preparation of Fraction IV—1.

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Pyrazole Studies

VI *. Variation of the Basic Properties of Pyrazolones with Substitution in the Pyrazolone Nucleus

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The pK_B-values of a series of substituted pyrazolones have been determined by potentiometrical titration with perchloric acid in glacial acetic acid.

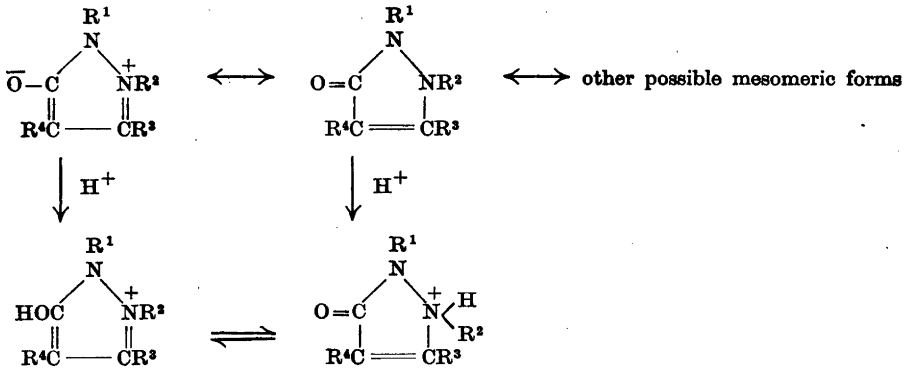
It is shown that electron releasing and electron attracting groups as substituents at different positions in the pyrazolone nucleus have an effect on the pK_B-values, in opposite direction when placed at N_1 or C_2, in the same direction when placed at C_4, the value of pK_B being partly determined by the participation of the polar phenol betain structure in the total resonance hybride. Only when the pyrazolone has the possibility of acquiring an antipyrin structure (*i.e.* only when it is not doubly substituted at C_4) is it possible to determine the pK_B-value in the way described.

The dipole moment of 3-antipyrin (1-phenyl-2 : 5-dimethylpyrazol-3-one) and 1-phenyl-3-methoxy-5-methylpyrazole have been measured. The value found for 3-antipyrin shows that the polar phenol betain structure is more abundantly represented in this substance than in the isomeric antipyrin (1-phenyl-2 : 3-dimethyl-pyrazol-5-one). The values found for pK_B of the two substances corroborate this result.

In a previous paper (Veibel *et al.*[1]) we showed that it is possible to determine pK_B for substituted pyrazolones by titrating them with perchloric acid, using glacial acetic acid as solvent. We decided to use this method for investigating the variation of the basic properties of the pyrazolone nucleus with substitution of different hydrocarbon groups for hydrogen, located at the positions 1, 3 or 4. Table 1 gives a summary of the results obtained, included some few pyrazolones where hydrogen at C_4 has been substituted with a halogen atom instead of with a hydrocarbon radical.

Remembering that only such pyrazolones which are able to acquire an antipyrin structure are titratable as bases (Veibel *et al.*[1]) we have in Table 1 formulated the pyrazolones as phenol betains, understanding by this the total resonance hybride:

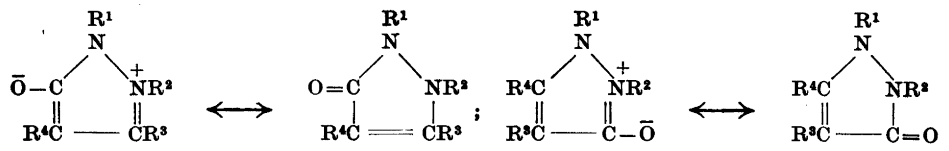
* Part V: *Acta Chem. Scand.* **7** (1953) 119.



A comparison of I, II, III, and VIII shows that the substitution of a methyl group for hydrogen at C_3 increases the basic strength of the pyrazolone (0.6 units in pK_B), that of a tertiary butyl group too, but less (0.4 units in pK_B), and that of a phenyl group has practically no effect. In terms of the theory of hyperconjugation a methyl group is an electron releasing group, a tertiary butyl group too, but less. The phenyl group, on the other hand, is an electron attracting group. The two alkyl groups will tend to increase the participation of the phenol betain structure in the resonance hybride, thus increasing the basic character of the pyrazolone. The phenyl group might be expected to act in the opposite direction, but the value found for pK_B of VIII gives no indication of either electron releasing or electron attracting effect of the phenyl group at C_3. A comparison of II with IV, V and VI shows that substitution of an alkyl group, or an alkenyl group, for a hydrogen atom at C_4 slightly reduces the basic character of the pyrazolone. This may mean that an electron releasing group as substituent at the carbon atom which is neighbour to the negative end of the conjugated system of double bonds has an effect in the opposite direction of the effect caused by a similar group at the carbon atom which is neighbour to the positive end of the conjugated system. Regarding the non-polar antipyrine structure it means that an electron releasing substituent at the carbon-end of the conjugated system $O{=}\overset{|}{C}{-}\overset{|}{C}{=}\overset{|}{C}{-}$ will increase the basic properties of the pyrazolone, whereas a similar substituent at the neighbouring carbon atom will decrease the basic properties. A comparison of the couples I—VIII and II—VII shows that the effect of a phenyl group as substituent is greater at C_4 than at C_3. It is seen (II—IV—VII; X—XI—XIV) that the effect of the electron attracting phenyl group at C_4 is in the same direction as and somewhat greater than the effect of the electron releasing alkyl groups. Finally, IX shows that substitution of two alkyl groups for two hydrogen atoms at C_4, which prohibits the establishing of an antipyrin structure, will cause a weakening of the basic properties of the pyrazolone to such a degree that the substance is untitratable, at all events by the method applied here.

II—X—XV show that the effect of substitution of methyl for hydrogen at the nitrogen atom is a considerable increase of the basic properties. A comparison of the couples I—II and II—X shows that the effect of a methyl

Table 1. Summary of pyrazolones investigated.



I—XXVIII: XXIX—XXXIII;

	R^1	R^2	R^3 (at C^3)	R^4 (at C^4)	MV at 50 % neutralisation	pK_B	M.p. found	M. p. lit.
I	H	H	H	H	184	11.5	165°	165°[2]
II	H	H	CH_3	H	151	10.9	216—18°	215°[3]
III	H	H	$(CH_3)_2C$	H	162	11.1	200°	210°[25]
IV	H	H	CH_3	C_2H_5	160	11.1	227—28°	228°[4] / 230°[5]
V	H	H	CH_3	n-C_3H_7	167	11.2	207—08°	209—10°[6] / 211°[4]
VI	H	H	CH_3	C_3H_5	164	11.2	195—96°	195°[7]
VII	H	H	CH_3	C_6H_5	183	11.4	208—10°	—
VIII	H	H	C_6H_5	H	177	11.4	232—33°	236—37°[8]
IX	H	—	CH_3	$(C_2H_5)_2$	>400	>15	104—05°	105.5°[9]
X	CH_3	H	CH_3	H	119	10.3	117°	117° [9a]
XI	CH_3	H	CH_3	C_2H_5	126	10.5	94—95°	—
XII	CH_3	H	CH_3	n-C_3H_7	127	10.5	84—85°	—
XIII	CH_3	H	CH_3	C_3H_5	127	10.5	71—72°	—
XIV	CH_3	H	CH_3	C_6H_5	147	10.9	175°	—
XV	CH_3	H	C_6H_5	H	150	10.9	206°	207° [9b]
XVI	C_6H_5	H	CH_3	H	176	11.3	127°	127°[10]
XVII	C_6H_5	H	CH_3	C_2H_5	186[1] / 177[2]	11.5[1] / 11.3[2]	111° / 82°	108°[11] / 80°[11]
XVIII	C_6H_5	H	CH_3	n-C_3H_7	179	11.4	101—02°	101—02°[12]
XIX	C_6H_5	H	CH_3	C_6H_5	209	11.9	199—200°	—
XX	C_6H_5	H	C_6H_5	H	228	12.2	137°	137—38°[13]
XXIa	C_6H_5	H	CH_3	Cl	287	13.2	153°	153°[14]
XXIb	C_6H_5	H	CH_3	Br	293	13.3	122°	122°[14]
XXII	C_6H_5	—	CH_3	CH_3, Cl	>420	>15	68°	68°[14]
XXIII	C_6H_5	CH_3	CH_3	H	169	11.2	113°	113°
XXIV	C_6H_5	CH_3	CH_3	$(CH_3)_2CH$	189	11.6	101—03	101—03°
XXV	C_6H_5	H	CH_3	pyrazolonyl	275	13.0	destr.	destr. [15]
XXVI	C_6H_5	—	CH_3	C_3H_5 pyrazolonyl	>410	>15	161—62°	161—62°[16]
XXVII	C_6H_5 / C_6H_5	H / H	CH_3 / CH_3	-CH=pyrazolonyl / =CH-pyrazolonyl	312 / >400	13.8 / >15	185°	180°[17]
XXVIII	C_6H_5	H	CH_3 (at C_4)	diphenylethenyl (at C_5)	230	12.2	220°	—
XXIX	C_6H_5	H	H	CH_3	226	12.2	167°	167°[18]
XXX	C_6H_5	H	C_2H_5	CH_3	236	12.3	172°	172°[19]
XXXI	C_6H_5	H	H	C_6H_5	273	12.9	256°	256°[19]
XXXII	C_6H_5	CH_3	H	CH_3	190	11.6	113°	113°[20]
XXXIII	C_6H_5	(OCH_3 at C_3)	H	CH_3	244	12.5	B.p. 16 mm 154—56°	150—60°[20]

[1] Anhydrous. [2] 1 mole of water of crystallisation.

group at C_3 and at N_1 is sensibly the same. In this series the effect of substitution of hydrogen with alkyl or aryl at C_4 or methyl with phenyl at C_3 described above is found again.

In XVI—XX the same effect of substituting alkyl or aryl groups for hydrogen at C_3 or C_4 is observed. Here the effect of a phenyl group at C_3 is somewhat greater than at C_4. Possibly this is due to some form of interaction between the phenyl groups at N_1 and at C_3, the N_2-atom facilitating the displacement of electrons somewhat more than the C_5-atom.

The effect of a phenyl group at N_1 is a decrease of the basic properties (compare II and XVI). The change in pK_B is, however, only 0.4, considerably less than the effect of substitution of a phenyl group for a hydrogen atom in *e. g.* aniline (change of $pK_B \sim 3.7$).

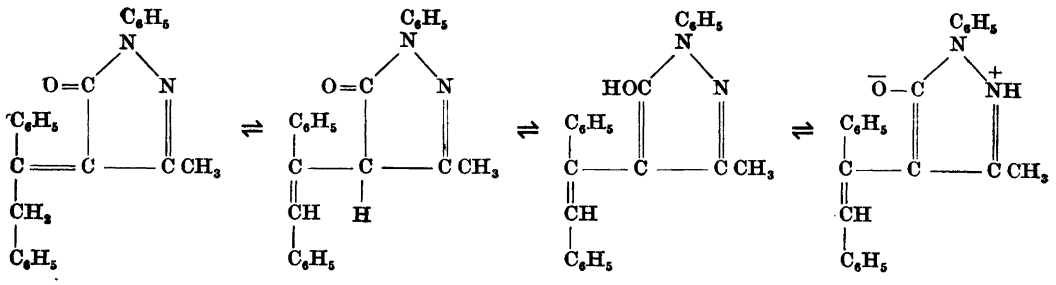
From XXI a and b we learn that the effect of halogen atoms (electron attracting atoms) is considerably greater than the effect of a phenyl group. There is no significant difference between the effect of chlorine and of bromine. XXII verifies the result found in IX, that double substitution at C_4 will make the pyrazolone untitratable.

The pK_B-value found for antipyrin (XXIII) is in accordance with the assumption that the antipyrin structure (or the phenol betain structure) is responsible for the basic properties of the pyrazolones, pK_B being for antipyrin within the limit of error identical with pK_B for 1-phenyl-3-methylpyrazol-5-one. XXIV shows that the effect of an alkyl-substitution at C_4 is the same as that found for monosubstitution at C_4 in pyrazolones not substituted at N_2.

XXV—XXVIII are examples corroborating the results obtained with less complicated pyrazolones. XXV is the bis-pyrazolone of XVI. It is titratable, but pK_B has increased from 11.3 to 13.0. 1 mole of the bis-pyrazolone claims 2 moles of perchloric acid. XXVI is the bispyrazolone of XVII. As it is doubly substituted at C_4 it cannot acquire antipyrin structure and, consequently, it is untitratable.

In XXVII we investigated a bis-pyrazolone with a methin group interposed between the two pyrazolone nuclei. The result is that at C_4 we have in one nucleus a double bond, in the other a single bond. The substance claims one mole of perchloric acid per mole of bis-pyrazolone, the pK_B of the molecule being 13.8. It seems natural to locate the basic properties on the pyrazolone nucleus with a single bond to the methin group and to regard the other nucleus as untitratable.

XXVIII is an example of a pyrazolone with double bond to a substituent at C_4, *viz.* a 1:2-diphenylethylidene group. The pyrazolone is titratable with perchloric acid and it may also be titrated in ethanolic solution with 0.1 *N* sodium hydroxide, comp. Veibel *et al.*[21]. The titration curves are given in Fig. 1. From the curves the equivalent point may be determined according to Gran[22]. We find equivalent weights 354 and 352, calculated 352.4. The titratability of the substance means that it behaves not as a 1:2-diphenylethylidene, but as a 1:2-diphenylethene-substituted pyrazolone, a hydrogen atom having been transferred from the substituent to the pyrazolone nucleus, creating by this a double bond in the substituent, conjugated with the double bonds in the pyrazolone nucleus:



XXVIII

XXIX—XXXIII are some substituted pyrazol-3-ones. XXIX, XXX and XXXI are isomers of XVI, XVII and XX. It is seen that the pK_B-values of the pyrazol-3-ones are superior to the corresponding values for the pyrazol-5-ones by 0.7—0.9, *i. e.* the pyrazol-3-ones are less basic than the pyrazol-5-ones. For the pyrazol-5-ones we have made the assumption that a phenol betain structure is responsible for the basic character of the substances. If this assumption is valid for the pyrazol-3-ones too, it seems natural to consider the fact that the distances between the positive and the negative charges is shorter in the pyrazol-3-ones than in the pyrazol-5-ones. This difference may explain the difference in basic strength.

XXIII and XXXII are antipyrin and 3-antipyrin. Here the difference in pK_B is only 0.4, and whereas the difference between the pK_B-values for XVI and XXIII is only 0.1, we find a difference of 0.6 between the pK_B-values for XXIX and XXXII. The electron releasing methyl group at N_2 in XXXII has caused a considerable increase in the participation of the phenol betain

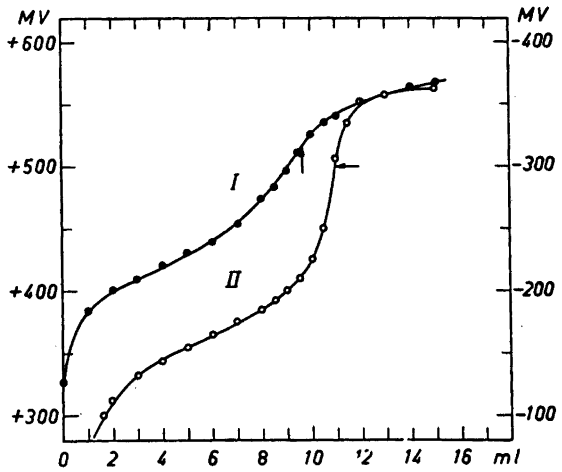
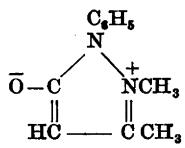


Fig. 1. Titration curves for XXVIII. I: Titration with 0.1 N perchloric acid (right hand ordinate). II: Titration with 0.1 N sodium hydroxide (left hand ordinate). The arrows indicate the end-points calculated from the curves.

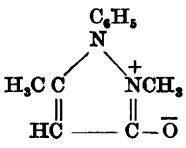
structure in the resonance hybride. A comparison of XXXII and XXXIII, the 3-antipyrin and 1-phenyl-3-methoxy-5-methylpyrazole tends to corroborate the hypothesis discussed here. For XXXIII no phenol betain structure is possible, and not only is the substance less basic than 3-antipyrin, but it is a weaker base than 1-phenyl-5-methylpyrazol-3-one too.

A further corroboration is obtained by comparing the dipole moments of XXXII and XXXIII with that of antipyrin (XXIII). The values found are:



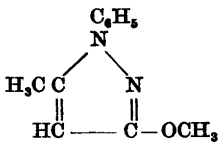
XXIII

5.48 D (25°)[23]



XXXII

5.69 D (24°)



XXXIII

1.80 D (27°)

As above the distance between the electric charges must be greater in XXIII than in XXXII. With the following bond lengths:

C—O 1.43 Å; N—C 1.47 Å; N—N 1.40 Å; N = C 1.23 Å[24]

and the valence angle 110° (Jensen and Friediger [23]) the distance is approximately 3.77 Å in XXIII and < 2.66 Å in XXXII. Jensen and Friediger estimate from the value of the dipole moment of XXIII that the participation of the phenol betain structure in the resonance hybride is 30—35 %. As the value calculated for the dipole moment of XXXII must be smaller than that calculated for XXIII and a greater value is actually found, this means that the participation of the phenol betain structure in the resonance hybride of XXXII is considerably greater than 35 %.

EXPERIMENTAL PART

The substances I, II, IV—VI, VIII, IX, XVI—XX, XXV—XXVII and XXIX—XXXIII were prepared by current methods and showed with a few exceptions the m. p. s indicated in the litterature. XXIa, XXIb and XXII were placed at our disposal by Gunnel Westöö, Ph. D., to whom we want to express our thanks. XXIII and XXIV were commercial products. The other substances were prepared as indicated below (all m. p.s are uncorrected; all microanalyses by Mr. A. Grossmann or Mr. W. Egger, Department of Organic Chemistry, The University, Copenhagen).

3-tert. Butylpyrazol-5-one (III). 10 ml of ethanol were added to a mixture of 7.5 g of ethyl trimethylacetoacetate (ethyl pivalylacetate) and 4 ml of 60 % hydrazine hydrate. A clear solution resulted. The reaction between the keto ester and hydrazine starts slowly at room temperature, causing a slight increase in temperature and a deposition of crystals. After 2—3 hours the crystals are filtered off, washed with water and air-dried. Yield 4.2 g with m.p. 200° (Dayton [24] indicates m.p. 210° without describing the synthesis). Recrystallised from aqueous ethanol 5.1 g with m.p. 200—201° = 82 % were obtained. (Found: N 20.18. Calc. for $C_7H_{12}ON_2$ (140.2): N 19.99.)

3-Methyl-4-phenylpyrazol-5-one (VII). 7.5 g of 50 % hydrazine hydrate are added to 15.4 g of ethyl α-phenylacetoacetate. Some ml of ethanol are added in order to increase the mutual solubility of the components. Upon slight heating the reaction starts and proceeds then regularly. After cooling a raw product was isolated by suction (30 %,

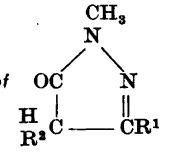
m.p. 208—210°), washed with ethanol and recrystallised from the minimum amount of ethanol. The m.p. was unchanged 208—210°, the yield of pure product was 3.5 g = 27 %. (Found: C 68.96; H 5.69; N 16.13. Calc. for $C_{10}H_{10}ON_2$ (174.2): C 68.92; H 5.78; N 16.09.)

1-Methyl substituted pyrazol-5-ones (X—XV). For the preparation of these pyrazolones an aqueous *N* solution of methyl hydrazine was prepared as ollows: A suspension of 1 mole of barium hydroxide octahydrate in 500 ml of water was heated to 100° with mechanical stirring. A solution of 1 mole of hydrazine sulphate in 150 ml of water was heated to 70° and added to the suspension of barium hydroxide, precipitating barium sulphate immediately. In order to complete the precipitation and to obtain the barium sulphate in a filtratable form 300 ml of water was added and the suspension stirred at 70° for a further hour, after which the barium sulphate was filtered off and washed on the filter with hot water, the filtration flask being chilled in ice-water in order to reduce the loss of methyl hydrazine during the filtration. The combined filtrate and washings were made up with water to 1 000 ml. This stock solution was used for the preparation of the 1-methyl-pyrazolones.

A mixture of 0.2 mole of (α-substituted) ethyl acetoacetate or ethyl benzoylacetate and 200 ml *N* methyl hydrazine solution is heated to 70° and mechanically stirred for the time indicated in Table 2. After cooling the solution is extracted twice with low boiling ligroin; from the aqueous layer the water is removed *in vacuo* at a bath temperature of 45—50°. The residue is dissolved in chloroform and dried with anhydrous sodium sulphate. The crude pyrazolone is isolated by evaporation of the chloroform; it is purified by fractionation in a vacuum, preferably from a sausage flask, by recrystallisation from ethyl acetate, methanol or ethanol, or by sublimation.

1,4-Diphenyl-3-methylpyrazol-5-one (XIX). 4 g of ethyl α-phenylacetoacetate and 2 g of phenylhydrazine are mixed, forming a homogenous mixture which soon becomes turbid, the mixture at the same time heating up to become luke-warm. When again cooled down to room temperature it solidifies to a semi-solid mass which is dissolved in 25 ml of ethanol. The solution is cooled in ice water, depositing crystals which are filtered off. Yield 3.1 g with m.p. 88—90° (dec.); from the filtrate 1.2 g of crystals with m.p.

Table 2. Preparation of



	R^1	R^2	Reaction time, h.	Yield %	Purified by	M.p.	B.p.	% C		% H		% N	
								Calc.	found	Calc.	found	Calc.	found
X	CH_3	H	0.5	51	dest.	117°	$130°_{19mm}$	—	—	—	—	—	—
XI	CH_3	C_2H_5	10	60	recryst. ethyl ac.	94—95°	$137°_{15mm}$	59.95	59.91	8.63	8.48	19.99	19.85
XII	CH_3	C_3H_7	10	65	recryst. ethyl ac.	84—85°	150_{15mm}	62.30	62.33	9.15	9.09	18.17	18.18
XIII	CH_3	C_3H_5 (allyl)	5	50	recryst.	71—72°	—	63.12	63.15	8.02	7.90	18.41	18.42
XIV	CH_3	C_6H_5	10	70	recryst. aq. methanol	175°	—	70.19	70.40	6.43	6.33	14.89	14.72
XV	C_6H_5	H	10	65	recryst. ethanol	206°	—	—	—	—	—	—	—

198—199°, recrystallised from ethanol 199—200° were isolated. The low melting product is the phenylhydrazone of ethyl α-phenylacetoacetate, the high melting substance is the corresponding pyrazolone. Total yield 76 % (52 % phenylhydrazone and 24 % pyrazolone). (Phenylhydrazone of ethyl α-phenylacetoacetate, Found: C 72.63; H 6.90; N 9.37. Calc. for $C_{18}H_{20}O_2N_2$ (296.4): C 72.94; H 6.80; N 9.45.)

The phenylhydrazone looses ethanol spontaneously, being converted into the pyrazolone, slowly at room temperature (some days), fast at 140° where 0.8968 g loose 0.1376 g during 5 hours, then retaining its weight on prolonged heating. The loss is 15.35 %; calculated for the loss of 1 mole of ethanol 15.55 %.

The pyrazolone may be prepared without isolation of the phenylhydrazone by heating the mixture of ethyl α-phenylacetoacetate and phenylhydrazine to 140° for 1 hour as soon as the spontaneous reaction is over. Yield from 4 g of ethyl α-phenylacetoacetate and 2 g of phenylhydrazine 3.2 g = 64 %. (1,4-Diphenyl-3-methylpyrazol-5-one, Found: C 76.95; H 5.72; N 11.34. Calc. for $C_{16}H_{14}ON_2$ (250.3): C 76.79; H 5.64; N 11.19.)

1-Phenyl-3-methyl-4(1″:2″-diphenylethenyl)pyrazol-5-one (XXVIII). This substance is prepared by heating equimolar amounts of 1-phenyl-3-methylpyrazol-5-one and desoxybenzoin on the steam bath for 3—4 hours. The solid condensation product is filtered off, washed with ether (to remove unreacted desoxybenzoin) and recrystallized from glacial acetic acid. Yield 50 %, m.p. 220°. (Found: C 80.90; H 5.79; N 7.99. Calc. for $C_{24}H_{20}ON_2$ (352.4); C 81.79; H 5.72; N 7.95.)

Determination of the dipole moments of 1-phenyl-2 : 5-dimethylpyrazol-3-one (XXXII) *and 1-phenyl-3-methoxy-5-methylpyrazole* (XXXIII), Table 3. Thanks are due to Mr. G. Nygaard, M.Sc., and Professor K. A. Jensen, Department of Organic Chemistry, University of Copenhagen, for carrying out the measurements and the calculation of the dipole moments.

Table 3. Dipole moments of XXXII and XXXIII.

	Mole fraction of substance	d of the solution	ε	P	
XXXII M = 188.2	0.00974	0.8784	2.7192	638.5	T = 24° P^∞ = 730 P_E (calc.) = 50.7 P^∞_{0+A} = 679; P^∞_0 = 672 (P_A presumed to be 15 % of P_E)
	0.00468	0.8753	2.4914	679.3	
	0.00238	0.8741	2.3879	719.9	
	$\mu = 0.01273\ \sqrt{P^\infty_0 \cdot T} = 5.69$ D				
XXXIII M = 188.2	0.0302	0.8879	2.4845	122.9	T = 27° P^∞ = 127 P_E (calc.) = 52 P^∞_{0+A} = 75; P^∞_0 = 67 (P_A presumed to be 15 % of P_E)
	0.0220	0.8824	2.4243	124.0	
	0.0151	0.8760	2.3778	(133.5)	
	$\mu = 0.01273\ \sqrt{P^\infty_0 \cdot T} = 1.80$ D				

Thanks are due to *Laurits Andersens Fond* and to *Otto Mønsteds Fond* for grants facilitating the carrying out of this investigation.

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Factors Affecting the Hydrolysis and Dimerisation of Metal Ions

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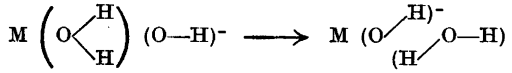
A correlation of associated ion species in solution is at present hampered by uncertainties concerning the ability of the associating ligand to penetrate the hydration sheath of the metal ion. This is particularly evident where the association is predominantly electrostatic (see, *e.g.*, Williams [1]). In metal-hydroxyl ion systems, however, the situation is probably rather simpler. As Davies [2] has pointed out, it is highly probable that in these complexes the hydroxyl ion is directly adjacent to the metal ion, such an arrangement being effected by a transition



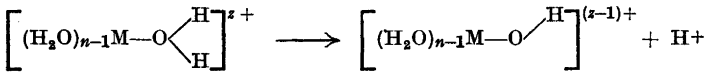
(I) (II)

It thus appears that correlation of the stabilities of these species may be possible.

THE FIRST MONOMER HYDROLYSIS PRODUCT, $MOH^{(z-1)+}$

The formation of this species by a mechanism such as the above would rule out application of the N. Bjerrum ion association theory [3] to determine dissociation constants for purely electrostatic systems, for although the theory may apply to (I), the observed constant would relate to (II). Davies [2] has demonstrated the non-applicability of the Bjerrum theory in cases where electrostatic association is to be expected between the metal and hydroxyl ions.

The hydrolysis process may be pictured as a proton dissociation step:



The tendency of the hydrated ion to lose a proton should therefore be related to the strength of the metal ion-water linkage of the first coordination sphere.

Table 1. Data on

For definitions of symbols, see text. r = ionic radius [6] after Pauling except where marked x, these being experimental values. ΔH_s data are experimental values given by Bernal and Fowler [7], except those in parentheses, which are estimates by these authors. ΔG values are calculated $= -RT \ln 55.5\ \beta_1'$, using quoted values of β' or figures obtained from conversion of $\varkappa_1'$ to β_1', assuming $K_w = 10^{-14.0}$. log $\varkappa_1'$ values given in parentheses are open to question on theoretical

Ion	r in Å	ΔH_s kcals.	N	log $\varkappa_1'$	Medium	Method employed	Ionic Strength
Na^+	0.95	—114	3	~14.7	OH^-	(a) kinetic	
						(b) conductance	0
Li^+	0.60	—136	3	~14.1	$0H^-$	conductance	0
						(8a) kinetic;	
Ba^{2+}	1.35	—346	6	—13.36	OH^-	E.M.F.	0
Sr^{2+}	1.13	—376	6	—13.05	OH^-	solubility	0
						(8a) kinetic;	
Ca^{2+}	0.99	—410	6	—12.70	OH^-	solubility	0
Mg^{2+}	0.65	—490	6	—11.41	Cl^-	E.M.F.	0
Be^{2+}	0.31	—608	4	— 6.52	ClO_4^-	E.M.F.	1.0
La^{3+}	1.15	—768	6	~10.7	(OH^-)	solubility	0
Ce^{4+}	1.01		6	+0.72	ClO_4^-	spectrophotometric	2.0
U^{4+}	0.97		6	— 1.49	ClO_4^-	spectrophotometric	0.5
Pu^{3+}	1.01*		6	— 6.95)	ClO_4^-	E.M.F.	0
Tl^+	1.49	—107	2	—13.18	OH^-	solubility	0
Tl^{3+}	0.95		4	— 1.14	ClO_4^-	E.M.F.	3.0
In^{3+}	0.81	—980	6	— 3.85	Cl^-, Br^- I^-	E.M.F.	0
Ga^{3+}	0.62		6	(— 3.43)	NO_3^-	E.M.F.	varied
Al^{3+}	0.50	—1 149	6	— 4.89	ClO_4	kinetic	0
Pb^{2+}	1.21		4	— 8.78	NO_3^-	E.M.F.	0
Sc^{3+}	0.81	(~ —980)	6	— 4.93	ClO_4	E.M.F.	1.0
V^{3+}	0.65*	(~ —830)	6	(— 2.92)	SO_4^{2-}	E.M.F.	small
Cr^{3+}	0.65*		6	— 3.90	ClO_3^-	kinetic	0
Mn^{2+}	0.80*	—479	6	(~ — 9.5)	$CH_3\ CO_2^-$	distribution	varied
Fe^{3+}	0.67*	(~ —1 185)	6	— 3.05	ClO_4^-	E.M.F.	3.0
Fe^{2+}	0.75*	—500	6	— 9.50	ClO_4^-	E.M.F.	1.0
Co^{2+}	0.72*	504	6	(—10.20)	Cl^-	E.M.F.	small
Ni^{2+}	0.69*	516	6	(—10.64)	Cl^-	E.M.F.	small
Ag^+	1.26	162	2	~ —11.7	(OH^-)	solubility	small
Cu^{2+}	0.70*	536	4	— 7.97	NO_3^-	E.M.F.	0
Hg^{2+}	1.10	480	2	— 3.70	ClO_4^-	E.M.F.	0.5
Cd^{2+}	0.97	462	4	(~ — 8.5)	$CH_3\ CO_2^-$	distribution	varied
Zn^{2+}	0.74	528	4	(— 9.60)	SO_4^{2-}	E.M.F.	small
Th^{4+}	0.02	(~ 1 540)	6				

Only those ions whose radii are known are included in this Table. Other recent hydrolysis plutonium(IV) [40],

Thus the heat of formation of this bond could be tried as a function of the logarithm of the acid dissociation constant of the aquo ion, log$\varkappa_1'$, where

$$\varkappa_1' = \frac{[MOH^{(z-1)+}][H^+]}{[M^{z+}]}$$

(using the recent terminology of the Swedish schools [4]). These heats are not known, but a useful average estimate that probably gives the correct relative

MOH$^{(z-1)}$ *systems.*

grounds, owing to neglect of factors such as possible polymerisation, complex formation with anions, *etc.*; these systems particularly merit fresh examination. ΔS values are calculated from Cobble's equations [44], giving ΔH values from $\Delta H = \Delta G + T\Delta S$. p. = polymerisation. e = electrostatic.

Temp. in °C	Reference No.	ΔS cals./deg.(calc.	ΔG kcals.	ΔHkcals. (calc.)	f_c (Fig. 2a)	f_c (Fig. 2b)	State of solution
25	8(a), (b)	— 22	~ — 1.42	— 7.0	—		
25	8(b)	— 40	~ — 2.27	— 14.1	—		
25	8(a), 9	+ 27	— 3.26	+ 4.7	—		
25	10	+ 29	— 3.68	+ 5.1	—		
25	8(a), 11	+ 34	— 4.13	+ 5.9	—		
25	12	+ 43	— 5.92	+ 6.9	—		
25	13	+ 58	—12.60	+ 4.7	0.35	.31	p. (ref. 13)
25	14	+ 22	~ — 6.89	— 0.3		.33	
25	15	+ 10	—22.48	— 19.5			p. (ref. 15)
25	16	+ 12	—19.48	— 15.9	.47		p. on ageing (ref. 16)
?	17	+ 22			.37		
25	11 (b)	— 3	— 3.52	— 4.4	.55	.51	
25	18	+ 23	—19.98	— 13.0	.61		no p. (ref. 18)
25	19	+ 28	—15.68	— 7.4	.48	.35	
25	20	+ 37	(—16.80)	— 5.8	.43		p. (ref. 21)
15	22	+ 40	—14.30	— 2.5	.29	.31	p. (ref. 23)
18	24	+ 29	— 9.27	— 0.9	.58		p. (ref. 24)
25	25	+ 28	—14.79	— 6.4	.43	.35	p. (ref. 25)
25	26	+ 33	(—17.53)	— 7.7	.46	.14	
15	22	+ 33	—15.62	— 6.0	.42		p. on ageing (ref. 27)
20	28	+ 40	— 8.48	+ 3.2	.38		
25	29	+ 32	—17.28	— 7.7	.48	.42	p. (ref. 29)
25	30	+ 40	— 8.53	+ 3.3	.39		no p. (ref. 30)
25	31	+ 41	(— 7.56)	+ 4.7	.26		
25	31	+ 42	(— 6.97)	+ 5.6	.22		
25	32	— 11	~ — 5.52	— 8.7	.65	.65	
18	33	+ 41	—10.36	+ 1.7	.45	.50	p. (ref. 33)
25	34	+ 31	—15.28	— 6.0	.71	.76	no p. (ref. 34)
20	28	+ 34	(~ — 9.72)	+ 0.2	.39	.53	
25	35	+ 40	(~ — 8.40)	+ 3.5	.35	.44	
		+ 10				.52	p. (ref. 36)

studies are those on tin(II) [37], vanadium(IV) [26], uranium(VI) [38], neptunium(V) [39], and (V) [41] and (VI) [42].

orders may be obtained by dividing the heat of hydration of the ion, ΔH_s, by the characteristic coordination number, N, of J. Bjerrum [5]. This latter quantity indicates the number of energetically closely bound ligands found in a metal coordinate system, and is not necessarily the same as the maximum coordination number.

Figure 1 shows a plot of $\frac{\Delta H_s}{N}$ against $\log \varkappa_1'$ for a number of metal ions, from the data of Table 1. It should be noted that the $\varkappa_1'$ values are not all comparable, owing to many of them having been obtained under different

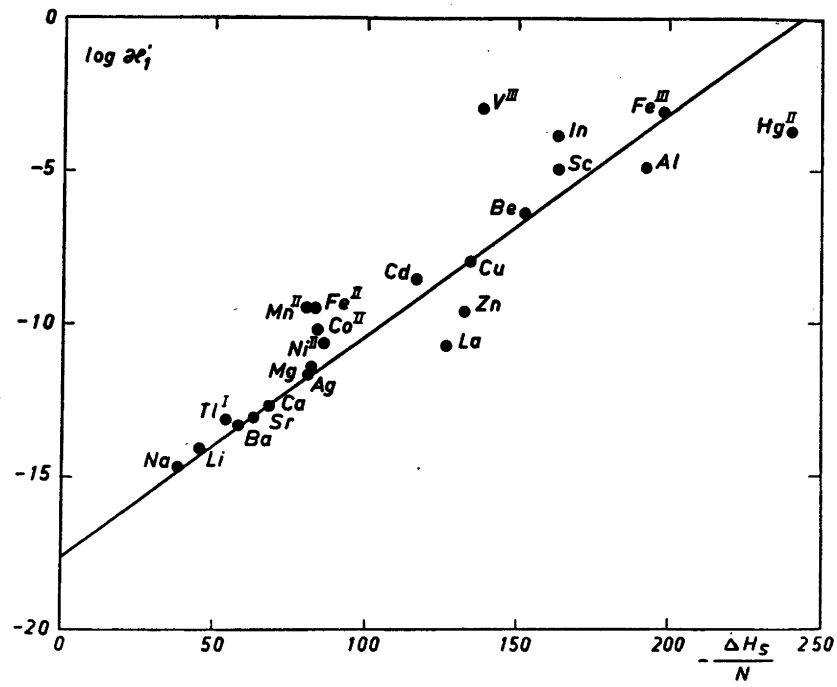


Figure 1. Correlation of aquo ion acid dissociation constants with heats of hydration.

conditions of medium, temperature, and experimental accuracy, but nevertheless the plot is reasonably linear.

Assuming the correctness of this empirical relation, it is possible to predict the order of expected $\varkappa'$ values where these are uncertain. Mn^{2+}, Fe^{2+}, Co^{2+}, and Ni^{2+} should have log $\varkappa_1' \sim -11$. The reported values for these latter ions may be too high, for experimental difficulties are considerable (see also later comments). The indications are that Th^{4+} has a log$\varkappa_1'$ value ~ 1, although not too much reliance may be placed on this, for the heat of hydration may be rather lower than that quoted. It is by no means certain that thorium is as strongly acid as this value of log$\varkappa_1'$ would imply (see, for example, the work of Kraus and Holmberg [43]).

If, as suggested above, the OH^- ion in these hydrolysis complexes is adjacent to the cation, it should be possible to set up a simple coulomb expression to describe their behaviour where the association is purely electrostatic. Davies [2] has proposed an empirical expression equivalent to

$$\log \beta_1' = 0.607 \frac{z^2}{r} - 1.150$$

to correlate the association constant

$$\beta_1' = \frac{[MOH^{(z-1)+}]}{[M^{z+}][OH^-]}$$

with r, the ionic radius in Å, and the valence, z, in electrostatic cases. It would seem, however, that a term $\frac{z \cdot 1}{a}$, where a is the distance between the centres of charge on the metal ion and the hydroxyl ion, would emphasise the concept of coulombic attraction. To try this approach, the inter-charge distance was taken as $a = r + 0.74$, with satisfactory results. This presumes that although the metal ion could in principle approach quite close to the oxygen atom of OH^- along the O—H bond axis, it is highly probable that it could not come any closer to the centre of negative charge than the covalent radius of the oxygen atom, *viz.*, 0.74 Å. Figure 2a shows a plot of log β_1' against $\frac{z}{r + 0.74}$ for the alkali and alkaline earth metals, where electrostatic association is to be expected. The linearity of the plot appears to justify the relation as an empirical criterion for solely electrostatic bonding forces.

Some support for this relation may be derived as follows. A theoretically more satisfactory quantity to correlate with $\frac{z}{a}$ would be the heat of formation of the M—OH bond (necessarily assuming that the effective dielectric constant for use in the coulomb expression does not vary too greatly from case to case). However, the thermodynamic quantities required for the evaluation of this heat, *viz.*, the heat of removal of one water molecule from the hydration shell of the solvated M^{z+} ion and the heat of hydrolysis *, ΔH, for the reaction

$$[M(H_2O)_n]^{z+} + OH^- \longrightarrow [M(OH)(H_2O)_{n-1}]^{(z-1)+} + H_2O,$$

are not known. Nevertheless, it is perhaps not unreasonable to expect that the heats of removal of one water molecule would follow the order of the heats of removal of one OH^- ion. The former quantity has been approximated to earlier in this paper as $\frac{\Delta H_s}{N}$, which itself is linearly related to $\log \varkappa_1'$, and thus to $\log \beta_1'$ ($\varkappa_1 = K_w \beta_1'$). Hence the use of $\log \beta_1'$ in the coulomb expression is somewhat justified. It is apparent that $\frac{\Delta H_s}{N}$ versus $\frac{z}{r + 0.74}$ may also be used as a criterion for electrostatic forces (Figure 2b).

Figures 2a and 2b include a number of systems which deviate markedly from the plot, and these may be considered to be 'covalent' to an extent measured by the deviation. Considering Figure 2a (the same type of definition would apply to Figure 2b), the electrostatic contribution, E, may be defined by

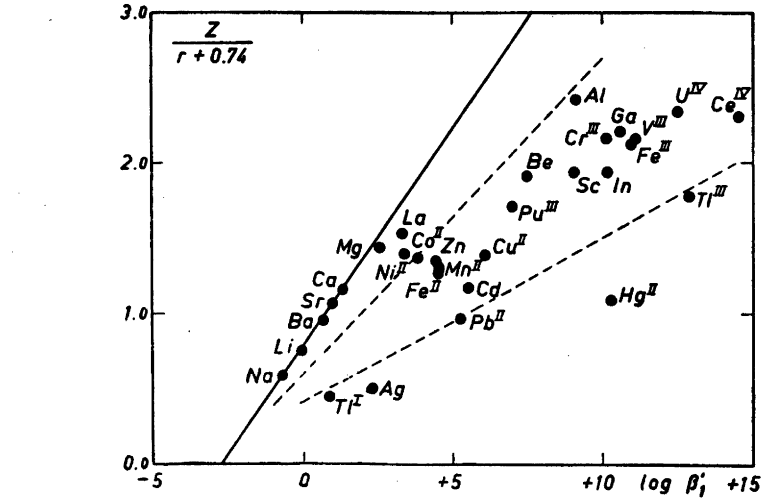
$$E = (\log \beta_1')_E - (\log \beta_1')_E^0$$

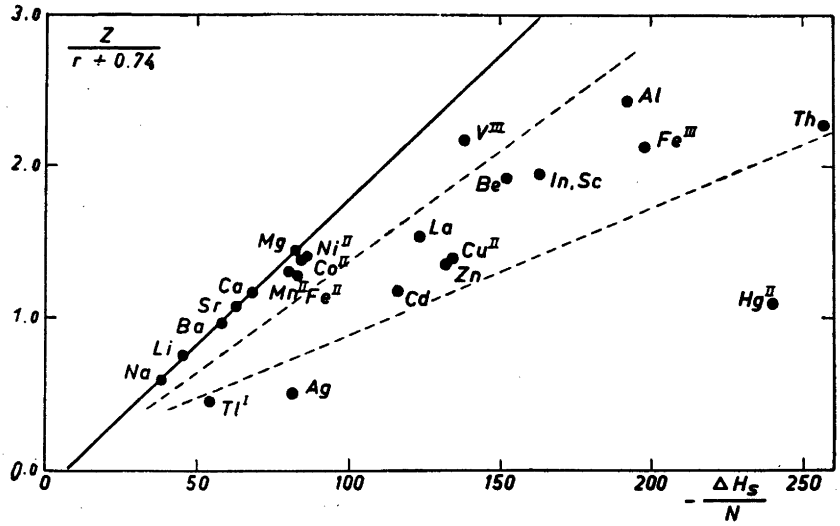
the difference between the 'electrostatic' $(\log \beta_1')_E$ given by the plot of Figure

* ΔH may be estimated from

$$\Delta H = \Delta G + T\Delta S,$$

using known ΔG values and calculated values of ΔS from Cobble's recent empirical equations [44]. Such estimates are included in Table 1, but it is difficult to assess their reliability until more accurate experimental data are forthcoming.





Figures 2a and 2b. Criteria for solely electrostatic association forces in metal hydrolysis species $MOH^{(z-1)+}$.

2a for a given r value and $(\log \beta_1')_E$ at $\frac{z}{r+0.74}=0$. The 'covalent' contribution, C, is expressed by

$$C = \log \beta_1' - (\log \beta_1')_E.$$

The covalent fraction, f_C, is then

$$f_C = \frac{C}{E+C} = \frac{\log \beta_1' - (\log \beta_1')_E}{\log \beta_1' - (\log \beta_1')_E^0}$$

Values of this function for various hydroxyl systems are included in Table 1. The differences in f_C from Figures 2a and 2b are not important in most cases, for the same qualitative trends are shown in both Figures, and f_C has only been defined to show relative characteristics. The observed trends are not unexpected: decreasing covalence accompanies the orders $Tl^{III} \rightarrow In \rightarrow Ca \rightarrow Al$, and $Hg^{II} \rightarrow Cd \rightarrow Zn$. A few differences are noticeable in the two sets of f_C values, however. The positions of V^{3+} and La^{3+} differ on the $\frac{\Delta H_s}{N}$ and log β_1' plots (they deviate from the relationship of Fig. 1). This may be due to inaccuracies in the ΔH_s estimate (Bernal and Fowler [7] stress the approximate nature of their calculations for the +3- and +4-valent ions) and also, in the case of V^{3+}, possibly to the $\log \varkappa_1'$ value (the calculations of Meites [26] neglect formation of anion complexes and polynuclear species). Behaviour comparable with Sc, Cr, and Fe^{III} is to be expected of V^{III}. Lanthanum, however, is probably highly polar. Differences are also apparent in the case of Fe^{2+} and Mn^{2+}. From other considerations (see below) it would appear that the $\frac{\Delta H_s}{N}$ plot is more reliable here, and that the Fe^{2+}, Co^{2+}, Ni^{2+} and Mn^{2+} hydroxyl systems are all highly polar, as Figure 2b suggests.

THE FIRST DIMER HYDROLYSIS PRODUCT, $M_2OH^{(2z-1)+}$

It is interesting to see how the tendency for the dimerisation reaction

$$M^{z+} + MOH^{(z-1)+} \longrightarrow M_2OH^{(2z-1)+}$$

to occur is related to the covalence of the M—OH bond. Figures 2a and 2b illustrate how the MOH systems may be broadly divided into three groups (though of course without implying discontinuity): (1) strongly electrostatic, (2) partly covalent, and (3) strongly covalent. Metals in groups (1) and (3) show no tendencies to dimerise, but there is marked polymerisation in group (2), containing partly covalent systems. Table 2 shows how the value of log K_1'', defined by

$$K_1'' = \frac{[M^{z+}]\,[MOH^{(z-1)+}]}{[M_2OH^{(2z-1)+}]}$$

appears to vary directly with $\frac{1-f_C}{r}$ for ions of a given valence ($1-f_C = E$, the electrostatic fraction). This observation may be discussed as to its possible causes. Two effects may govern the extent of dimer formation through $-\underset{\underset{H}{|}}{O}-$ bridges:

(1). Where the M—OH linkage is electrostatic, the high dielectric constant of water will prevent significant triple ion formation [45] of the type $M^{z+}\,OH^{-}M^{z+}$, particularly where $z > 1$. The Fe^{2+} system may be mentioned again here.

Figure 2b indicates it to be electrostatic, implying no dimerisation possibilities, while the log β_1' plot suggests some covalence. Since Fe^{2+} does not form polynuclear complexes [30], this suggests that the system is electrostatic, and that the quoted value of log β_1' is too high, *i. e.* the aquo ion is a weaker acid than supposed.

It would seem, therefore, that dimerisation is only possible when some covalent forces are operating. On the other hand, increased participation of the oxygen bonding electrons in the metal-oxygen linkage as covalence increases would bring about a reduction in the electron density associated with the oxygen atom. This would have an effect of opposing dimerisation, for the tendency of a second metal atom to set up a similar bond with the oxygen atom would be diminished *. These two effects, acting in opposition, imply the existence of a maximum in dimerisation tendencies. The alkali and alkaline earth metals do not dimerise; while mercury(II) and thallium(III) do not dimerise either, presumably because of their relatively high covalence. In between these extremes, metals such as beryllium, aluminium, gallium, scandium, chromium(III), iron(III), etc. dimerise markedly.

(2). Professor J. Bjerrum has suggested to me that steric effects could also operate. With a highly polar M—OH linkage, the metal ion may be able to "move" to allow access to the oxygen atom by a second metal ion (for a triple ion, the configuration of minimum potential energy is a linear one). With increasing covalence, the angle MÔM probably decreases towards the value found in many strongly covalent oxygen compounds, *viz.*, ~ 100—$110°$, and the result of this would be to make dimerisation less likely for large ions than for small ones, other factors being equal. There would be insufficient room for two large ions to give a small MÔM angle. Ionic charge would also have some effect, it may be supposed, but there is insufficient data to test this.

This discussion indicates the basis of a working rule: that as the size of the metal ion and the covalence of the M—OH bond increase, the tendency to form dimers decreases. (This presumes, not unreasonably, that the M—OH bonds in the dimer partake of somewhat similar character to those in the monomer system). The properties of systems yet to be examined may thus be predicted. Within the series Cu^I, Ag^I, Au^I, the Ag—OH bond is quite covalent (Table 1), and this, together with the large radius of the Ag^+ ion (1.26 Å), can be expected to prevent significant dimer formation. The same would hold for

* Somewhat of interest here, and in agreement with the views expressed, are the recent comments of Hazel, McNabb and Santini [46] on the strong condensation tendencies of protomated vanadates. They point out that $[—\overset{|}{\underset{|}{V}}—O—\overset{|}{\underset{|}{V}}]^{4-}$ should be favoured over $[—\overset{|}{V}—O—H]^{2-}$, since vanadium is more electropositive than hydrogen; whereas with the corresponding phosphate ions, $[—\overset{|}{\underset{|}{P}}—O—\overset{|}{\underset{|}{P}}]^{4-}$ and $[—\overset{|}{P}—O—H]^{3-}$, the condensation process does not occur at room temperature, presumably because of the similar electronegativities of phosphorus and hydrogen. In the terms of the discussion given above, this is roughly the same as saying that the P—O bond is very much more covalent than the V—O bond, and hence in the former case polymerisation tendencies are considerably reduced.

Table 2. Dimerisation of metal ions through hydroxyl bridging. All determinations made in perchlorate medium.

Ion	log K_1''	Ionic strength	Temperature in °C	Reference No.	f_c (Fig. 2a)	$\frac{1-f_c}{r}$
Hg^{2+}	too small for measurement	0.5	25	34	0.71	0.26
Pb^{2+}	0.48	0	18	24	0.58	0.35
Cu^{2+}	1.52	0	18	33	0.45	0.78
Be^{2+}	3.01	1.0	25	13	0.35	2.10
Tl^{3+}	too small for measurement	3.0	25	18	0.61	0.41
Sc^{3+}	3.87	1.0	25	25	0.43	0.70

Au^{I}. Similar considerations may possibly apply to Tl^{I} *. In Group IV b of the Periodic Table, Pb → Sn → Ge, the slight dimerisation of lead(II) proceeds to colloidal characteristics in germanium(II) [48].

Interpolation of the data in Table 2 suggests that Cd^{2+} dimerises in the manner discussed to an extent between that of Cu^{2+} and Pb^{2+}, while Zn^{2+} does so rather more strongly than copper. In^{3+} would show very similar characteristics to Sc^{3+}.

SUMMARY

The state of solution of metal ions is discussed, and the acid dissociation constant of the aquo ion is correlated with the heat of hydration of the ion. The nature of the metal ion — hydroxyl ion bond is considered, and shown to be characterised in cases of electrostatic linking by a simple coulomb expression involving either the stability of the hydrolysis complex or the heat of hydration of the metal ion. Covalent contributions in other metal systems are estimated by their deviations from these empirical criteria, and later used in a discussion of the possible factors governing the formation of metal dimers through hydroxyl bridges. In this examination, the dimerisation characteristics of metal ions are related to the covalence of the metal-hydroxyl bond and the metal ionic radius. Some predictions of behaviour in systems yet to be examined are made.

I thank Professor J. Bjerrum for many helpful criticisms and his continued interest in this enquiry.

APPENDIX

The close correlation between the structures of solid hydroxides and the state of hydrolysed species in solution is of some interest. Thus it may be said in general that only those metals whose solid hydroxides have hydroxyl bonds linking the metal ion are able to polymerise in solution through hydroxyl bridging. (See Wells [49] for a classification of

* George, Rolfe and Woodward [47] have held that the Tl^{I}-OH system is electrostatic, but their evidence is not conclusive, as they acknowledged. Moreover, they did not consider the possibility of partial covalent character, discussed here.

solid hydroxides). Metals forming ionic lattices (*e. g.* alkali and alkaline earth metals excluding beryllium) show no dimerisation in the solution phase, while highly covalent systems would contain discrete molecules in their lattices, and here again there would be no polymerisation in solution. From this viewpoint, Mn(II), Fe(II), Co(II), Ni(II), and Cd should show no polymerisation tendencies, since the hydroxides of these metals have the CdI_2 ionic layer structure. (It should be remembered that the dimerisation expectations of cadmium mentioned above are based largely on a rather inaccurate determination of the first hydrolysis constant. Experimental studies are shortly to be undertaken to settle this point.)

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Crystal and Molecular Structure of 1,1,4,4-Tetrachloro*cyclo*hexane

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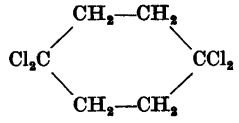
A crystal structure determination of tetrachloro*cyclo*hexane of m.p. 125° C led to a monoclinic unit cell containing 4 molecules with $a = 10.16$; $b = 7.73$; $c = 11.41$; $\beta = 91°$. The space group is $C2/c$ and the molecules are centro-symmetrical. Fourier projections along b and c confirmed the structure formula corresponding to 1,1,4,4-tetrachloro*cyclo*hexane which is in agreement with the first preparation of this compound from 1,4-*cyclo*hexanedione and phosphorous pentachloride [1]. The Cl—C—Cl angle is about 4° *smaller* than the "tetrahedral" angle. This is believed to result from the repulsion between the chlorine atoms and the nearest hydrogen neighbours. The angle between the (a) C—Cl bond and the chief axis of the carbon ring is 4° compared with the angle 7—8° found in the case of CHCl groups. The general features of the structure may be understood from an inspection of the b and c projections reproduced in Fig. 4a and 4b.

In the course of a series of structure determinations of tetrahalogeno-*cyclo*hexanes which is being carried out in our laboratory we have investigated the structure of tetrachloro*cyclo*hexane, m. p. 125° C, a substance first prepared by Kishner [1] more than sixty years ago from 1,4-*cyclo*hexanedione and phosphorus pentachloride. The structure formula should therefore be



Besides testing the validity of this conclusion we hoped to be able to study the molecular structure in some detail. Parallel to the X-ray analysis an electron diffraction investigation was started which already in its earlier stages made it very probable that the structure formula given above is the correct one. A report of the electron diffraction investigation will be published shortly [2].

The substance used in both investigations was obtained by direct chlorination of *cyclo*hexane in very much the same way as that described by Riemschneider and Drescher [3]. Crystals were grown from ethyl ether and turned

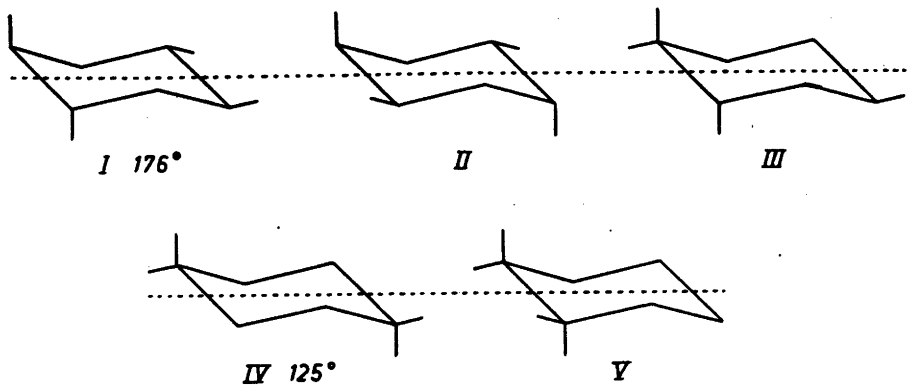


Fig. 1. Models of tetrachlorocyclohexanes all giving projections of the same kind along the dotted line. I : 1a,2a,4e,5e; II : 1a,2e,4a,5e; III : 1ae,2a,4e; IV 1ae,4ae; V 1ae,2ae.

out to be monoclinic. From Weissenberg and rotation photographs the following parameters of the unit cell were derived: $a = 10.16$ Å; $b = 7.73$ Å; $c = 11.41$ Å; $\beta = 91°$. The space group is $C2/c$ and the density calculated assuming the unit cell to contain *four* molecules is 1.65 g/cm³ which corresponds closely to the densities found for other tetrachloro*cyclo*hexanes.

The Fourier projection of the molecule along the *b*-axis is shown in Fig. 2a; in Fig. 2b the projection obtained by Hassel and Wang Lund [4] of the molecule

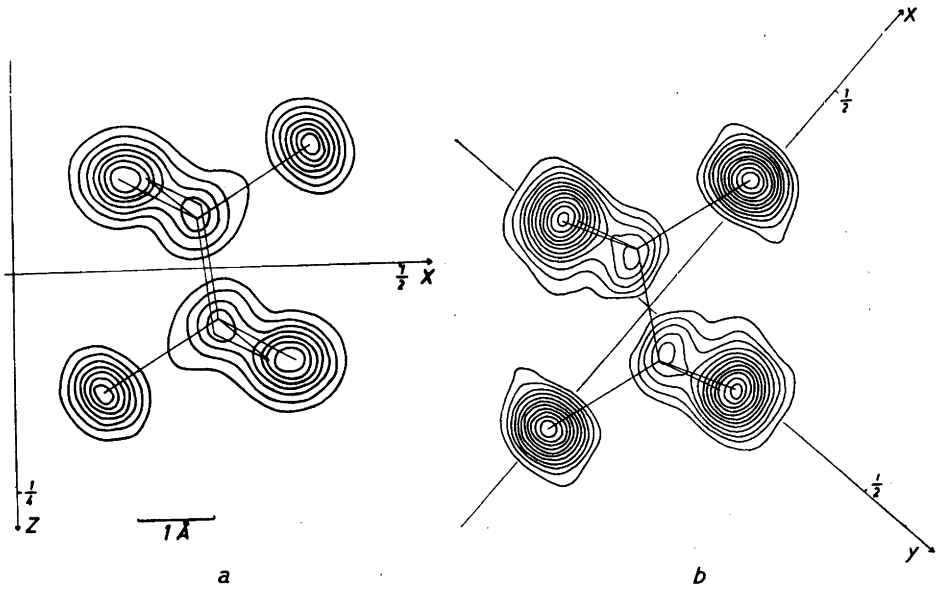


Fig. 2. a) Fourier projection of the 1ae, 4ae-tetrachlorocyclohexane molecule along the b-axis. b) corresponding projection of the 1a, 2a, 4e, 5e-tetrachlorocyclohexane molecule.

Table 1. Atomic coordinates of one molecule.

	x	y	z
Cl_{1a}	+ 0.112	+ 0.063	+ 0.139
(Cl_{4a}	+ 0.388	+ 0.437	− 0.139)
Cl_{1e}	+ 0.363	− 0.091	+ 0.110
(Cl_{4e}	+ 0.137	+ 0.591	− 0.110)
C_1	+ 0.261	+ 0.079	+ 0.057
(C_4	+ 0.239	+ 0.421	− 0.057)
C_2	+ 0.243	+ 0.095	− 0.075
(C_5	+ 0.257	+ 0.405	+ 0.075)
C_3	+ 0.170	+ 0.255	− 0.108
(C_6	+ 0.330	+ 0.245	+ 0.108)

of 1a, 2a, 4e, 5e-tetrachloro*cyclo*hexane * (m. p. 176° C) has been reproduced for comparison. The striking correspondence between the two projections does not, however, prove unambiguously that the substance is the 1,1,4,4-tetrachlorocompound. All the molecules represented in Fig. 1 may of course give projections of the same kind. Among them there is even one, the 1a, 2e, 4a, 5e compound (II) which has a center of symmetry like the 1,1,4,4 compound (IV). The final proof for the correctnes of the 1,1,4,4 structure was delivered by the working out of a Fourier projection along the *c*-axis (compare Fig. 3).

From the two refined Fourier maps all coordinate values for the chlorine atoms could be directly obtained. In the case of the z coordinate of the 1e chlorine atom, however, the accuracy may be somewhat inferior to the accuracy of the others because of overlapping with carbon atom 3 (6). For this carbon atom, on the other hand, the x and y coordinates could be determined with considerable accuracy. The other coordinates had to be evaluated under the assumption that the carbon ring is of the classical "chair" form with strictly tetrahedral valency angles. The C—C distances were assumed to be 1.54 Å, the C—Cl distances 1.78 Å. The coordinates are listed in Table 1. The number of observed reflexions, the intensity of which has been measured and used for the evaluation of the reliability factors were 52 for the h 0 l zone, 36 for the h k 0 zone, compared with the number of theoretically available reflexions (CuK-radiation) 77 and 58 respectively. The reliability factors thus obtained were 0.13 (h 0 l) and 0.14 (h k 0). The observed and computed structure factors are listed in Table 3.

Table 2.

	Ideal model	4° a-def. 1° e-def.	X-ray result
$Cl_{1a}-Cl_{1e}$	2.98	2.83	2.83
$Cl_{1a}-Cl_{4a}$	4.99	5.13	5.13
$Cl_{1a}-Cl_{4e}$	4.86	4.96	4.98
$Cl_{1e}-Cl_{4e}$	6.28	6.27	6.27

Computed Cl—Cl distances in an "ideal" and in a distorted model described in the text, both compared with directly observed distances.

* For the ε and $\varkappa$ bonds the new designations a (axial) and e (equatorial) suggested by Barton, Hassel, Pitzer and Prelog [7] have been adopted.

Table 3. List of observed and computed structure factors.

hkl	F_{obs}	F_{calc}	*hkl*	F_{obs}	F_{calc}
002	7.7	+ 7.5	$60\bar{1}\bar{0}$	3.3	+ 2.7
004	21.8	−23.0	$60\bar{1}2$	2.0	+ 2.2
006	5.3	− 4.0			
008	5.1	+ 6.3	802	4.7	+ 5.9
00 10	< 2.1	+ 0.1	804	5.7	− 5.5
00 12	1.8	− 2.0	806	4.1	− 4.5
00 14	< 1.3	0	808	< 2.0	+ 1.3
			8010	< 1.5	+ 1.0
200	10.6	−10.9			
400	13.9	−14.5	$80\bar{2}$	3.9	− 3.8
600	2.6	− 1.6	$80\bar{4}$	4.4	− 4.8
800	7.3	+ 7.8	$80\bar{6}$	4.0	+ 3.0
10 00	< 2.1	− 1.1	$80\bar{8}$	2.6	+ 1.9
12 00	< 1.6	− 0.9	$80\bar{1}\bar{0}$	3.1	− 1.9
202	1.8	− 2.7	10 02	< 2.1	− 1.4
204	9.1	+ 9.1	10 04	< 2.0	+ 1.6
206	4.4	+ 3.3	10 06	1.6	+ 2.6
208	4.4	− 5.4	10 08	< 1.4	− 0.6
2010	< 2.1	− 0.5			
2012	2.6	+ 3.8	10 $0\bar{2}$	< 2.1	− 1.4
2014	< 1.2	+ 0.2	10 $0\bar{4}$	2.6	− 1.4
			10 $0\bar{6}$	< 1.8	+ 1.5
$20\bar{2}$	11.0	− 9.8	10 $0\bar{8}$	2.4	+ 1.7
$20\bar{4}$	7.3	− 6.6			
$20\bar{6}$	5.1	+ 4.9	12 02	2.0	− 2.2
$20\bar{8}$	9.1	+ 8.7	12 04	< 1.3	+ 0.7
$20\bar{1}\bar{0}$	< 2.1	− 0.5			
$20\bar{1}2$	3.7	− 4.2	12 $0\bar{2}$	2.9	+ 3.1
$20\bar{1}4$	< 1.3	+ 0.1	12 $0\bar{4}$	1.3	+ 1.5
402	4.9	− 5.5	020	11.5	+14.1
404	10.8	+11.9	040	5.9	− 5.6
406	< 1.8	+ 1.7	060	11.9	−10.1
408	6.6	− 7.0	080	2.9	− 2.4
4010	2.0	− 2.1			
4012	1.6	+ 1.6	200	12.8	−11.0
			400	15.5	−15.0
$40\bar{2}$	12.0	+11.2	600	2.2	− 1.7
$40\bar{4}$	14.8	+15.2	800	7.5	+ 8.3
$40\bar{6}$	6.0	− 5.1	10 00	− 1.8	− 1.1
$40\bar{8}$	8.9	− 8.7	12 00	− 1.3	− 1.2
$40\bar{1}\bar{0}$	< 2.1	+ 0.2			
$40\bar{1}2$	1.6	+ 2.0	110	1.8	+ 2.1
			130	3.2	+ 3.5
602	< 1.7	+ 0.3	150	2.4	+ 2.0
604	2.4	− 3.2	170	− 1.8	− 1.0
606	< 2.0	− 0.8	190	1.8	− 2.0
608	4.4	+ 5.2			
6010	1.8	+ 2.3	220	1.6	− 0.7
6012	1.8	− 2.5	240	1.8	+ 1.8
			260	2.9	+ 2.8
$60\bar{2}$	3.3	− 1.4	280	− 1.6	− 0.4
$60\bar{4}$	2.4	+ 1.8			
$60\bar{6}$	< 2.0	− 1.5	310	2.7	+ 3.2
$60\bar{8}$	2.9	− 2.1	330	2.7	− 2.7

hkl	F_{obs}	F_{calc}	*hkl*	F_{obs}	F_{calc}
350	4.5	− 3.3	750	3.3	+ 2.9
370	− 1.8	− 0.6	770	− 1.6	+ 0.0
390	1.6	+ 1.7			
			820	5.1	+ 5.5
420	5.9	− 6.3	840	3.8	− 3.4
440	− 1.6	+ 1.8	860	3.6	− 4.1
460	4.9	+ 5.3			
480	1.8	+ 2.1	910	3.7	+ 3.1
			930	− 1.8	+ 1.2
510	4.0	− 4.4	950	− 1.6	− 0.6
530	− 1.6	− 2.6	970	− 0.9	− 1.4
550	− 1.8	− 0.1			
570	− 1.8	+ 2.0	10 20	− 1.8	+ 0.3
590	0.9	+ 1.7	10 40	− 1.6	+ 1.5
			10 60	− 0.9	+ 1.0
620	3.8	− 3.7			
640	− 1.8	+ 1.0	11 10	2.5	+ 2.6
660	− 1.8	+ 0.7	11 30	− 1.6	− 0.1
680	2.0	+ 1.6	11 50	1.8	− 1.8
			12 20	2.0	− 1.4
710	2.5	− 3.3			
730	− 1.8	+ 1.3	13 10	1.6	− 1.7

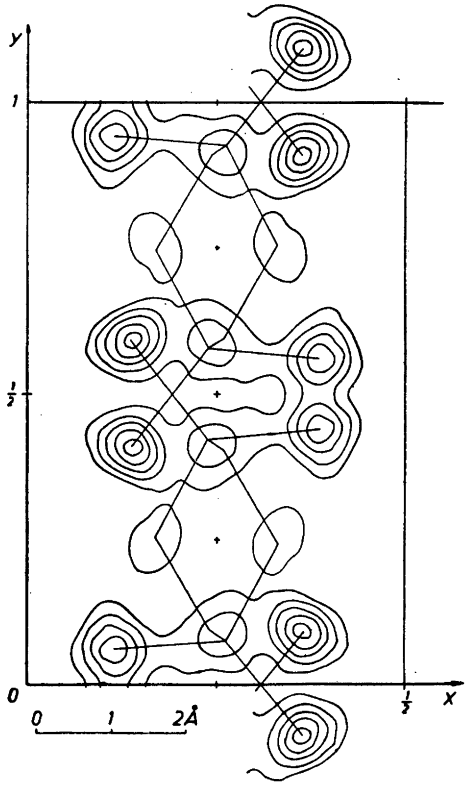


Fig. 3. Fourier projection of the 1ae, 4ae-tetrachlorocyclohexane molecule along the c-axis.

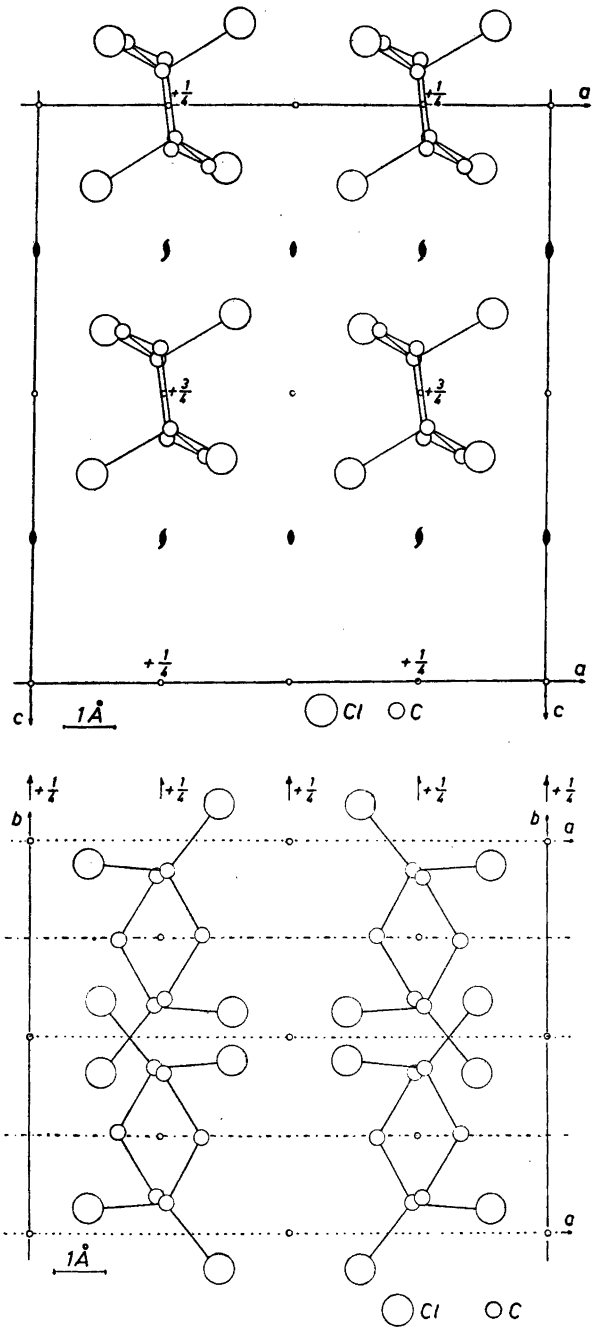


Fig. 4. Projections of the structure, a) along the b-axis, b) along the c-axis.

The assumed C—Cl distance of 1.78 Å is about one hundreth of an Ångström shorter than the mean value found in previous structure determination of chlorinated *cyclo*hexanes in which *one* chlorine atom only is attached to each carbon atom. A certain shortening of the bond in CCl_2 groups appears reasonable. In the case of methylene chloride the microwave spectroscopical value [5] is 1.7724 ± 0.0005 Å, approximately half way between the bond length in carbon tetrachloride and methyl chloride. In the present structure determination the value derived for the Cl—C—Cl angle will depend to some extent on the length attributed to the C—Cl bond. With the value 1.78 Å the angle becomes 105.3°, should it be one hundreth of an Ångström shorter the angle would become 106.1°. It therefore appears rather certain that the angle is in fact somewhat smaller than the "tetrahedral" angle. The explanation of this "compression" of the angle may, we think, be found in the repulsion exerted on the two chlorine atoms by the nearest (a) hydrogen atoms of the same molecule. In the case of Cl_{1a} the repelling hydrogen atoms would be the 3a and 5a atoms, and for the other chlorine atom (Cl_{1e}) the hydrogen atoms 2a and 6a. Such a view appears to be strengthened by the fact that the best agreement between observed and calculated Cl—Cl distances is obtained by assuming that the chlorine atoms all remain in the plane containing the carbon atoms 1 and 4 and the chief axis of the carbon ring. The angle between the C_1—Cl_{1a} direction and this axis thus becomes 4° (compared with 7 or 8° previously found in CHCl groups [4,6]) and the acute angle between the chief axis and the C_1—Cl_{1e} bond 1° smaller than it would be in an undistorted structure. It may be added that the distortions from an "ideal" structure just described are in good agreement with the results obtained in the course of the electron diffraction investigation [2]. The extent of agreement obtained between observed and computed Cl—Cl distances on the basis of the model just described may be judged from Table 2. That the bending angle of the (a) C—Cl bond away from the trigonal axis is smaller for CCl_2 groups than for CHCl groups appears rather natural as stated above, when the distance between the (e) chlorine atom and its nearest hydrogen neighbours is also being considered.

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The Enzymatic Synthesis of Ureidosuccinic Acid in Rat Liver Mitochondria

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It has been shown that the pyrimidine orotic acid (uracil-4-carboxylic acid) is rapidly synthesized *in vitro* in rat liver slices[1]. As orotic acid is the only free pyrimidine shown to be extensively utilized for nucleic acid synthesis in the rat and as the existing evidence points to orotic acid as either itself being a direct intermediate in the biogenesis of pyrimidines or very closely related to a direct intermediate, an investigation of the enzymatic synthesis of orotic acid in rat liver should prove very fruitful for the general problem of pyrimidine biogenesis. In a previous investigation [2] it was shown that aspartate, CO_2 and USA * were intermediates in orotic acid synthesis in rat liver slices. Of these substances USA had earlier been implicated in pyrimidine biogenesis by the work of Wright *et al.*[3,4], who demonstrated that the acid was an intermediate in pyrimidine synthesis in *Lactobacillus bulgaricus 09*.

Our earlier results with liver slices led us to the following formulation of the first step in the biogenesis of orotic acid [2]:

$$NH_3\ (?) + CO_2 + \text{L-aspartate} \rightarrow \text{L-ureidosuccinate}$$

In the present paper the *de novo* synthesis of USA in rat liver mitochondria is demonstrated. Ammonia is shown to be one of the precursors in the synthesis. The mechanism of the reaction has been studied in isolated liver mitochondria and it is suggested that a glutamic acid derivative (*e. g.* UGA), ATP and Mg^{++} are required for the synthesis of USA. The reaction thus shows a great resemblance to the synthesis of citrulline from ornithine, CO_2 and ammonia, as studied by Grisolia and Cohen [5].

* The following abbrevations are used in the present paper: Ureidosuccinic acid: USA; Ureidoglutaric acid (= carbamyl glutamic acid): UGA; Adenosine triphosphate: ATP; Tris (hydroxymethyl) aminomethane : Tris.

EXPERIMENTAL

Preparation of rat liver mitochondria. The differential centrifugation method of Schneider and Hogeboom [6] with 0.25 *M* sucrose as homogenizing medium was used. "Versene" (ethylenediaminetetraacetic acid), 0.01 *M* at pH 7.8, was included in all isolation media, since Slater and Cleland [7] have demonstrated that the addition of this chelating agent increases the stability of the mitochondria.

Two rats were killed by decapitation, the livers immediately removed and chilled on ice. All the following operations were carried out at around 0°.. The livers were homogenized in a large Potter-Elvehjem apparatus with a plastic pestle in a volume of *ca.* 50 ml sucrose solution. The homogenate was diluted to 110 ml with the same medium. All following centrifugations were carried out in the head of the multispeed attachement of a refrigerated International centrifuge.

In a first centrifugation at 1 000 r.p.m. (100 g*) for 15 min. cellular debris, nuclei, etc. were removed. The supernatant from this run was centrifuged at 15 000 r.p.m. (18 000 g) for 15 minutes and the supernatant discarded. The sedimented mitochondria, which microscopically contained very few cell nuclei were gently suspended in sucrose and recentrifuged at 15 000 r.p.m. for 10 minutes. The sediment from this centrifugation was used for the experiments. It could be stored for several hours before use without evident impairment of its synthetic activity. Immediately before use the mitochondria were evenly suspended in isotonic KCl, giving a final volume of 10—15 ml.

Mitochondria prepared in this way were usually employed in about 10 experiments. Since the capacity of the mitochondria to synthesize USA varied some from one preparation to another, mitochondria from the same preparation were used for obtaining each curve in the graphs presented.

Incubation. All experiments were conducted in 200 ml Erlenmeyer flasks at 37° in an atmosphere of O_2:CO_2 (95 % : 5 %). Unless otherwise indicated incubation was carried out over a period of two hours in a total volume of 15 ml. About 4 mg of mitochondrial nitrogen were employed in each experiment.

Determination of enzyme activity. No specific method for the determination of USA in the presence of a large excess of UGA was available. Therefore the experiments were carried out with isotopic precursors (N^{15}-L-aspartic acid and N^{15}-ammonium chloride), and the amount of USA formed was determined by the isotope dilution technique. 200 μmoles of non-labeled D,L-USA was added as carrier at the end of each experiment, the USA was reisolated by a chromatographic technique and recrystallized to constant isotope content. As the amount of N^{15}-precursors in the experiments was very large as compared to the pools of the same unlabeled substances in the mitochondria (assuming rapid and complete mixing between the added precursors and the mitochondrial aspartate and ammonia), and as the amount of formed L-USA was very small as compared to the amount of added carrier D,L-USA the following approximation is valid:

$$\mu\text{moles USA formed} = \frac{200 \cdot a}{b}$$

where a = xs N^{15} in reisolated USA, and b = xs N^{15} in the precursors. Both NH_4Cl and aspartic acid contained throughout the experiments 32 % excess N^{15}. Hence: μmoles USA formed = $6.25 \cdot a$.**

Reisolation of USA. At the end of the experiment the reaction was stopped by immersion of the flasks in a boiling water bath for 3 minutes. Two hundred μmoles D,L-USA, dissolved in 1 ml of 0.4 *N* NaOH, were added and the coagulated proteins were filtered off with the aid of some celite. The solution was brought to pH 10—11 with a few drops of

* This centrifugal force was considerably lower than what is generally used for removal of cell nuclei. However, already at 200 g great losses of mitochondria took place under the experimental conditions used.

** The calculation does not take into consideration the possibility of non labeled aspartate and ammonia being formed from glutamate in the experiments, where the latter acid was included in the medium. There is some evidence that a slight dilution of the isotopic precursors did take place through this mechanism. The amounts of USA obtained by the calculation represent thus minimum values.

2 *N* NaOH and added to the top of a Dowex 2 column (200—400 mesh Cl^- form, diam. 2 cm, length 10 cm). After all the solution had penetrated into the ion exchange resin the column was washed with 50 ml of water and then with 0.005 *N* HCl until the effluent became distinctly acid (*ca.* 300 ml). From that point on another 100 ml of 0.005 *N* HCl were run through the column and the effluent discarded. The solvent was changed to 0.04 *N* HCl and all the USA was eluted by 100 ml of this acid. If orotic acid had been included in the medium, it could be obtained from the column by further elution with 100 ml of 0.5 *N* HCl.

Two drops of Kjeldahl indicator were added to the effluent containing the USA, the solution was neutralized with *N* NaOH and evaporated to dryness *in vacuo*. The residue was dissolved in a total of 3 ml of water, filtered and transferred to a centrifuge tube. The solution was acidified with two drops of concentrated HCl, seeded with a very small crystal of USA, and crystallization allowed to take place in the refrigerator over night. The crystals were centrifuged, washed twice with 0.5 ml of ice cold water and recrystallized from 1 ml hot water. The crystals from this second crystallization had generally the same isotope content as their mother liquor and this isotope content as determined in the mass spectrometer was used for the calculation of enzyme activity.

The method was tested by addition of 1.0 and 2.0 μmoles of N^{15}-USA to heat coagulated mitochondria in the medium of Table 1. After reisolation in the way described the recovery of the USA was calculated from isotope analysis to be 96 and 103 % respectively.

The method outlined above permitted a relatively rapid determination of the different factors influencing the USA synthesizing capacity of mitochondria. Up to 15 ion exchange columns were run parallell and the rate of flow through them was accelerated by air pressure. At the conclusion of each run the columns were washed first with 250 ml of 4 *N* HCl and then with water to remove the excess HCl. They were then immediately ready for use in a new experiment.

D,L-*Ureidosuccinic acid* and L-*ureidoglutaric acid* (carbamyl glutamic acid) were synthesized according to Nyc and Mitchell [8]. The syntheses of *N^{15}*-L-*aspartic acid*, *N^{15}*-L-*ureidosuccinic acid* and *C^{13}*-L-*aspartic acid* have been described earlier [2].

Table 1. USA synthesis in different cell fractions.

Cell fraction	mg N	μmoles USA synthesized	μmoles USA synthesis/mg N	% recovery
Whole homogenate	156	19.6	0.126	100
"Cell nuclei"	51	3.1	0.061	16
Mitochondria	26	9.3	0.358	47
Cell sap + microsomes	68	*ca.* 0.2		
Mitochondria + cell sap + microsomes	94	9.7	0.103	49

The values are calculated for one rat liver. An aliquot from each fraction suspended in 2 ml sucrose was incubated in the following medium: 0.006 *M* N^{15}L-aspartate * and $N^{15}H_4Cl$, 0.012 *M* $NaHCO_3$, 0.015 *M* L-glutamate, 0.007 *M* L-UGA. 0.008 *M* $MgCl_2$ *, 0.001 *M* ATP *, 0.04 *M* phosphate buffer and 0.04 *M* tris buffer, pH 7.4 *. Gas = O_2:CO_2 (95 % : 5 %), volume = 15 ml, KCl to isotonicity *.

* All salts, unless otherwise indicated, were potassium salts. $MgCl_2$ was added only immediately before the start of the experiment. — Ba-ATP (purchased from the Sigma company) was suspended in ice cold water and dissolved with a minimum amount of 0.5 *N* HCl. A slight excess of Na_2SO_4 was added, the solution neutralized to pH 7 and centrifuged. — The buffers were chosen such as to give the desired pH after saturation with the gas used. — No consideration was taken of the osmotic pressure of phosphate in the calculation of isotonicity (K. W. Cleland, *Nature* **170** (1952) 497).

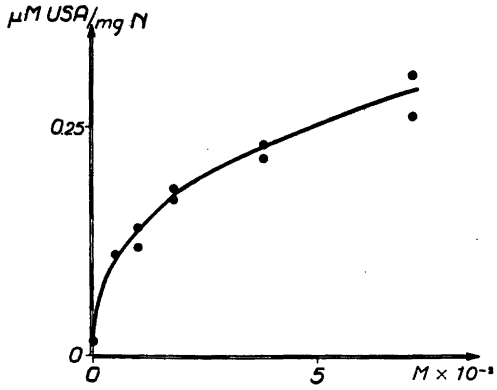

Fig. 1. USA synthesis in the presence of glutamate. Each flask contained aspartate, ammonium chloride, $NaHCO_3$, $MgCl_2$, ATP and buffers as in Table 1. L-*Glutamate as indicated on the abscissa.*

RESULTS

Localization of USA synthesis. Early experiments to demonstrate USA synthesis in cellfree systems using the same substrate as in the slice experiments did not meet with success. It was found, however, that whole homogenates either prepared with isotonic KCl or sucrose could synthesize USA quite rapidly, if glutamate in relatively high concentrations was included in the medium. When USA synthesis was studied in different fractions of a sucrose homogenate the results of Table 1 were obtained. In these experiments part of the glutamate was substituted by UGA to obtain optimal synthesis (see below).

It can be seen that the largest amount of USA formed per mg nitrogen was obtained in the mitochondrial fraction. The relatively high synthesis in the "nuclear" fraction is probably explained by inclusion of mitochondria and whole cells in this fraction. The experimental setup presumably did not allow ATP synthesis in the supernatant fraction and therefore the failure to synthesize USA in the reported experiment is not conclusive. However, only insignificant synthesis was obtained in the supernatant, also when 3-phosphoglyceric acid was added to provide ATP. Furthermore, very little stimulation was observed, when mitochondria were combined with supernatant. It was never possible to recover more than 75 % of the activity of the whole homogenate in the different cellular fractions. *Under the experimental conditions* of Table 1 no more than traces of orotic acid were synthesized in the mitochondria, a fact which might favourable influence the accumulation of USA in the present system *.

Requirement for glutamate or UGA. Fig. 1 demonstrates the requirement of the reaction for glutamate. It can be seen that the enzyme system is not yet saturated at a concentration of glutamate of 0.07 *M*. Succinate + UGA can substitute for glutamate in the reaction (Fig. 2, curve II). Saturation of the enzyme system is obtained around 0.02 *M* UGA and 0.015 *M* succinate. The

* U. Lagerkvist (unpublished) has found that *under the proper conditions* the synthesis of orotic acid from USA is localized in rat liver mitochondria.

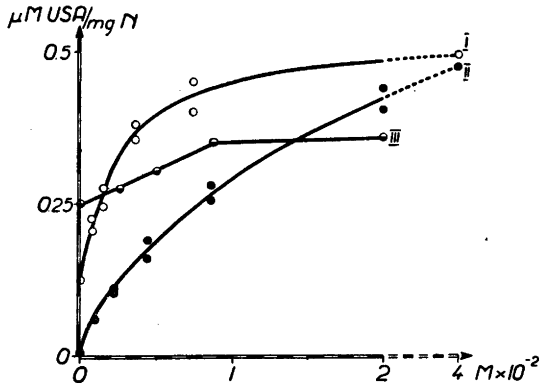

Fig. 2. UGA and USA synthesis. Medium: 0.02 M aspartate; ammonium chloride, $NaHCO_3$, $MgCl_2$, ATP and buffers as in Table 1.

Curve I: 0.015 M L-glutamate, L-UGA as indicated on the abscissa.
Curve II: 0.015 M succinate, L-UGA as indicated on the abscissa.
Curve III: 0.015 M succinate, 0.01 M L-UGA, L-glutamate as indicated on the abscissa.

function of glutamate in Fig. 1 seems to be a double one: partly it serves as a substrate for combustion, partly it serves as the source of a derivative that is necessary for the reaction. The first function can be fulfilled by succinate, the second one by UGA. UGA or succinate separately, however, even at concentrations of 0.1 *M* only permit small amounts of USA synthesis.

Curve I in Fig. 2 demonstrates the influence of varying concentrations of UGA in the presence of glutamate. It is interesting to compare curves I and II in Fig. 2. The same concentrations of glutamate and succinate respectively are used in the two curves. Curve I shows a steeper slope and earlier saturation of the enzyme with UGA than curve II. Curve III shows the slight stimulation of USA synthesis exerted by glutamate at optimal succinate and slightly suboptimal UGA concentrations. When both UGA and succinate

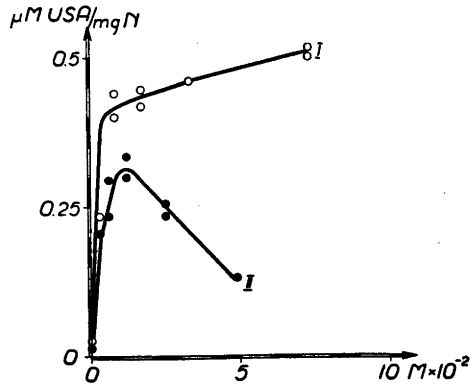

Fig. 3. Requirement of oxidizable substrate. Medium: 0.02 M aspartate; ammonium chloride, $NaHCO_3$, $MgCl_2$, ATP and buffers as in Table 1.

Curve I: 0.007 M L-UGA, L-glutamate as indicated on the abscissa.
Curve II: 0.02 M L-UGA, succinate as indicated on the abscissa.

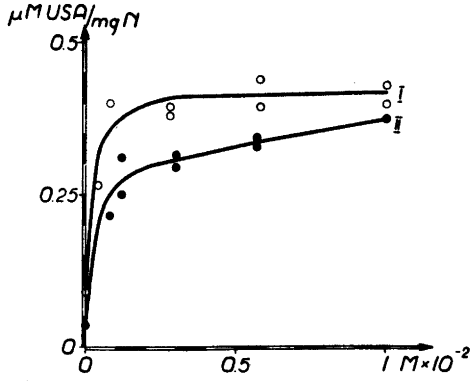

Fig. 4. Ammonia and USA synthesis. Medium: 0.02 M aspartate; $MgCl_2$, ATP and buffers as in Table 1.

Curve I: 0.015 M L-glutamate, 0.007 M L-UGA, $N^{14}H_4Cl$ as indicated on the abscissa.

Curve II: 0.015 M succinate, 0.02 M L-UGA, $N^{14}H_4Cl$ as indicated on the abscissa.

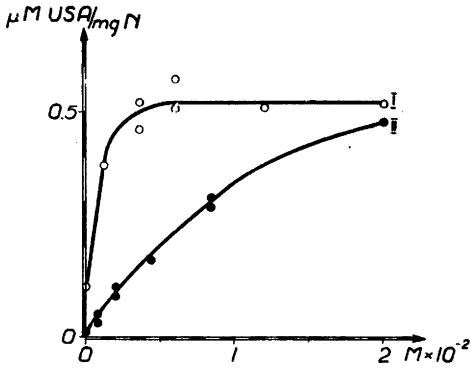

Fig. 5. Aspartate and USA synthesis. Ammonium chloride, $NaHCO_3$, $MgCl_2$, ATP and buffers as in Table 1.

Curve I: 0.015 M L-glutamate, 0.007 M L-UGA and N^{14}-aspartate as indicated on the abscissa.

Curve II: 0.015 M succinate, 0.02 M L-UGA and N^{14}-aspartate as indicated on the abscissa.

concentrations were optimal, glutamate no longer showed this stimulating effect.

Fig. 3 shows the influence of varying concentrations of glutamate (curve I) and succinate (curve II) at constant UGA concentration. The apparent inhibiting effect of larger concentrations of succinate cannot be explained without further experiments.

Influence of ammonia and aspartate. The dependence of USA synthesis on the simultaneous presence of ammonia and aspartate in the medium is demonstrated by Figs. 4 and 5. In these experiments only one of the two precursors contained N^{15}. The concentration of the non isotopic precursor varied in the presence of a constant amount of the labeled precursor. Isotope incorporation into USA did thus in the aspartate experiments (Fig. 5) only take place from $N^{15}H_4Cl$ and in the ammonia experiments (Fig. 4) from N^{15}-aspartate. In the degradation experiments related below it was demonstrated that each of the precursors donated its nitrogen almost exclusively to only one of the two nitrogens of the USA. In the calculation of the amount USA formed the isotopic excess observed in the reisolated USA was therefore multiplied with 12.5 instead of 6.25.

Curves I and II in Figs. 4 and 5 represent experiments in which glutamate and succinate respectively were used as oxidizeable substrates. In Fig. 5 curve I saturation of the enzyme with aspartate apparently occurs at much lower concentrations than in curve II. One explanation for this might be that the presence of glutamate inhibits the disappearance of aspartate by transamination reactions.

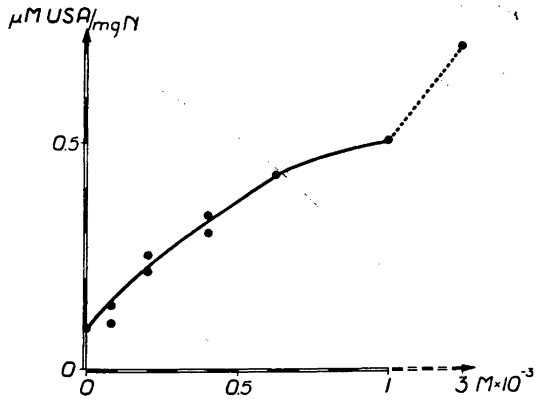

Fig. 6. ATP and USA synthesis. Aspartate, ammonium chloride, $NaHCO_3$, $MgCl_2$, glutamate, UGA and buffers as in Table 1. ATP as on the abscissa.

ATP and Mg^{++}. The demonstrated requirement of an oxidizable substrate is best explained on the basis of an ATP dependence of the reaction studied. A small amount of ATP (which can be replaced by ADP or AMP) was always included in each experiment and the influence of varying concentrations of ATP is demonstrated by Fig. 6. The ATP concentration (0.001 *M*) chosen in all the experiments, where ATP was not varied, was not optimal. The influence of Mg^{++} is demonstrated by Fig. 7. At this point it cannot be decided with certainty whether Mg^{++} was necessary for the regeneration of ATP or whether it directly participated in the synthesis of USA.

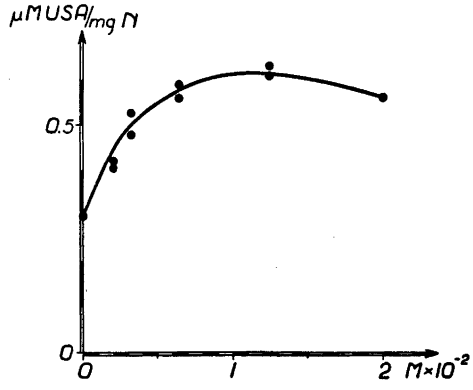

Fig. 7. $MgCl_2$ and USA synthesis. Aspartate, ammonium chloride, $NaHCO_3$, ATP, glutamate, UGA and buffers as in Table 1. $MgCl_2$ as on the abscissa.

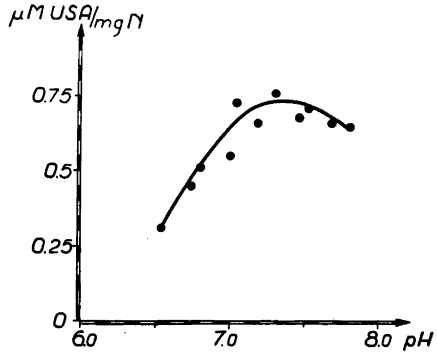

Fig. 8. Influence of pH. Aspartate, ammonium chloride, $NaHCO_3$, $MgCl_2$, ATP, glutamate and UGA as in Table 1. 0.04 M phosphate and tris buffers at varying pH. The pH was measured at the end of the experiment.

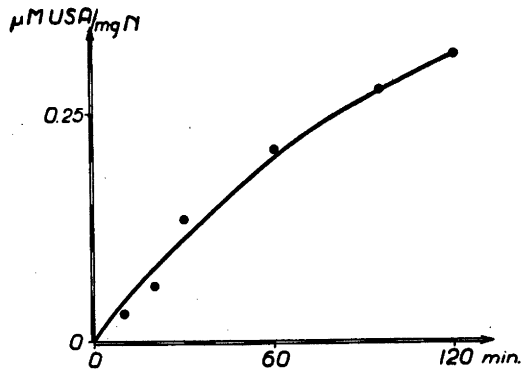

Fig. 9. Time curve of USA synthesis. Substrate as in Table 1.

pH optimum. The pH curve of Fig. 8 shows a rather broad maximum around pH 7.4. It must be kept in mind that the curve represents the pH optimum of a sum of several different reactions (*e. g.* oxidation of glutamate and USA synthesis).

The *time curve* of Fig. 9 demonstrates a linear reaction rate for 1—1.5 hours, after which time a slight reduction in the rate occurred.

Fig. 10 shows that a straight line is obtained when USA synthesis is plotted against increasing *amounts of mitochondria.*

Degradation of USA. The present system did not allow stoichiometric studies. Instead the direct transformation of aspartate and ammonia into USA was demonstrated by degradation of USA after administration of each of the isotopic precursors.

In each experiment mitochondria from one liver were incubated in 75 ml of the media specified in Table 2. After two hours 0.8 mmoles carrier USA was added, and the USA was reisolated in the way outlined earlier. After two recrystallizations a small part of the USA was analyzed for isotope, while the

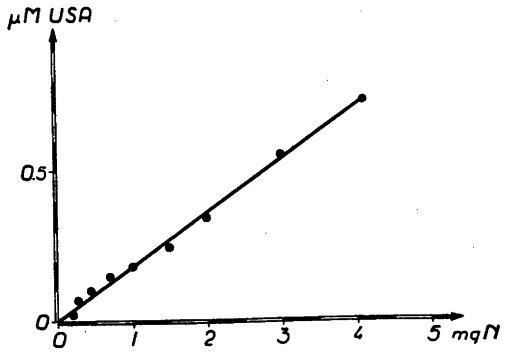

Fig. 10. USA synthesis with different amounts of enzyme. Substrate as in Table 1.

Table 2. Degradation of USA after incubation with $N^{15}H_4Cl$ (32 % excess N^{15}) and N^{15}-1,4-C^{13}-L-aspartate (31.9 % N^{15} and 16.1 % C^{13}).

Precursor	Atom % excess N^{15} in			Atom % C^{13} in		N^{15} / C^{13} in	
	USA	carbamino-N	aspartate-N	USA	aspartate from USA	precursor	aspartate from USA
Ammonium chloride	0.293	0.525	0.058				
Aspartate	0.265	0.039	0.498	0.233	0.284	1.92	1.76

The same medium was used as in Table 1 with the exception that in the experiment with labeled NH_4Cl nonlabeled aspartate was used, and in the experiment with labeled aspartate the ammonium chloride contained no isotope. The degradations are described in the text, the carbamino group is represented by the isotope value of ammonia, the aspartate moiety of USA by the isotope values of the Cu-aspartate.

rest was hydrolyzed in 5 ml concentrated HCl at 100° for 24 hours. In this way the carbamino nitrogen of USA was obtained as ammonia, while the part of the molecule that corresponds to aspartic acid was obtained as aspartic acid. The subsequent separation and isolation of the derived ammonia and aspartic acid involved the same steps as described earlier in the corresponding degradation of orotic acid [2]. After purification, ammonia and copper aspartate were analyzed for isotope.

Table 2 presents the results of experiments with $N^{15}H_4Cl$ and N^{15}-1,4-C^{13}-L-aspartate respectively. It can be seen that the N^{15} from ammonia is incorporated into the carbamino nitrogen of USA, while that of aspartate forms the other nitrogen atom of the acid. Furthermore, in the aspartate experiment the N^{15}/C^{13} ratios in the precursor and in the aspartate derived by degradation from USA are almost the same, indicating that aspartic acid is utilized as an intact molecule.

DISCUSSION

From the data in Table 1 it can be calculated that the rat liver homogenate obtained from one animal synthesized USA at the rate of ca 200 μmoles per day. One can furthermore very approximately calculate that one rat liver contains about 100 μmoles of nucleic acid pyrimidines. The USA synthesis of the liver should therefore suffice to renew all the nucleic acid pyrimidines of the liver in 12 hours. The estimations of the "half life times" of nucleic acids in rat liver have arrived at values of around one week. The amount of USA synthesis demonstrated in Table 1 is thus far more than sufficient to provide for all pyrimidine synthesis in rat liver, even if one assumes that part of the USA is diverted to acid soluble pyrimidine derivatives with a high turnover rate.

The present experiments confirm the earlier finding [2] that aspartic acid is involved in orotic acid biogenesis, probably *via* USA. They furthermore establish that ammonia is directly involved in USA synthesis and thus is the

precursor of N_1 of orotic acid. In liver slices we had found that the isotope from N^{15}-ammonia even in short time experiments is almost evenly distributed between N_1 and N_3 of orotic acid, which made us reluctant to accept ammonia as a direct precursor of N_1. We can now explain these earlier results by the probability that N^{15}-ammonia in liver slices gives rise very rapidly to N^{15}-aspartate through amination of a dicarboxylic acid. The present interpretation also fits excellently with earlier results from *in vivo* experiments with rat liver obtained by Lagerkvist [9], who could demonstrate that the incorporation of isotope from N^{15}-ammonia into position 1 of polynucleotide uracil was between 3—4 times greater than that into position 3.

As to the mechanism of the reaction the similarity between USA synthesis and citrulline synthesis is apparent. For the latter case Grisolia and Cohen [5] have demonstrated that the fixation of ammonia and carbon dioxide proceeds in two steps: in the first step a labile phosphorylated derivative ("compound X") is formed from UGA, ammonia, CO_2 and ATP in the presence of Mg^{++}. The second reaction involves the formation of citrulline from ornithine + compound X, while UGA is regenerated. In recent experiments Grisolia and Cohen [10] have demonstrated that instead of UGA, formylglutamic, acetylglutamic, chloroacetylglutamic or propionylglutamic acids can be utilized in citrulline synthesis.

The formation of USA also involves a fixation of ammonia and CO_2, which proceeds with the aid of ATP and a glutamic acid derivative. UGA can act as this glutamic acid derivative, though relatively high concentrations are needed to saturate the enzyme. It is of interest to note that considerably smaller amounts of UGA are sufficient, if the oxidation of glutamate is used as an energy source than if succinate is used (curves I and II in Fig. 2). Furthermore relatively small amounts of glutamate still slightly stimulated USA synthesis in the presence of optimal amounts of succinate and slightly suboptimal amounts of UGA (curve III, Fig. 2). This might indicate that a glutamic acid derivative that differs from UGA is more directly involved in the synthesis. Matters are, however, complicated by the demonstrated inhibition of USA synthesis by succinate at higher concentrations, and it is hoped that experiments with soluble enzymes now in progress will answer this question.

The following over all mechanism for the synthesis of USA is thus suggested:

$$NH_3 + CO_2 + \text{L-aspartate} \xrightarrow[\text{ATP, } Mg^{++}]{\text{L-UGA or other glutamate derivative}} \text{L-ureidosuccinate}$$

It should be pointed out in this connection that the above mechanism has been suggested by Wright [11] already in 1951 in a review article, though at that time no experimental evidence for the reaction existed.

Another theory that has been brought forward at different times [12–14] is that arginosuccinic acid or a compound derived from it might be involved in pyrimidine biogenesis. This acid can be enzymatically synthesized from citrulline + aspartic acid in rat liver. If it were an intermediate in the synthesis of USA from aspartic acid this would explain the fixation of NH_3 and CO_2 during

the reaction. In experiments, where N^{15}-citrulline was incubated in liver slices together with orotic acid under the conditions described earlier [2], it was found, however, that no N^{15}-orotic acid was derived from citrulline. This experiment tends to exclude arginosuccinate as an intermediate in orotic acid synthesis at least in *rat* liver slices.

SUMMARY

The synthesis of ureidosuccinic acid from aspartic acid, ammonia and CO_2 was studied in rat liver mitochondria. Under the conditions chosen it was found that the presence of glutamic acid or ureidoglutaric acid + succinic acid was necessary for the reaction, which also depended upon ATP and Mg^{++}. The resemblance between ureidosuccinic acid synthesis and citrulline synthesis is pointed out.

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Studies on Conidendrin *

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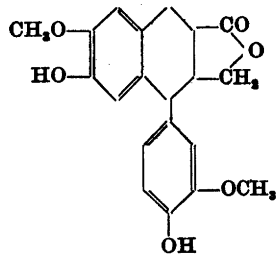
Hypobromite oxidation of α-conidendrin dimethyl ether gives among other products a neutral substance which is shown to be 6,7-dimethoxy-1-(3′,4′-dimethoxyphenyl)-2-naphthaldehyde. β-Conidendrin dimethyl ether is very stable to hypobromite. The diol corresponding to β-conidendrin dimethyl ether has been prepared.

During his studies on "sulphite-liquors lactone", conidendrin (I), Holmberg [1] oxidised α-conidendrin dimethyl ether with hypobromite and obtained two acids later identified by Erdtman [2] as 6-veratroylveratric acid (II) and 6,7-dimethoxy-1-(3′,4′-dimethoxyphenyl)-1,2,3,4-tetrahydro-2,3-naphthalene-dicarboxylic acid (III), and in addition a neutral compound $C_{16}H_8O(OCH_3)_4$ which according to Erdtman was probably a carbonyl compound. The structure of this product was never elucidated.

Conidendrin, formerly a substance of purely academic interest is now available in very large quantities due to the observation that the waste liquor from sulphite pulping of *Tsuga heterophylla*, "Western hemlock", contains unusually large amounts of conidendrin, and the excellent work done by the chemists of The Crown Zellerbach Company, Camas, which resulted in a convenient method of isolating conidendrin from this material [3]. Conidendrin is therefore now a potential technical raw material and one suggested utilisation is the production of the nor-conidendrin compounds possessing good anti-oxidising properties [4–7].

Attempts have been made to increase the yield of the dicarboxylic acid (III) which appeared to be an interesting intermediate for various synthetic products, by varying the procedure employed by Holmberg, *e. g.* by using hypochlorite or hypoiodite instead of hypobromite. Oxidation with permanganate was also attempted: under no conditions was the yield increased, but a reasonable amount of Holmberg's neutral compound was obtained and the structure of this substance has now been determined. The correct composition

* Part XVII in the series: *Constitution of resin phenols and their biogenetic relations.* Part XVI *Acta Chem. Scand.* **6** (1952) 1147 (erroneously numbered XV). Part XV *Acta Chem. Scand.* **5** (1951) 936.



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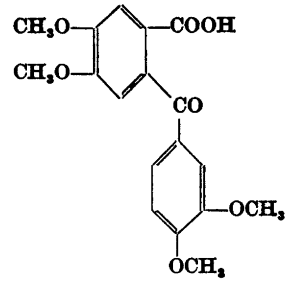

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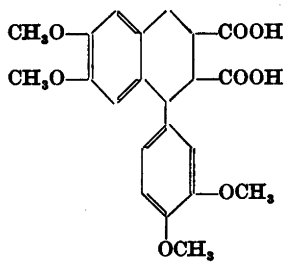

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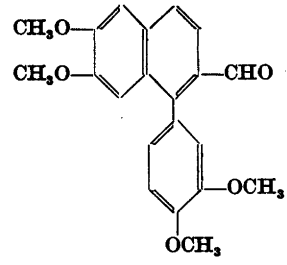

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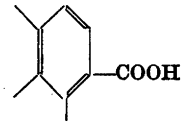

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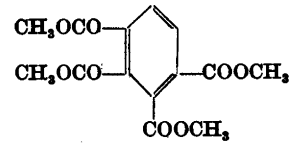

VI

was found to be $C_{17}H_8O(OCH_3)_4$ and the substance yielded a 2,4-dinitrophenylhydrazone and an oxime. The latter could be dehydrated by acetic anhydride to a nitrile, which was hydrolysed to the corresponding carboxylic acid. The substance, consequently, must be an aldehyde. Direct oxidation of this very sparingly soluble aldehyde to the carboxylic acid with permanganate under various conditions was at first unsuccessful, the compound being revocered unchanged or being completely destroyed. Ultimately it was found that the acid could be obtained by oxidation in pyridine solution.

In order to locate the carboxyl group in this acid, which from its composition appeared to be a phenyl naphthalene carboxylic acid, it was demethylated and the crude reaction product oxidised with permanganate. An acid was obtained, which was methylated with diazomethane, and the resulting ester was found to be identical with prehnitic acid tetramethyl ester (VI) prepared as described by Smith and Carlson [8]. The ester exhibited the peculiar purple coloration on exposure to light described by these authors. The colour was

quite distinct after 10—15 minutes' irradiation with ultraviolet light and disappeared on melting or recrystallisation.

The isolation of prehnitic acid proves conclusively that the structure of the acid is 6,7-dimethoxy-1-(3',4'-dimethoxyphenyl)-2-naphthoic acid (V). This acid was also found to be identical with a synthetic sample recently prepared by Walker [9] and kindly furnished by him. The corresponding aldehyde, Holmberg's "neutral substance", is consequently 6,7-dimethoxy-1-(3',4'-dimethoxyphenyl)-2-naphthaldehyde (IV). Holmberg reported a yield of about 6 % but this may be increased by slight modification of the original method. Holmberg mixed a solution of α-conidendrin dimethylether in alkali with a hypobromide solution and heated the mixture on a boiling water-bath. If, however, the solutions are heated almost to the boiling point prior to the mixing a 10—12 % yield is obtained.

If, instead of α-conidendrin dimethyl ether, the β-isomer was oxidised with hypobromite no aldehyde or only traces of the aldehyde (IV) and a little 6-veratroylveratric acid were obtained, the bulk of β-lactone being recovered unchanged. When the phenyltetralin-dicarboxylic acid (III) was subjected to hypobromite oxidation it was also largely recovered unchanged and only traces of 6-veratroylveratric acid could be isolated. Thus it is obvious that the oxidation of α-conidendrin dimethyl ether (or rather the corresponding hydroxy acid) follows three distinctly different routes; the first leading to the aldehyde (IV), the second to the dicarboxylic acid (III) and the third to 6-veratroylveratric acid but there may, of course, be a common primary reaction stage.

Haworth and Wilson [10] have reduced α-conidendrin dimethyl ether with lithium aluminium hydride and obtained a diol m. p. 167—168° identical with (+) *iso*-laricresinol dimethyl ether. The same diol has now been obtained from the dimethylester of Holmberg's phenyltetraline dicarboxylic acid (III). Hence this acid retains the configuration of the α-conidendrin series and it is obviously formed by direct oxidation of the $-CH_2OH$ group to —COOH. As expected β-conidendrin dimethylether on reduction with lithium aluminium hydride furnished a different diol (m. p. 140—142°).

EXPERIMENTAL *

Composition of Holmberg's "neutral substance". The aldehyde (IV) was purified by distillation *in vacuo* and by several crystallizations from glacial acetic acid and ethanol. M.p. 182—183°. (Found: C 71.84, 71.13; H 6.18, 5.91; OCH_3 35.6. Calc. for $C_{21}H_{20}O_5$ (352.4): C 71.58; H 5.72; OCH_3 35.23).

6,7-Dimethoxy-1-(3',4'-dimethoxyphenyl)-2-naphthaldehyde-2,4-dinitrophenylhydrazone. Hot solutions of the aldehyde (IV) (0.5 g) and of 2,4-dinitrophenylhydrazine (0.7 g) in glacial acetic acid were mixed and heated on a water-bath for 15 minutes. The red precipitate was collected while hot and crystallized from glacial acetic acid. M. p. 289—291° (decomp.). (Found: OCH_3 22.9). Calc. for $C_{27}H_{24}N_4O_8$ (532.5): OCH_3 23.3.

6,7-Dimethoxy-1-(3',4'-dimethoxyphenyl)-2-naphthaldehyde oxime. A solution of the aldehyde (IV) (1 g) and of hydroxylaminehydrochloride (1 g) in absolute ethanol (50 ml) and pyridine (4 ml) was boiled under reflux for two hours. The solution was evaporated to dryness and the residue washed with water. The product (1 g) was crystallized from ethanol. M. p. 185—186°. (Found: N 3.68). Calc. for $C_{21}H_{21}NO_5$ (367.4): N 3.81.

* All melting points uncorrected.

6,7-Dimethoxy-1-(3',4'-dimethoxyphenyl)-2-naphthoic acid nitrile. A solution of the oxime above (0.7 g) in acetic anhydride (20 ml) was heated under reflux for 1.5 hours. Water (50 ml) was added carefully and the mixture heated on a water-bath for half an hour and then poured into a large volume of water (450 ml). On cooling a slightly yellow precipitate was formed. Crystallization from ethanol (animal charcoal) gave a colourless product. M.p. 185—186° depressed on mixing with the starting material to 165—170°. (Found: C 72.46; H 5.42; N 3.92; OCH_3 35.77. Calc. for $C_{21}H_{19}NO_4$ (349.4): C 72.19; H 5.48; N 4.01; OCH_3 35.53).

6,7-Dimethoxy-1-(3',4'-dimethoxyphenyl)-2-naphthoic acid. Method A: The nitrile above was boiled with ethanolic potassium hydroxide (ethanol 20 ml, potassium hydroxide 8 g) for three days. Some ammonia was still being evolved but the mixture was cooled and acidified with conc. hydrochloric acid (10 ml). The heavy precipitate (containing hydrated silica from the glass vessel) was extracted with boiling ethanol and the combined extracts precipitated with water. The product was dissolved in dilute sodium hydroxide separated from some undissolved material, acidified with dilute hydrochloric acid and the precipitate crystallized from ethanol. M. p. 202—203°.

Method B: A mixture of the aldehyde (IV) (3 g), dissolved in pyridine (60 ml) containing a little sodium hydroxide (2 ml) (2 *N*), and potassium permanganate (1.1 g) in water (60 ml) was heated on a water-bath for half an hour with occasional shaking, cooled and acidified with a mixture of conc. sulfuric acid (50 ml) and water (150 ml). Sodium bisulphite was added to dissolve the manganese sludge and the remaining product was dissolved in dilute sodium hydroxide and precipitated with acid y elding the acid (V) (2.8 g), m. p. 202—203° after crystallization from ethanol. The m. p. was undepressed when the compound was mixed with the acid from the nitrile. (Found: C 68.8; H 5.7; OCH_3 33.8; Eqv. weight 376. Calc. for $C_{21}H_{20}O_6$ (368.4): C 68.47; H 5.47; OCH_3 33.7; Eqv. weight 368).

Methyl ester of the above acid. The acid (V) was dissolved in methanol and treated with an excess of diazomethane in ether. The solvent was evaporated and the residual ester crystallized from methanol. M. p. 151.5—152.5°. (Found: OCH_3 40.33. Calc. for $C_{22}H_{22}O_6$ (382.4): OCH_3 40.58).

Oxidation of 6,7-dimethoxy-1-(3',4'-dimethoxyphenyl)-2-naphthoic acid (V) to 1,2,3,4-benzenetetracarboxylic acid. The acid (V) (1 g) was heated with pyridine hydrochloride (5 g) at 180° for 2 hours. The mixture was then poured into dilute hydrochloric acid (200 ml) and continously extracted with ether for 12 hours. On evaporation of the ether layer a light brown product (0.8 g) was obtained. This was immediately dissolved in water (100 ml) containing potassium hydroxide (0.1 g) and finely ground potassium permanganate (9 g) was added. The mixture was heated on a water-bath for 2 hours with mechanical stirring. Excess permanganate was destroyed with ethanol and the solution filtered. The filtrate, acidified with hydrochloric acid, was passed through a cation exchanger (IR 120) which was then washed with hydrochloric acid (4 *N*) and the eluate evaporated to dryness when a solid product (0.4 g) was obtained.

Crystallization from hydrochloric acid (1 : 1) gave a product m. p. 215—225° (decomp.). (Found: Eqv. weight 64.5. Calc. for $C_{10}H_6O_8$ (254): Eqv. weight 63.5).

The substance was dissolved in methanol and treated with an excess of diazomethane in ether. The ester was obtained in almost quantitative yield. M. p. from methanol 126—126.5° undepressed when the compound was mixed with an authentic specimen of tetramethyl-1,2,3,4-benzenetetracarboxylate.

iso-Lariciresinol dimethylether. The dimethylester of the phenyltetralindicarboxylic acid (III)[2] (0.5 g) was reduced with lithium aluminium hydride (0.5 g) following the procedure of Haworth and Wilson [6]. The product was crystallized from methanol. M. p. 175.5—176.5° and 172—175° when mixed with the diol (m. p. 171—172°) below, $[\alpha]_D^{20}$ +23° (*c* 1.172 in chloroform).

For comparison α-conidendrin dimethylether was also reduced and the product crystallized from methanol. M.p. 171—172°, $[\alpha]_D^{20}$ +21° (*c* 1.930 in chloroform) or +29° (*c* 2.552 in acetone). Haworth and Wilson give m. p. 167—168°, $[\alpha]_D^{20}$ + 18° in chloroform.

Diol from β-conidendrin dimethyl ether. A slight modification of the method above was used when the β-conidendrin dimethyl ether was reduced. After decomposing the metal alkoxide it was necessary to extract with hot benzene to get the organic material in solution. The crude product melted at 126—129°. After crystallization from benzene it

melted at 82—84°, solidified if the melt was held at 105—110° for some time, shrank from 132°, and there were some signs of a new solidification before it melted at 140—142°. $[\alpha]_D^{20}$ + 46.2° (*c* 2.33 in chloroform). (Found: C 67.56; H 7.56; OCH_3 32.36. Calc. for $C_{23}H_{32}O_6$ (388.4): C 68.02; H 7.26; OCH_3 31.95.)

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Studies on Humic Acids

V *. The Reaction of p-Benzoquinone with Alkali

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p-Benzoquinone, treated with alkali yields hydroquinone and amorphous "quinone humic acids". The amount of hydroquinone increases with increasing alkalinity. With weak alkali polyhydroquinones are probably formed, *e. g.* $[C_6H_2(OH)_2]_n$ which are oxidised by quinone to partly quinonoid products with formation of hydroquinone (15—30 %). Investigations of the humic acid itself seems to support this theory. With strong alkali over 50 % hydroquinone is formed and the polymeric acid should correspondingly be more highly oxidised. The results obtained with these products were, however, inconsistent.

Many quinones when treated with alkali undergo disproportionation leading to the corresponding hydroquinones and amorphous polymerisation products, "quinone humic acids". The nature of these acids is obscure, but they are obviously related to the "humic acids" formed on alkaline oxidation of the corresponding phenols and, according to Eller [1], also to the natural humic acids obtained from soil, peat and brown coal. Eller suggested that all these products are polymeric hydroxyquinones and one of us (H. E.), that the polymerisation essentially involves the formation of diphenyl linkages [2]. There are numerous examples of the facile formation of diphenyl derivatives from simple phenols and quinones.

In a very simplified manner the polymerisation of *p*-benzoquinone in the absence of other oxidising substances, *e.g.* air, may be discussed in the following way.

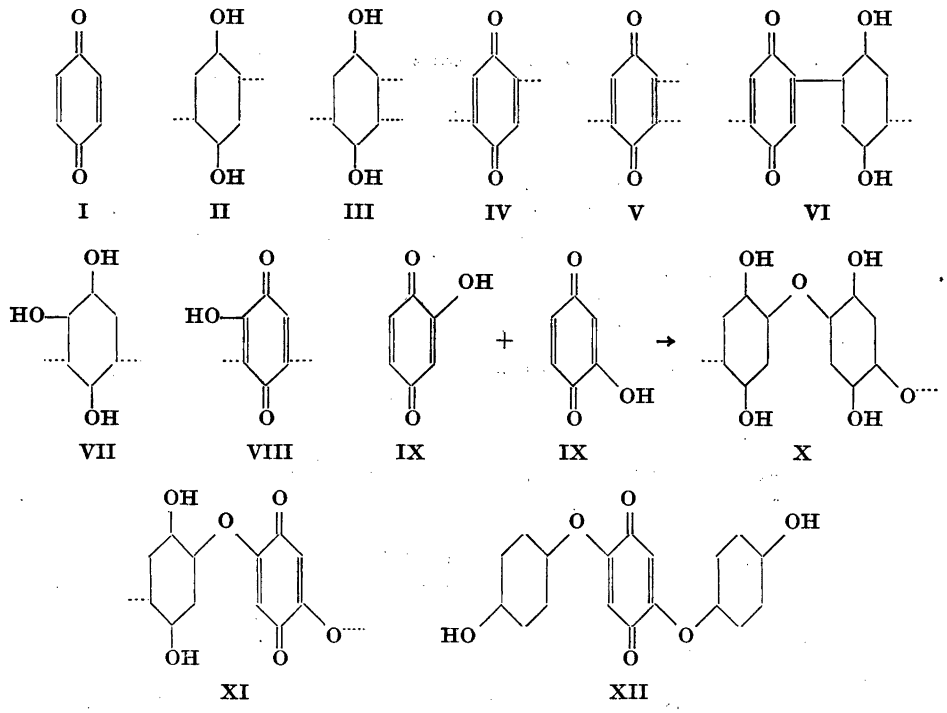
Quinone ($C_6H_4O_2$) (I) may be transformed into a linear polymer II (or more probably into a three dimensional polymer containing elements of type III) without formation of hydroquinone: n $C_6H_4O_2 \rightarrow (C_6H_4O_2)_n$. Dehydrogenation of such polymers to a more or less quinonoid stage by unchanged quinone would require the simultaneous formation of up to 50 % hydroquinone:

$$2n\ C_6H_4O_2 \rightarrow (C_6H_2O_2)_n + n\ C_6H_4(OH)_2.$$ (Polymers IV, V.)

* Continuing the series *Studies on the Formation of Complex Oxidation and Condensation Products of Phenols, Proc. Roy. Soc. (London)*, **A 143** (1933) 177, 191, 223, 228.

All intermediate degrees of oxidation (*e.g.* VI) are possible and yields of hydroquinone varying between 0 and 50 % would therefore be theoretically possible.

Complications due to nuclear hydroxylation may be expected and for the formation of polymer VII, $(C_6H_4O_3)_n$, (which is equivalent to IV as regards degree of oxidation) we have the following balance: $2n\ C_6H_4O_2 + n\ H_2O \rightarrow (C_6H_4O_3)_n + n\ C_6H_4(OH)_2$ requiring the formation of 50 % hydroquinone. Complete oxidation to polymer VIII would increase the quantity of hydroquinone, to a possible maximum yield of about 67 %.



Flaig [3] assumed that the oxidation of hydroquinone in alkali to a humic acid proceeds *via* hydroxyquinone (IX) which is polymerised in a way "resembling the Thiele acetylation" to X. We have no reason to believe that the humic acids produced by oxidation of phenols and decomposition of quinones in alkali have fundamentally different structures. The polymer X has the same composition as polymer VII and if obtained by anaerobic polymerisation of quinone would require the simultaneous formation of about 50 % hydroquinone (if completely oxidised to XI, 60 %). As will be shown below quinone actually may be polymerised by weak alkali with formation of only about 15—20 % hydroquinone and this makes Flaig's mechanism of humic acid formation improbable. Moreover polymer XI would presumably not be capable of existence in alkaline solution but would suffer hydrolysis with formation of

2,5-dihydroxyquinone, a compound which has never been observed as a product of the alkaline decomposition of quinone. Diels and Kassebart's interesting quinone trimeride (XII) obtained from quinone by the action of pyridine is easily hydrolysed by alkali to hydroquinone and 2,5-dihydroxyquinone [4]. The success of this trimerisation is due to the specific participation of pyridine in the process, as recognised by Diels.

It is true that *p*-xyloquinone and thymoquinone on treatment with alkali do not yield typical humic acids but undergo some other type of reaction. Di-, tri- and tetra-quinone, however, yield typical humic acids when treated with alkali (Erdtman, Granath and Schultz, forthcoming publication).

From the above discussion it is evident that the yields of hydroquinone actually obtained on alkaline polymerisation of quinone are of importance for the understanding of the polymerisation process and the structure of the polymers. Consequently we have studied this problem in some detail and some of the results are recorded in Table 1.

Table 1. Per cent hydroquinone formed from quinone on polymerisation with alkali. In all experiments 0.01 mole quinone was added to 10 ml sodium hydroxide solution.

Normality of sodium hydroxide solution	Time of reaction	Per cent hydroquinone
15	120 min	63
10	15 »	52
10	24 hours	51
5	24 »	39
2	15 min	35
1	15 »	28
1	24 hours	25
0.5	15 min	23
0.5	24 hours	21
0.25	24 »	20
0.1	24 »	— *
NaAc	20 days	14 **

* Solution almost neutral. Partial decomposition only.
** Details see below.

As seen from Table 1 the yield of hydroquinone varies greatly. Strongly alkaline media favour the formation of hydroquinone (increase the degree of oxidation of the polymerisation product). The yield of hydroquinone is independent of the amount of alkali used in the experiments, except at low alkali concentrations when the effect of the neutralisation of the alkali by the humic acid is noticeable.

Reaction of quinone with methanolic sodium hydroxide also furnishes hydroquinone, *e.g.* with 2 *N* sodium hydroxide 34 % as compared with 35 % obtained with aqueous sodium hydroxide.

It was of course of interest to see whether the formation of hydroquinone could be depressed below 20 %, (the minimum reached in our experiments with sodium hydroxide) by employing still weaker alkali, and quinone was therefore treated with a saturated sodium acetate solution in the presence of

undissolved sodium acetate. Under these conditions the reaction was slow. A yield of only 14—15 % hydroquinone was obtained and in this case the hydroquinone was contaminated with a little 2,5,2′5′-tetrahydroxydiphenyl (about 1—2 % of the quinone). This observation provides an interesting indication of the probable route of polymerisation.

The results recorded in Table 1 support our assumption that quinone humic acids are formed by nuclear C-C-coupling. In weak alkali (low yields of hydroquinone) the polymeric product appears to contain mainly elements of types II and III with relatively small amounts of structures IV, V, VII and VIII.

In stronger alkali when high yields of hydroquinone are obtained the polymers probably possess a higher degree of oxidation. This may be due to a higher percentage of quinonoid nuclei and/or structures of the types VII and VIII.

As already mentioned, pure phenol ether structures (X, XI) are improbable but diphenyleneoxide groups may well be formed especially after the acidification of the reaction mixtures. The observation that humic acids which have been dried in air or even stored under water for some time exhibit a decreased reactivity may perhaps be explained by such complications.

The amorphous nature of the humic acids and their derivatives is easily understood on the basis of the above considerations. They obviously possess a very complex and irregular structure and the number of possible atropisomerides is very great.

The humic acids dissolve in alkali giving green or brown solutions which absorb oxygen with the greatest of ease yielding deep brown solutions. This phenomenon is particularly pronounced with the "sodium acetate humic acid" and is probably due to the oxidation of the primary polymers to products containing a larger percentage of quinonoid nuclei.

In our studies on the chemistry of the polymeric products we encountered all the hardships inherent in humic acid research. Strictly reproducible results could never be obtained, especially with the products obtained with strong alkali. However, as expected, the product obtained with sodium acetate gave less confusing results, some of which are therefore recorded.

Methylation with dimethyl sulphate and alkali in the absence of oxygen yielded products containing 20—25 % methoxyl increased by further methylation with diazomethane to 24—25 %. (A polymer of type VI with OCH_3 instead of OH requires 25.5 % methoxyl.) The analytical data for hydrogen and carbon give little information. A methylated polymer II requires 70.6 % C and 5.9 % H and methylated VI 69.4 % C and 4.1 % H. Most of our products had 66.2—68 % C and 4.1—4.5 % H.

Similar result were obtained on reductive acetylation with zinc and acetic anhydride. The light coloured products contained 50—51 % acetoxyl increased to 56 % on renewed reductive acetylation. (An acetylated polymer II requires 61.4 % acetoxyl, 62.5 % C and 4.2 % H. A "monoacetate" of II requires 39.3 % acetoxyl, 64.0 % C and 4.0 % H. Found: 62.1—62.6 % C and 4.4—4.7 % H.)

When hydrolysed with alkali in the absence of air, solutions were obtained which absorbed oxygen with the development of a green colour soon changing to brown.

The results obtained with these humic acid preparations are at least qualitatively in harmony with the view that they consist essentially of partly oxidised polymers of the types II and III.

EXPERIMENTAL

Reaction of p-benzoquinone with alkali: Quinone (1.1 g) was very finely powdered and added under hydrogen to oxygen free sodium hydroxide solutions (10 ml) of varying concentrations and the mixture vigorously shaken. After the decomposition of the quinone the solutions were acidified with 2 *N* sulphuric acid allowing no contact with air as long as the solution was alkaline. If necessary, the humic acid suspension was boiled briefly in order to facilitate filtration. The precipitate was carefully washed with 1 % sulphuric acid and the filtrate and washings exhaustively extracted with ether. The light brown ether solution was dried (Na_2SO_4), filtered through a short column of aluminium oxide to remove a small amount of brown impurities and the ether evaporated. The residue was almost pure hydroquinone as shown by the melting point and mixed melting point with an authentic specimen. The experiments were run in duplicate, the results varying within one or two per cent. Compare further Table 1.

Reaction of quinone with sodium acetate: Finely powdered quinone (1.1 g) was mixed with powdered anhydrous sodium acetate (8.2 g) and water (10 ml). The mixture was shaken vigorously under hydrogen and then allowed to stand for twenty days, and worked up as described above. The hydroquinone did not appear to be quite pure and the experiment was run on a larger scale employing 55 g quinone. The hydroquinone fraction was acetylated and the acetate distilled in a vacuum. A residue was obtained which on trituration with methanol crystallized. After recrystallization the product was obtained pure (m. p. 168—169°) and was identified as dihydroquinone tetraacetate by mixed m. p. with an authentic specimen as well as by conversion to diquinone.

Preparation of "sodium acetate humic acid": Quinone (30 g) was treated as in the previous experiment with sodium acetate (250 g) and water (300 ml). The mixture was poured into water (4 l) containing concentrated hydrochloric acid (250 ml). As soon as possible the precipitated humic acid was filtered (no suction!) or centrifuged. The creamy material was suspended in dilute (1 %) hydrochloric acid and centrifuged, the process being repeated several times. Finally, the humic acid was washed once with distilled water. (Repetition causes considerable losses due to peptisation.) The humic acid was partly dehydrated on porous tile and used for further experiments in this state (containing 50—70 % water). When dry the humic acid dissolves slowly even in alkali.

Methylation of "sodium acetate humic acid": Fresh, moist humic acid (about 4 g) was dissolved in methanol (25 ml) and dimethyl sulphate (4 ml) added, followed by the dropwise addition (under hydrogen) of sodium hydroxide solution (4 ml, 40 %), with stirring and external cooling. After the completion of the reaction the mixture was acidified and the precipitate collected and carefully washed. The air-dried product was extracted with light petroleum in order to remove traces of hydroquinone dimethyl ether. Part of the preparation was dried *in vacuo* over phosphorous pentoxide at room temperature and analysed. The bulk of the material was dissolved in dioxan and treated with a large excess of diazomethane in ether. After 24 hours light petroleum was added to precipitate the humic acid which was then dried *in vacuo* over paraffin wax and phosphorous pentoxide.

The methoxyl content of the products obtained by methylation with dimethyl sulphate and alkali varied somewhat. Found: OCH_3, 24.9, 23.4, 20.9, 20.4, 20.6, 22.0, 20.0, 20.3, 20.7 %. Remethylated (CH_2N_2) products (prior to remethylation 20.3, 20.7 % OCH_3) contained 24.7, 24.5 % OCH_3.

Attempts were made to reduce the „sodium acetate humic acids" (prior to acidification) with sodium amalgam (under hydrogen). The colour decreased very considerably but colourless solutions were never obtained. Direct methylation of the reaction mixtures with dimethyl sulphate and alkali yielded alkali insoluble products which were extracted with petroleum ether to remove the hydroquinone dimethyl ether. The insoluble products were lighter coloured than the methylated nonreduced humic acids. Found: OCH_3, 27.2, 27.6 %. This is far less than expected for a methylated polymer II and the result is difficult to explain. Possibly nuclear hydrogenation has occurred.

Samples of "sodium acetate humic acids" which had been stored for three months under water, in the presence of a little toluene to avoid microbial infection, on methylation with dimethyl sulphate and alkali gave products with a decreased methoxyl content (15–16 %).

Reductive acetylations: Fresh, moist "sodium acetate humic acid" (6 g) was suspended in acetic anhydride (300 ml), containing four drops of pyridine. The mixture (cooled externally) was stirred mechanically and zinc dust added until the colour of the resulting solution no longer decreased. The brown solution was filtered with stirring into a large volume of ice cold water. The light yellowish brown precipitate was filtered and washed carefully with water, dried in air and extracted with ether which dissolved only a very small amount of material. Part of the product was dried *in vacuo* over phosphorous pentoxide at room temperature and the residue subjected to two renewed acetylations. Found after the first acetylation 50.5, after the second 55.8 and after the third acetylation 56.1 % acetoxyl. The products were not "homogeneous" and when the product containing 56.1 % acetoxyl was dissolved in chloroform and fractionately precipitated with ether fractions were obtained containing between 48 and 57 % acetoxyl. The acetoxyl content increased with increasing solubility in the solvent mixture. Preparations made from humic acids which had been stored under water for 3–6 months had only 43–46 % acetoxyl content. (One reductive acetylation only.)

A methylated "sodium acetate humic acid" containing 22.8 % OCH_3 was also subjected to reductive acetylation. The product was found to contain 23.0 % acetoxyl and 13.3 % methoxyl a result which is not easy to interpret.

As mentioned in the introduction, humic acids prepared with strong alkali gave inconsistent results. As a rule methylation and reductive acetylation experiments yielded preparations containing less methoxyl and acetoxyl than those obtained from the "sodium acetate humic acid".

The reaction of quinone with 10 *N* NaOH in the presence of methanol yielded a humic acid which after reductive acetylation contained methoxyl (5.5 % OCH_3, 38.5 % $OCOCH_3$) indicating that some addition of methanol had taken place during the formation of the humic acid.

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Low-molecular Carbohydrates in Algae

IV *. Investigation of Pelvetia canaliculata

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The investigation of the brown alga *Fucus vesiculosus*, in which mannitol, l-mannitol β-glucoside, 1,6-mannitol di-(β-glucoside) and l-mannitol acetate were found, was reported in Part I[1] of this series. It was of interest to study other brown algae and in the present paper a similar investigation of *Pelvetia canaliculata* is reported.

This alga contained, in addition to D-mannitol, another sugar alcohol, D-volemitol, in yields of about 1,3 and 0.85 % respectively. Mannitol is quite a common constituent of algae and although volemitol has been isolated from some cryptogams, the mushroom *Lactarius volemus* [2] and lichens of the genera *Dermatocarpon* [3,4] and *Endocarpon* [4], it has not previously been found in algae.

A mixture of monoglucosides of mannitol and volemitol, representing about 0.2 % of the alga, was also isolated. From this mixture, l-mannitol β- glucopyranoside, identical to the specimen from *Fucus vesiculosus*, was obtained in a pure state. Another substance, m.p. 182—184° and specific rotation —17° (Water) on hydrolysis yielded equimolecular amounts of volemitol and glucose. The analysis was consistent with the formula of a volemitol glucoside monohydrate. The substance consumed 7 moles of periodic acid with the formation of 5 moles of formic acid, indicating that the substance is (1 or 7)-D-volemitol-β-D-glucopyranoside. Crystalline preparations of volemitol glucoside, melting gradually between wide limits (*e.g.* 140—160°) but chromatograhpically indistinguishable from the specimen above, were also obtained. Similar results with periodate oxidation suggest a mixture of 1- and 7-volemitol glucosides. A further reason for this assumption is advanced below.

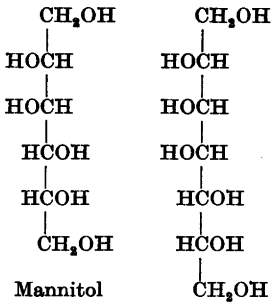
Finally, diglucosides of mannitol and volemitol were obtained. The mannitol diglucoside could not be distinguished chromatographically from that isolated from *Fucus vesiculosus*. The volemitol diglucoside, amorphous, was chromatographically but not chemically pure. On acid hydrolysis, it yielded two moles of glucose and one of volemitol. The values obtained for the consumption of periodic acid and formation of formic acid were inconsistent

* Part III. *Acta Chem. Scand.* 7 (1953) 1218.

with any formula but their ratio (1.56) is rather close to that calculated for the 1,7-volemitol diglucoside (1.6). On periodate oxidation no formaldehyde was formed, and these facts, together with the specific rotation, —13° (Water) are consistent with the formula of 1,7-volemitol di-(β-glucopyranoside).

In all the isolated glucosides, primary hydroxyl groups are glucosidically linked. In mannitol and volemitol the primary hydroxyl groups are very similar, having identical configurations on two adjacent carbon atoms. It is therefore reasonable to assume that they should be almost equivalent in their reactions with enzymes, and that the glucose should be almost statistically distributed between these groups. According to this theory, which is consistent with the yields obtained, both 1- and 7-volemitol monoglucoside should occur in the alga. Although only one of them has been isolated in a state of purity, this is a further argument, in addition to the experimental, for the occurrence of both substances.

In addition to these substances, the presence of sucrose and a small amount of glucose and fructose was demonstrated chromatographically.



EXPERIMENTAL

The alga was collected at Lofoten, Norway, in July and August 1952. The coarsely ground alga (630 g) was extracted in a continuous extractor with ether for 2 days and with methanol for 16 days. The methanol extract was concentrated to dryness under reduced pressure, the residue treated with water, and undissolved material removed by filtration. Lead acetate was added to the aqueous solution, the precipitate filtered and the excess of lead precipitated with hydrogen sulphide. Salts were removed by treatment with the Amberlite resins IR 120 and IR 4B and the solution on concentration to dryness gave a light yellow syrup (19.1 g).

Half of the carbohydrate fraction was dissolved in 1 % aqueous ethanol (100 ml) and added to the top of a carbon-Celite column (35 × 4.5 cm). The column was eluted with the same solvent (3 000 ml) followed by aqueous ethanol (4 000 ml), the concentration of which was increased continuously from 1 to 25 %. The eluate, collected in fractions, was investigated by paper chromatography (Solvents: butanol-ethanol-water, 4 : 1 : 5 and ethyl acetate-acetic acid-water, 3 : 1 : 1), and similar fractions combined and concentrated. The main fractions in the order of their elution were

Mannitol, 3.4 g,
Mannitol and volemitol, about 50 % of each, 1.7 g,
Volemitol, 2.2 g,

Mannitol monoglucoside, 0.1 g,
Mannitol and volemitol monoglucosides, 0.5 g,
Volemitol monoglucoside 0.1 g.
Mixture of several substances, sucrose recognized by R_F-value and colour reactions.
Mannitol diglucoside, 0.03 g,
Mannitol and volemitol diglucosides 0.05 g,
Volemitol diglucoside, 0.03 g.

Sugar alcohols. The mannitol and volemitol, both recrystallised from methanol, melted at 163—164° and 152—153°, undepressed on admixture with authentic samples. They showed positive rotations in borax solutions and thus both belonged to the D-series.

Mannitol monoglucoside. The mannitol monoglucoside was crystallised from aqueous methanol, m.p. 137—139°, undepressed on admixture with the l-mannitol β-glucoside obtained from *Fucus vesiculosus*. The same substance was obtained from the mixture of monoglucosides by chromatography on thick filter paper.

Volemitol monoglucoside. Chromatographically pure preparations of volemitol monoglucoside were obtained by the separation on the carbon column, by separation of the mixed monoglucosides on thick filter paper (Whatman 3 MM, three successive 24 hour developments with ethyl acetate-acetic acid-water solvent) or by spontaneous crystallisation from a solution of mixed glucosides in aqueous ethanol. Some fractions were amorphous, others crystalline but with a varying range in melting points, *e. g.* 140—153°, 155—168° and 182—184°. On the paper chromatograms, the preparations behaved identically. The specimen melting at 182—184° was obtained from the mixed glucosides by spontanous crystallisation and subsequent recrystallisation from aqueous ethanol. The m. p. was unchanged on further recrystallization so it was considered to be a pure substance. $[\alpha]_D^{20}$ −17° (water, $c = 2$). On hydrolysis it yielded glucose and volemitol. The glucose was characterised by paper chromatography and its ready fermentation with baker's yeast. The volemitol was isolated by chromatography on thick filter paper and recrystallised from ethanol. M. p. 151.5—152.5°, undepressed on admixture with authentic volemitol. The ratio of glucose to volemitol, 1 : 1.2 was determined by quantitative paper chromatography [5], and the analysis was consistent with the formula of a volemitol monoglucoside monohydrate. (Found: C 39.5; H 7.24. Calc. for $C_{13}H_{26}O_{12} \cdot H_2O$ (392.3): C 39.8; H 7.19.) On periodate oxidation with 0.1 *M* solution at pH 3.9 and room temperature overnight, the substance consumed 7.1 ± 0.2 mole periodate. By oxidation with sodium metaperiodate under similar conditions, 5.1 ± 0.1 mole of formic acid were formed. A sample, m.p. 140—153°, obtained by separation on thick filter paper, gave similar values.

Mannitol diglucoside. The sample obtained from the separation on the carbon column yielded mannitol and glucose on hydrolysis. On paper chromatography in different solvents (*e. g.* ethyl acetate-acetic acid-water, three successive 24 hours developments) it could not be distinguished from the 1,6-mannitol di-(β-glucoside) from *Fucus vesiculosus*. It was not investigated further.

Volemitol diglucoside. The hydrolysis of the chromatographically pure sample from the carbon column yielded glucose and volemitol in the proportion 2 : 1.1. On periodate oxidation, with conditions as for the monoglucoside, 5.87 mole of periodate were consumed and 3.76 mole of formic acid (but no formaldehyde) were formed. These figures indicate that the substance is not pure, and the specific rotation, −13° (water, $c = 2$) is significant only by its sign and relative magnitude.

SUMMARY

The carbohydrates in the brown alga *Pelvetia canaliculata* have been investigated. The sugar alcohols mannitol and volemitol and mono- and di-β-glucosides of these substances in which glucose is linked to a primary hydroxyl group, have been isolated.

The authors are indebted to Professor N. A. Sörensen, Trondheim, for his interest in this investigation, to *Norsk Institutt for Tang- og Tare-forskning*, Trondheim, for a gene-

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Studies on the Chemistry of Lichens

V *. The Furanoside Structure of Umbilicin

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The D-arabitol D-galactoside, umbilicin, isolated from *Umbilicaria pustulata* as described in part II[1] of this series, was later shown to be a constituent of several lichens of the order Gymnocarpeae (Part IV). The substance has the specific rotation —80° in water, indicating a β-glycosidic structure. The results of the periodate oxidation of umbilicin during which 10 moles of oxidant were consumed, indicate that umbilicin is either 3-D-arabitol β-D-galactopyranoside (I) or a galactofuranoside (II). Furanosides of aldohexoses are rather rare in Nature so we considered the former structure more probable. In order to elucidate the structure, I was synthesised from lactose by degradation to 3-D-arabinose β-D-galactopyranoside according to Zemplén [2] and finally reduction with sodium borohydride. The amorphous substance could be hydrolysed to galactose and arabitol, but the R_F-value was less than that of umbilicin and a furanosidic structure must now be considered. The rate of acid hydrolysis of umbilicin was rapid which is characteristic of furanosides [3]. In the hydrolysate of fully methylated umbilicin the presence of a substance having the same R_F-value and colour reactions as 2,3,5,6-tetra-*O*-methyl-galactose but different from those of 2,3,4,6-tetra-*O*-methyl-galactose was demonstrated.

The former substance was prepared by Haworth, Ruell and Westgarth [4] from the liquid methyl galactoside obtained by cold treatment of galactose with methanolic hydrogen chloride. We used crystalline ethyl β-galactofuranoside [5] as starting material and obtained the galactose tetramethyl ether as a liquid with the specific rotation —32° in water. The somewhat higher value obtained by Haworth *et al.* indicates that their substance contained some galactopyranose tetramethyl ether. Humphreys, Pryde and Waters [6] characterised the galactofuranose tetramethyl ether by transformation into the corresponding galactonic amide. The yield in this series of reactions is rather low however, and adopting the method of Boissonnas [7] for the identification of methylated glucoses, we reduced the furanoside to 2,3,4,6-tetra-*O*-methyl-D-galactitol, and transformed the latter to the diazoate, m.p. 99—100°.

* Part IV, *Acta Chem. Scand.* **7** (1953) 591.

** Svenska Sockerfabriks AB Research Fellow 1950—1953.

Finally, a larger amount of methylated umbilicin was hydrolysed and the galactose tetramethyl ether and the arabitol tetramethyl ether separated by distillation. The galactose tetramethyl ether was reduced to the D-galactitol derivative and azoylated, yielding derivatives identical with authentic specimens. The arabitol tetramethyl ether was obtained as an oil, the azoate of which has not crystallized.

The periodate oxidation of umbilicin, at 50° and pH 3.5, was studied as a function of time in an endeavour to learn more about the structure. The periodate consumption increased continously however, and no turning point was observed. A similar result was obtained when ethyl β-D-galactofuranoside was subjected to periodate oxidation under the same conditions.

EXPERIMENTAL

(All melting points uncorrected.)

3-D-Arabitol β-D-galactopyranoside. A solution of 3-D-arabinose β-D-galactopyranoside [2] (2.5 g) in water (5 ml) was dropped into a solution of sodium borohydride (0.2 g) in water (3 ml). The addition was made over five minutes and after a further fifteen minutes' excess borohydride was destroyed with acetic acid. The reaction mixture was concentrated to dryness and the residue acetylated with acetic anhydride (30 ml) and pyridine (15 ml), poured into water and extracted with chloroform. The chloroform solution was washed with water, dried over calcium chloride and concentrated. The yield of syrupy acetate was almost quantitative. The substance did not crystallise even after innoculation with umbilicin acetate. The product obtained after deacetylation with sodium ethoxide in ethanol was still amorphous and had a lower R_F-value than umbilicin, using butanol-ethanol-water (4 : 1 : 5) as solvent. The presence of arabitol and galactose in the hydrolysate of the substance was demonstrated by paper chromatography.

Hydrolysis of umbilicin. The hydrolysis of umbilicin at 100° in 0.01 *N* hydrochloric acid was followed polarimetrically. The value of $10^5 \times k$ expressed in minutes and Brigg's logarithms was determined to 2 200, a value of the magnitude characteristic for furanosides [3] and about one hundred times greater than expected for a pyranoside.

Ethyl β-galactofuranoside tetramethyl ether. Ethyl β-galactofuranoside (5.0 g) and powdered sodium hydroxide (23 g) were added to anhydrous peroxide-free dioxan (210 ml). Methyl sulphate (27.2 ml in total) was added with vigorous stirring, as the temperature was raised to 70°. The intention was to carry out the methylation as described previously[3], but the temperature was raised too quickly for the present mixture and the solid material melted and formed hard lumps. Water (15 ml) was added and the methylation continued in the liquid two-phase system. The dioxan phase was concentrated and the residue, combined with small amounts of methyl ethers isolated from the aqueous phase, was subjected to a second methylation with methyl sulphate (14 ml) and sodium hydroxide (11.5 g) in dioxan (150 ml). Following methylation, the dioxan solution was filtered off and the filter cake washed with dioxan. The combined solutions were concentrated, the residue dissolved in chloroform (50 ml) and washed with water (10 ml). The chloroform solution was dried over anhydrous magnesium sulphate, filtered and concentrated. The residual oil amounted to 6.21 g, part of which was distilled at 2—3 mm and 120—122°. (Found: C 54.3; H 9.13. Calc. for $C_{12}H_{24}O_6$ (264.3): C 54.3; H 9.17.)

2,3,5,6-Tetra-O-methyl-D-galactose. The crude, methylated ethyl galactoside (4.00 g) was dissolved in 0.2 *N* sulphuric acid (120 ml) and kept at 100° for 22 hours. The solution was neutralised with sodium hydrogen carbonate, concentrated to 30 ml and extracted with chloroform (2 × 10 + 8 × 5 ml). The extract was dried over magnesium sulphate, the solvent distilled off and the residual sirup distilled under reduced pressure. Yield 2.96 g, b.p. 113—114° at 0.35 mm. $[\alpha]_D^{20}$ −32° (Water, c = 2.3). The overall yield from the ethyl galactoside was 81 %. (Found: OCH_3 51.9. Calc. for $C_{10}H_{20}O_6$ (236.3): OCH_3 52.3. Molecular weight, by hypoiodite oxidation [9], 231.)

2,3,5,6-Tetra-O-methyl-D-galactitol. Raney nickel (100 mg) was added to a solution of 2,3,5,6-tetra-*O*-methyl-D-galactose (1.037 g) in water (10 ml) and the pH of the mixture

adjusted to 7.2 with sodium hydroxide. After hydrogenation for 10 hours at 120° and 155 atm. the mixture was filtered. Hypoiodite titration [9] showed that the reduction had reached 96 % of completion. The filtrate was concentrated to a syrup, dissolved in chloroform, filtered through a layer of aluminium oxide and reconcentrated. The residue, a colourless oil (1.00 g) was distilled. Yield, 0.87 g, b.p. 122—124° at 0.25 mm. The distillate crystallised, and recrystallisation from benzene-light petroleum (b.p. 40—50°) yielded hygroscopic needles, m.p. 41—41.5°, $[\alpha]_D^{20}$ —24° (Water, $c = 2$). Tipson and Levene [10] prepared the same substance by similar methods. Their preparation melted at 83—84° and had the specific rotation —26.8° (water). However, not until the conclusion of this work were we able to obtain the substance in this higher melting modification.

*2,3,5,6-Tetra-*O*-methyl-*D*-galactitol 1.4 diazoate.* To the galactitol tetramethyl ether (121 mg) in purified, dry pyridine (4 ml), azoyl chloride (300 mg) was added and the mixture kept at 40° for 12 hours and then at 100° for 24 hours. The reaction mixture was allowed to cool, water (0.5 ml) slowly added and most of the pyridine distilled off under reduced pressure. The residue was dissolved in chloroform, and the azoic acid which separated was filtered off. The chloroform solution was washed twice with 0.5 *N* sulphuric acid, precipitating further amounts of azoic acid which were filtered off, and finally washed with sodium hydrogen carbonate solution. The chloroform was distilled off and the crude azoate (310 mg), dissolved in dry benzene, was added to the top of a column (2.5 × 13 cm) of acid treated aluminium oxide. The column was developed with dry, alcohol-free chloroform (300 ml) giving a wide zone in the middle of the column and two narrow zones near the top. The main zone was extracted with methanol, yielding the crude azoate (255 mg), which was recrystallised from ethanol. M. p. 99—100°. (Found: N 8.30. Calc. for $C_{36}H_{38}O_8N_4$ (654.7): N 8.57).

Methylation of umbilicin. Umbilicin octaacetate [11] (2.49 g) was methylated twice with methyl sulphate (8.8 ml) and sodium hydroxide (8.6 g) in pure dioxan (85 ml), as previously described [8]. The oil obtained on concentrating the dioxan-benzene solution was dissolved in chloroform and washed with a small amount of water, yielding on concentration under reduced pressure a pale yellow oil (1.58 g). A small amount of this substance decomposed on attempted distillation, 160° and 0.01 mm.

*Hydrolysis of octa-*O*-methyl-umbilicin.* Methylated umbilicin (530 mg) was hydrolysed in 0.5 *N* sulphuric acid (10 ml) for 16 hours at 100°. The hydrolysate was neutralised with barium carbonate and filtered through a short column of aluminium oxide. The filtrate was concentrated and the residual syrup partitioned between chloroform (10 ml) and a few drops of water. The syrup obtained on concentrating the chloroform phase (442 mg) on paper chromatograms (Solvent: benzene-light petroleum, 1 : 1, saturated with water. Reagent: aniline hydrogen phtalate) gave only one spot, indistinguishable from that of authentic 2,3,5,6-tetra-*O*-methyl-D-galactose. On distillation of this syrup two fractions were obtained. The first (212 mg) b.p. 80—100° at 0.25 mm was a thin syrup, containing 13 % of galactose tetramethyl ether, determined by hypiodite titration. The main constituent of this fraction must be an arabitol tetramethyl ether. The second fraction (195 mg), b.p. 106° at 0.25 mm, was more viscous and consisted of galactose tetramethyl ether of 99 % purity, determined as above, $[\alpha]_D^{20}$ —32° (Water, $c = 3.8$). The main part of this fraction (189 mg) was hydrogenated as described above, yielding 175 mg of distilled product, b. p. 116—118 at 0.2 mm. Recrystallisation from benzene-light petroleum afforded crystals melting at 40.5—41.5°, but owing to the poor reproducibility of this value, a mixed m.p. determination with authentic material was considered insignificant. Instead, a sample (46 mg) was azoylated as described above, yielding a product (30 mg) melting at 97—99°, undepressed on admixture with 2,3,5,6-tetra-*O*-methyl-D-galactitol 1,4-diazoate.

SUMMARY

The furanoside configuration of umbilicin has been shown by the isolation of 2,3,5,6-tetra-*O*-methyl-D-galactose from the hydrolysate of the fully methylated product. The galactose tetramethyl ether was characterised by reduction to the corresponding D-galactitol derivative and subsequent azoylation.

One of us (B. W.) wishes to thank for a grant from W. Roos' fund.

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Structure of an Acidic Xylan Isolated from Birch Wood Holocellulose

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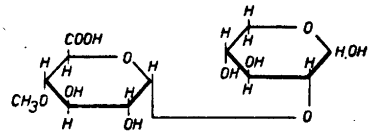
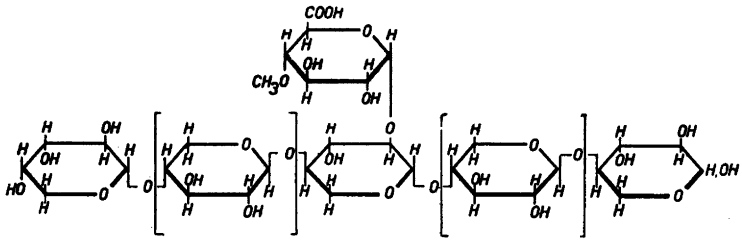
In a previous work Gustafsson *et al.*[1] have proved chromatographically that the carbohydrates from birch wood (*Betula verrucosa*) are built up chiefly from glucose (58 %) and xylose (38 %) and from small amounts of galactose, arabinose, mannose and rhamnose all together about 4 %. It was found that birch wood also contains other compounds similar to carbohydrates, which however, were not identified. To investigate the nature and origin of these compounds holocellulose was prepared from birch wood according to Chanda *et al.*[2]. The holocellulose was extracted with *N* sodium hydroxide and an acidic xylan fraction was isolated from the extract by precipitating with acetic acid. The raw xylan was purified as a copper complex. The purified xylan fraction was hydrolysed, and a paper chromatographic analysis showed that the hydrolysate contained xylose and monomethylaldobiuronic acid only. The monomethylaldobiuronic acid was isolated, after a graded hydrolysis, by elution from a cellulose column. A paper chromatographic study proved that the R_F values and colour reactions of the acid were the same as for 2-*O*-(4-*O*-methyl-α-D-glucuronosido)-D-xylose, which has been isolated by Jones[3] from spruce wood. The methoxyl and carbonyl content was determined and found to be in agreement with the theoretical values. It was thus considered that the aldobiuronic acid is identical with 2-*O*-(4-*O*-methyl-α-D-glucuronosido)-D-xylose I.



The 4-*O*-methylaldobiuronic acid mentioned above has previously been isolated not only from spruce wood but also from aspen wood (*Populus tremuloides*)[4] and from *Eucalyptus regnans*[5]. 4-*O*-methyl-glucuronic acid has been found in several plant gums such as mesquite[6] and myrrh[7] gums.

By oxidation of the xylan fraction in question, with 0.3 *M* sodium periodate according to Chanda [2] it was shown that the periodate uptake was about one mole per $C_5H_8O_4$ unit. The reaction however, was not complete, and one xylose unit per 22 $C_5H_8O_4$ units remained unoxidized. The formic acid release from the periodate oxidation of xylan was determined in principle according to Halsall *et al.* [8], and the yield of formic acid produced was 0.142 moles per $C_5H_8O_4$ unit. A viscometric determination in formamide of the molecular weight according to Husemann [9] ($K_m = 8 \cdot 10^{-3}$) gave a DP value of 24—25 units. Titration of the xylan with sodium hydroxide gave an equivalent weight o 3 140, which corresponds to 24 $C_5H_8O_4$ units. Uronic acid determination according to Whistler *et al.*[10] gave 0.06 moles of CO_2 per $C_5H_8O_4$ unit, and a methoxyl micro determination according to Zeissel [11] 0.06 moles per $C_5H_8O_4$ unit.

From the above consideration it seems justifiable to assign the acidic xylan fraction the following structure II:



The xylan forms a straight chain built up from about 22 xylopyranose units. The xylopyranose units are joined together with 1,4-β linkages. In the main chain there is a single branching point, and the side chain is formed from one 4-methylglucuronic acid residue only, which is linked to the main chain by a 1,2 bond. No conclusions as to the position of the branching point on the main chain can be drawn.

This xylan fraction is only a part of the birch wood hemicelluloses, which probably contain other types of xylans too. From the literature on wood hemicelluloses it is evident that the xylans from plants belonging to different botanical systematic groups vary considerably in respect to their structure and composition. On the other hand it seems probable that *e. g.* all hardwoods contain xylan of the same type as the acidic birch wood xylan.

EXPERIMENTS

Preparation of birch holocellulose. 200 g (dry-matter 89.5 %) of birch wood (*Betula verrucosa*) flour extracted with acetone was suspended in 8 800 ml of water containing 1 000 g of acetic acid (98 %) and 1 000 g of sodium chlorite. The mixture was heated to 60° C and kept at that temperature for one minute. Sodium acetate (40 g) was added, and the reaction mixture was transferred to a thermostat at 30° C and kept there with occasional shaking for 20 hours. The brownish yellow mother liquor was filtered off through a glass filter, and the holocellulose was washed with cold water to remove the salts. After that the water was displaced by acetone. The yield was 77.5 % of the original wood.

	Ash %	Pentosan %[12]	CO_2 %[10]	$-OCH_3$ %[11]	Lignin %[13]
Birch wood	0.33	24.7	0.84	6.8	20.4
Birch holocellulose	0.98	29.7	1.35	3.23	4.0

Isolation and purification of xylan. 100 g (dry-matter 88.9 %) of holocellulose was extracted with *N* sodium hydroxide in a nitrogen atmosphere for 20 hours at room temperature. The mixture was filtered, and the residue was washed with water. The filtrate and the water used for washing were poured together and the pH of the solution was adjusted to 5.5 with acetic acid. Part of the dissolved polysaccharides was thereby precipitated. The precipitate was centrifuged and washed twice with ethanol-water (1 : 1) and then twice with ethanol in order to displace the water. Finally the ethanol was displaced with ether. The precipitate was dried *in vacuo* at 50° C. The yield was 18.5 g (Xyl. I).

10 g (Xyl. I) was dissolved in *N* sodium hydroxide solution and was precipitated with Fehling's solution as a copper complex. The polysaccharide copper complex was filtered off, and the complex was decomposed with 1.5 *N* hydrochloric acid. The undissolved part was centrifuged off, and washed first with ethanol : water : hydrochloric acid (45 : 40 : 10) to remove the copper salts, then with ethanol : water mixtures in with the ethanol content was increased for each washing, and finally with pure ethanol and ether. The precipitate was dissolved in *N* sodium hydroxide and reprecipitated with acetic acid. The precipitate was washed and dried as before. Yield 5.9 g (Xyl. II).

	Ash %	Lignin %[13]	$-OCH_3$ %[11]	CO_2 %[10]	$[\alpha]_D^{20}$ (NaOH)
Xyl. I	—	+	1.84	1.78	−80.0 (c = 0.5)
Xyl. II	0.39	0	1.65	2.01	−77.0 (c = 2.5)

Isolation of 4-methylglucuronosidoxylose. 3 g (Xyl. II) was dissolved in 72 % sulfuric acid and the solution was diluted with water to form a 4 % solution. The solution was boiled for 6 hours and neutralized with barium carbonate. Ba^{++} ions were then removed by an ion-exchange resin (Amberlite IRC 50). The solution was evaporated to dryness, and the residue was extracted with boiling methanol. The clear methanolic solution was concentrated to a yellowish syrup. Examination with different solvents on paper chromatogram such as butanol : ethanol : water (4 : 1 : 5), butanol : pyridine : benzene : water (5 : 3 : 1 : 3) and ethyl acetate : acetic acid : formic acid : water (18 : 3 : 1 : 4), showed, on development with aniline oxalate and *p*-anisidine hydrochloride, the presence of xylose and 4-methylglucuronosidoxylose only. As substances for comparison pure specimens of D-xylose and 4-methylglucuronosidoxylose were used. Aniline oxalate gave an orange spot with 4-methylglucuronosidoxylose, the spot having an intensive orange fluorescence in ultraviolet light.

The 4-methylglucuronosidoxylose was isolated by elution from a cellulose column, ø 22 mm and 340 mm high. Butanol : ethanol : water (2 : 3 : 5) was used as eluant, and the fractions were collected in test tubes by using an automatic fraction collector. The contents of the tubes that indicated only 4-methylglucuronosidoxylose on paper chromatogram were then grouped and the solvent was distilled off *in vacuo*. The residue was light yellow and hygroscopic. Hypoiodite oxidation of the isolated substance calculated as $C_{12}O_{11}H_{20}$ indicated a purity of 97 %. Hydrolysis with *N* sulfuric acid for 6 hours gave only traces of xylose and unhydrolysed 4-methylaldobiuronic acid, as was the case with the substance used for comparison. (Found: OCH_3 8.87. Calc. for $C_{12}O_{11}H_{20}$ (340): OCH_3 9.10.)

Periodate oxidation of xylan. 300 mg (Xyl. II) was suspended in 25 ml of water and treated in the dark with 25 ml of 0.3 *M* sodium metaperiodate at room temperature. Samples were drawn from the solution at intervals. The rotation and amount of periodate consumed (per $C_5H_8O_4$) were determined. The following results were obtained:

Time	20.5 h	90.0 h	164.0 h	228.0 h
$[\alpha]_D^{20}$	64.7°	83.3°	89.7°	85.3°
$\frac{NaJO_4}{C_5H_8O_4}$	0.73	1.11	1.25	1.39

A similar oxidation was also carried out at pH 3.8 in a solution buffered with sodium acetate. The periodate consumed after 91 hours per $C_5H_8O_4$ unit was 1.043 moles of $NaJO_4$.

After oxidation the sodium periodate was destroyed with ethylene glycol. The solution was dialysed against tap water, concentrated to a syrup, and hydrolysed. Analysis by paper chromatography showed the presence of 4.4 % xylose calculated on the original xylan, which means that approximately one xylose unit per 22 $C_5H_8O_4$ units had escaped oxidation.

300 mg (Xyl. II) was suspended in 30 ml of water and 10 ml of 16 % potassium chloride solution and 20 ml 0.3 *M* sodium metaperiodate were added. The oxidation was carried out in the dark at room temperature. Samples were taken at intervals and the remaining sodium periodate was destroyed with ethylene glycol. The formic acid liberated was titrated with 0.01 *N* sodium hydroxide and found to be constant after 120.0 h *i.e.* 0.142 moles per $C_5H_8O_4$ unit.

Equivalent weight by titration. The xylan (Xyl. II) was titrated after dialysis. 100.0 mg (Xyl. II) consumed 3.12 ml of a 0.0102 *N* sodium hydroxide solution, corresponding to an equivalent weight of 3 140.

Viscosity determination. The viscosities of xylan (Xyl. II) in formamide were measured in an Ubbelohde viscometer at 20° C.

C g/100 ml	Solution	Solvent	$\eta sp/C$	DP
0.0958	422.4	414.9	0.19	24
0.1868	430.3	414.9	0.20	25

SUMMARY

An acidic xylan fraction was isolated from birch wood *(Betula verrucosa)* holocellulose. By hydrolysis it gave xylose and 4-*O*-methyl-(2-*O*-glucurono-sido)-D-xylose only. The xylan forms a straight chain of about 22 xylopyranose units. The xylopyranose units are joined together with 1,4-β linkages. In the main chain there is a single branching point, and the side chain is formed from one 4-methyl-glucuronic acid residue only. This has been linked to the main chain by a 1,2 bond.

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The Structure of Liquids

III *. Diatomic Molecules

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When gases consisting of diatomic molecules, like oxygen, nitrogen and chlorine, are condensed to liquids, it might be expected that their molecules persist also in the liquid state and form the basic elements of the structure of the liquids. The experimentally determined atomic distribution curves for oxygen, nitrogen and chlorine seem, however, if they are correct, to indicate a rather complex structure [1–3].

Sharrah and Gingrich have determined atomic distribution curves for liquid oxygen at 62° K and 89° K[1], and the two curves show a marked resemblance. The main difference is a marked flattening of the maxima observed at 89° K compared with the corresponding maxima at 62° K.

In the atomic distribution curve for oxygen at 89° K peaks are observed at:

1.3 2.2 3.4 4.2 5.0 6.0 and 7.0 Å

Sharrah and Gingrich admit that the interpretation of some of these peaks, if real, is somewhat difficult. They suggest as a possible explanation the presence of ozone in the sample, produced by the strong X-ray irradiation of the liquid. As another possibility they mention the occurrence of O_4 molecules. The authors point out that the peak observed at 3.4 Å may have some relation to the distance of 3.48 Å between O_2 molecules in double-molecules, reported by Vegard [4] to be present in the γ-form of crystalline oxygen.

Recently Kanda and Suguwara [5] have shown that ozone is not produced in significant quantities, and as the experimental X-ray intensity curve is determined for a limited range of s values ($s = 4\pi \sin \Theta/\lambda$), an error is introduced which may account for the maximum at 2.2 Å.

The distances between neighbouring peaks in the atomic distribution curve are:

0.9 1.2 0.8 0.8 1.0 and 1.0 Å.

The maxima are therefore approximately periodic, and their mean distance is 0.95 Å. This seems to indicate the presence of a very marked general ripple. If this is the case, a peak should be found at about 0.24 Å. This part of the

* Parts I and II: see reference [7].

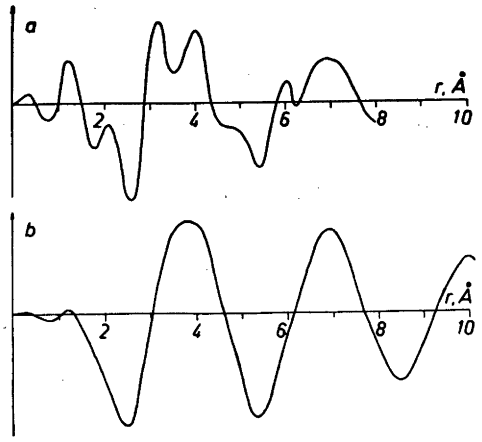

Fig. 1. Liquid oxygen.
a. Atomic distribution curve. $4\pi r^2 (\varrho(r) - \varrho_0)$.
b. Electronic distribution curve. $\sigma(r)$.

curve is unfortunately not given in the publication. A computation of the inner part was therefore undertaken, using the intensity curve published by Sharrah and Gingrich. The result is given in Fig. 1 a, and from this curve it is seen that a maximum is really found between 0.2 and 0.3 Å. A period of the general ripple, $p_r = 0.95$ Å, should correspond to an error in the intensity curve at $s = 2\pi/p_r = 6.6$ Å^{-1} or $\sin\Theta/\lambda = 0.53$. The occurrence of a maximum at 0.2—0.3 Å in the atomic distribution curve indicates that this error should be positive. The intensity curve of Sharrah and Gingrich really possesses a small maximum at about $\sin\Theta/\lambda = 0.53$ Å. It is therefore quite likely that the effect of a relative small positive error here is sufficiently increased to account for the observed general ripple by the introduction of the factor $1/f^2$.

An electronic distribution curve for oxygen at 89° K has been computed from the intensity curve of Sharrah and Gingrich. The result is given in Fig. 1 b.

Both curves of Fig. 1 represent the difference between the actual density of the liquid, and a uniform electronic, respectively atomic density of the sample.

A comparison reveals that curve 1 a must contain a term of period 0.95 Å. The amplitude of this term falls off with increasing r. For very small values of r it also seems to decrease, and the maximum expected at 0.24 Å is shifted a little towards greater r-values. The atomic distribution is computed from the equation [1,6]:

$$4\pi r^2(\varrho(r) - \varrho_0) = \frac{2r}{\pi} \int_0^{s_0} s \cdot i(s) \cdot \sin(rs) \cdot \mathrm{d}s$$

where $i(s)$ is derived from the experimental data, s_0 is the upper limit of the determined intensity range, $\varrho(r)$ is the actual atomic density, and ϱ_0 is the average atomic density. It is readily seen that the general ripple may be caused

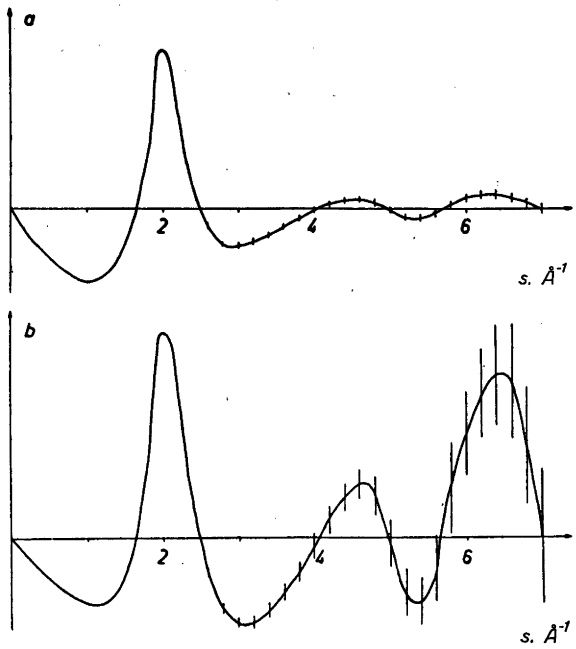

Fig. 2. Intensity curves for liquid oxygen.
a. $s \cdot I_{m+l}$ (s)
b. $s \cdot i$ (s)

by a comparatively broad maximum at about $s = 6.6$ Å^{-1} in the intensity curve. A certain width of this maximum is required to make the amplitude decrease with increasing r. The reduction of the amplitude and shifting of the position of the first maximum to greater values of r are effected by the factor r on the right side of the above equation.

A positive error in the intensity-curve at about $s = 6.6$ Å^{-1} will therefore well account for the general ripple of the atomic distribution curve.

Fig. 2 a represents the term $s.I_{m+l}(s)$ from which the electronic distribution is computed. $I_{m+l}(s)$ is obtained from [7]:

$$I_{m+l}(s) = I_{\text{corr}}(s) - I_B(s)$$

where $I_{\text{corr}}(s)$ is the corrected experimentally determined intensity, and $I_B(s)$ is the sum of the coherent and incoherent scattering from the oxygen atoms.

Fig. 2 b represents $s \cdot i(s)$ from which the atomic distribution is computed. This term is given by:

$$s \cdot i(s) = s \cdot I_{m+l}(s)/f^2$$

where f is the atomic scattering factor.

The vertical lines in the curves indicate the influence of a small, constant error in $I_{m+l}(s)$.

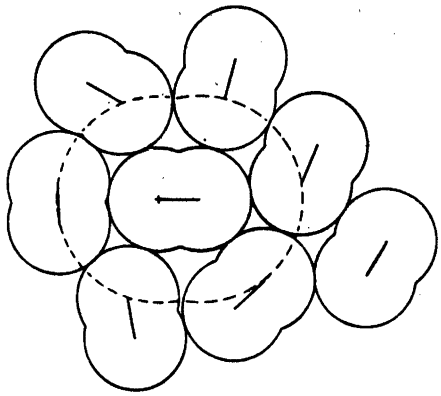

Fig. 3. 2-dimensional packing of "diatomic molecules".

In curve 2 b the magnitude and width of the maximum at $s = 6.6$ Å^{-1} are very great. It is readily seen that a small experimental error may greatly influence the computation of the atomic distribution curve.

It is most likely that a strong general ripple, deforming the atomic distribution curve, has made an interpretation of the structure rather problematic.

The electronic distribution curve 1 b is very simple; it has only three maxima in the interval of 0—8 Å. The first one is a quite small and sharp peak at 1.23 Å. The next maximum has a great width, from about 3.0 to 4.5 Å, and the top is unusually flattened. The third peak is located at about 6.9 Å.

The position of the first maximum corresponds well to the spectroscopically observed distance between the atoms in the oxygen molecule, 1.22 Å.

From X-ray crystallographic data it is known that the distance between oxygen atoms in direct contact but not bounded together, is about 3.1—3.2 Å. The peak between 3.0 and 4.5 Å is therefore, as indicated in Fig. 3, due to distances between the nearest neighbours of oxygen molecules.

The third peak, at 6.9 Å, probably represents the second peak caused by packing of the oxygen molecules. Assuming the ratio between the distances to the first and second neighbours in the packing of oxygen molecules to be the same as in molten metals and liquid inert gases, between 1.8 and 1.9, the position of the first peak caused by the packing should be 3.6—3.8 Å, when the second one is found at 6.9 Å. The actual position of the second and greatest peak in the distribution curve is at about 3.8 Å.

The diatomic oxygen molecules may then, as a first rather rough approximation, be considered as spheres. It should therefore be necessary to consider the properties of the systems formed when solid spheres are packed together, before a more detailed discussion of the structure of liquid oxygen is attempted.

The atomic and electronic distribution curves for liquid nitrogen shown in Fig. 4 have been computed from an experimental intensity curve given by Sharrah [2]. The atomic distribution curve, Fig. 4 a, seems to contain a term of period *ca.* 1 Å. The electronic distribution curve has only three maxima.

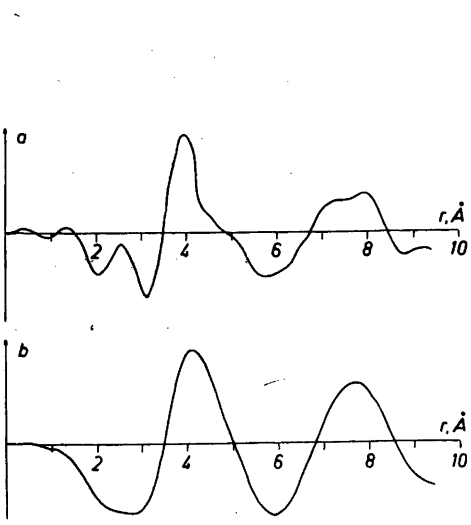

Fig. 4. Liquid nitrogen.
a. Atomic distribution curve. $4\pi r^2 (\varrho(r)-\varrho_0)$.
b. Electronic distribution curve. $\sigma(r)$.

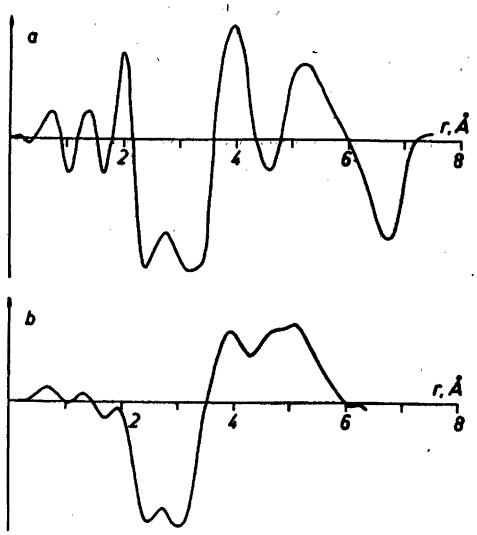

Fig. 5. Liquid chlorine.
a. Atomic distribution curve. $4\pi r^2 (\varrho(r)-\varrho_0)$.
b. Electronic distribution curve. $\sigma(r)$.

The first maximum between 1.0 and 1.54 Å is very small, and probably represents the intramolecular distance, while the two larger maxima at 4.1 and 7.7 Å represent the first and second packing distances as previously seen by oxygen. The ratio of the distances is 1.88, corresponding to a packing of spheres.

The distribution curves for liquid chlorine shown in Fig. 5 have been computed from an experimental intensity curve given by Gamertsfelder [3]. Both curves contain ripple terms and no attempt of interpretation has been made.

From the three above examples it is seen that the existing experimental X-ray scattering data for liquid diatomic molecules are quite unsatisfactory for determination of correct atomic distribution curves. This seems to be caused in part by the limited s-range and in part by insufficient accuracy of the data at even moderate s-values. In most cases, however, it is possible to compute an electronic distribution curve, the interpretation of which is rather simple.

SUMMARY

The two preceding papers of this series deal with systematic errors in the distribution curves of monoatomic liquids. In this paper the distribution curves of diatomic molecules are considered, using liquid oxygen, nitrogen, and chlorine as examples. It is shown how the atomic distribution curves emphasize the systematic errors compared with the electronic distribution curves.

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Potentiometric and Spectrophotometric Studies on 8-Quinolinol and Its Derivatives. X. Solubility of Some Metal Chelates of 8-Quinolinol-5-Sulphonic Acid in Aqueous Solution

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In the preceding paper of the present authors [1] the stability of some metal chelates of 8-quinolinol-5-sulphonic acid in aqueous solutions was investigated. The results demonstrated that the sulphonic acid group has practically no effect on the stability. On the other hand, it is well known that the metal chelates of this reagent are more soluble than those of 8-quinolinol [2]. The solubility of metal chelates of 8-quinolinol-5-sulphonic acid, however, seem not to have been determined before.

As a dibasic acid, 8-quinolinol-5-sulphonic acid (H_2A) has two types of sparingly soluble metal chelates: $M(HA)_2$ and MA as was observed by Claus and Posselt [3] long ago. We have observed, however, that although it is obviously possible to precipitate $M(HA)_2$ between pH's 4—8 this requires an excess of reagent, so rendering the potentiometric method useless for the determination of the solubility product. Therefore we have investigated only chelates of the type MA, except in the case of barium and strontium where the conditions required were attainable.

RESULTS

The potentiometric method used has been recently described by Ekman and Näsänen [4]. Equilibrium was frequently reached after one day but in some cases a very great tendency to supersaturation existed and precipitation occasionally only began after one week. In this respect, cobalt chelate, espesially, offered difficulties. In connection with cobalt chelate a slow darkening of the solution was observed when sodium hydroxide was added in excess. The maximum at about 360 mμ was shifted towards longer wavelengths and the maximum extinction increased considerably. Generally the measurements are made after 1—2 weeks' standing.

In some cases the precipitate was so soluble that the potentiometric method gave very inaccurate results. This was especially true in the case of copper

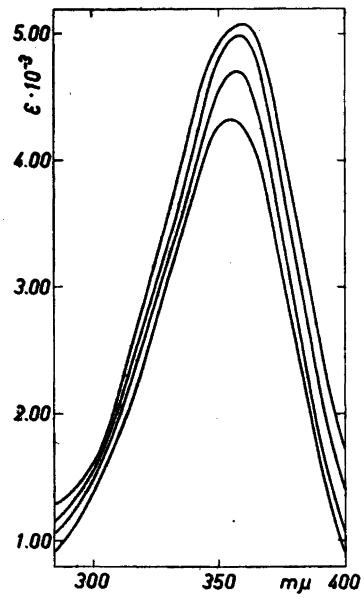

Fig. 1. Molar extinction coefficients of 8-quinolinol-5-sulphonic acid chelates of the alkaline earth metals. From the highest curve downwards: $c_{BaCl_2} = 0.0999$. *pH = 10.5.* $c_{SrCl_2} = 0.0998$. *pH = 10.6.* $c_{CaCl_2} = 0.0945$. *pH = 10.5.* $c_{MgCl_2} = 0.0929$. *pH = 9.6.* $c = 1.48 \cdot 10^{-4}$.

chelate. Therefore we also used a spectrophotometric method for checking. In these measurements the metal ion concentration and pH used were so high that, according to the values of the stability constants, practically all the reagent was in the form MA, and sufficient reagent was added to produce precipitation. When equilibrium was reached the ultraviolet spectrum of the saturated solution was measured. If dilution for extinction measurement was necessary, this was made with a solution of the metal salt in question in such a manner that the metal ion concentration remained constant. The pH of the saturated solution was checked and the effect of addition of sodium hydroxide studied. Thus determination of the concentration of MA was possible if the molar extinction coefficient was known. These were determined in mixed solutions of metal salt and reagent. The pH and the concentration of metal ion were so high that practically all the reagent was present as the chelate MA. The concentration of the reagent was so low that no precipitation occurred.

In Fig. 1 we see the molar extinction coefficients of the alkaline earth metal chelates between 290—400 mμ and in Figs. 2 and 3 those of the other metal chelates investigated. When the concentration of MA in saturated solution is known, we obtain the solubility product from the equation

$$S_2 = [M^{++}]\ [A^{=}] = [MA] / k_1 \qquad (1)$$

where the values of the stability constants k_1 may be obtained from the preceding paper of the present authors [1].

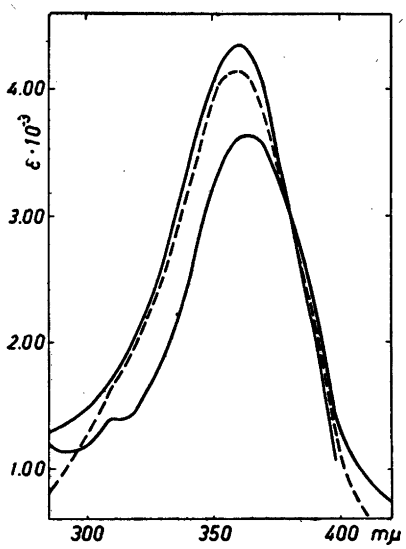

Fig. 2. Molar extinction coefficients of 8-quinolinol-5-sulphonic acid chelates of some divalent metals. Upper full line: $c_{Co(NO_3)_2}$ = *0.00975. pH = 7.3. Lower full line:* $c_{Cu(ClO_4)_2}$ = *0.0095. Broken line:* c_{NiCl_2} = *0.00974. pH = 7.4.*

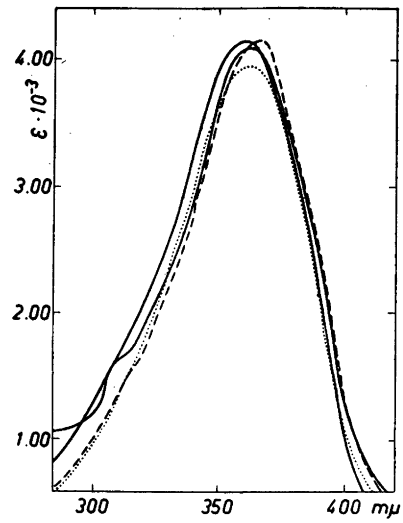

Fig. 3. Molar extinction coefficient of 8-quinolinol-5-sulphonic acid chelates of some divalent metals. Upper full line: c_{MnCl_2} = *0.01. pH = 8.2. Lower full line:* $c_{Cd(ClO_4)_2}$ = *0.00958. pH = 8.0. Broken line:* $c_{Pb(NO_3)_2}$ = *0.00990. pH = 7.5. Dotted line:* $c_{Zn(NO_3)_2}$ = *0.00922. pH = 7.2.*

The analysis of the precipitates obtained gave the formula $M(HA)_2$ or MA without water of hydration. The chelates investigated were of the colours (without drying):

$Ba(HA)_2$	white	CdA	yellowish
BaA	yellowish	ZnA	»
$Sr(HA)_2$	white	CoA	brownish yellow
SrA	yellow	NiA	greenish
CaA	»	CuaA	»
MgA	yellowish	PbA	yellowish
MnA	»		

The potentiometric results are presented in Table 1, where c_M is the stoichiometric concentration of the metal ion, c that of the reagent and c_B that of the sodium hydroxide added. The electrolyte added is signified after the symbol of the chelate.

Table 1. Potentiometric determination of the solubility product of some metal chelates of 8-quinolinol-5-sulphonic acid at 25° C. $S_1 = [M^{++}]\,[HA^-]^2$. $S_2 = [M^{++}]\,[A^=]$.

$Ba(HA)_2$.	pH	$\sqrt{I}$	pS	$Sr(HA)_2$.	pH	$\sqrt{I}$	pS
KCl.	3.71	0.44	6.26	KCl.	3.98	0.44	5.69
$c = 1.17 \cdot 10^{-3}$	3.74	0.50	6.17	$c = 1.17 \cdot 10^{-3}$	3.99	0.49	5.65
$c_B = 5.86 \cdot 10^{-3}$	3.76	0.66	6.12	$c_B = 5.86 \cdot 10^{-3}$	4.02	0.74	5.58
$c_M = 6.24 \cdot 10^{-3}$.	3.82	0.86	6.02	$c_M = 6.24 \cdot 10^{-3}$.	4.08	0.96	5.51
	3.86	1.05	5.98		4.10	1.16	5.52
	3.84	1.29	6.11		4.08	1.38	5.66
BaA.	7.96	0.14	5.59	SrA.	6.97	0.14	6.62
KCl.	8.46	0.14	5.60	KCl.	7.57	0.14	6.61
$c = 2.49 - 2.67 \cdot 10^{-3}$	8.47	0.46	5.06	$c = 2.49 - 2.67 \cdot 10^{-3}$.	7.48	0.30	6.19
$c_B = 3.56 - 4.68 \cdot 10^{-3}$	8.65	0.70	4.76	$c_B = 3.56 - 4.42 \cdot 10^{-3}$	7.74	0.71	5.73
$c_M = 5.94 - 11.22 \cdot 10^{-3}$.	8.65	0.75	4.74	$c_M = 6.96 - 11.21 \cdot 10^{-3}$.	7.91	1.10	5.48
					8.02	1.56	5.31
CaA.	6.51	0.14	7.09	MgA.	7.11	0.43	6.58
KCl.	7.12	0.34	6.53	KCl.	6.52	0.69	6.22
$c = 2.49 - 2.67 \cdot 10^{-3}$	7.34	0.53	6.21	$c = 2.50 - 31.3 \cdot 10^{-3}$	6.17	0.89	6.07
$c_B = 3.56 - 4.42 \cdot 10^{-3}$	7.60	0.84	5.85	$c_B = 3.74 - 46.9 \cdot 10^{-3}$	5.92	1.19	5.79
$c_M = 5.93 - 11.06 \cdot 10^{-3}$	7.64	1.25	5.74	$c_M = 4.63 - 58.1 \cdot 10^{-3}$.	5.51	1.52	5.76
MnA.	4.78	0.33	8.75	CdA.	3.41	0.19	10.90
$MnCl_2$.	4.59	0.70	8.20	$NaNO_3$.	3.77	0.37	10.36
$c = 2.68 \cdot 10^{-3}$				$c = 2.74 - 2.80 \cdot 10^{-3}$.	3.45	0.50	10.24
$c_B = 5.10 \cdot 10^{-3}$.				$c_B = 3.75 - 4.94 \cdot 10^{-3}$.	3.48	0.70	10.09
$c_M = 0.036 - 0.167$.				$c_M = 1.21 - 2.39 \cdot 10^{-3}$.	3.49	0.94	10.01
ZnA.				CoA.			
$NaNO_3$.	3.50	3.48	10.89	$Co(NO_3)_2$.	3.91	0.523	10.52
$c = 1.67 \cdot 10^{-3}$.	3.68	3.68	10.83	$c = 2.90 \cdot 10^{-3}$	3.85	0.710	10.34
$c_B = 3.56 \cdot 10^{-3}$.	3.86	3.86	10.27	$c_B = 5.50 \cdot 10^{-3}$.			
$c_M = 5.60 \cdot 10^{-3}$.	3.87	3.87	10.08	$c_M = 0.169$.			
	3.92	3.92	9.92				
NiA.	3.26	0.14	11.76	PbA.	2.41	0.13	13.58
KCl.	3.62	0.45	10.97	$NaNO_3$.	2.46	0.29	13.18
$c = 2.49 \cdot 10^{-3}$	3.65	0.71	10.81	$c = 2.61 \cdot 10^{-3}$.	2.51	0.40	12.83
$c_B = 4.42 \cdot 10^{-3}$.	3.67	1.06	10.73	$c_B = 0.00$.	2.59	0.81	12.30
$c_M = 1.09 \cdot 10^{-2}$.	3.58	1.58	10.90	$c_M = 5.82 \cdot 10^{-3}$.	2.62	1.16	12.11

The spectrophotometric results are presented in Table 2. In those cases where the agreement with the potentiometric results was good only one experiment was made. In the case of MnA and CoA the agreement was inferior. In the case of CuA the potentiometric method was useless. Therefore in those cases several measurements were made.

DISCUSSION

The maxima of the spectra of MA in the case of the alkaline earth metals seem to change regularly from about 354 mμ for MgA towards longer wavelengths, being 356 mμ for CaA, 358 mμ for SrA and 359 mμ for BaA. The maximum value of the molar extinction coefficient increases in the same order. For the other chelates the maximum occurs at about 262 mμ for Cd, Co and

Table 2. Spectrophotometric determination of the solubility products of some metal chelates of 8-quinolinol-5-sulphonic acid at 25° C. $S_2 = [M^{++}]\,[A^=]$.

	$c \cdot 10^3$	c_M	$[MA] \cdot 10^3$	$\sqrt{I}$	pS_2
BaA	2.23	0.151	0.217	0.67	4.81
SrA	2.23	0.151	0.077	0.67	5.73
CaA	2.23	0.143	0.194	0.66	6.00
MgA	2.28	0.169	3.81	0.72	6.11
MnA	2.68	0.0363	1.25	0.33	8.90
»	2.68	0.0363	1.27	0.33	8.90
»	2.68	0.167	2.12	0.70	8.30
»	23.1	0.231	2.55	0.83	8.20
CdA	2.68	0.0348	0.168	0.32	10.60
PbA	2.68	0.0359	0.0074	0.33	12.80
ZnA	2.68	0.0335	1.06	0.32	10.49
CoA	2.90	0.092	2.51	0.52	10.40
»	2.90	0.169	2.69	0.71	10.32
»	3.85	0.233	3.38	0.84	10.25
»	7.16	0.288	3.56	0.95	10.27
NiA	2.38	0.185	8.00	0.75	10.86
CuA	2.68	0.0345	1.45	0.32	13.50
»	2.68	0.0950	1.69	0.53	13.17
»	2.68	0.159	2.14	0.68	13.00
»	2.68	0.220	2.19	0.81	12.98

Zn and at about 265 mμ for Ni and Cu. The maximum value of the molar extinction coefficient increases, however, in the order Cu, Zn, Cd, Pb, Mn, Ni, Co.

The results of Table 1 can be represented by the equation

$$pS_1 = pS_{1,0} - \frac{3.05\sqrt{I}}{1 + a\sqrt{I}} + BI \qquad (2)$$

or

$$pS_2 = pS_{2,0} - \frac{4.07\sqrt{I}}{1 + a\sqrt{I}} + BI \qquad (3)$$

Using the reasonable value $a = 1.5$, we have calculated the parameters by the method of least squares. These data are presented in Table 3. The values for copper, cobalt and manganese chelates are obtained from spectrophotometric measurements.

Table 3. The thermodynamic solubility products of 8-quinolinol-5-sulphonic acid chelates of some divalent metals at 25° C.

	$pS_{1,0}$	B	$pS_{2,0}$	B	Added electrolyte
Ba	6.99	0.26	6.09	0.151	KCl
Sr	6.45	0.30	7.10	0.068	»
Ca	—	—	7.47	− 0.035	»
Mg	—	—	7.60	—	»
Mn	—	—	9.79	− 0.14	$MnCl_2$
Cd	—	—	11.39	0.21	$NaNO_3$
Zn	—	—	11.30	0.25	»
Co	—	—	11.46	0.44	$Co(NO_3)_2$
Ni	—	—	12.10	0.28	KCl
Cu	—	—	14.34	0.15	$Cu(ClO_4)_2$
Pb	—	—	13.96	− 0.13	$NaNO_3$

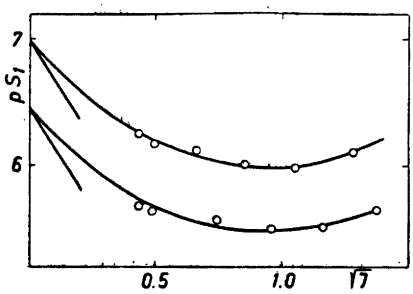

Fig. 4. Solubility products $[Ba^{++}]\,[HA^-]^2$ *(upper curve) and* $[Sr^{++}]\,[HA^-]^2$ *(lower curve) as functions of ionic strength.*

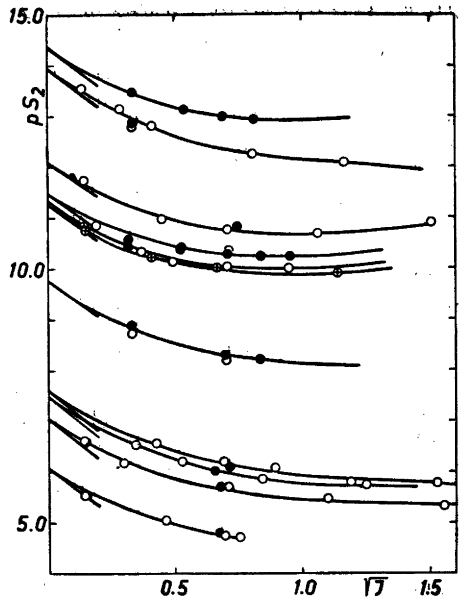

Fig. 5. Solubility products ($[M^{++}]\,[A^{=}]$) *as functions of ionic strength. From the top:* BaA, SrA, CaA, MgA, MnA, ZnA, CdA, CoA, NiA, PbA, CuA. *Spectrophotometric:* ●. *Potentiometric:* ○.

In Figs. 4 and 5 the solubility products are represented as functions of ionic strength. We see from Fig. 5 that the potentiometric and spectrophotometric results agree relatively well. The smaller differences may in part be due to the different composition of the medium.

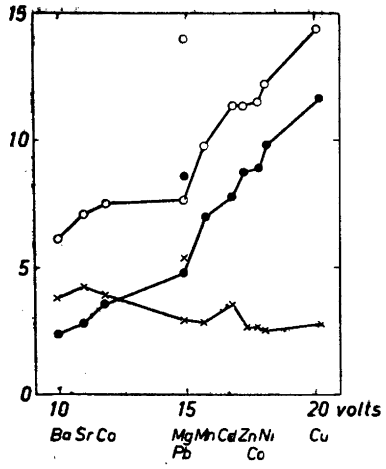

Fig. 6. Ionization potential of gaseous metal atom and $pS_{2.0}$ (○), $\log k_{1.0}$ (●), *and log* $[MA]_0$ (×).

In Fig. 6 log $k_{1,0}$, $pS_{2,0}$ and log [MA] ($I = 0$) are represented as functions of the second ionization potential of gaseous metal atoms. As is seen from the figure, and still better from Table 2, the solubility [MA] of different metal chelates varies considerably and does not seem to be a simple function of the ionization potential. Therefore in this case the solubility product is not quite parallel with the stability constant.

Some of these chelates are recommended for the gravimetric analysis of metals in question [5]. Although our analyses show definite composition for these compounds (at about 170° C they seem to lose all water of hydration) they have hardly any importance in this respect because of their slow and frequently incomplete precipitation [5]. In the latter respect, only lead chelate is a total exception as may be seen from Table 2. It must also be observed that in all our measurements an excess of metal salt has been present.

SUMMARY

The solubility products of certain 8-quinolinol-5-sulphonic acid chelates of some divalent metals have been determined. Also the concentration of the chelate molecule in saturated solution has been determined spectrophotometrically. The ultraviolet spectra of the chelates have been determined. The solubility products decrease in the order: Ba, Sr, Ca, Mg, Mn, Cd, Zn, Co, Ni, Cu, but the concentration of the neutral molecule in saturated solution does not follow the same order.

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Oxidation of Toluene with Dilute Nitric Acid at High Temperature and Pressure

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It was shown already by Fittig [1] that benzoic acid was formed by boiling toluene with nitric acid. Konowalow [2] used dilute acid at 125—130° C and Sachsse [3] carried out the oxidation at 120—150° C. Coblenz and Walker [4] maintained toluene in contact with nitric acid of about 40 % strength at about 100° C for several days and Seydel [5] carried out the reaction at 110° C and 5 kg/cm² in the presence of catalysts. Askenasy, Elöd and Trogus [6] studied this reaction at high temperatures under oxygen pressure and obtained a 65—70 % yield of benzoic acid. The yields are otherwise poor in spite of long reaction times and very much by-products are formed.

This paper summarizes some results, obtained during research work performed in order to find new ways of oxidizing aromatic hydrocarbons and make this process adaptable to industrial manufacture [7].

The oxidation of toluene with dilute nitric acid is a rather slow process at ordinary pressure, and temperatures around 100° C. The reaction is, however, very fast at higher temperatures and pressures. It was shown that temperatures around 200° C and pressures around 30—50 kg/cm² should be used in order to obtain a good yield of a comparatively pure product. Under such conditions, the conversion is complete in a few minutes and the process may be run with a very good capacity. The concentration of the nitric acid is preferably kept within the limits 10—50 %. The reaction is difficult to start if the concentration is lower than 10 %, and it might be difficult to control if the concentration is higher than 50 %. Even at concentrations of 10—50 % certain steps must, however, be taken in order to control the reaction. A suitable charging of the reaction vessel by adding the nitric acid or both the reactants continuously is necessary. If the acid is added continuously to the organic compound during the course of the reaction, a low concentration of the nitric acid in the reaction mixture will obviously be maintained all the time, even if a comparatively concentrated acid is added. Under these circumstances no excess of acid can arise in the reaction mixture until possibly when the last portion of acid is added. The process has also been developed to a continuous one by charging acid and toluene with high pressure pumps

and continuously discharging *via* a regulating device in timed relation to the addition of new starting material. In order to control the reaction, it is necessary to let out the gases formed, continuously or intermittently, if the pressure should show a tendency to rise too high.

A very important problem connected with this process is to restrict the formation of nitrated by-products. The nitration rate increases with increasing concentration of nitric acid in the reaction mixture. It is, however, possible to charge comparatively concentrated acid if this is made continuously as described above, keeping the concentration in the reaction mixture at a low level and avoiding excess of acid. The nitric acid supplied should be consumed by the oxidation reaction as rapidly as possibly. Both temperature and pressure must be kept at a high level, because this means an increase in the rapidity of the oxidation reaction, which is much more important than the increase in the rapidity of the nitration. An increase in pressure especially was shown to depress the amount of nitrated by-products in the crude benzoic acid. When oxidizing toluene along these lines, it was possible to obtain a yield of 85—90 % of a crude benzoic acid, containing about 10 % of nitrobenzoic acids and about 1 % of picric acid. When the nitric acid is in this way consumed very quickly and is present in less than the equivalent amount and at low concentration during the main part of the reaction period, the corrosive action is very much reduced.

The nitrobenzoic acid mixtures were found to contain mainly the *p*-isomer. The acids could be separated by fractional distillation of the crude acid mixture or of the esters. The nitro compounds could also be removed quantitatively by reduction with sulfur dioxide in alkaline solution [10].

There are, however, further compounds formed as by-products and intermediate products in this process. From crude benzoic acid made in a pilot plant by continuous oxidation of toluene with 33 % nitric acid at 200° C and 30 kg/cm² there were isolated about 0.5 % of mononitro toluenes, primarily the *o*-isomer and about 0.1 % of benzaldehyde. It has also been possible to isolate phenylnitromethane from the crude acid. This compound was prepared by Konowalow [2] by reacting toluene with nitric acid at 100—105° C for 24 hours. It is also known that by heating at 225° C this compound is decomposed, giving benzaldehyde, water and nitrogen oxides. Actually, when the above crude benzoic acid was heated at 230° C for 2 hours, benzaldehyde distilled in a yield of approximately 1 % of the weight of the acid and some nitrogen dioxide was formed. It was also found that phenylnitromethane could be oxidized to benzoic acid in good yield, using dilute nitric acid at high temperature and pressure.

Askenasy, Elöd and Trogus [6] considered phenylnitromethane to be an intermediate step in the oxidation of toluene to benzoic acid with nitric acid although they did not isolate this compound from their reaction products. As phenylnitromethane has now been isolated from the reaction product and has been oxidized to benzoic acid, it seems likely that it is an intermediate in this process. The *o*- and *p*-nitrobenzoic acids are doubtless formed by oxidation of the corresponding mononitrotoluenes, which are formed primarily at the beginning of the experiments when the nitric acid concentration is comparatively high. *p*-Nitrotoluene gives an almost quantitative yield in such condi-

tions, whereas *o*-nitrotoluene is oxidized to a mixture of *o*-nitrobenzoic acid, and picric acid [7]. *o*-Nitrotoluene is oxidized comparatively slowly and it is formed in larger amount than the *p*-isomer when toluene is nitrated. This explains the fact that mainly *o*-nitrotoluene was found in the crude benzoic acid and very little of the *p*-isomer. Some *m*-nitrobenzoic acid is, of course, also formed by oxidation of *m*-nitrotoluene. This nitrotoluene is, however, formed in very small amounts and most of the *m*-nitrobenzoic acid present has evidently been formed by nitration of benzoic acid with dilute nitric acid.

Ethyl benzene was also oxidized in the same way, giving crude benzoic acid in 75—80 % yield at 170—190° C and 30—40 kg/cm. Finally *isopropylbenzene* was oxidized to crude benzoic acid.

EXPERIMENTAL

Apparatus and experimental procedure. The oxidations were performed in a stainless steel autoclave according to a general procedure reported in a previous paper [8]. The yields reported above do not include small quantities of benzoic acid, dissolved in the mother liquor and washing water. Most of the experiments were batch-wise but benzoic acid was also made continuously in the following way: The autoclave was charged with a small quantity of toluene, about 10 % of the autoclave volume, together with the equivalent amount of nitric acid. This first charge was then heated under stirring until the reaction started. Then more of the components were charged. When the autoclave had been filled to the discharge level, a discharge pipe was opened and the same quantity of reaction product was automatically discharged per time unit as was supplied in the form of starting materials.

Picric acid. This contaminant was determined by precipitation with copper sulphate solution in ammonia or colorimetrically at smaller concentrations. The picric acid could be removed from the crude benzoic acid by leaching with water.

Nitrobenzoic acids. These compounds were determined polarographically in aniline solution on samples free from picric acid or with correction for picric acid according to a method developed in this laboratory [9]. Different methods for separation of the nitrobenzoic acids from the benzoic acid have been investigated. Sublimation and crystallisation from different solvents did not give complete separation, nor did reduction with iron and hydrochloric acid. Reduction with SO_2 in alkaline solution was, however, found to be a very efficient method for removing the nitrobenzoic acids completely [10]. Another way to remove the nitrobenzoic acids, which also made it possible to isolate them, was fractionation under vacuum. When a crude acid, containing 15 % of nitrobenzoic acid, was distilled in a short laboratory column at 100 mm Hg, there was obtained an 80 % yield of distillate in the form of white benzoic acid with less than 0.01 % of nitrobenzoic acid and a distillation residue, of mainly *p*-nitrobenzoic acid. (Melting point 235—236° C, equ.w. 165.7.) When such distillations were made on a technical scale, however, the residue contained a little more benzoic acid. Attempts were made to isolate the nitrobenzoic acids from such a residue by crystallization from ethyl alcohol and by esterification. From the mixture of ethyl esters the *p*-isomer could be isolated in a rather pure form by fractionation.

Benzaldehyde and nitrotoluenes. By the vacuum fractionation of 450 kg of crude benzoic acid, made in a pilot plant by continuous oxidation of toluene with 33 % nitric acid at 30 kg/cm² and 200° C, there were obtained 60 kg as a first fraction. This fraction was redistilled and gave 14.6 kg of a fraction, boiling up to 185° C at 100 mm Hg. The temperature then remained constant and only benzoic acid distilled. The product, boiling up to 185° C was oily and had a red to brown color. One kg was redistilled in a laboratory column with 3 plates at 10—12 mm Hg and five fractions were cut. They were analysed for benzaldehyde by sodium bisulphite and iodine. In addition to this the benzaldehyde was precipitated with phenylhydrazine and identified by the melting point as benzaldehydphenylhydrazone after two crystallisations from ethyl alcohol. The nitrotoluenes in fraction 2 were determined as amines after reduction of the corresponding

fraction with Zn and HCl. The amines were fractionated and *o*- and *p*-toluidines were identified through their boiling points and the melting points of their hydrochlorides. Fraction 3 was redistilled after removing acid compounds with alkali and then investigated in a polarograph. The boiling point and the polarographic data indicated, that *o*-nitrotoluene was the main product. Table 1 shows the result of this destillation:

Table 1.

Fraction No.	Boiling point, °C, at 11 mm Hg	Percent by weight	Composition
1	60—68	3	Mainly benzaldehyde
2	68—96	7	Benzaldehyde 10 %, *o*-nitrotoluene about 70 %, *p*-nitrotoluene about 10 %
3	96—131	11	Mainly *o*-nitrotoluene
4	131—135	25	Benzoic acid, somewhat contaminated with nitrotoluenes
5	135	45	Benzoic acid (m.p. 122.5—123.5 Eq.wt. 122.7)
Residue		2	
Losses		7	

Phenylnitromethane. The above mentioned crude benzoic acid, made continuously in a pilot plant, was extracted with toluene, the toluene was evaporated and the residue was dissolved in diethyl ether and treated with saturated sodium bicarbonate to remove strong acids and then with saturated Na_2CO_3 and 2 *N* NaOH. The solution in NaOH was acidified, and the oily product which then separated was distilled at 21 mm Hg. The main part of the oil boiled at 108—118° C and had the following data:

$n_D^{20} = 1.5323$, $D_4^{20} = 1.1587$ (Phenylnitromethane boiling point at 4 mm Hg 118—119° C, $n_D^{20} = 1.5323$, $D_0^{20} = 1.1598$). The oil was investigated polarographically, which also indicated that it mainly consisted of phenylnitromethane.

According to a procedure described by Black and Babers [11] phenylnitromethane was made in a fairly good yield by reacting benzyl cyanide with methyl nitrate to phenylnitroacetonitrile, converting this to phenylnitroacetic acid with sodium hydroxide and treating the said acid with concentrated hydrochloric acid. The phenylnitromethane boiled at 97—99° C (5 mm Hg) and had n_D^{20} 1.5327 and D_0^{20} 1.1607. It was oxidized with 15 % HNO_3 in a 20 % equivalent excess at 180—200° C and 20—25 kg/cm² according to the general procedure described above. From 34 grams of phenylnitromethane there was formed 28 grams of a product with the following analyses: Eq.wt. 132, N (Dumas) 2.36 %. This was dissolved in ammonia and purified by reduction with SO_2. After precipitating and washing there was obtained a white product with the following analyses: Eq.wt. 121.0, melting point 123° C, nitrobenzoic acid about 2 % (polarographically). Investigation in ultra-violet spectrophotometer showed that the product was mainly benzoic acid.

Oxidation of toluene. Continuous oxidation of toluene to benzoic acid was carried out with 15 % nitric acid at 175—190°. In one case the reaction was run with a toluene excess of 30 %, 3.5 % nitric acid being discharged and the final product containing 11 % nitrobenzoic acid as by-product. The corrosive effect on the autoclave, which was made of stainless steel (45 % Cr, 5 % Ni), was only 0.2—0.3 g/m²/h. If the same reaction was carried out with a nitric acid excess of 60 %, the concentration of the discharged nitric acid amounted to 10 %, the content of nitrobenzoic acid in the crude reaction product was 21 % and the corrosive effect was greater than in the preceding example. Yields of more than 80 % have been obtained in such oxidation experiments.

Oxidation of ethyl benzene. Ethyl benzene ($D_4^{20} = 0.867$, $n_D^{20} = 1.4944$) was oxidized with a 30 % surplus of nitric acid. The result may be seen from Table 2.

Table 2.

Nitric acid concentration %	Temperature °C	Pressure kg/cm²	Crude product			
			Yield %	Eq.wt.	Melting point °C	Nitrobenzoic acid %
15	166	21	62	131.6	116—117	12
15	156	36	69	123.9	119—120	7
15	168	41	80	123.7	118—119	6
15	168	42	80	128.4	115—116	11
15	191	40	69	122.2	118.5—119.5	7
30	150	32	79	129.1	115—117	11
30	162	36	74	125.9	115—117	10

The products were identified in ultra-violet spectrophotometer as mainly benzoic acid.

Oxidation of isopropyl benzene. This oxidation was made with nitric acid of 15 % concentration in a 20 % excess at 185—195 °C and 35 kg/cm². From 60 g of the hydrocarbon there was obtained 45 g of a compound with equiv. weight 127 and N (Dumas) 1.20 %. This was purified by reduction with SO_2 and a 75 % yield was obtained of a white product, which was identified in ultra-violet spectrophotometer as mainly benzoic acid.

SUMMARY

Toluene was oxidized with dilute nitric acid at 30—50 kg/cm² and 170—200°C to give crude benzoic acid in a yield of more than 80 %. The optimal conditions for this oxidation were studied. Nitrobenzoic acids, mainly the *p*-isomer, were formed as by-products together with small amounts of picric acid. Furthermore it was possible to isolate benzaldehyde, nitrotoluenes and phenylnitromethane in crude benzoic acid from continuous oxidation. Phenylnitromethane was oxidized to benzoic acid with dilute nitric acid at 170—200° C under pressure.

Ethyl benzene and *iso*propyl benzene were oxidized in the same way as toluene giving crude benzoic acid in good yields.

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The Distribution of Compounds Formed in the Reaction between Ethylene Oxide and Water, Ethanol, Ethylene Glycol, or Ethylene Glycol Monoethyl Ether *

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Addition of ethylene oxide to compounds containing an active hydrogen atom leads to a series of consecutive reactions. A theory is developed for the distribution of the compounds formed. The assumption is made that all steps except the first one are kinetically identical. It is shown that the distribution is determined by the proportion between the starting materials and by the ratio of the identical rates of the higher steps to the rate of the first one. The theory is tested by experiment and also by recalculation of previously published data. The agreement is satisfactory.

Compounds containing an active hydrogen atom react with ethylene oxide to give a series of consecutive, irreversible, competitive reactions, in which a reactive hydroxyl group is formed in every step. With ethanol, for instance, the reactions are:

$$C_2H_5OH + CH_2CH_2O \longrightarrow C_2H_5OCH_2CH_2OH$$

$$C_2H_5OCH_2CH_2OH + CH_2CH_2O \longrightarrow C_2H_5OCH_2CH_2OCH_2CH_2OH \text{ } etc.$$

From both theoretical and technical points of view it is important to know the distribution of compounds obtained under various conditions. Before this problem is considered, a few words must be said concerning the molecular mechanism of the reaction. Ingold [1] recently gave a survey of this question. It seems to be agreed that the reaction is a nucleophilic substitution by means of the reactive group on a carbon atom of ethylene oxide or its conjugate acid.

* Part of this work was presented at the XIIIth International Congress of Pure and Applied Chemistry, Stockholm 1953.

Under acid or neutral conditions the nucleophilic reagent in the example cited above is the undissociated alcohol, but under alkaline conditions the reagent is the corresponding base, *i.e.* the alcoholate ion. The various mechanisms will affect the distribution of the products formed. The simple, uncatalysed reaction will first be considered, and the treatment will then be modified for the catalysed reactions.

Flory [2] in 1940 calculated the distribution of products in the reaction of ethylene oxide with a compound with a reactive group. He made the assumption, that all steps in the reaction sequence are kinetically identical and showed that a Poisson distribution results.

Natta *et al.* [3–5] considered the distribution of products in a series of consecutive competitive reactions where the various steps proceed with different rates. He arrived at formulae in which the concentrations of the various products were obtained as functions of the concentration of the remaining, unchanged starting material. In principle the same formulae were derived as early as 1906 by Abel [6] who considered the stepwise saponification of esters of polyvalent alcohols. Martin and Fuchs [7] obtained the same formulae for the case of the stepwise chlorination of alkanes.

All of these authors, except Flory, derived their expressions assuming second order kinetics and a constant volume throughout the reaction. Fuoss [8] discussed four consecutive reactions and adopted a method using reaction probabilities for the derivation of the distribution of compounds. His results were identical with those obtained by the kinetic method, as pointed out by Potter and Macdonald [9].

The assumption of Flory that all steps are kinetically identical is, in most cases, an over-simplification. On the other hand the formulae of Natta are very cumbersome, so it seemed justified to find a reasonable approximation. As is seen from the reaction scheme the reactive hydroxyl group is bound to an oxyethylene group in every step except the first one. It therefore seemed chemically justified to assume that all steps except the first one proceed with equal rate.

In the technical manufacture of glycol products, ethylene oxide is generally added continuously to the well-mixed, liquid batch, and this is also the practice in laboratory experiments. For the derivation of equations it was therefore considered justified, not to use the common kinetic arguments, but rather a probability method similar to that used by Fuoss. It is assumed that the mixing is ideal, so that concentration gradients do not occur. We first derive general equations and then introduce the appropriate simplifications. The following notation is used:

N_i		molecule with i added ethylene oxide molecules
m		number of moles of ethylene oxide
n_{00}		» » » » starting material
n_i		» » » » N_i in the reaction product
k_i		velocity constant for the reaction of N_i with ethylene oxide
c_i	$= k_i/k_0$	named distribution constant as proposed by Natta [5]
v	$= m/n_{00}$	average number of moles of ethylene oxide per mole of starting material

If the hydroxyl groups of all the reacting species are equally reactive, the reaction probability of each species is equal to its mole fraction. If dm moles of ethylene oxide react, the amount reacting with species N_i thus equals $\mathrm{d}m \frac{n_i}{\Sigma n_j}$. If the reactivities are not equal, the reaction probabilities are proportional to the velocity constants. We then get, for the amount reacting with species N_i, the analogous expression $\mathrm{d}m \frac{k_i n_i}{\Sigma k_j n_j}$. We thus obtain the following system of differential equations:

$$\frac{\mathrm{d}n_0}{\mathrm{d}m} = -\frac{k_0 n_0}{\Sigma k_j n_j} \tag{1}$$

$$\frac{\mathrm{d}n_i}{\mathrm{d}m} = \frac{k_{i-1} n_{i-1}}{\Sigma k_j n_j} - \frac{k_i n_i}{\Sigma k_j n_j} \tag{2}$$

$$\Sigma n_j = n_{00} \tag{3}$$

$$v = \frac{m}{n_{00}} \tag{4}$$

Equation (1) gives the decrease of the starting material and equation (2) the increase of the compounds formed. Equation (3) expresses the fact that the number of reacting molecules is constant throughout the reaction. Equation (4) defines v. Dividing equation (2) by equation (1):

$$\frac{\mathrm{d}n_i}{\mathrm{d}n_0} = \frac{c_i n_i - c_{i-1} n_{i-1}}{n_0} \tag{5}$$

This can be evaluated to the recursion formula (6) which gives n_i as a function of n_{i-1} and n_0:

$$n_i = c_{i-1} n_0^{c_i} \int_{n_0}^{n_{00}} n_{i-1} n_0^{-c_i - 1} \mathrm{d}n_0 \tag{6}$$

Solution of this integral leads to different results dependent upon the values of the distribution constants c_i. If all reactive groups are equally reactive, all c_i are equal to unity. Using equation (4) we obtain Flory's formula:

$$\frac{n_i}{n_{00}} = e^{-v} \frac{v^i}{i!} \tag{7}$$

If all reactive groups have different reactivities, all c_i are different and we obtain a formula equivalent to that of Natta:

$$\frac{n_i}{n_{00}} = (-1)^i \prod_{j=1}^{i-1} c_j \sum_{j=0}^{i} \frac{1}{\prod_{\substack{k=0 \\ k \neq j}}^{i} (c_j - c_k)} \left(\frac{n_0}{n_{00}}\right)^{c_j} \tag{8}$$

Finally, if we assume that all hydroxyl groups bound to an oxyethylene group have identical reactivity, which differs from that of the reactive group of the starting material, all distribution constants are equal (= c) but different from unity. Keeping in mind that $\Sigma c_i n_i$ then equals $n_0 + c(n_{00}-n_0)$ we obtain, by integrating equation (1) and using equation (6), the following expressions:

$$v = c\ln\frac{n_{00}}{n_0} - (c-1)\left(1-\frac{n_0}{n_{00}}\right) \tag{9}$$

$$\frac{n_i}{n_{00}} = \frac{c^{i-1}}{(c-1)^i}\left\{\frac{n_0}{n_{00}} - \left(\frac{n_0}{n_{00}}\right)^c \sum_{j=0}^{i-1}\frac{1}{j!}\left[(c-1)\ln\frac{n_{00}}{n_0}\right]^j\right\} \tag{10}$$

Equation (9) gives the interdependence of n_0/n_{00}, c, and v. It is seen, that it is not possible to obtain n_0/n_{00} as an explicit function of v and c. Equation (10) gives n_i/n_{00} as a function of the concentration of the unchanged starting material, n_0/n_{00}, with c as a parameter. These formulae are somewhat complicated to use, but much easier to handle than formula (8). Their usefulness will be tested in the experimental part.

During the preparation of this paper a preliminary communication by Gold [10] came to our notice. This author presents, in a different form, the same formulae as our equations (9) and (10), but no derivation is given.

Till now we have assumed uncatalysed conditions. In the case of acid catalysis exactly the same reasoning may be applied leading to identical equations. Generally the values of the distribution constants c_i change, as the reactivities of the reactive groups towards the conjugate acid of ethylene oxide may be different from those towards the neutral oxide.

In the case of alkaline catalysis the equations must be modified. It is assumed that we may disregard the reaction of the undissociated molecules in comparison with the corresponding anions. In that case we can apply the same reasoning as above to the anions and get:

$$\frac{dn_i}{dm} = \frac{k_{i-1}^- n_{i-1}^-}{\Sigma k_j^- n_j^-} - \frac{k_i^- n_i^-}{\Sigma k_j^- n_j^-} = \frac{c_{i-1}^- n_{i-1}^-}{\Sigma c_j^- n_j^-} - \frac{c_i^- n_i^-}{\Sigma c_j^- n_j^-} \tag{11}$$

where n_i^- is the number of moles of N_i^-, the anion corresponding to N_i and $c_i^- = k_i^-/k_0^-$. The velocity constants k_i^- refer to the reactions of the anions with ethylene oxide. In order to determine n_i^- we study the protolytic equilibrium of the reactive molecules, which may be put thus:

$$N_i \rightleftharpoons N_i^- + H^+$$

If the equilibrium constant is K_i, the concentration of H^+ is h, and the concentration of the catalyst is a, we have:

$$\frac{n_i^- \cdot h}{n_i - n_i^-} = K_i \tag{12}$$

$$\Sigma n_j^- = a \tag{13}$$

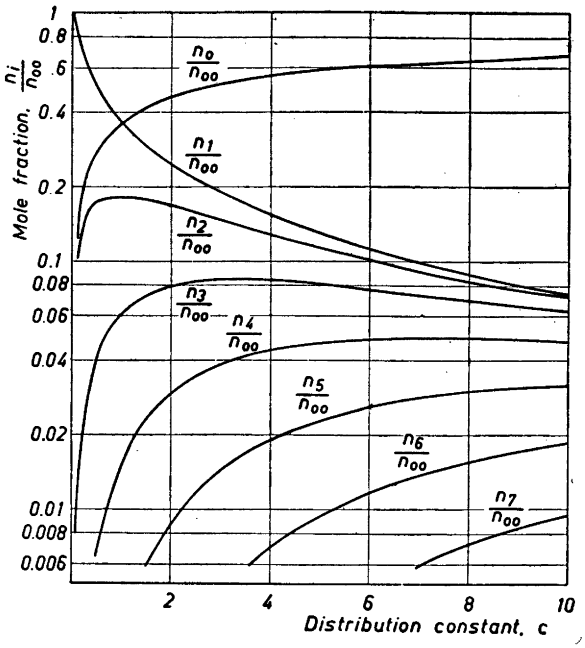

Fig. 1. Distribution of compounds formed by the addition of one mole of ethylene oxide to one mole of reactive substance; n_i/n_{00} = mole fraction of compound with i moles of ethylene oxide added; c = distribution constant.

n_i^- may be neglected in comparison with n_i and we get:

$$n_i^- = a \frac{K_i n_i}{\Sigma K_j n_j} = a \frac{c_i' n_i}{\Sigma c_j' n}, \text{ where } c_i' = K_i/K_0 \qquad (14)$$

When substituting the value of n_i^- from (14) in (11), this equation takes the same form as equation (2) and may be solved in the same way. We obtain solutions corresponding to equations, (8) to (10), where the products $c_i^- c_i'$ are substituted for the constants c_i. Here c_i^- is a measure of the reactivity of the anions towards ethylene oxide and c_i' a measure of the acidity of the hydroxyl groups in comparison with the corresponding properties of the starting material.

Thus it is seen that equations of the form (8) to (10) account for the distribution of compounds formed in the reaction of ethylene oxide with substances with an active hydrogen atom, with or without acid or alkaline catalysts. Fig. 1 gives a graphical representation of the equations (9) and (10) for various values of c with $v = 1$, *i.e.* equimolecular amounts of starting material and ethylene oxide.

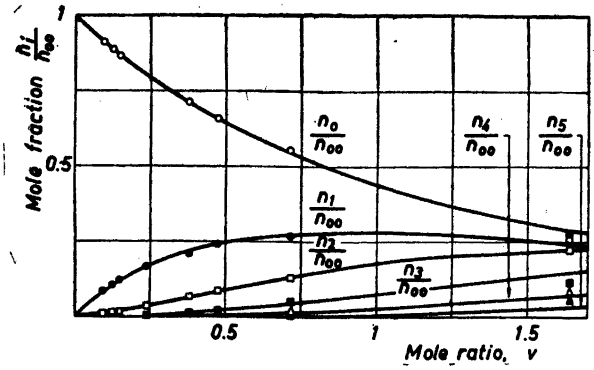

Fig. 2. Distribution of compounds formed by the addition of ethylene oxide to water with sulphuric acid as catalyst; n_i/n_{00} has the same meaning as in Fig. 1; v = mole ratio of reactants, ethylene oxide / water.

EXPERIMENTAL VERIFICATION

Previous work

Only a few papers have been published concerning the reaction of ethylene oxide with reactive molecules, where the reaction product was analysed with sufficient accuracy to be of value for the testing of the formulae given above.

Matignon, Moureu, and Dodé [11] studied the hydration of ethylene oxide with sulphuric acid as catalyst. They analysed the reaction product by distillation and the composition of the fractions by measuring their refractive indices. The percentages of ethylene oxide used up for the formation of the various compounds were reported. The amount of unchanged starting material (*i. e.* water) was not determined, but can be calculated by difference. In Fig. 2 the points correspond to the experimental data, corrected for losses during the analyses (5 %). The curves are calculated with equations (9) and (10) on the assumption that $c = 1.66$. The curves fit the points fairly well.

Recently the hydration of ethylene oxide was experimentally studied also by Davis, von Waaden, and Kurata [12]. As their paper reached us only when our paper was ready for publication, comparison between our formulae and their results is not made. However, qualitative agreement at least seems to exist between their work and ours.

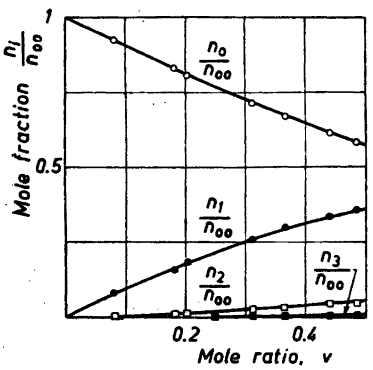

Fig. 3. Distribution of compounds formed by the addition of ethylene oxide to methanol; n_i/n_{00} and v have the same meaning as in previous Figures.

Natta and Simonetta [4] gave results for the reaction between ethylene oxide and methanol or monomethyl ethers of ethylene glycol and diethylene glycol. The reactions were carried out in an autoclave at various temperatures without added catalysts. The analyses of the products were made as in the work by Matignon, Moureu, and Dodé but no details were given. In the case of the glycol ethers most of the ethylene oxide used remained unreacted, so the yields of the reaction products were low and seem uncertain. These results are therefore not used to test our equations. The data concerning the reaction between methanol and ethylene oxide are treated as above and the results are plotted in Fig. 3. The effect of the temperature variations has not been considered. The theoretical curves are calculated on the assumption that $c = 0.55$. The agreement between experiment and theory seems good in this case too.

The reaction between ethylene oxide and phenol with an alkaline catalyst was studied by Miller, Bann, and Thrower [13] and by Patat, Cremer, and Bobleter [14]. Both groups of investigators found that the monophenyl ether of ethylene glycol is exclusively formed before the ethylene oxide reacts with the glycol hydroxyl group. Then a mixture of the monophenyl ethers of polyoxyethylene glycols results which, according to the former group of authors, has a distribution of the Poisson-series type. In the papers mentioned it is assumed that the preferential formation of the first member of the product series is caused by phenol being more acidic than the various glycol ethers. The latter may all be considered equally acid and therefore the distribution constants c'_i in equation (14) are equal and very small, thus giving also to the constant c of equations (9) and (10) a low value. It is easily seen from the equations and also from Fig. 1 that, for $v = 1$ and c approaching 0, n_0/n_{00} also approaches 0 and n_1/n_{00} approaches unity. The phenyl ether of glycol is thus preferentially formed and acts as starting material when further amounts of ethylene oxide are added. The compounds formed are distributed according to a Poisson series as their reactivities are equal to that of the glycol ether.

Present work

Procedure. Experiments were carried out on the reaction between ethylene oxide on the one hand and water, ethylene glycol (henceforth simply named glycol), ethanol, or monoethylether of ethylene glycol (henceforth ethylglycol) on the other. With water, experiments were made without catalyst and also with oxalic acid, carbonic acid, sodium carbonate, or sodium hydroxide as catalyst. In the experiments with the alcohols the corresponding sodium alcoholates were the catalysts. The experiments with water without catalyst and with carbonic acid as catalyst were carried out in a 2 litre autoclave equipped with stirrer and kept at the desired temperature by means of circulating oil. The ethylene oxide was charged in portions from a steel container and introduced into the autoclave by heating the container in a water bath. The ethylene oxide could not be charged in one portion as this would lead to excessive pressure and temperature. All the other experiments were carried out in a glass apparatus at atmospheric pressure. The ethylene oxide was evaporated from a glass vessel and introduced at the bottom of the reaction vessel by means of a glass filter. The liquid material was stirred by the gas bubbles and kept at constant temperature by means of a regulated bath. The reaction vessel was also equipped with a reflux condenser, cooled to $-15°$ C to prevent evaporation. The amount of starting material was accurately weighed at the beginning of the experiment and the amount of ethylene oxide reacted was determined as the increase in weight, at the end of the experiment. The experimental errors in these experiments were smaller than those of the autoclave runs.

Equimolecular amounts of the reaction components ($v = 1$) were used in most cases, but in some cases this led to such large amounts of the higher products that it was found impossible to separate the components. In these cases a lower proportion of ethylene oxide was used. The analysis of the reaction product was carried out by distillation. Each fraction contained at most two components. Their proportions were calculated from the hydroxyl content, determined by acetylation, and in some cases also from water analyses according to Fischer, and ethylene glycol analyses by periodate oxidation. Where equimolecular amounts of the reaction components were used the fractionations were made directly on a 40 ml sample of the product in a spinning band column according

Table 1. Reaction between ethylene oxide and various substances. Experimental conditions, and experimental and theoretical distribution of compounds in reaction product.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Expt. No.	Substance		Ethylene oxide, g	Catalyst		Temp. °C	Mole ratio v	a)	n_0/n_{00}	n_1/n_{00}	n_2/n_{00}	n_3/n_{00}	n_4/n_{00}	n_5/n_{00}	$\sum \frac{n_i}{n_{00}}$	$\sum \frac{in_i}{vn_{00}}$	c'	c''	c
	Nature	g		Nature	g														
1	Water	100.00	244.75	Oxalic acid	0.30	85	1.001	E	0.460	0.262	0.163	0.075	0.028	0.016	1.003	0.990	1.95	1.85	
								T	0.456	0.257	0.169	0.079	0.028	0.008					1.9
2	»	1 000.0	223	CO_2	b)	105	0.0912	E	0.915	0.074	0.008	0.001			0.998	1.009	1.66	3.0	
								T	0.918	0.074	0.008	0.001							2.5
3	»	571.0	279.5	—	—	100	0.2002	E	0.839	0.132	0.026	0.003			1.000	0.964	2.69	2.2	
								T	0.837	0.131	0.028	0.004							2.5
4	»	1 000.0	245.85	Na_2CO_3	2.65	85	0.1006	E	0.921	0.066	0.013	0.003			1.003	0.998	6.63	4.4	
								T	0.919	0.065	0.014	0.002							5.5
5	»	1 000.2	241.3	NaOH	1.00	85c)	0.0986	E	0.931	0.041	0.016	0.005	0.002		0.997	1.015	12.0	15.5	
								T	0.932	0.044	0.017	0.005	0.001						13
6	Glycol	150.20	106.20	Na	0.30	85	0.996	E	0.375	0.341	0.185	0.097			0.998	1.005	1.04	1.14	
								T	0.369	0.368	0.183	0.061							1
7	Ethanol	114.5	108.8	»	1.0	65	0.994	E	0.476	0.234	0.146	0.076	0.032	0.026	0.991	1.019	2.15	2.19	
								T	0.479	0.234	0.162	0.081	0.031	0.010					2.2
8	»	115.1	108.6	»	1.0	65	0.987	E	0.485	0.225	0.166	0.072	0.056		1.003	1.006	2.27	2.33	
								T	0.481	0.234	0.161	0.080	0.031						2.2
9	Ethylglycol	141.23	69.65	»	0.75	65	1.009	E	0.372	0.362	0.187	0.088			1.010	0.991	1.06	1.03	
								T	0.365	0.368	0.186	0.062							1

a) E = experimental values; T = theoretical values calculated with v of column 8 and c of column 20.
b) The pressure of the carbon dioxide was 1 atm.
c) During the first part of the experiment the temp. was 65° C.

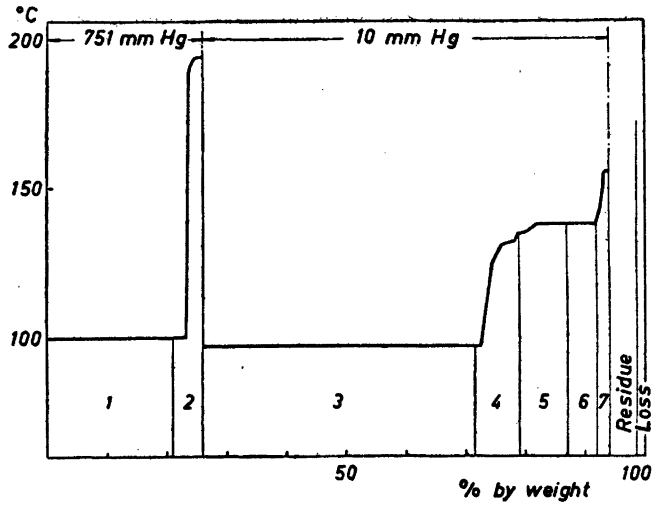

Fig. 4. Distillation curve for product obtained from reaction between ethylene oxide and water with sodium carbonate as catalyst, Experiment No. 4.

to Björkman and Olavi [15]. This column has about 25 theoretical plates. Where a lower proportion of ethylene oxide was used too much of the starting material remained unreacted to give accurate data for the components. In these cases most of the unchanged starting material was first distilled off in a Podbielniak "Hyper-Cal" distillation apparatus with about 50 theoretical plates and a take-off of 10 % of the total reflux. A sample of 400—500 g was used. The distillation was interrupted when the temperature in the distillation vessel was *ca.* 25° C above that of the starting material. The amount of distillate

Table 2. Analytical distillation of 43.59 g of product from the reaction between ethylene oxide and water, Experiment No. 4, Fig. 4.

Fraction No.	Amount g	% OH	Composition		Summarised composition	
			Amount, g	Compound	Compound	Amount, g
1	9.2218		9.2218	Water	Water	10.0396
2	2.1407	33.87	0.8178	»	Glycol	22.8498
			1.3229	Glycol	Diglycol	7.8040
3	19.9146		19.9146	»	Triglycol	2.2748
4	3.2664	42.60	1.5133	»		
			1.7531	Diglycol [a]		
5	3.2638	32.75	0.0990	Glycol		
			3.1648	Diglycol		
6	2.1980	32.08	2.1980	»		
7	1.0229	28.98	0.6881	»		
			0.3348	Triglycol [b]		
Residue	1.94	21.7	1.94	»		
Sum	42.9682					
Loss	0.62					

[a] Abbreviation used for diethylene glycol.
[b] » » » triethylene glycol.

was determined as loss of weight of the liquid in the distilling flask. Part of this residue was then subjected to fractionation in the spinning band column.

Materials. The organic reagents used were carefully purified. The ethylene oxide was practically free from acetaldehyde (less than 0.01 %). The ethanol was made "super dry" by distillation from a solution of magnesium ethoxide in ethanol [16]. The glycol and its ethyl ether were carefully fractionated in the Podbielniak distillation apparatus. The sodium alcoholate catalysts were prepared *in situ* by addition of metallic sodium to the starting materials.

Conditions and Results. The experimental conditions and results are summarised in Table 1. Before these are discussed the details of the calculations of one experiment are given as an illustration of the method used. In Experiment No. 4, 245.85 g of ethylene oxide was reacted with 1 000.0 g of water with 2.65 g of dry sodium carbonate as catalyst. The molecular proportions of the reactants, v, is calculated as 0.1006. Of the reaction product, 400.0 g was distilled in the Podbielniak apparatus giving 136.8 g of residue. Of this, a sample of 43.59 g was distilled in the spinning band column. The distillation curve is shown in Fig. 4 and the results including analyses and calculated amounts of individual compounds are given in Table 2. The residue from the distillation is counted in with the highest glycol. The amounts of water charged and distilled off in the Podbielniak apparatus are recalculated to correspond to the amount fractionated in the final distillation. In order to compensate for the distillation loss the sum of the fractions was used instead of the amount distilled. Water distilled off = (400.0 − 136.8) (42.97/136.8) = 92.71 g. The total amount of water in the reaction product thus was 102.75 g. Water charged = (1 000.0/18.02) (400.0/1 248.5) (42.97/136.8) = 5.5844 moles. The amounts of the products are converted to moles and the values of the various mole fractions n_i/n_{00} calculated (Table 1, columns 10 to 15). The sum of these mole fractions should equal unity and the sum of the expressions in_i/n_{00} should equal v. As is seen from Table 1, columns 16 and 17, the agreement is satisfactory.

The experimental results in Table 1 were obtained in the way described. Of the various values for c, c' (column 18) is calculated from v and n_0/n_{00} by means of equation (9), c'' (column 19) from n_0/n_{00} and n_1/n_{00} by iteration in equation (10), and c (column 20) is a chosen mean value. In combination with the experimental value of v (column 8), this mean value is used for the calculation of the theoretical values of n_i/n_{00} (columns 10 to 15) given below the experimental values.

It is of interest to compare the values of the distribution constants c determined by distribution experiments with those that can be calculated from determinations of the individual velocity constants. Table 3 gives velocity constants determined at 25° C on dilute solutions of ethylene oxide in the respective reagent with sodium hydroxide as catalyst [17]. The constants are second order constants reduced to unit molar concentration of catalyst. The quotients of the appropriate constants are compared with those determined from the distribution experiments.

Table 3. Velocity constants at 25° C and distribution constants for the reaction between ethylene oxide and various compounds with sodium hydroxide as catalyst.

Compound	Velocity constant lit · min^{-1} · mole^{-1}	Ratio between velocity consts.	Distribution constant, c
Water	$1.26 \cdot 10^{-4}$	4.80	13
Glycol	$6.05 \cdot 10^{-4}$		
Diglycol	$1.27 \cdot 10^{-3}$	2.10	1
Ethanol	$1.29 \cdot 10^{-3}$	0.47	2.2
Ethylglycol	$6.10 \cdot 10^{-4}$	1.00	1
Ethyldiglycol	$6.10 \cdot 10^{-4}$		
Diglycol	$1.27 \cdot 10^{-3}$	2.08	2 [a])

[a]) Expected value.

DISCUSSION

Comparison between theoretical and experimental results shows that the theory presented accounts fairly well for the course of the reactions between ethylene oxide and reactive molecules. Especially significant is the result, that the distribution of products in the reaction with glycol or its ethyl ether closely follows a Poisson series, *i.e.* $c = 1$, see Table 1, Experiment Nos. 6 and 9. This is a direct proof of the assumption that all hydroxyl groups bound to an oxyethylene group have the same reactivity.

The value of the distribution constant c and, with it, the amounts of the higher glycol products increase with increasing alkalinity, Table 1, Experiment Nos. 1 to 5. This was also found to be the case by Davis, von Waaden, and Kurata [12]. The large difference between the results with sodium carbonate (Experiment No. 4) and sodium hydroxide (Experiment No. 5) may seem noticeable. However, from published data [18] for the velocity constants of the reaction between ethylene oxide and water, it may be calculated that, with the amount of sodium carbonate catalyst used, the catalysed and uncatalysed reactions are of comparable importance. Thus the value of c is intermediate between the values for uncatalysed and hydroxyl ion catalysed reactions.

A comparison between the quotients in Table 3 of the kinetical rate constants and the distribution constants c brings out some points of interest. The values agree in the case of the pair, ethylglycol — ethyldiglycol. For the pair ethyldiglycol — diglycol, the value predicted for c is 2 because the latter compound has two hydroxyl groups of the same reactivity as that of the single one of the former. This is in agreement with the experimental value from the velocity constants, 2.08. In the other cases there are discrepancies. Apart from experimental errors, these may have two causes. Firstly, the influence on the c-values of the differences in acidity of the various compounds are not accounted for by the kinetic experiments. Secondly, the rate constants were measured in different media which also differ from the media in the distribution experiments. Moreover, the medium changes during the reaction in the latter experiments, but it seems reasonable to assume that the distribution constants remain unchanged. The discrepancies ought to diminish as one passes to higher members in a reaction series, as is found to be the case for ethylglycol — ethyldiglycol. It should perhaps be observed that the rate constants given were determined at a much lower temperature than that used in the distribution experiments. For the early steps of the reaction series this difference may also have some influence on the velocity constant ratio.

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Short Communications

A Method of Detection of N-Dimethylamino Acids on Paper

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In 1950 Bowman suggested reductive methylation as a method of determining N-terminal amino acids in peptides [1]. Shortly afterwards Ingram [2,3] was able to show its applicability to smaller peptides. The detection of the N-dimethylamino acids formed was based upon the buffering action of these compounds — a method suffering from the disadvantage of being insufficiently specific. We therefore consider it worth while to publish a direct and more reliable procedure which has been used by us for some time.

The detection of the spots is based upon conversion of the dimethylamino acids to betaines by means of methyl iodide:

$$(CH_3)_2N—CHR—COOH + CH_3J \rightarrow (CH_3)_3\overset{+}{N}—CHR—COO^- + H^+ + J^-$$

The iodide remaining in the spot after evaporation of excess methyl iodide may be detected in two ways. Oxidation with chlorine in vapor phase yields iodine which can be detected by spraying with a starch solution (*cf.* Rydon and Smith [4]). An alternative method which we find preferable involves precipitation of silver iodide followed by reduction with a photographic developer. The sensitivity varies considerably, depending on the dimethylamino acids and on the chromatographic system used, but is high enough for the detection of concentrations larger than 5—10 $\mu g/cm^2$. The reaction is particularly sensitive to basic (and sulphur containing) dimethylamino acids where the sensitivity may be higher than that of the ninhydrin reaction of the corresponding unsubstituted amino acids. The darkness of the background varies within wide limits depending on the paper used but all common brands tried have given satisfactory results.

The dried paper is placed in a desiccator, containing a beaker with methyl iodide and left for two hours at 35—40°. The beaker should be placed above the paper to ensure a satisfactory exposure of the paper to the methyl iodide vapor. When the reaction is complete the paper is dried in an oven at 100° C for a few minutes. It is then immersed in an ammoniacal 0.1 *N* silver nitrate solution. The excess of silver nitrate is removed by careful washing with distilled water. The spot may now be developed by a rapid photographic developer (*e. g.* prepared according to the formula of Kodak D 72).

The method may be illustrated by the separation and identification of a mixture of dimethyl*iso*leucine, dimethylleucine, and dimethylphenylalanine (Fig. 1). The paper (Whatman No. 1) had been treated with 0.02 *M* sodium tetraborate solution and dried before application of the dimethylamino acids. The paper chromatogram was developed by a solvent system consisting of 87 % tertiary butanol and 13 % 0.02 *M* sodium tetraborate buffer for a minimum time of 40 hours.

A great number of other amino acids have been methylated, chromatographed and identified in mixtures with untreated amino acids. Amino acids and dimethylamino acids can be detected on the same chromatogram by the multiple dipping technique [5]. The paper is first dipped in a 0.2 % ninhydrin-acetone solution and the color developed in the usual way. The spots are marked with a pencil. The paper is now treated with methyl iodide vapor as described above, dipped in silver nitrate solution and then washed with a strong ammonia solution. The colored spots gradually fade and disappear. The



Fig. 1. Paper chromatogram of dimethyl leucine (1), dimethyl isoleucine (2), dimethyl phenylalanine (3), and a mixture of these acids (4). For details see text.

subsequent treatment with photographic developer reveals the dimethylamino acids, the basic and sulphur containing amino acids as black spots. The identification is simplified by the fact that a dimethylamino acid in butanol-acetic acid-water-systems travels in front of and not widely separated from the corresponding unsubstituted amino acid.

Finally we wish to point out the possibility of using the proposed method for detection of all kinds of substances capable of reacting with methyl iodide with formation of iodide ions (amines, organic sulphides *etc.*). The method has also been used by us to detect ninhydrin-negative impurities in commercial amino acid samples and contaminants obtained in eluates from ion exchange resins.

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The Use of ^{110}Ag in Quantitative Paper Chromatography of Sugars

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In connection with other investigations it was necessary to determine quantitatively very small amounts of paper-chromatographically separated reducing sugars. The method of Trevelyan *et al.* [1] was used, involving the precipitation in the spots of metallic Ag from $AgNO_3$-acetone solution. The present communication deals with some preliminary studies on the conditions under which reducing sugars can be determined quantitatively with the aid of ^{110}Ag incorporated in $AgNO_3$.

Methods: The sugars were separated in descending chromatograms on sheets of Whatman No. 4 or 54 filter paper, size 30 × 50 cm. The temperature was 20° C and the duration of the chromatography was 24 hours. Two solvents were used: (a) *n*-butanol, pyridine, and water in the ratio 3:2:1.5, and (b) ethyl acetate, glacial acetic acid, and water in the ratio 60:17:17.5[2].

Ag of high purity grade was activated in the Norwegian uranium reactor to a specific activity of about 1.5 mC ^{110}Ag/g Ag. The activated Ag was dissolved in pure nitric acid and the nitrate was prepared. It was then found suitable to dilute this nitrate ten times with inactive $AgNO_3$. The experiments included two aldoses: glucose and galactose, two ketoses: fructose and sorbose, and two disaccharides: maltose and lactose. These sugars were determined singly or in different combinations. It should be mentioned that glucose and fructose were only separated with solvent (b) and that glucose and galactose were not separated from the same solution with these solvents. Small volumes (3—10 μg in one ml) of the sugars to be tested were applied to the papers with an Agla micrometer syringe. In some cases larger quantities, up to 40 μg were used, the spots being allowed to dry between successive 1 μl loadings of the paper. The position of the separated spots were identified with the aid of standard solutions placed on narrow edge strips of the chromatographic paper. R_F values could not be used as the solvent front leaves the paper in about 9 hours and the R_F values in such a case are not sufficiently reproducible. Around the spots,



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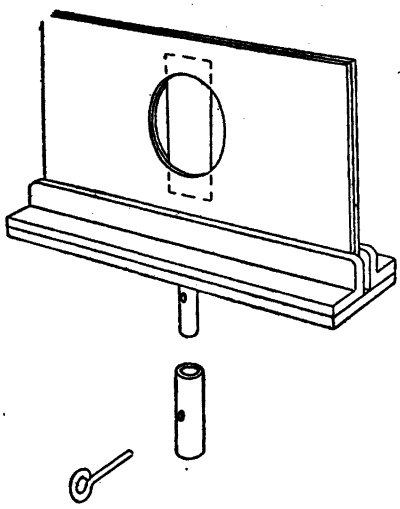

Fig. 1. Frame for developing the spots.

strips of identical sizes, 4 × 10 cm, were cut out. These strips were dipped in the $AgNO_3$-acetone solution and dried at 20° C. They were then sprayed with a 0.5 *N* solution of sodium hydroxide in aqueous ethanol. In order to attain reproducible conditions a special, simple turnable frame (Fig. 1) was designed. From the sprayer, the position of which was fixed, a well definied volume of NaOH solution could be uniformly distributed on the paper strip. At a given time ("development-time") following the spraying, the strip was immersed into 6 *N* NH_3. After washing in running water and air drying, the spots were cut out into squares of equal sizes, the radioactivity of which was determined. When the spots were large compared with the standard size chosen, they were measured in two parts. The background, which is due to the reduction ability of the paper and probably also to the starting hydrolysis of the cellulose, was determined from a square of equal size cut from the vicinity of the spot. The most reproducible values were obtained when the total β- and γ-radiations were determined, *i. e.* without shielding. A thin windowed (2 mg mica/cm²) Geiger counter-tube was used.

Results: The reduction values, expressed as counts per minute, are found to be rectilinear functions of the amount of sugar applied (Fig. 2a), whence it is possible to determine quantitatively the sugars studied in amounts down to about 3 μg and up to about 40 μg (the higher concentrations not included in the figure). Since there is no simple stoichiometric relationship between the amount of sugar applied and the reduction value (*cf.* the different and lower values for the two ketoses as compared to the aldoses), the determination has to be performed relative

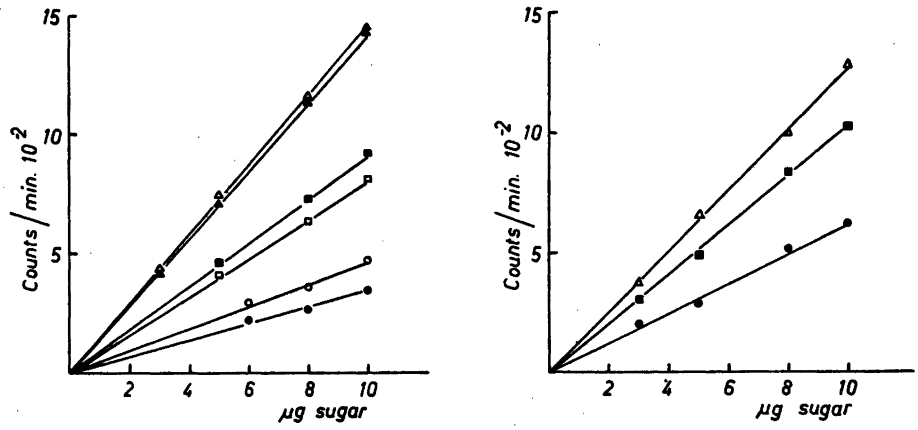

Fig. 2a and b. Reduction values (counts/min.) as a function of the amount of different sugars. △ *glucose,* ▲ *galactose,* ■ *fructose,* □ *sorbose,* ○ *lactose,* ● *maltose. 10 secs. in $AgNO_3$-acetone. The dots represent a mean value of at least six different determinations. Washed with 6 N NH_3. a. Development-time 5 minutes. Subtracted background 50 counts/min. b. Development-time 12 minutes. Subtracted background 200 counts/min.*

to an identically treated standard. It should be mentioned that the ketoses require a longer development-time than the aldoses and the disaccharides an even longer time. Experiments with a varied development-time has disclosed that a maximum reduction value is reached after 5 minutes in the case of the monoaldoses, after 10 minutes in the case of the ketoses, and after 10—12 minutes in the case of the disaccharides. However, even at the maximum value, the difference between aldoses and ketoses is retained. This fact is in agreement with the results of experiments using the method of Wallenfels *et al.*[3], where fructose and sorbose required treatment in steam for 1.5 minutes whereas a greater amount of aldoses could be developed in 10—20 seconds. The longer development-time required in the case of the ketoses can be explained on the basis of the chemical structure and mode of reaction. It is more difficult to explain why the disaccharides require such a long time for complete development. It could be expected that the mode of reaction of glucose and maltose is identical and that the reduction value per weight of the disaccharide is half that of the monosaccharide. This relation is obtained after 12 minutes development (Fig. 2b). With these extended development-times the background becomes correspondingly higher and the values therefore less reliable. — When the method of Wallenfels *et al.*[3] is applied, the spots of maltose and glucose are developed at equal rates.

A complicating factor is the experience that, in washing with 6 *N* NH_3, a part of the Ag is extracted. This is particularly evident in the case of tri- and oligosaccharides, where the washing produced a total decoloration of the spots. Since this extraction does not occur in 2 *N* NH_3 it seems most likely that the extraction is due not to the presence of Ag_2O but to the oxidation and dissolution of the finely dispersed Ag.

Conclusion: ^{110}Ag can be used for chromatographic determinations of reducing sugars. The isotope is useful for investigations of reaction conditions in chromatographic methods involving development with Ag. The fact that di- and monoaldoses give reduction values proportional to the number of reducing groups (Fig. 2b), indicate the possibility of estimating oligosaccharides even in cases where standards can be obtained only of the mono- and disaccharide of the series.

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1. Trevelyan, W. E., Procter, D. P. and Harrison, J. S. *Nature* **166** (1950) 444.
2. Löfgren, N., Larsson, N. and Holmström, B. *Unpublished results.*
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Ruminant Bloat and Foaming Activity of Clover

ARTTURI I. VIRTANEN and F. R. WILLIAMS

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Many explanations have been proposed for ruminant bloat, in which thousands of cows die each year. As the bloating can be stopped, and the animal cured with anti-foaming agents as oil if given in good time, it is most likely that bloat is caused by the intense formation of foam in the rumen. Solid particles from the contents of the rumen gather into this foam, and the passage for gas escape becomes accordingly blocked. The great amounts of gas which are normally formed by fermentations in the rumen thus cause the bloat.

In order to find out if a great amount of substances with strong foaming activity are found in red clover which in Finland is the most dangerous cause of bloat during the latter part of summer, we made some determinations on the foam producing qualities of clover and timothy. The method used was the following.

Measurement of foam produced by shaking of crushed samples of plants in water. Parallel experiments with different amounts of water showed that a 5 % suspension of crushed fresh clover in water was the most satisfactory concentration for examination

to an identically treated standard. It should be mentioned that the ketoses require a longer development-time than the aldoses and the disaccharides an even longer time. Experiments with a varied development-time has disclosed that a maximum reduction value is reached after 5 minutes in the case of the monoaldoses, after 10 minutes in the case of the ketoses, and after 10—12 minutes in the case of the disaccharides. However, even at the maximum value, the difference between aldoses and ketoses is retained. This fact is in agreement with the results of experiments using the method of Wallenfels *et al.*[3], where fructose and sorbose required treatment in steam for 1.5 minutes whereas a greater amount of aldoses could be developed in 10—20 seconds. The longer development-time required in the case of the ketoses can be explained on the basis of the chemical structure and mode of reaction. It is more difficult to explain why the disaccharides require such a long time for complete development. It could be expected that the mode of reaction of glucose and maltose is identical and that the reduction value per weight of the disaccharide is half that of the monosaccharide. This relation is obtained after 12 minutes development (Fig. 2b). With these extended development-times the background becomes correspondingly higher and the values therefore less reliable. — When the method of Wallenfels *et al.*[3] is applied, the spots of maltose and glucose are developed at equal rates.

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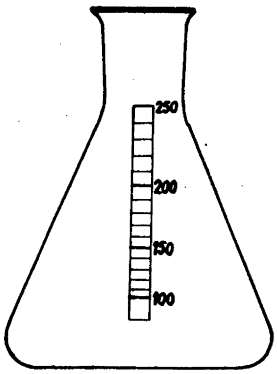

Fig. 1. Markings scratched on glass.

of foaming. The plant material was crushed as much as possible in the same manner in all the experiments. 100 ml water + 5 g crushed plants was put in a 250 ml flask, and the flasks were shaken in a flask shaker, always at the same speed (approx. 300 per min). The froth produced was quite stable, approx. 1/3 of the volume still remaining after standing overnight. Figures on the volume of the foam were fairly well reproducible for triplicate samples.

In all experiments a 5 % suspension was used. Graphs were plotted against time of shaking at constant speed. Volume of froth was read off directly from the graduations marked on the flasks (Fig. 1). All samples were done in triplicate.

Maximum amount of foam was usually produced during 1—3 hours of shaking. In some cases it was equivalent to the foam produced in parallel experiments by a 0.01 % solution of Baker saponin.

Fig. 2 shows the formation of foam in different experiments. All the investigated samples of red clover contained a great amount of foam-producing substances. As a general tendency we may, however, note that the samples taken in August have had a greater foaming activity than those taken in September. In the last samples (19/9) the activity has been only about half of that in the samples from August. It is to be observed that the pasture season in Finland generally ends in the middle of September. It is unfortunate that we have no determinations of red clover from the earlier part of summer when bloat is very scarce.

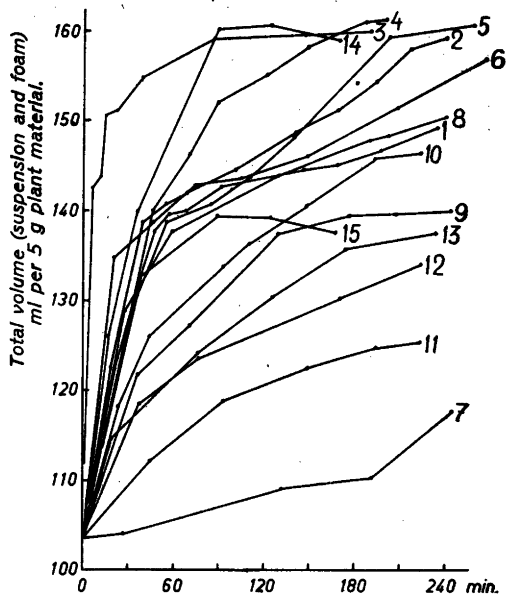

Fig. 2. Foaming of crushed clover, grass, and hay.

1. Red clover, 13.8.1952;
2. Red clover, 15.8.1952;
3. Baker saponin, 0.01 % solution;
4. Red clover, 21.8.1952;
5. Red clover (2nd year, 3rd crop), 27.8. 1952;
6. Red clover (1st year, 3rd crop), 27.8.1952;
7. Grass, 3.9.1952;
8. Red clover (1st year, 3rd crop), 3.9.1952;
9. Red clover (1st year, 3rd crop), 11.9.1952;
10. Red clover (2nd year, 3rd crop), 11.9. 1952;
11. Grass, 11.9.1952;
12. Red clover (2nd year, 3rd crop), 19.9. 1952;
13. Red clover (1st year, 3rd crop), 19.9. 1952;
14. Hay (clover), 12.12.1952;
15. Hay (timothy), 12.12.1952.

The few samples of fresh grasses (mostly timothy) studied (7, 11) showed much lower foaming activity than fresh clover. With hay of clover and timothy — when the high drymatter content (about 90 %) of hay (expts. 14 and 15) compared with fresh clover and timothy (drymatter content about 20 %) is taken into account —

much less foam was formed than with corresponding fresh samples. In addition the froth which had been formed with hay of both types was much less stable, and commenced subsiding immediately after removal from the shaker, the subsidence being almost complete after about 1 hour. These observations agree with the well known fact that grass without leguminous plants does not cause bloat, and that clover made into hay does not do it either. The untenableness of the froth formed with hay is an interesting phenomenon, and suggests that in addition to the decrease of the saponin content also other changes influencing the quality of the foam occur in the drying of clover.

The attempt to produce bloat experimentally with two cows by giving them about 25 g of commercial saponin failed. We did not therefore publish our above-mentioned results, as more knowledge about the substances with foaming activity in clover was needed, as well as animal experiments. The commercial saponin was apparently as foam-producing as that found in clover (*cf.* curve 3).

The present publication of our results depends on that Lindahl *et al.*[1] have recently published a communication about their preliminary investigations on the role of alfalfa saponin in ruminant bloat. They have effected bloat in sheep, goat, and heifer by saponin isolated from this leguminous plant. The amounts of alfalfa saponin which they have used in animal experiments (15—55 g to sheep, and 75 g to heifer) are considerably greater than the amount of commercial saponin which we gave to our cows, so that our negative result may depend on this. It is, however, noteworthy that Lindahl *et al.* did not effect any perceptible bloat in sheep with commercial saponin (from the yucca plant).

Our observations about the great amounts of foam-producing substances (or substance) in fresh clover, and their scarcity in fresh grass (timothy), and in hay made of clover and grass give good support to the conception that bloat in ruminants is caused by an intense formation of foam in the rumen, and that this formation prevents the escape of gas from the rumen. The animal experiments of Lindahl *et al.* with alfalfa saponin have proved the correctness of this idea.

1. Lindahl, I. L., Cook, A. C., Davis, R. E. and Maclay, W. D. *Science* **119** (1954) 157.

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On the Formation of Phlorizin in Normal- and Low-Nitrogen Apple Maidens

ARTTURI I. VIRTANEN and KRISTIAN OLAND

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In this laboratory, the influence of the nitrogen content of cells on their enzymatic activity with microorganisms has been previously investigated. It then appeared that some enzymes lose a large, or even the largest, part of their activity when the N-content of the cells falls by 20—40 %, while other enzymes retain their activity rather well. The mutual relation between the enzymes in a low-N cell thus differs entirely from that in normal-N cells. With *Aerogenes* bacteria for instance, Virtanen and Alonen [1] noted that low-N cells form fermentation products from sugar in quite different proportions than normal-N cells.

One of us [2], has recently given an account of investigations on the influence of nitrogen fertilizing on the nitrogen content, and various nitrogen fractions, of apple maidens. These investigations showed that the maiden can grow relatively well during one summer without N-nutrition, although the nitrogen content falls to about half that of the maiden grown with nitrate. In the roots, the drop in nitrogen is even greater (*cf.* Table 1). It is thus possible to investigate the influence of the lowering of the nitrogen content on the metabolic reactions and enzymatic activity with higher plants as well.

Table 1 shows analyses of different nitrogen fractions in maidens, stocks and roots of the apple trees. Plant 5 was given no nitrogen fertilizers and the leaves of Plant 2 were sprayed with urea solution eight times during the summer. However, this did not influence the N-content of the plant, so that both Plant 5 and Plant 2 represent low-N plants. Plants 12 and 18 were fertilized with nitrate, which they readily absorbed, as can be observed from the nitrogen analyses of the plants. These plants represent high-N plants. The growth of the plants appears on Table 2.

When apple maidens were extracted with 70 % alcohol and the alcohol evaporated, we observed a copious crystalline precipi-

much less foam was formed than with corresponding fresh samples. In addition the froth which had been formed with hay of both types was much less stable, and commenced subsiding immediately after removal from the shaker, the subsidence being almost complete after about 1 hour. These observations agree with the well known fact that grass without leguminous plants does not cause bloat, and that clover made into hay does not do it either. The untenableness of the froth formed with hay is an interesting phenomenon, and suggests that in addition to the decrease of the saponin content also other changes influencing the quality of the foam occur in the drying of clover.

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Table 1. Composition of N-fractions in "Maiden", "Stock", and "Roots" of appletrees without and with nitrate (mg N/100 g. dry matter).

Plant N:o	Plant part	Total org. N	"Protein N"	Soluble N	Amino N	Amide N
5	Maiden	642	575	67	36.5	9.9
(No N)	Stock	328	296	32	18.6	4.8
	Roots	413	380	33	19.9	4.4
2	Maiden	536	470	66	31.5	8.8
(Urea)	Stock	313	282	31	18.3	5.8
	Roots	455	425	30	19.7	5.6
12	Maiden	1 000	806	194	91.6	55.1
(NO_3)	Stock	969	651	318	129.8	85.3
	Roots	1 608	984	624	225.5	164.1
18	Maiden	891	699	192	61.2	28.2
(NO_3)	Stock	971	691	280	105.8	70.7
	Roots	1 521	1 009	512	193.1	133.6

tate which had been formed in the extract of low-N stocks and roots. In corresponding extracts of high-N plants, a precipitate of this kind was not observed. The precipitate was separated and recrystallized several times, its melting point being 114—115° C (uncorrected). This substance did not contain any nitrogen and gave in analysis 53.02 % C and 5.90 % H. It contained an aromatic ring, phenolic hydroxyl groups, and a CO group, and from the molecular weight determinations, seemed to contain 2 benzene rings with 3 hydroxyl groups. The CO group apparently belonged to the carbon chain which linked the rings. At this stage we noticed that phlorizin is a known compound in the bark of the apple tree and the mixed melting point and other characteristics showed our substance to be identical with phlorizin. The paper chromatograms (*cf.* below) were also identical.

Quantitative determinations showed that 5.2 % of phlorizin on a dry weight basis, could be found in low-N stocks, and 1.2 % in high-N stocks. Lack of plant material prevented us from further quantitative determinations of phlorizin. According to approximate observations during the evaporation of ethanol, the lower value may be valid for stock and roots of all high-N plants, while the higher value is valid for the corresponding parts of all low-N plants.

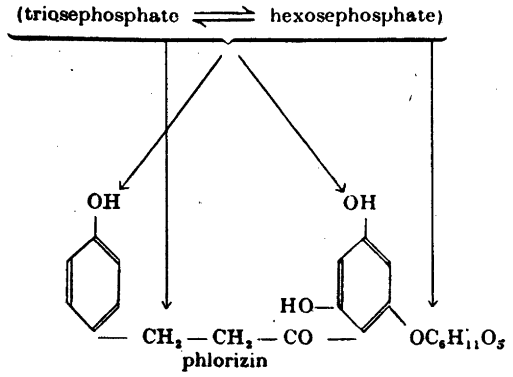
It is possible to separate and identify phlorizin using paper chromatography. With 70 % ethanol we found the R_F value to be 0.82, and with phenol-water 0.69. Chromatograms were developed by spraying with benzidine, prepared according to Horrocks [3]. Spots appeared upon warming at 100° C for 10—15 minutes.

As phlorizin is formed most freely in low-N plants it is probable that nitrogen deficiency brings about changes in the mutual relations between the enzymes in apple maidens, in consequence of which the phlorizin synthesis increases. Phlorizin is formed from the products of CO_2-assimilation, and it can be calculated that

Table 2. Initial weights of plants and production during experimental time.

	Plant				
	5 (No N)	2 (Urea)	7 (Urea)	12 Nitrate	18 Nitrate
Initial weight of rootstocks, g. dry m.	34.3	30.4	46.6	23.0	28.8
Increase in rootstocks, g. dry m.	4.8	7.9	5.3	8.5	5.3
Weigth of maidens, g. dry m.	6.7	7.7	6.9	11.1	10.0

3.5 hexose molecules are needed to form one phlorizin molecule, irrespective of whether the synthesis occurs from the triose, or earlier, stages, or from the hexose stage.



Accordingly, the increased phlorizin synthesis brings about a decrease of sugar and of soluble carbon compounds in general in the plant sap, since phlorizin is very difficult to dissolve in water. This is obviously of importance for the plant, since lack of nitrogen has reduced the normal synthesis of amino acids to a minimum, and the utilization of carbon compounds in amino acid synthesis has thus become smaller.

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Removal of an *Iso*propylidene Group in 1,2—5,6-Di-*iso*propylidene-D-glucofuranose with a Sulphonic Type of Resin

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α-1,2-*Iso*propylidene-D-glucofuranose is a very useful compound for the preparation of other derivatives of D-glucose. In the past, it has usually been prepared from 1,2—5,6-di-*iso*propylidene-D-glucofuranose by careful hydrolysis with dilute aqueous hydrochloric acid [1], aqueous acetic acid [2] or nitric acid and ethyl acetate [3]. The preferential removal of the 5,6-*iso*propylidene group is due to its greater susceptibility to acid as compared with the other.

In some synthetic work carried out in this laboratory it was found that the preferential removal of the 5,6-*iso*propylidene group in the di-*iso*propylidene derivative was effected in a few hours at room temperature on treatment with a sulphonic type of resin. This method which gives a good yield of the desired compound is also very convenient.

The use of an acidic resin for the hydrolysis of sugar derivatives has been reported previously, *e. g.* the hydrolysis of alkyl glycosides [4], N-glycosides [5] and now recently for the removal of an *iso*propylidene group in the synthesis of some methylated galacturonic acids [6].

Experimental. 1,2-5,6-di-*iso*propylidene-D-glucofuranose (8 g) was dissolved in water (320 ml) and Amberlite IR 120-(H) (16 g in the hydrogen form) were shaken at room temperature for 3.5 hours. The resin was filtered off, washed with water, the filtrate concentrated in vacuum to dryness and a white material was obtained (6.7 g). It was recrystallised from ethyl acetate. Small amounts of insoluble material, which was probably D-glucose and starting material, were filtered off. The recrystallised material (4.6 g) had m.p. 156—158° C and $[\alpha]_D^{20} = -11.3°$ (*c*, 1.25 in water). The literature reports m. p. 156—157° C and $[\alpha]_D^{20} = -11.8°$.

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Differences in the IR Absorption of the Stereoisomers of Certain Dibasic Carboxylic Acids

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Fliel and Kofron [1] have examined the infrared spectra of the optical isomers of the acid phthalic ester of *p*-ethylphenylmethyl-carbinol. Their results show that the (+), (−), and racemic forms have identical spectra in chloroform solution but that those of the crystalline forms, taken as nujol mulls, exhibit significant differences. The absence of free OH stretching frequency and at the same time a shift in the main carbonyl frequency was most interesting. This effect, however, was not general for all substances of the type in question, and the authors came to the conclusion that only when the (+) and (−) forms alone cannot dimerize, changes in the main carboxylic frequencies may be expected. — In order to obtain more evidence about the infrared absorption of optically active substances and stereoisomers in general certain simple α,α′-disubstituted dicarboxylic acids and some other optically active substances have been examined.

Although it is quite obvious from the theoretical point of view that the (+) and (—) forms should give identical spectra in solution, it may be possible that the racemic compounds show some differences in concentrated solutions owing to stronger bonds of the hydrogen-bond type. Concentrated (nearly saturated) solutions of some optically active and racemic substances have thus been investigated in order to give possible support to the assumption mentioned above. The α,α′-dimethylsuccinic and α,α′-dimethylglutaric acids as well as mandelic acid were examined in chloroform solution, whilst tartaric acid, alanine and leucine were studied in aqueous solutions between 7 and 11 μ. For α,α′-dimethylsuccinic, mandelic and tartaric acid and the amino acids no differences in position or intensity of the absorption bands were found between the racemic and optically active form. In the case of α,α′-dimethylglutaric acid, however, a slight difference was observed in the broad maxima at 10.7 μ (930 cm^{-1}), commonly



Fig. 1. I: Racemic α,α′-dimethylglutaric acid in chloroform solution. II: (+)-α,α′-dimethylglutaric acid in chloroform solution.

ascribed to out of plane bendings of OH[2]. The question whether this difference (Fig. 1) is significant enough to be regarded as due to the influence of a presumable racemic compound is left open until further evidence concerning similar effects can be offered. — Using solid samples of the dicarboxylic acids on the other hand more pronounced differences between the racemic and the optically active forms could be observed, the following shifts of main frequencies being noted. The main carbonylic stretching at 5.84 μ for the active α,α′-dimethylsuccinic acid with a slight shoulder at 5.77 μ has in the racemic form given place to two distinct maxima, one at 5.77 μ and the other at 5.88 μ. The active form of α,α′-dimethylglutaric acid has its main carbonyl frequency at 5.86 μ, at which value this band remains even in the racemic form, the only difference being that a slight shoulder appears at 5.80 μ. The spectra in general show other differences as well, unexpectedly pronounced for succinic acid but, on the contrary, very slight for glutaric acid. — No differences between the (+) and (—) forms as observed by Gore and Petersen [3] could be found.

As might be expected most interesting differences were observed in the spectra of the

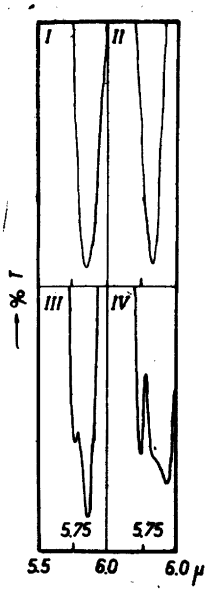

Fig. 2. I: Meso α,α'-dimethylsuccinic acid. II: Meso α,α'-dibromosuccinic acid. III: Racemic α,α'-dimethylsuccinic acid. IV: Racemic α,α'-dibromosuccinic acid.

meso and racemic forms of the same dicarboxylic acid. Both the optically inactive stereoisomers of the α,α'-dimethyl derivatives of succinic, glutaric and adipic acid were investigated, and they all showed in solid forms differences in the main frequencies. The changes were not quite similar for the different homologues, but it could be established that the effects diminished with increasing length of the carbon chain.

The racemic form of α,α'-dimethylsuccinic acid shows a double peak at 5.77 μ and 5.87 μ, while the *meso* form has a single peak at 5.85 μ. The racemic form of α,α'-dimethylglutaric acid has the carbonyl peak at 5.86 μ with a slight shoulder at 5.80 μ. The *meso* form on the other hand has the corresponding peak at 5.78 μ with a shoulder at 5.85 μ. The shift in the absorption frequencies of the two forms of α,α'-dimethyladipic acid is too small to be determined with certainty. Both the absorption bands occurred at 5.85 μ, the racemic form, however, with a slight shift to longer wavelength.

Due to the insolubility of the *meso* forms of α,α'-dimethylsuccinic and α,α'-dimethyladipic acid in solvents suitable for infrared absorption measurements a complete investigation of the homologues in solution could not be performed. A comparison of the spectra of the two optically inactive forms of α,α'-dimethylglutaric acid in saturated chloroform solution, however, showed the carbonyl stretching frequency of the *meso* form to be at 5.79 μ and that of the racemic form at 5.83 μ.

From these experiments with the dicarboxylic acids the general conclusion can be reached that where a difference *does occur*, the carbonyl frequency of the racemic form is shifted to longer wavelength. More valuable and useful conclusions could be drawn by comparison of the *meso* and racemic forms of different substitution derivatives of the same acid. The behaviour of the main carbonyl absorption band seems to be characteristic for the stereoisomeric forms of acids being derived from the same dicarboxylic acid and only differing in the substituents. It should be observed that in this investigation these substituents are approximately of the same type. The carbonyl bands of α,α'-dimethylsuccinic and α,α'-dibromosuccinic acid are shown in Fig. 2. The double peaks are probably characteristic for the racemic forms of α,α'-disubstituted succinic acids, whereas the *meso* forms show single peaks. As both the isomers of α,α'-dibromosuccinic acid in dioxan solution gave well defined single peaks at 5.73 μ and 5.75 μ, respectively, it is obvious that the changes mentioned above were not caused by any decomposition products of the substances. It seems reasonable to the authors to explain the existence of double peaks here as due to steric effects, as such explanations have in other connections already been proposed by Duncanson [4] for some derivatives of succinic acid.

Experimental. The solid samples of the methylsubstituted acids were examined as solid pressed potassium bromide plates according to Schiedt and Reinwein [5]. The bromo compounds were investigated as nujol mulls. The spectrophotometer used was a Perkin Elmer model 21. Detailed results and discussions will be published later in connection with further work.

The authors are indebted to Professor Arne Fredga, who kindly placed most of the substances at our disposal. This work has been financially supported by grants from the *Rockefeller* and *Nobel Foundations*, which is gratefully acknowledged.

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A New Glycoside from *Furcellaria fastigiata*

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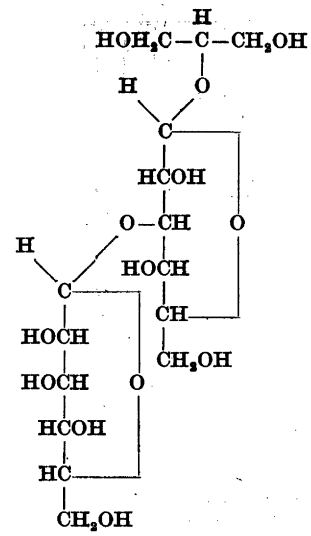
As part of our investigations of the low-molecular carbohydrates in algae, the red alga *Furcellaria fastigiata* has been investigated. The glycerol α-galactoside, floridoside, first isolated by Colin and Guéguen [1] from *Rhodymenia palmata* and recently shown by Putman and Hassid [2] to be 2-glycerol α-D-galactopyranoside, was obtained. In addition to this substance another carbohydrate, slower than floridoside on the carbon column and with an R_F value in the disaccharide region, was isolated. It did not crystallise but was purified *via* its highly crystalline acetate derivative. It was non-reducing and on hydrolysis yielded galactose, mannose and glycerol, the proportions of which, determined by quantitative paper chromatography [3], were 1:1:1. The mannose and galactose were characterised as the phenyl hydrazone and α-methylphenyl hydrazone, respectively. As floridoside was found in the same plant, it was probably a mannoside of this substance, the high specific rotation of the acetate indicating an α-mannoside. On periodate oxidation it consumed only 2 moles of oxidant, with the formation of 1 mole of formic acid, hence the hydroxyl group on carbon atom 3 in the galactose must be glycosidically linked and the substance should have the structure I.



I

This was finally shown by a degradation according to Barry [4], *i.e.* treatment of the periodate oxidised glycoside with phenyl hydrazine, when the oxidised units are split off. Floridoside, identical to an authentic specimen, was obtained in a satisfactory yield (50 %), in agreement with the postulated structure.

3-Floridoside α-mannoside nonaacetate: M.p. 153–154°, $[\alpha]_D^{20}$ +103° (Chloroform, $c = 2$). (Found: C 49.6; H 5.52. Calc. for $C_{33}H_{46}O_{22}$ (749.7): C 49.9; H 5.85.)

The author is indebted to *Statens Naturvetenskapliga Forskningsråd* for financial support and to *Institutt for Tang- og Tareforskning*, Trondheim, for a generous gift of the alga.

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The Growth of *Clostridium pasteurianum* in Synthetic Nutrient Solution

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Various experiments have been made with anaerobic *Clostridia* which fix molecular nitrogen. Until now, nutrient solutions to which potato extract has been added have generally been used, as the bacterium could not be made to grow successfully with synthetic nutrients. This has made it more difficult to obtain reliable results in such experiments as investigation of secretion of nitrogen compounds, or determination of the requirements of Mo or other trace elements, as many unknown factors are included in potato extract. In a recent paper [1] on the inhibition by N_2O of biological nitrogen fixation and the uptake of combined nitrogen we mentioned that we have been able to make *Clostridium pasteurianum* grow in synthetic nutrient solution by adding different vitamins to the solution. In this paper we will give a detailed account of the vitamin requirements of *Clostridium*.

The bacterial strain, *Cl. pasteurianum* W5, used in our experiments was kindly provided by Dr. H. L. Jensen, Lyngby, Danmark.

Basal nutrient solution: 0.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.1 g NaCl, 10 mg $FeSO_4 \cdot 7 H_2O$, 25.2 mg $Na_2MO_4 \cdot 2 H_2O$, 20 g glucose, 1 000 ml distilled water. The nutrient solution had to be clarified by filtration as the growth was determined by turbidity measurements. The solution without sugar was sterilized in an autoclave for 20 min at 120° C.

Glucose and vitamins were added to a sterile mineral salt solution, which was then poured into test-tubes, 10 ml in each. The test-tubes were sterilized for 15 min at 115° C. The pH was regulated so that it should be 7.4 in the tubes before inoculation. Bacteria for inoculation were grown in 1 l Erlenmayer flasks in a water-bath at 30° C. Air was displaced from the flasks with nitrogen gas (washed with sulphuric acid and alkaline pyrogallol solution). The nutrient solution contained all the 8 vitamins which will be mentioned below. From this culture 10 ml were inoculated into 500 ml nutrient solution without any addition of vitamins. With the inoculation was added such an amount of vitamins that the bacteria grew fairly well. The cultivation took place under the same conditions as the earlier one.

From this cultivation 1 ml was inoculated into each of the 10 ml testsolutions in tubes. The test-tubes were placed in a 12 l vacuum desiccator from which air was removed with a suction-pump. The desiccator was then filled with nitrogen gas and alkaline pyrogallol solution placed on the bottom of the desiccator. The nitrogen procedure was then repeated three times. Growth temperature was 30° C, growth period 7 days.

Vitamins used: 1. Folic acid, 2. Ca-pantothenate, 3. Pyridoxin, 4. *p*-Aminobenzoic acid, 5. Riboflavine, 6. Thiamine, 7. Niacin, 8. Biotin.

Vitamins used in different experiments can be seen in Table 1. After 7 days of growth, 1 ml was transferred from each experiment to a new nutrient solution of the same kind. In this way the vitamins which had been added with the inoculation were gradually excluded.

Measuring of the growth. In each experiment there were 5 parallel test-tubes. In 4 of them growth was measured with the Klett-Summerson photometer, filter 42, and the 5th tube used for inoculation into the following 5 tubes containing sterile nutrient solution.

The inoculation was continued until growth had ceased in all 5 test-tubes, when the experiment was finished. After 7 inoculations even those experiments in which the bacteria still grew were discontinued. In these cases the total period of growth comprised 7 weeks. Experiments 6 and 1 + 2 were inadvertently finished after 5 weeks, although the bacteria were still growing.

The figures in Table 1 show photometer readings. The readings are mean values from the readings of 4 tubes. 0 = no growth.

The results of the experiments indicate: Only folic acid has maintained even growth after all the 7 inoculations.

Thiamine has maintained growth after 5 inoculations. The experiment was then inadvertently finished. Since the mixture of riboflavine, thiamine, niacin, and biotin has not been able to maintain growth after the 4th inoculation, thiamine obviously cannot maintain growth alone.

Table 1. Vitamin requirements of Clostridium pasteurianum.

Vitamins added to the basal nutrient solution	1. inoculation	2. inoculation	3. inoculation	4. inoculation	5. inoculation	6. inoculation	7. inoculation
1. Folic acid	64	111	156	161	121	122	104
2. Ca-pantothenate	101	100	116	58	0	0	0
3. Pyridoxin	66	79	150	125	107	69	0
4. *p*-Aminobenzoic acid	72	0	0	0	0	0	0
5. Riboflavine	132	74	0	0	0	0	0
6. Thiamine	110	51	122	104	111	finished	
7. Niacin	62	0	0	0	0	0	0
8. Biotin	61	0	0	0	0	0	0
Combinations:							
1 + 2	110	135	181	187	121	finished	
3 + 4	80	68	72	0	0	0	0
5 + 6	119	0	0	0	0	0	0
7 + 8	68	0	0	0	0	0	0
1 + 2 + 3 + 4	102	130	163	144	124	135	115
5 + 6 + 7 + 8	112	102	115	0	0	0	0
1 + 2 + 3 + 4 + 5 + 6 + 7 + 8	108	116	118	118	118	148	131

4 mg of each of the vitamins 1, 2, 3, and 4, and 20 mg of each of the vitamins 5, 6, and 7, and 10 μg of vitamin 8, were weighed in volumetric flasks and diluted to 100 ml, 20 ml of this solution were put in 1 l nutrient solution.

In nutrient solutions containing *p*-aminobenzoic acid, riboflavine, niacin, and biotin growth has not occurred after the 2nd or 3rd inoculation.

Pantothenic acid and pyridoxin have not been able to maintain growth after the 5th and 6th resp. inoculations.

The experiments with mixtures of vitamins have given results which conform with those obtained with separate vitamins. Only mixtures containing folic acid have maintained even growth during the whole experiments (1 + 2, 1 + 2 + 3 + 4, and 1 + 2 + 3 + 4 + 5 + 6 + 7 + 8; the first one was indeed finished inadvertently after the 5th inoculation, but judging by experiment 1 growth would have been obvious even after this).

On the basis of our experiments folic acid is a decisive factor for the growth of *Clostridium pasteurianum*

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Equilibrium between the aa and ee Conformations of „*Trans*"-1,2-Dichlorocyclohexane

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Electron diffraction observations [1] on "*trans*"-1,2-dibromocyclohexane indicated the presence of approximately equal amounts of the *aa* and *ee* conformations in the gaseous state. Recent dipole moment measurements on "*cis*"- and "*trans*"-1,2-dichlorocyclohexane (both vapour and solution) lead to similar results [2]. We considered the question so important that we started electron diffraction investigations on the 1,2-chlorine derivatives.

The substance designated as "*trans*" was prepared from cyclohexene and chlorine. The product was distilled in a Podbielniak column at 50 mm Hg at the constant temperature of 99° C, it had the refractive index n_D of 1.4885 at 25.5° C. The second substance was kindly placed at our disposal by Dr. Henry C. Stevens, Ohio, U.S.A. and is identical with the "*cis*" compound used for the dipole moment measurements[2].

The radial distribution curves $(\frac{\sigma(r)}{r})$ obtained are reproduced in Fig. 1 together with line diagrams giving the most important interatomic distances calculated for models corresponding to *1a2a*, *1e2e* and *1a2e* configurations. These distances were computed not for strictly tetrahedral valency angles but assuming in the *trans* compound a bending of the *a* C—Cl bonds away from the axis of the carbon ring amounting to 7° and an angle between neighbouring *e* C—Cl bonds of 73.5° (instead of 70.5°). In the case of the "*cis*" compound a corresponding bending of the *a* bond of 8° was assumed.

Curve A of Fig. 1 cannot be explained by only *one* of the line diagrams and must therefore correspond to a mixture of two configurations. As we have all reason to believe that the substance used is a pure chemical substance it must be the "*trans*" compound and consist in the vapour state of a mixture of nearly equal amounts of the *aa* and the *ee* conformations. Curve B is in good agreement with the line diagram drawn for the *ae* configuration ("*cis*" compound).

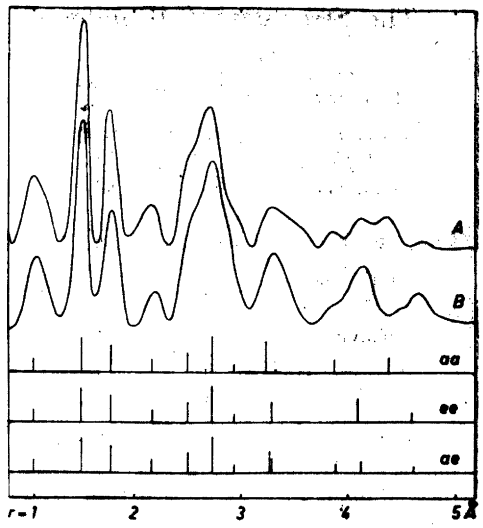

Fig. 1. Curve A: $\frac{\sigma(r)}{r}$*-curve of "trans"-1,2-dichloro cyclo hexane. Curve B:* $\frac{\sigma(r)}{r}$*-curve of "cis"-1,2-dichloro cyclo hexane.*

The results obtained for both *trans* 1,2-dihalides so far studied thus indicate very strongly the presence of nearly equal quantities of the *aa* and *ee* conformations. From an earlier electron diffraction investigation [3] on the vapour of the 1,2-dichloro-4,5-dibromocompound with possible conformations *aa-ee* and *ee-aa* the conclusion was drawn that the conformation having chlorine atoms in *a* positions predominates. These results cannot — it appears — be brought in accordance with simple considerations regarding the interaction between neighbouring not bonded atoms.

We want to express our sincere thanks to Dr. Henry C. Stevens for his kindness in placing a sample of the „*cis*" compound at our disposal.

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The Structure of Bromine 1,4-Dioxanate

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The structure of the solid compound containing equal numbers of bromine and 1,4-dioxan molecules has been determined by X-ray analysis. The monoclinic structure — space group C_{2h}^3-C2/m — is based on a *c*-centered unit cell containing *two* molecules of each species and having the dimensions: $a = 9.65$, $b = 9.05$, $c = 4.25$. The β angle is 91.4°. It was not necessary to take advantage of the fact that the corresponding iodine compound has a similar unit cell in order to derive approximate coordinates for the bromine atoms. The signs of the structure factors derived from these bromine coordinates alone were used when carrying out the Fourier syntheses which led to the electron density maps reproduced in Fig. 1. The calculation of the signs of structure factors using atomic coordinates of bromine, oxygen and carbon derived from these maps did not alter any of the signs used in the *c*-axes synthesis. The atomic coordinates are:

Br	$x = 0.1125$,	$y = 0$,	$z = 0.100$
O	$x = 0.3795$,	$y = 0$,	$z = 0.319$
C	$x = 0.422$,	$y = 0.133$,	$z = 0.466$

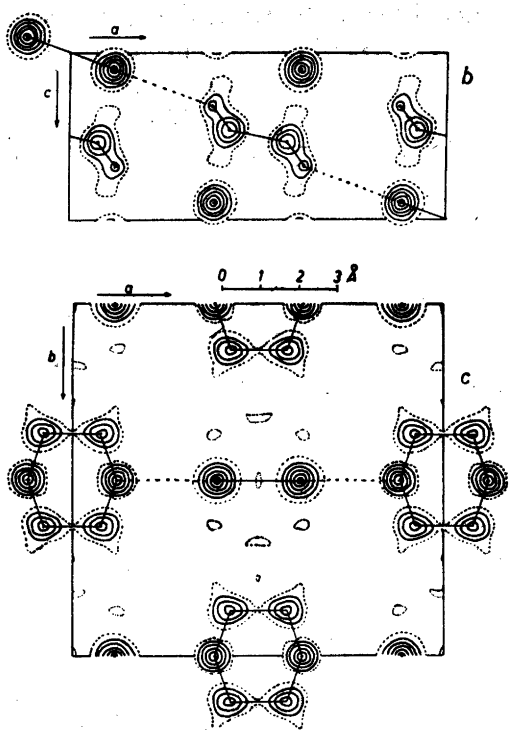

Fig. 1. Electron density maps projected: b. along b *axis, c. along* c *axis. Interval between drawn contour lines about six times larger for bromine than for carbon and oxygen atoms.*

The Br-Br distance within the bromine molecule is found equal to 2.31 Å, a value slightly larger than that of the free molecule (2.28). The line joining the oxygen atoms of two neighbouring dioxan rings contains the two bromine atoms of a Br_2 molecule. The O-Br distance is only 2.71 Å. This is the most striking feature of the whole structure as it indicates a very strong interaction between the bromine and oxygen atoms. The O-Br bond is *not* situated in the plane containing the oxygen atom and the two adjacent carbon atoms. The consequences of these findings will be discussed in a forthcoming, more detailed publication.

Taking into account the dominating influence of the bromine atoms on the diffraction of the X-rays it cannot be expected that the coordinates of the carbon or oxygen atoms are very accurate. Nevertheless the observed distances and angles within the ("chair"-formed) dioxan ring appear rather acceptable: C—C = 1.54, C—O = 1.41 and the angles C—C—O and C—O—C 111° and 116.6°, respectively.

In order to establish whether the structures of molecular compounds between 1,4-dioxan and halides of electropositive elements are based also on strong interaction between halogen and oxygen, work has been started on such compounds. Preliminary results indicate that the structure of the compound with mercuric chloride may have a structure resembling the structure of the bromine compound.

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Methylal as a Solvent in Lithium Aluminium Hydride Reductions

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The number of solvents used in lithium aluminium hydride reductions is very limited and only one of them — diethyl ether — has found more general use. Tetrahydrofuran has proved quite a good medium and a few other solvents such as di-*n*-butyl ether, N-ethyl-morpholine, bis (β-ethoxyethyl)ether (diethyl carbitol) and dioxan have been used for special purposes.

In the course of some other work the need arose for a lowboiling solvent with solvent properties differing from those of diethyl ether. It seemed that methylal (formaldehyde dimethyl acetal), well known as an excellent solvent for a number of organic substances, was a suitable choice. The nonreduction of the acetal group by lithium aluminium hydride has been established by Prins [1], Julian, Meyer and Ryden [2] and Marwel and Hill [3]. The low boiling point of methylal (42°) allows the rapid removal of the solvent from the products and makes it less apt to cause rearrangements. As a solvent it is superior to diethyl ether in a great many instances. *E. g.* does it dissolve to an appreciable extent polycarboxylic acids and polycarbonyl compounds. Glycols and other polyhydric alcohols which often are the reaction products in the reductions concerned are also generally more soluble in methylal than in ether and thus the isolation of products is facilitated. Another advantage is that the set-up with a Soxhlet or continuous extractor often can be omitted. (For experimental details in general see Brown, W. G. *Organic Reactions* 6 (1951) 469.)

Like acetals in general methylal is stable towards alkaline aqueous solutions but is rapidly hydrolyzed by acid solutions especially by mineral acids. This would seem to be a serious drawback in the use of methylal as the aluminium hydroxide formed in the decomposition of the intermediate compounds by water very often is dissolved in dilute sulphuric acid. If, however, the reaction products are stable towards alkali the aluminium hydroxide may be dissolved in dilute aqueous sodium hydroxide. Otherwise it may be dissolved in a solution of sodium potassium tartrate. As mentioned below it is in some cases not necessary to dissolve the hydroxide at all.

The total solubility of lithium aluminium hydride in methylal has not been determined but on refluxing a slurry of the hydride for four hours and clarifying the resulting solution by sedimentation a reagent was obtained containing 5.6 g hydride per 100 g methylal, *i. e.* about a 1.3 *M* solution. This concentration is usually more than adequate for normal purposes. In practice, however, it is more convenient to use a slurry as is the case when ether is the solvent.

A few reductions which we have performed in order to determine the yields are listed in Table 1.

As will be seen the yields are about the same as when the reduction takes place in diethyl ether. It should be mentioned in this connection that methylal apparently has good solvent characteristics for the intermediate metal complexes, a fact which contributes to the good overall yields. It seems therefore safe to recommend methylal as a solvent for lithium aluminium hydride reductions especially in those cases where reactants or products are sparingly soluble in ether.

The methylal used was purified in the following way: A good commercial grade

Table 1.

Reactant	Product	Per cent yield in methylal	Per cent yield in ether
Anthranilic acid	*o*-Aminobenzyl alcohol	95	97 [4]
Phenylacetic acid	β-Phenylethyl alcohol	98—99	92 [4]
Phtalic anhydride	Phtalyl alcohol	99—100	87 [5]
Adipic acid	Hexanediol-1,6	78	Not reported
Sorbic acid	Sorbyl alcohol	92	92 [4]
*Cyclo*pentanone	*Cyclo*pentanol	92	85 [6]
Benzamide	Benzylamine	82	85 [7]

methylal was distilled through a 40 cm Widmer column rejecting head and tail fractions. The distillate was treated overnight with metallic sodium and thereafter refluxed for four hours with freshly cut sodium. Finally it was treated with a small amount of lithium aluminium hydride and distilled through the Widmer column, again rejecting small head and tail fractions.

A typical reduction was performed as follows: 0.01 mole of the substance to be reduced was dissolved in 50 ml methylal. This solution was added to a briskly stirred slurry of lithium aluminium hydride (the calculated amount with 100 % excess) in 25 ml methylal at a rate so as to keep the reaction mixture in gentle reflux. Thereafter was refluxed for one hour, cooled and the excess hydride decomposed by cautiously adding water. A further amount of water was added dropwise until a granular white mass had formed at the bottom of the flask. The methylal layer was decanted and the residue extracted with further amounts of methylal. The extracts were collected, dried with anhydrous sodium sulphate and the solvent distilled off. Usually the residue consisted of a very pure reduction product having nearly the correct melting point or refractive index.

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Kinetics and Equilibria in Flavoprotein Systems

I. A Fluorescence Recorder and its Application to a Study of the Dissociation of the Old Yellow Enzyme and its Resynthesis from Riboflavin phosphate and Protein

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For the first time we are now able to study in detail the kinetics of the reversible reaction between a coenzyme and its apoprotein. We have made use of the disappearance of the fluorescence of flavin mononucleotide when it combines with the specific protein to form the "old yellow enzyme". The fluorescence was recorded with a highly sensitive apparatus that enabled us to record "on" and "off" velocities in the formerly inaccessible concentration region, $10^{-6}-10^{-9}$ *M*. The combination reaction was found to be of the second order, the dissociation reaction of the first order. The influence of pH, salt concentrations and temperature will be reported later.

A few data may be found in the literature on the equilibrium constants of coenzymes with their specific proteins,

$$K = \frac{[\text{Coenzyme}] \times [\text{Protein}]}{[\text{Coenzyme-Protein}]}$$

These values are mainly based upon determinations of overall reaction velocities with excess of substrate, and varied concentrations of coenzymes and proteins. However, kinetic data on the combination and dissociation velocities, according to the equation:

$$\text{Coenzyme} + \text{protein} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{coenzyme-protein}$$

have been lacking, but for one exception. By using rapid spectrophotometry refined to the limits of amplification and stability, Theorell and Chance [1] obtained an approximate value for k_1 in the reaction:

$$2\ \text{DPNH} + \text{ADH (alcohol dehydrogenase)} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (\text{DPNH})_2\text{—ADH}.$$

* Fellow, Norwegian Research Council.

k_1 was computed on the assumption that the two DPNH molecules were bound independently and that the effective molarity of ADH was twice its actual concentration. This assumption may or may not be completely valid. It was not possible to determine k_2 because this would have required dilutions exceeding the practical limits of spectrophotometry. k_2 thus had to be calculated from k_1 and a rather uncertain value of the equilibrium constant K, according to the relationship:

$$K = \frac{k_2(\text{sec}^{-1})}{k_1(M^{-1} \times \text{sec}^{-1})}$$

It should be pointed out that even in the simpler situation when one molecule of coenzyme combines with one molecule of protein, as is the case for the "old yellow enzyme", it can by no means be taken for granted that the combination and dissociation reactions should follow the simple law of mass action, since flavin nucleotides [2] and pyridine nucleotides (see *e. g.* [3]) both are attached to their proteins by more than one linkage.

The velocity constants, k_1 and k_2, are of decisive importance for the overall reaction velocity in the case of "mobile" coenzymes. For instance, in the system DPN + ethanol (both in excess) + ADH, the limiting factor for the reaction velocity is the dissociation velocity (k_2) of $(DPNH)_2$—ADH.

It was desirable to find a more sensitive method than spectrophotometry in order to study the kinetics of the formation and dissociation of coenzyme-enzyme compounds at high dilutions. This would give conveniently low "on" velocities and high enough degrees of dissociation for studying "off" velocities and equilibria.

Fluorescence measurements were found to be around 1 000 times more sensitive than spectrophotometry and in addition to cover a much wider range of concentrations: with our apparatus 10^{-5}—10^{-9} M.

The old yellow enzyme in its oxidized form was found to be a very suitable compound for kinetic studies with the aid of our fluorescence recorder.

In a preliminary communication [4] we have briefly reported some of the results already obtained. We have made use of the fact that the fluorescence of FMN (flavin mononucleotide) is quenched by coupling to the protein [5].

In this paper we shall describe the fluorescence recorder used in these studies, and a means of purifying commercial FMN. The data and calculations of some typical "on reactions" (FMN + apoenzyme → FMN-apoenzyme /O.Y.E./) and "off reactions" (FMN-apoenzyme → FMN + apoenzyme) will be reported.

THE RECORDING FLUORESCENCE METER

Fig. 1 is a block diagram of the different parts of the apparatus.

Optical parts. Stabilized 220 V A.C. (1) is connected with the mercury lamp (3) over the choke (2). In order to avoid stray light the lamp is entirely enclosed in a water-cooled housing (4) equipped with water inlet and outlet (5). The adjustable condensor lens (6) makes the light parallel and sends it through the filter (7), which has a maximal transmission around 360 mμ, thus letting the strong mercury lines at 366 mμ pass through nearly unabsorbed

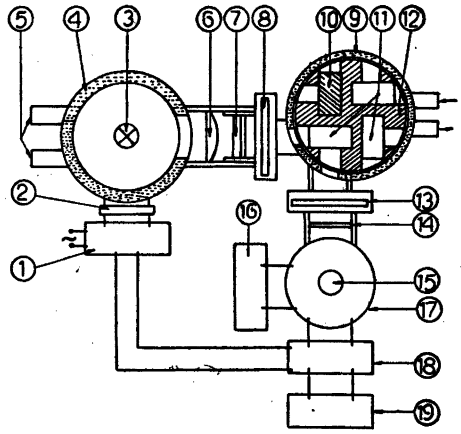

Fig. 1. Fluorescence recording instrument.

1. Stabilizer, Philips, 0—1 000 VA
2. Choke for the lamp
3. Mercury lamp, Philips, H.P. 80 watt (outer bulb removed)
4. Water cooled lamp housing
5. In- and outlets for tap water
6. Condenser lens, f = 65 mm, diameter 40 mm
7. UV filter "Jena UG 2"
8. Entrance slit selector
9. Thermostated water
10. Plexiglas standard
11. Cuvettes, maximum size 20 × 30 mm
12. Cylindrical cuvette holder
13. Exit slit selector
14. Filter for visible light, "Jena GG 5"
15. Photomultiplier RCA 1 P21
16. Battery for 15, 10 × Tudor 90 V
17. Housing for photomultiplier
18. Preamplifier and Power Supply, see Fig. 3
19. Recorder Speedomax, Leeds and Northrup

and minimizing the visible light. The entrance slit selector (8) is a revolving brass disc with eight rectangular openings, 17 mm high, and 1, 2, 3, 4, 5, 6, 7 or 10 mm wide. The entering light intensity used can thus be varied tenfold. The plexiglas standard prism (10), made of colorless, "nonfluorescent" plexiglas and the three cuvettes (11) are located in a revolving aluminium cylinder with windows at right angles for light entrance and exit. The cylinder is surrounded by circulating thermostated water of desired temperature and covered by an aluminium disc with holes for the addition of solutions to the cuvettes. The hole can be shut to exclude external light. Three cuvettes can thus be used intermittently, readings being taken on cuvettes and standard by turning the cylindrical cuvette holder (12). The dark current value is obtained in intermediate positions. The fluorescence light is allowed to fall on an exit slit selector (13), which is similar to (8) and has openings of 17 mm height

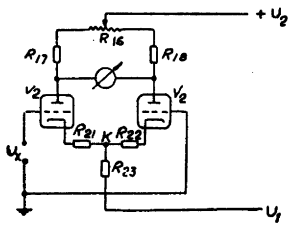

Fig. 2. Simplified diagram showing the general principle of preamplifier.

and 1—6 mm width. The light filter (14) transmits only visible light to the photomultiplier (15) in its housing (17). The distance from the condenser lens to the center of the cuvette is 7 cm, from the cuvette to the photomultiplier 8 cm.

Electrical parts. The preamplifier (18) for the recorder (19) was built especially for the purpose by Mr. K. O. Särnesjö at this institute. A simple diagram is given in Fig. 2. It is "push-pull coupled" and works in the following way. Suppose a positive voltage, U_x, is imposed on the grid of the triode, V_1, the anode current through V_1 and the voltage at point K will increase. This results in an increased negative voltage on the grid of V_2, thereby decreasing its anode current. It can be shown that if the fixed resistance, R_{23}, is sufficiently high the anode current will decrease in V_2 about as much as it will increase in V_1. With this arrangement the voltage variations at point K are kept very small, and negative current feed-back is avoided. The reverse feed-back function is taken care of by resistors R_{21} and R_{22}.

One of the favourable properties of this push-pull coupled amplifier is its high stability. The operating point of the tubes, V_1 and V_2, is determined by the high resistance R_{23} and the fixed grid bias U_1. If U_1 and R_{23} are chosen so that the voltage drop over R_{23} is compensated by U_1, the operating point becomes fairly independent of moderate variations in the power supply or the parameters of V_1 and V_2.

Fig. 3 shows a complete wiring-diagram of the amplifier with photomultiplier, recorder and power supplies. As can be seen, the sensitivity, S, of the apparatus can be regulated at the amplifier within the limits 0.1 V to 100 V per full deflection (10 inches) of the recorder. For checking the performance, a standard cell is provided, and adjustment can be made for full scale deflection of the recorder in the 1 volt range by regulating the variable resistor R_{19}.

The sensitivity of the apparatus for the fluorescence of FMN may be expressed as follows: At sensitivity, $S = 0.1$ V/10 inches, anodic resistance of photomultiplier = 100 kohm, slit openings 6 mm, the recorder makes a deflection of 2 to 2.5 inches for a 10^{-9} M solution of FMN. Under these conditions the recording system is still very stable. If needed, it would be possible to increase the sensitivity still more by increasing the anodic resistance above 100 kohm. However, the limiting factor in the practical use of the apparatus was the fluorescence of the cuvettes and the buffers used. Common glass cuvettes fluoresce too strongly, and thus we had to use cuvettes of "non-fluorescent" glass. The fluorescence of these cuvettes containing distilled water caused deflections around 3 inches at $S = 0.1$ V/10 inches and slit openings 1 and 6 mm. Under these conditions 10^{-8} M FMN adds 3 inches to the

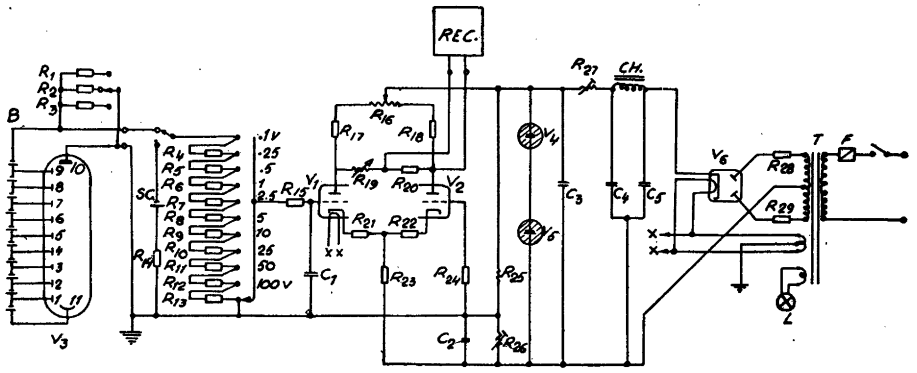

Fig. 3. Wiring-diagram.

R_1 — 20 kohm 1/2 W
R_2 — 50 kohm 1/2 W
R_3 — 100 kohm 1/2 W
R_4 — 600 kohm 1/2 W
R_5 — 200 kohm 1/2 W
R_6 — 100 kohm 1/2 W
R_7 — 60 kohm 1/2 W
R_8 — 20 kohm 1/2 W
R_9 — 10 kohm 1/2 W
R_{10} — 6 kohm 1/2 W
R_{11} — 2 kohm 1/2 W
R_{12} — 1 kohm 1/2 W
R_{13} — 1 kohm 1/2 W
R_{14} — 50 kohm 1/2 W
R_{15} — 620 kohm 1/2 W
R_{16} — 1 kohm (pot.) 1 W
R_{17} — 30 kohm 1 W
R_{18} — 30 kohm 1 W
R_{19} — 20 kohm (pot.) 1 W
R_{20} — 1 kohm 1/2 W
R_{21} — 4 kohm 1/2 W
R_{22} — 4 kohm 1/2 W
R_{23} — 30 kohm 1 W
R_{24} — 1.5 M 1/2 W
R_{25} — 10 kohm 6 W
R_{26} — 3.7 kohm (to be adjusted) 2 W
R_{27} — to be found by testing
R_{28} — 160 ohm 1 W
R_{29} — 160 ohm 1 W

C_1 — 2 000 pF
C_2 — 16 μF 150 V
C_3 — 16 μF 350 V
C_4 — 25 μF 350 V
C_5 — 25 μF 350 V

V_3 — 1P21
V_1, V_2 — E80CC
V_4 — VR150
V_5 — VR105
V_6 — EZ40
L — Lamp, 6,3 V
T — Transformer 2 × 260V, 60 mA
CH — Choke 20 H, 60 mA
F — Fuse 0.5 A
Rec. — Recorder Speedomax 0—10 mV Leeds & Northrup
S. C. — Standard cell for checking recorder sensitivity
B — Battery, 10 × 90 V Tudor

deflection. Of course the salts and buffer substances used had to be pure enough not to cause inconvenient increase of the blank fluorescence. So far it has not been necessary to subject any of the substances employed to further purification. It might be mentioned that our tap water gives a considerable fluorescence.

Of course, the apparatus can be adjusted so as to use the fluorescence level of the blank (cuvette + buffer) as base line instead of the dark current level. However, in this way it is difficult to test for changes in the standard deflec-

tion. The plexiglas standard gave a deflection of about 5 inches at $S = 0.25$ V/10 inches and slit openings 1 and 6 mm. Variations of the deflections were corrected for by measurements of the fluorescence of the standard. For each experiment the deflection of a known solution of FMN was also determined. The relationship between the deflections caused by the plexiglas standard and fresh solutions of FMN has remained the same as long as the apparatus has been in use (six months). This strongly suggests that the fluorescence of the plexiglas standard has kept constant.

MATERIALS AND METHODS

The preparation of the old yellow enzyme (O. Y. E.)

"Gelbes Rohferment" was prepared from brewer's bottom yeast, kindly supplied by Hamburgerbryggeriet, Stockholm. We used the procedure of Warburg and Christian [6] but omitted their methanol and chloroform treatments which involve rather heavy losses. A product which was about 25 % pure was obtained from "Rohferment" by adsorption on $Al(OH)_3$(Cγ) and elution [7] followed by precipitation between 50 and 60 % saturation of ammonium sulphate, dialysis, and repeated adsorption on Cγ and elution. A further purification of the 25 % pure product could be accomplished by electrophoresis in the Tiselius apparatus, using phosphate buffer of pH 7. The best fraction thus obtained contained 5.3 γ FMN per mg of protein and was 83 % pure as calculated from the data obtained for the crystalline enzyme [8]. The electrophoretic pattern showed some colorless components to be present in amounts roughly corresponding to the degree of purity found by the above analysis of the FMN content. Apoenzyme was prepared from this preparation and from a preparation which was only 25 % pure, and the rates of combination with FMN were compared under different conditions. The rates were found to be the same with both preparations. Furthermore, the addition of serum albumin to an apoenzyme did not affect the reaction velocity. Accordingly, we felt confident in using such 25 % pure preparations for our kinetic measurements after having exhausted our supply of the 83 % pure product.

We had available some "Gelbes Rohferment" which was kindly given to H. T. by Otto Warburg as early as 1935. It was of interest to compare O.Y.E. prepared from this product with O.Y.E. prepared from our yeast. It was found that the aluminum-hydroxide and ammonium sulphate purification procedures, when applied to either of the "Rohferments", gave products with very similar degrees of purity and electrophoretic patterns. The two yellow proteins from Berlin and Stockholm had the same sedimentation constants ($S_{20°} = 5.7-5.8$ in the analytical ultracentrifuge, Spinco) in excellent agreement with the sedimentation constant, $S_{20°} = 5.76$, reported in 1936 [9]. Furthermore, the kinetic data obtained in dissociation and recombination experiments with the FMN protein systems from the two preparations gave indistinguishable results. It may be concluded that the O.Y.E. in the form of dry "Rohferment" keeps for at least 19 years.

Apoenzyme was prepared according to the method of Warburg and Christian [10]. We have observed that the apoenzyme is less stable in glass-distilled water than in a dilute (~ 0.01 *M*) sodium-phosphate or ammonium sulphate solution.

Flavin mononucleotide

We have used FMN purified from a commercial product, kindly supplied by Sigma Chemical Company. When this product was subjected to paper electrophoresis in 0.05 *M* ammonium acetate at pH 7 (5 mA, 100 Volts per 22 cm paper), about 10 % of the flavin material did not move in the electric field (called component 1), 80 % (component 2) moved with the same rate, as FMN prepared from O.Y.E., 2.6 cm/hour. 10 % moved faster than FMN, 3.0 cm/hour (component 3). Component 2 contained as much phosphorus as FMN, 1 P per 1 riboflavin, whereas component 3 contained about twice as much. Components 2 and 3 had the same absorption spectrum as riboflavin. Component

2 combined with apoenzyme at the same rate as did FMN, whereas component 3 behaved quite differently. The "synthetic" compound of component 2 and the apoenzyme had the same dissociation velocity constant in 0.01 *M* acetate buffer, pH 3.72, as had O.Y.E.

These experiments give additional evidence to prove that the main component (~ 80 %) of the commercial product is identical with natural FMN. Component 3 may be a riboflavin diphosphate.

The ammonium salt of FMN was not very stable, even when light was kept out carefully. After some weeks of occasional use, a large percentage of FMN was inactive. The deterioration was observed as an increased proportion of fluorescence that did not disappear upon the addition of apoenzyme ("rest fluorescence").

The part of FMN which had deteriorated did not move in our paper electrophoresis experiments. The unchanged FMN and the immobile spot could be washed out separately and the amount of yellow material determined spectrophotometrically at 445 mμ. The deteriorated FMN could thus be determined in two ways: by the rest fluorescence observed in the presence of excess apoenzyme (the usual method), and by paper electrophoresis of the deteriorated solution. The two methods gave similar results. Thus, a solution which was calculated to contain 51 % deteriorated FMN from titration with excess of apoenzyme, was found to contain 57 % deteriorated FMN by paper electrophoresis. The small disagreement could be due to the fact that the electrophoresis experiment was carried out four days after the titration experiment. The fluorescence and the optical density at 445 mμ of a 50 % deteriorated FMN solution was not less than that of a fresh solution. We have not yet investigated the product formed when FMN deteriorates. Obviously the phosphoric acid group is split off, with or without the ribityl group. Usually we did not use solutions of FMN which contained more than 15 % non-coupling flavin material. The light absorption coefficient, β, was taken as 2.7×10^7 cm^2/mole[11].

In order to calculate the concentration of FMN, it was necessary to subtract the fraction of inactivated flavin material from the value found by spectrophotometry.

FMN was kept cold and in the dark as much as possible. During the experiments, the exposure to ultraviolet light was kept to a minimum by the use of small entrance slits. With the most frequently used slits, 1×17 mm, only 3 % of the total volume of the solution was exposed. Only in experiments with very low concentrations of FMN ($c < 10^{-8}$ *M*) was it necessary to use wider entrance slits. In experiments of longer duration than one minute, the solution was kept in the dark most of the time, readings being taken at suitable intervals.

The fluorescence of FMN was found to be proportional to its concentration in all solutions tested. This, of course, holds true only for solutions in which light absorption does not interfere. The quenching of the fluorescence of FMN by different anions has been described previously[4]. The quenching factor, QF, is defined as

$$QF = \frac{\text{The fluorescence in water}}{\text{The fluorescence in the solution.}}$$

The presence of O.Y.E. did not affect the fluorescence of FMN. Before any coupling had taken place at $t = 0$, the fluorescence of FMN was the same in the presence and absence of apoenzyme. It may be concluded that fluorescence measurements can be used to determine the concentration of FMN present in all mixtures of O.Y.E., apoenzyme and FMN.

EXPERIMENTS

Experiment 24 a. Determination of apoprotein concentration by titration with FMN, and calculation of the velocity constant of the "on reaction"

Conditions: Temp. 23°5. Sensitivity, *S*, = 0.5 V per 10 inches deflection was used in the FMN-additions 1—6, 1.0 V/10″ in addition 7. (A photomultiplier 4—5 times less sensitive than the one described was used in this experiment). Slits: entrance 1 mm, exit 6 mm. Cuvettes: 10 × 10 mm (plexiglas). Speed of recording paper, 3 inches per minute.

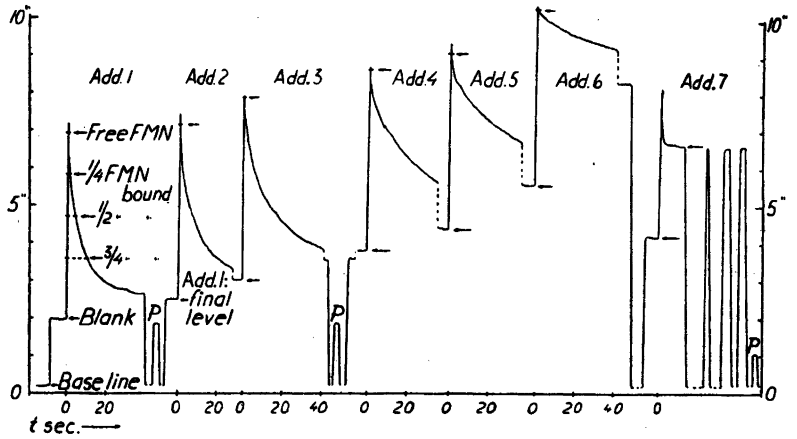

Fig. 4. Titration of apoenzyme with successive additions of FMN (expt. 24).

The levels of "free FMN" (see arrows) were calculated from the deflections caused by the same concentration of FMN without apoenzyme.

The final levels were checked repeatedly after each addition in order to ascertain that equilibrium was reached (indicated only after addition 7). The curves are direct copies of the original records.

P: deflection for plexiglas standard.

Buffer: 3.3 ml Na-phosphate, $\Gamma/2 = 0.1$, pH = 7.05. Apoenzyme: 0.2 ml. FMN solution: 5.55×10^{-4} *M* determined spectrophotometrically. Each addition = 1.96 μl.

The first deflection recorded in Fig. 4 is the blank caused by the fluorescence of the cuvette, buffer and apoprotein. The additions of FMN, from a stirring rod of glass, causes a sudden increase in fluorescence, corresponding to a deflection of about 5 inches. The period of the recorder, 1 sec. per 5 inches, has to be considered in rapid reaction cycles. Immediately after mixing the fluorescence begins to drop, due to the coupling of FMN to the protein. The fluorescence approaches asymptotically a level corresponding to the disappearance of 89 % of the initial fluorescence.

On the assumption that one molecule of FMN couples with one molecule of apoprotein [11] we can calculate the molarity of the apoprotein solution by adding the amounts of FMN coupled in the six first additions. On the seventh addition of FMN no decrease in fluorescence was observed. The results are summarized in Table 1.

Calculation of the "on reaction" velocity constant, k_1.

From the simple relationship

$$1 \text{ FMN} + 1 \text{ apoprotein} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} 1 \text{ O.Y.E.}$$

one would expect the "on reaction" to be of the second order. However, deviations could occur since the combination of FMN with the apoprotein involves the formation of (at least) two bonds, two unknown groups in the protein being attached to the phosphoric acid residue and the imino group (9) of the FMN[2]. It was therefore of considerable interest to calculate k_1 from different points on the experimental curves to test if it came out as a true constant. Since we knew that the rate of the off reaction was very low compared to the on reaction under the conditions used, we felt justified in neglecting

Table 1. "Titration" of apoprotein with FMN.

FMN Addition No.	FMN coupled, M	Per cent total rest fluorescence
1	0.296×10^{-6}	11
2	0.293 »	11
3	0.290 »	11
4	0.289 »	11
5	0.268 »	15
6	0.135 »	—
7	0	—

Total FMN coupled: 1.573×10^{-6} M.
The concentration of the undiluted apoprotein was
$= \frac{3.5}{0.2} \times 1.573 \times 10^{-6} = 27.5 \times 10^{-6}$ M.

the off reaction in calculating the rate constant, at least for the first three additions. We therefore used the simplified formula:

$$-\frac{dc}{dt} = k_1 \times [\underset{a}{\text{Protein}}] \times [\underset{b}{\text{FMN}}],$$

in its integrated form:

$$k_1 = \frac{2.3}{(a-b)t} \times \log \frac{b(a-x)}{a(b-x)}$$

The results are given in Table 2.

As may be seen from the table, the values for k_1 are nearly constant, the variations being within the experimental error. One may conclude that FMN and protein combine in such a way that the formation of one of the linkages greatly favours the formation of the other, so that the combination nevertheless follows the course of a second order reaction. The average value of k_1 from 1—3 is $= 10.6 \times 10^4$ $M^{-1} \times \text{sec}^{-1}$.

Table 2. Calculation of k_1 from different points on recombination curves (Fig. 4).

Addition No.	Fraction FMN bound	Time (sec)	k_1 $M^{-1} \times \text{sec}^{-1}$	k_1 average $M^{-1} \times \text{sec}^{-1}$
1	1/4	1.7	11.1×10^4	10.2×10^4
	1/2	4.7	9.9 »	
	3/4	10.0	9.7 »	
2	1/4	2.0	11.7 »	10.8 »
	1/2	5.5	10.6 »	
	3/4	12.3	10.0 »	
3	1/4	2.4	12.8 »	10.8 »
	1/2	7.3	10.6 »	
	3/4	18.2	9.0 »	

Experiment 24 b

The same apoenzyme was titrated under the same conditions with a solution of FMN which had been prepared some weaks earlier and occasionally exposed to light and to room temperature. It was made up to have the same optical density as the FMN used in expt. 24a and was found to have the same fluorescence. In the presence of excess apoenzyme it had 51 % rest fluorescence. Titration of the apoenzyme with this FMN gave the same value as in 24a, and the values for the velocity constant for the two first additions were 9.8 and 9.2 $\times$ 10^4. It may be concluded that the presence of the deterioration product of FMN does not disturb the on reaction. Nevertheless, the product decreases the accuracy of the experiment by increasing the fluorescence of the blank.

Experiment 51. Determination of dissociation velocity and equilibrium constant

Conditions: Temperature 23.5°. Sensitivity: $S = 0.25$ V/10 inches for expts. (a), (b) and (c); $S = 0.5$ for expt. (d). Slits: entrance 1 mm, exit 6 mm. Cuvettes: 10 $\times$ 10 mm (plexiglas). Speed of recording paper: 3 inches per minute. Buffer: 0.25 *M* with respect to Na-phosphate and 0.4 *M* with respect to NaCl. pH = 5.75. Total volume: 3.5 ml. O.Y.E. solution: 2.38 $\times$ 10^{-5} *M* as determined spectrophotometrically.

Table 3. Dissociation of O.Y.E. in different concentrations (Expt. 51 and Fig. 5). k_2 obtained from initial velocity at $t = 0$. "E" = O.Y.E.

Exp. 51	Initial Molarity of O.Y.E	Initial Velocity $-dc/dt$ $M \times sec^{-1}$	k_2 sec^{-1}	Per cent dissociation at equilibrium	Equilibrium constant, K M	$k_1 = \frac{k_2}{K}$ $M^{-1} \times sec^{-1}$
a	0.102 $\times$ 10^{-6}	12.3 $\times$ 10^{-10}	12.2 $\times$ 10^{-4}	23.5	0.75 $\times$ 10^{-6}	18 $\times$ 10^4
b	0.34 »	42.5 »	12.5 »	13.6	0.70 »	17 »
c	0.95 »	14.3 »	15.1 »	8.4	0.72 »	21 »
d	6.8 »	93 »	13.7 »	2.6	0.67 »	20 »
		Average:	13.5 $\times$ 10^{-4}		Average:	19 $\times$ 10^4

As seen from Table 3, four experiments, a, b, c and d were made with concentrations from 0.102 $\times$ 10^{-6} to 6.8 $\times$ 10^{-6} *M*.

From the equation, FMN-Protein $\xrightarrow{k_2}$ FMN + Protein, one would expect the off reaction to be of the first order, the reaction velocity being proportional to the enzyme concentration

$$-\frac{dc}{dt} = k_2 \times [\text{O.Y.E.}]$$

In one set of calculations we determined the initial velocities, $-dc/dt$ at $t = 0$, from tangents of the dissociation curves drawn at $t = 0$ (see Fig. 5 for expt. c), and obtained the equilibrium constants from the degrees of dissociation after equilibrium had been reached. The on reaction constant, k_1, was then calculated from the formula $K = \frac{k_2}{k_1}$. The results are summarized in Table 3.

k_1 was also calculated by using a method that is independent of the initial velocity at $t = 0$, but dependent on the equilibrium constant, K. The complete formula for a dissociation reaction with opposing "on" reaction was applied to different points on the curves obtained in expt. c, (see Fig. 5):

$$-dc_1/dt = k_2c_1 - k_1 \times c_2 \times c_3$$

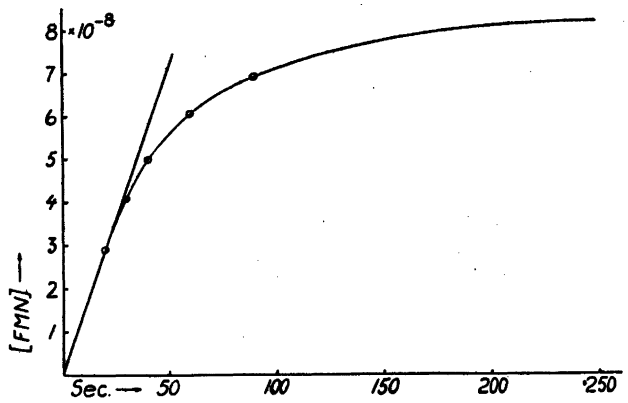

Fig. 5. Dissociation of O.Y.E., expt. 51 c. Ø Points used for the calculations of k_1 according to integrated formula.

where c_1 = [O.Y.E.], c_2 = [FMN], c_3 = [apoenzyme]. If the concentrations at $t = 0$ are $c°_1$, $c°_2$, $c°_3$, the integrated form of this equation is:

$$k_1 t = \frac{1}{2 \times a} \times \ln \frac{c_1 - \frac{2c°_1 + c°_2 + c°_3 + K}{2} - a}{c_1 - \frac{2c°_1 + c°_2 + c°_3 + K}{2} + a} + \text{constant}$$

$$\text{where } a = \sqrt{\frac{(2c°_1 + c°_2 + c°_3 + K)^2}{4} - (c°_1 + c°_2)(c°_1 + c°_3)}$$

We found for expt. c: $k_1 = 22.7 \times 10^4\ M^{-1} \times \text{sec}^{-1}$ after 20 seconds; 23.6×10^4 after 30 seconds; 21.8×10^4 after 40 seconds; 19.9×10^4 after 60 seconds and 17.8×10^4 after 90 seconds. Average: $21.2 \times 10^4\ M^{-1} \times \text{sec}^{-1}$. This value agrees with $k_1 = 21 \times 10^4$ found for expt. c by taking k_2 from the tangent at $t = 0$ and using the equation $K = \frac{k_2}{k_1}$, see Table 3.

In order to determine k_1 by an independent and direct method, we carried out an "on reaction" with FMN + protein in the same buffer and at the same temperature. The value obtained was $k_1 = 17 \times 10^4\ M^{-1} \times \text{sec}^{-1}$, in as good agreement as could be expected with the average value 19×10^4 from expt. 51.

DISCUSSION

The results reported above prove the perfect reversibility of the reaction between FMN and the protein of the O.Y.E. and, furthermore, that the combination reaction is of second order, whereas the dissociation reaction is of the first order under the conditions used in these experiments. Since it must be regarded as established that at least two groups in the FMN — the phosphoric acid residue and the imino group (9) — are bound to two sites in the protein, our results mean that the formation of one linkage so greatly favors the formation of the other one, that no half-coupled intermediates exist in detectable concentrations.

It should be pointed out that the leucoform of FMN or the $FMNH_2$-protein shows no fluorescence and therefore cannot be studied in this way. However, from data on the kinetic and equilibrium constants of the oxidized complex, compared with redox potential measurements to be made we hope to obtain the equilibrium constants of the reduced complex. The velocity constants, k_1 and k_2, are not directly obtainable from such measurements, but on the assumption that k_1 is the same for the oxidized and reduced compounds — as was the case with the DPN- and DPNH—ADH compounds — k_2 can be calculated. We may never be able to determine the dissociation velocity constant of $FMNH_2$-apoprotein (of O.Y.E.) directly, since the redox potential of O.Y.E., as compared with that of riboflavin, is very high. Thus the dissociation velocity of the reduced complex can be expected to be too low to be measured.

One may hope that accurate measurements of the kinetic data on O.Y.E. and other flavoproteins may give some clue to the chemical nature of the FMN-combining groups in the protein.

It may finally be mentioned that we have found fluorescence measurements to be very useful in following the reduction or oxidation velocities in pyridine nucleotide-enzyme systems, since DPNH, but not DPN is fluorescent. In the cases tested so far this fluorescence does not disappear when DPNH is combined with proteins.

Forthcoming papers will demonstrate the profound influence not only of pH and temperature, but also of anions like phosphate and chloride on the kinetics and equilibria of both flavin- and pyridine enzymes.

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A Method for Spectrophotometric Determination of Chenodesoxycholic Acid in Bile

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In 1924 Wieland and Revery [1] isolated a new bile acid from human bile, which they called antropodesoxycholic acid, but which proved to be identical with chenodesoxycholic acid, isolated at the same time from goose bile by Windaus, Bohne and Schwarzkopf [2]. This acid (3,7-dihydroxycholanic acid) could be prepared in relatively large amounts from human bile, but its physiological rôle does not seem to have been studied until the last few years. There has not even been a method for its quantitative determination in bile. Since 1951 there have been published some methods for separating the bile acids by columnar chromatography and determining the different fractions by means of titration (Bergström and Sjövall [3], Sjövall [4]) or infrared spectrometry (Wootton [5] Wootton and Wiggins [6]). It has been shown [6] that chenodesoxycholic acid is to be found in human bile in the same proportion as cholic acid (3,7,12-trihydroxycholanic acid) * while desoxycholic acid (3,12-dihydroxycholanic acid) forms a minor fraction and lithocholic acid (3-hydroxycholanic acid) is usually absent.

A perfect separation of the two dihydroxy acids is however not yet obtainable by chromatographic technique [4-6] and infrared spectrometry is not an ordinary laboratory method. It therefore seemed to be of importance to have such a colorimetric method available as would make it possible to determine the chenodesoxycholic acid in the presence of other bile acids. This was not the case with Saba's [7] different colorimetric methods — the only ones referred to in the literature.

Wieland and Revery [1] showed that chenodesoxycholic acid gave a positive Liebermann-Burchard reaction, and here one modification of this reaction is described, which may be used for quantitative determinations of chenodesoxycholic acid in an extract of hydrolysed bile and in the presence of the other common bile acids.

* According to Saba [7], however, this was already reported in 1939 by Ohyama and Nagaki in a Japanese journal: *Nippon-Shokakibyogakkai-Zasshi.* 38 (1939) 245.

METHOD

Reagents: Sodium hydroxide, 1 *N*; hydrochloric acid, approx. 2.5 *N* (titrated against the *N* hydroxide); ethyl ether, redistilled, peroxide-free; silver nitrate, approx. 0.05 *N* in 3 *N* nitric acid; light petroleum, b.p. 40—70° C, redistilled or Ed. XI; ethanol, 70 % (v/v); ethanol, absolute; ethyl acetate, *p. a.* or Ed. XI. (The grade of the ethyl acetate is very important. I have used the grade Ed. XI, b. p. 74—77° C, free from acid when tested with litmus paper. Technical ethyl acetate must usually be purified, *cf.* Gilman [8]); sulphuric acid, conc. *p. a.*; acetic anhydride, *p. a.*; chenodesoxycholic acid standard *: 1.00 mg/ml absolute ethanol (macro method) or 0.10 mg/ml absolute ethanol (micro method).

A. Hydrolysis and extractions:

1. Hydrolyse a suitable amount (about 0.2—1 ml fresh or 25—100 mg lyophilized bile) with 30 ml *N* sodium hydroxide for 5 hours at 110°—120° C.
2. Neutralize with an equivalent amount of the 2.5 *N* hydrochloric acid and acidify with one additional ml.
3. Dilute to about 50 ml with water and extract with 3 portions of 50 ml ethyl ether. Evaporate the combined extracts to about 50 ml and wash with water until the water gives no opalescence with the silver nitrate solution.
4. Evaporate to dryness and subject the residue to a three-stage counter-current extraction between 70 % ethanol and light petroleum 50 : 50. Evaporate the three combined ethanol extracts to complete dryness, dissolve in absolute ethanol and make up to volume (10.00 ml).

B. Colour reaction (macro method):

5. Evaporate an aliquot amount of the final alcohol extract (plus some standard solutions containing 0.2—1.5 mg chenodesoxycholic acid) to dryness in test tubes on water bath, then put the tubes into an oven at 100° C for about 5 minutes.
6. Prepare the EtS-reagent by mixing ethyl acetate and concentrated sulphuric acid in the proportion 15 : 1; cool if necessary under tap water to room temperature.
7. When the test tubes have cooled down to room temperature add 3.00 ml of the EtS-reagent to a set of the tubes and mix thoroughly with glass-rods.
8. Add 2.00 ml acetic anhydride to the tubes at appropriate intervals. Mix thoroughly again with the glass-rods.
9. Determine the absorbancy in a spectrophotometer at 6 150 Å in a 10 mm cuvette 12—15 minutes after the addition of the acetic anhydride to the tube.

Micromethod: Add to the samples and standards (containing 0.01—0.15 mg chenodesoxycholic acid) 1.00 ml EtS-reagent and at intervals 0.67 ml acetic anhydride. After 12—15 minutes read the absorbancy with a Pulfrich photometer, using 50 mm microcuvettes and filter S 61.

DISCUSSION OF THE METHOD

A. Hydrolysis and extractions. This part is in the main identical with the one ordinarily used for isolation of bile acids, see for instance Bergström and Sjövall [9]. The hydrolysis has proved to be complete — higher temperature, higher concentration of the sodium hydroxide or longer hydrolysing time has not given a higher yield. On the other hand, experiments with pure chenodesoxycholic acid show that nothing is destroyed during the process and at the same time that the extractions are quantitative. Only when working

* The author is indebted to Professor S. Bergström, Lund, for the samples of chenodesoxycholic acid and other bile acids tested in the experiments described in this paper.

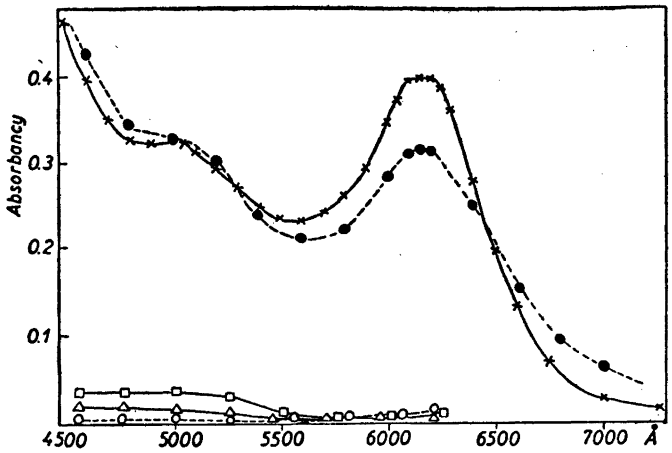

Fig. 1. Absorption curves. × *chenodesoxycholic acid (0.95 mg),* ● *bile extract, prepared as described in the text.* □ *cholic acid (1.0 mg),* △ *desoxycholic acid (1.0 mg),* ○ *lithocholic acid (1.0 mg). In this and following figures EtS = 15 : 1 means a reagent consisting of 15 parts ethyl acetate and 1 part sulphuric acid. EtS:Ac_2O = 3:2 means a mixture of 3.00 ml of the EtS-reagent in use and 2.00 ml acetic anhydride.*

with small amounts (less than 5 mg of the acid) is the recovery less than 95—100 %.

For the colour reaction it is absolutely necessary that the final extract should be quite free from hydrochloric acid and cholesterol. (The reaction will however unmask the presence of minute amounts of these substances, see below). Acidification is therefore recommended with a small amount of dilute hydrochloric acid. The washings with water and the counter-current distribution must not be omitted.

B. Colour reaction. a) The choice of reagents. Chenodesoxycholic acid dissolved in acetic anhydride gives a positive Liebermann-Burchard reaction after the addition of a few drops of concentrated sulphuric acid. The colour is not stable and therefore not suitable for colorimetry. In order to stabilize the colour a number of solvents were tried. With glacial acetic acid a promising absorption curve developed with a maximum at 5 100 Å, which in certain proportions of the reagents showed good colour-stability. However, both cholic acid and desoxycholic acid absorbed at the same wave-length to such an extent (10—20 % of chenodesoxycholic acid) that the method was unsatisfactory. This was also the case when chloroform was used as a solvent.

With ethyl acetate, used instead of glacial acetic acid, only a plateau was formed at 5 100 Å, but in addition it developed a broad-based absorption peak with maximum at 6 100—6 200 Å (Fig. 1). The reagents mixed in certain proportions gave a sufficiently stable colour; the presence of other bile acids had no influence and the absorbancy obeyed Lambert-Beer's law (Fig. 2). Thus, ethyl acetate could be regarded as an ideal solvent.

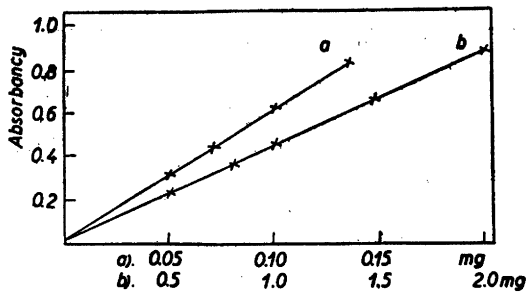

Fig. 2. Absorbancy of chenodesoxycholic acid. a) Micro method: Pulfrich-Photometer, Zeiss, 50 mm microcuvettes, filter S 61. EtS = 15:1; EtS:Ac_2O = 1.00 : 0.67. b) Macro method: Unicam SP 600, 10 mm cuvettes, 6 150 Å. EtS = 15 : 1; EtS:Ac_2O = 3 : 2. This means that the absorbancy for a given amount in the micro method will be the same as for an amount 15 times greater in the macro method.

The amount of sulphuric acid used in the colour reaction must necessarily be comparatively low. Before adding it, it therefore seemed better to mix it with either ethyl acetate or acetic anhydride. The last mentioned mixture is known from Schoenheimer-Sperry's method for determination of cholesterol[10] to be unstable even when kept in an ice-bath. The mixture with ethyl acetate proved to be more stable and was therefore chosen.

b) The amounts of the different reagents. For practical reasons the total volume of the reaction mixture was fixed at 5.00 ml for the determination in a 10 mm cuvette of the Beckman model (which needs 3—3.5 ml).

The composition of the reaction mixture was investigated in order to achieve a) a sufficiently high molar absorbancy and b) a stable absorbancy during a sufficiently long time. From Figs. 3 and 4 it will be seen that a) is mostly under the control of sulphuric acid and b) controlled by the ratio of ethyl acetate: acetic anhydride.

In Fig. 3 the amount of acetic anhydride is fixed at 2.00 ml, the ethyl acetate is nearly constant (2.73—2.86 ml) and the sulphuric acid varies between 0.27 and 0.14 ml. This is achieved by dissolving the chenodesoxycholic acid in 3.00 ml of different EtS-reagents (*i.e.* mixtures of ethyl acetate and sulphuric acids in proportions stated in the figure). Decreasing amounts of sulphuric acid give decreasing absorbancy, but on the other hand increasing amounts give decreasing stability. The best combination of high and stable absorbancy is achieved with the EtS-reagent 15:1.

In Fig. 4 the amount of sulphuric acid is constant and equal to that in 3.00 ml of EtS-reagent 15:1, *i.e.* 0.19 ml. The ethyl acetate (and hence the acetic anhydride too) is however varied as stated in the figure. It is seen that the amount (included the sulphuric acid) must be at least 3.0 ml. On the other hand increasing amounts of ethyl acetate give an inconsiderably increased stability, appearing at about the same absorbancy level and much later. The best reaction mixture will therefore be composed of 3.00 ml EtS-reagent 15:1 and 2.00 ml acetic anhydride.

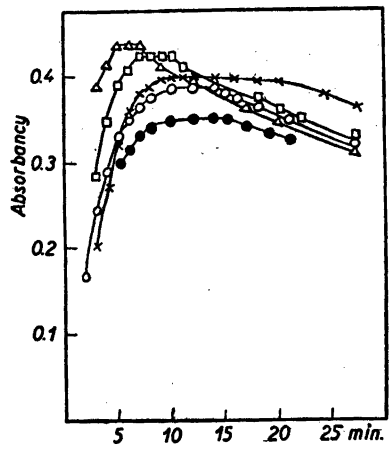

Fig. 3. The effect of the sulphuric acid on the absorbancy level (and stability). EtS: Ac_2O = 3 : 2. EtS-reagent: △ *10 : 1,* □ *12.5 : 1,* × *15 : 1,* ○ *17.5 : 1,* ● *20 : 1.*

c) The influence of external factors. The colour reaction may be performed in darkness or in light, but it is influenced by the external temperature. Fig. 5 shows that it is necessary to develop and read the colour at the same temperature. From the practical point of view it is difficult to use thermo-regulated cuvettes, and as far as I can see there is nothing to prevent work at room temperature. The temperature in the cuvette house of an Unicam spectrophotometer does not increase, not even after being in use for several hours.

d) The course of the reaction. When the reaction is performed in the way mentioned above, a series of different colours is developed. If the substrate is pure chenodesoxycholic acid a faint pink colour appears in the first minute, and in the second to third minutes it changes into dirty brown, soon shifting to dirty green. In the 5th—7th minutes the colour becomes more and more olive, which then remains but increases in intensity. The colour is maximal and stable from the 10th—12th until the 18th—20th minutes, then its intensity decreases slowly and after several hours it turns yellow.

When the substrate is a bile extract or chenodesoxycholic acid subjected to the whole procedure the maximum is reached in the 10th minute after the addition of acetic anhydride. It is stable no longer than till the 15th or 16th minute, and the yellow colour appears quicker, usually after 2—3 hours. The reason for this is possibly the presence of small amounts of hydrochloric acid (see below).

If the EtS-reagent is more than 2 hours old, the course of the reaction is altered. The maximum is reached later, and is stable for a shorter time. It is therefore recommended that a new reagent will be prepared after 2 hours; as seen below the reproducibility is sufficiently good to allow this. The course of the reaction is also altered if the EtS-reagent is added to the substrate more than 30 minutes before the acetic anhydride. In a typical series where

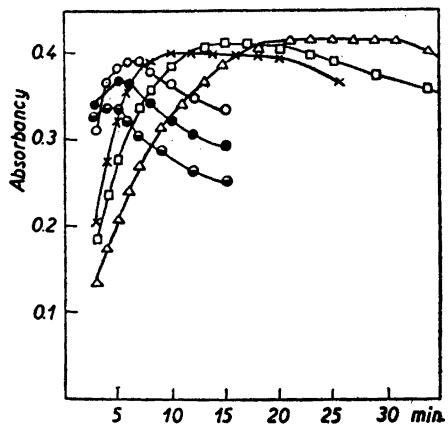

Fig. 4. The effect of the ratio of ethyl acetate to acetic anhydride on the stability (and absorbancy level). The EtS-reagent contains in every case 0.19 ml sulphuric acid (i.e. the same as in 3.00 ml EtS = 15 : 1).

△ *4.0 ml EtS + 1.0 ml* Ac_2O, □ *3.5 ml EtS + 1.5 ml* Ac_2O,
× *3.0 ml EtS + 2.0 ml* Ac_2O, ○ *2.5 ml EtS + 2.5 ml* Ac_2O,
● *2.0 ml EtS + 3.0 ml* Ac_2O, ◒ *1.5 ml EtS + 3.5 ml* Ac_2O.

the EtS-reagent was added 0, 30, 60 and 120 minutes before the addition of acetic anhydride the absorbancy was 0.400, 0.397, 0.388 and 0.373, respectively, for the same amount of chenodesoxycholic acid.

e) Specificity. Most bile compounds except the bile acids will have been eliminated before the colour reaction is performed and therefore without influence. The same is true of the added compounds. As mentioned above minor amounts of cholesterol or hydrochloric acid will influence the reaction. Cholesterol disturbs the stability, and the colour will deepen even after the 20th minute. Hydrochloric acid gives a quickly developing colour which reaches a maximum far below the expected and starts to drop in the 7th—8th minute and turns yellow before the 20th minute.

The final extract sometimes contains a faint amount of pigments; the main part has been destroyed during the hydrolysis. These small amounts in the extract may well be tolerated. It is not necessary and is theoretically wrong, to correct for the absorbancy of the pigments in the EtS-reagent, as its absorption curve changes after the addition of acetic anhydride. In larger amounts, however, the pigments will disturb the reaction, making impossible the simple method of performing the colour reaction on an unhydrolysed bile extract, even if this extract has been freed from cholesterol by the procedure described by Isaksson [11].

The common natural bile acids and some synthetic ones were tested, and from the results, listed in Table 1, it is seen that none of them showed a coloured product except chenodesoxycholic acid. This indicates the importance of the C_7—OH group for the reaction, which is in accordance with the results of

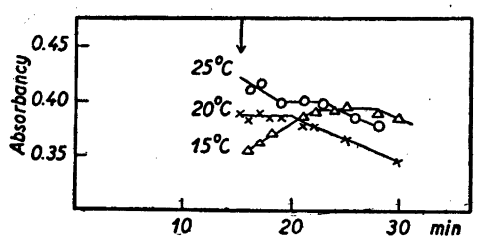

Fig. 5. The effect of temperature. The colours were first developed for 15 minutes at the given temperatures, then read at room temperature (20° C). EtS = 15 : 1, EtS:Ac_2O = 3 : 2.

Idler and Bauman [12]. They tested the Schoenheimer-Sperry modification [10] of the Liebermann-Burchard reagent and pointed out the effect of the position 7 on the reactivity of cholesterol derivatives and certain other sterols. In addition the present results show the inhibiting power of the C_{12}-substituent, either in the form of a hydroxyl or of a keto group.

Thus, the given method is specific for chenodesoxycholic acid among the common natural bile acids. The specificity is also seen in Fig. 1, where the absorption curve for an ordinary prepared extract from human bladder bile is given. In the upper part of the spectrum the course of the curve is nearly identical with that of pure chenodesoxycholic acid; below 5 500 Å the slopes differ owing to the presence of the other bile acids in the extract.

Table 1. The specificity of the colour reaction.

The determinations were made according to the macro method at 6 150 Å with an Unicam S. P. 600 spectrophotometer, using 10 mm cuvettes.

Common name	Structure	Absorbancy per mg substance			
		15 min. after the addition of Ac_2O	20	25	90
Natural bile acids:					
Lithocholic acid	3-monohydroxy	0.010 *	—	0.010	—
Hyodesoxycholic acid	3,6-dihydroxy	0.001	—	0.002	0.006
Chenodesoxycholic acid	3,7-dihydroxy	0.420	0.418	0.395	—
Desoxycholic acid	3,12-dihydroxy	0.000	—	0.001	0.005
Cholic acid	3,7,12-trihydroxy	0.006	—	0.006	0.005
Synthetic bile acids:					
—	3,7-dihydroxy-12-keto	0.009 *	—	0.008	0.006
Dehydrocholic acid	3,7,12-triketo	0.000	—	0.000	0.001
Apocholic acid	3,12-dihydroxy-$\Delta^{8:14}$	0.000	—	0.000	0.002
Sterol:					
Cholesterol	Δ^5-cholestenol	0.435	0.550	0.600	—

* The alcohol extract of the acid was faintly discoloured, owing to small quantities of impurities.

Table 2. Reproducibility of the whole method, when starting with the same or different amounts of lyophilized human bladder bile.

mg dry substance	25	50	100	150	200	Mean
No.	chenodesoxycholic acid as a percentage of dry substance					
66	—	15.0; 16.1; 16.2	—	—	—	15.8
68	—	12.3	14.7	—	—	13.5
72	—	—	12.9; 13.6	—	—	13.2
85	10.9	12.1; 12.1; 13.0	12.4; 12.6	—	—	12.2
97	—	16.1	16.6	—	—	16.4
112	19.2	18.9	20.2	19.1	—	19.4
117	16.1	14.4	15.2	—	14.8	15.1
125	—	—	19.3; 19.5	—	—	19.4
126	—	—	22.0; 23.6	—	—	22.8
127	—	—	17.5; 17.3	—	—	17.4

REPRODUCIBILITY

Colour reaction. Determinations according to the macro method in an Unicam SP 600 on 20 samples of the same amount (0.95 mg) of pure chenodesoxycholic acid gave absorbancy values within the range 0.388—0.403, mean value 0.395 with a standard error of ± 1.3 %. The determinations were performed during several days and in all 12 different preparations of EtS-reagent were used.

Determinations in duplicate on 26 different extracts gave a standard error of ± 2.4 % in the macro method, and 10 duplicate determinations according to the micro method gave a standard error of ± 4.9 %.

In 11 recovery experiments the recovery percentage ranged from 97 to 104 % of the amounts added. Here the pure chenodesoxycholic acid added varied between 10 and 200 % of the original amounts (0.4—0.9 mg) in the bile extract.

The whole method. In all, 29 extracts prepared from different amounts of dry substance from 10 human bladder biles were examined. The standard error was ± 4.8 %. The results are listed in Table 2. Four recovery experiments showed a recovery of 98 to 103 % (to one of the duplicates was added 5.0 mg chenodesoxycholic acid, giving the following results: 1) 22.3 and 28.2 mg, 2) 12.9 and 18.3 mg, 3) 19.3 and 24.3 mg, and 4) 17.5 and 22.0 mg).

This communication is part of an investigation on the differences in the chemical composition of normal and pathological bile, which will be reported elsewhere [13]. However, from the results listed in Table 2 it is seen that the amount of chenodesoxycholic acid is of the same magnitude as is known for cholic acid. This is in accordance with the findings of Wootton and Wiggins [6].

SUMMARY

1. A colorimetric method for the determination of chenodesoxycholic acid in bile is described.

2. The method is a modification of the Liebermann-Burchard reaction and is specific for chenodesoxycholic acid among the common bile acids.

3. The reaction may be performed on an extract of bile acids from hydrolysed bile with satisfactory accuracy.

This is part of an investigation partly supported by a grant from the *Swedish State Fund for the Promotion of Medical Research*. Thanks are due to Miss Ulla-Britt Josefsson for valuable assistance.

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Über einige Reaktionsprodukte von Dimethyldiäthoxysilan mit Aminoalkoholen

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Im folgenden wird die Darstellung der fünf Verbindungen I—V beschrieben

I $(CH_3)_2Si(O.CH_2.CH_2.NH_2)_2$
II $(CH_3)_2Si[O.CH_2.CH_2.N(CH_3)_2]_2$
III $(CH_3)_2Si[O.CH_2.CH_2.N(C_2H_5)_2]_2$
IV $(CH_3)_2Si[O.CH_2.CH_2.N(CH_3)_3J]_2$
V $(CH_3)_2Si[O.CH_2.CH_2.N(C_2H_5)_3J]_2$

Die Verbindungen I—III wurden durch Umsatz von Dimethyldiäthoxysilan mit den betreffenden Aminoalkoholen (β-Aminoäthanol, β-Dimethylaminoäthanol bzw. β-Diäthylaminoäthanol) im Überschuss erhalten. Um *beide* Äthoxygruppen des Dimethyldiäthoxysilans gegen Aminoalkoxygruppen auszutauschen, wurde ausserdem zu den Reaktionsgemischen etwas metallisches Natrium (das sich zu Alkoholat umsetzt) zugesetzt und der gebildete Äthylalkohol in dem Masse adbestilliert, wie er gebildet wurde. Die drei Verbindungen I—III sind Flüssigkeiten. Sie sind etwas löslich in Wasser, worin sie allmählich hydrolysiert werden. Die Hydrolyse erfolgt rascher, wenn zu den Lösungen starke Säuren oder Alkalien zugesetzt werden. IV und V kristallisieren gut und lösen sich leicht in Wasser.

Es wurde oben angenommen, dass die aus Dimethyldiäthoxysilan und β-Aminoäthanol gebildete Verbindung zwei Si—O—C-Bindungen gemäss der Formel I hat. Man kann aber im voraus nicht ohne weiteres eine Struktur mit zwei Si—N—C-Bindungen gemäss VI ausschliessen

VI $(CH_3)_2Si(NH.CH_2.CH_2OH)_2$

welche Verbindung möglicherweise durch Aminolyse des Dimethyldiäthoxysilans entstehen könnte. Die Struktur VI ist aber aus folgenden Gründen ausgeschlossen.

1) Dimethyldiäthoxysilan reagiert nicht mit einem primären Amin $R.NH_2$ in Gegenwart von Natriumalkoholat unter Bildung von $(CH_3)_2Si(NH.R)(OC_2H_5)$ oder $(CH_3)_2Si(NH.R)_2$.

2) β-Dimethylaminoäthanol und β-Diäthylaminoäthanol reagieren leicht mit Dimethyldiäthoxysilan, trotzdem dass sie keine N—H-Bindungen haben.

3) Die aus Dimethyldiäthoxysilan und β-Aminoäthanol erhaltene Verbindung reagiert nicht mit metallischem Natrium unter Wasserstoffentwicklung und kann daher keine Hydroxygruppen enthalten.

4) Die aus Dimethyldiäthoxysilan und β-Aminoäthanol erhaltene Verbindung reagiert heftig mit Methyljodid, was eher für eine freie Aminogruppe als für eine Si-NH-Bindung oder für eine freie Hydroxygruppe spricht.

Die Titrierbarkeit der Verbindung aus Dimethyldiäthoxysilan und β-Aminoäthanol mit Salzsäure (Methylrot) in wässrig-alkoholischer Lösung ist kein Beweis für die Formel I, denn eine Verbindung VI würde auch, wie viele andere Si-NH-Verbindungen, mit Salzsäure unter den erwähnten Bedingungen titrierbar sein [1]. Es ist sehr wahrscheinlich, dass eine Verbindung VI während der Titration mit Salzsäure unter Bildung von titrierbarem Äthanolamin zerlegt wird.

Eine Lichtabsorption, die I im ultraroten Gebiet bei 2,8—3,0 μ zeigt, rührt von den NH_2-Gruppen her. Sie verschwindet, wenn die Wasserstoffatome dieser Gruppen gegen Methylgruppen ausgetauscht werden.

BESCHREIBUNG DER VERSUCHE

Dimethyl-di(β-aminoäthoxy)silan (I). Ein Gemisch von 29,6 g (0,2 Mol) Dimethyldiäthoxysilan, 36,6 g (0,6 Mol) β-Aminoäthanol und 0,2 g Natrium wurde langsam in einem Kolben mit Vigreuxaufsatz und Destillationsrohr erhitzt bis kein Äthylalkohol mehr abdestillierte. Danach wurde fraktioniert. Es wurden 17,5 g I vom Sdp 116—116,5° (20 mm) erhalten. (Gef.: C 40,0; H 9,9; N 15,6; Si 15,7; Äquiv.-Gew. 89,8 *). Ber. für $(CH_3)_2Si(O \cdot CH_2 \cdot CH_2 \cdot NH_2)_2 = C_6H_{18}O_2N_2Si$ (178,3): C 40,4; H 10,2; N 15,7; Si 15,7; Äquiv.-Gew. 89,1).

Dimethyl-di(β-dimethylaminoäthoxy)silan (II). Diese Verbindung wurde in derselben Weise wie I aus 29,6 g (0,2 Mol) Dimethyldiäthoxysilan, 53,4 g (0,6 Mol) β-Dimethylaminoäthanol und 0,2 g Natrium dargestellt. Bei der Destillation des Reaktionsgemisches wurden 18,0 g II vom Sdp 113—115° (21 mm) erhalten. (Gef.: C 51,2; H 11,2; N 12,0; Si 12,2; Äquiv.-Gew. 118,0. Ber. für $(CH_3)_2Si[O \cdot CH_2 \cdot CH_2 \cdot N(CH_3)_2]_2 = C_{10}H_{26}O_2N_2Si$ (234,4): C 51,2; H 11,2; N 11,95; Si 12,0; Äquiv.-Gew. 117,2).

Dimethyl-di(β-diäthylaminoäthoxy)silan (III). 29,6 g (0,2 Mol) Dimethyldiäthoxysilan, 70,2 g (0,6 Mol) β-Diäthylaminoäthanol und 0,2 g Natrium ergaben, wie bei der Darstellung von I und II beschrieben worden ist, 40,6 g III vom Sdp 152—153° (20 mm). (Gef.: C 57,9; H 11,9; N 9,8; Si 9,7; Äquiv.-Gew. 145,4. Ber. für $(CH_3)_2Si[O \cdot CH_2 \cdot CH_2 \cdot N(C_2H_5)_2]_2 = C_{14}H_{34}O_2N_2Si$ (290,5): C 57,9; H 11,8; N 9,6; Si 9,7; Äquiv.-Gew. 145,2).

Additionsprodukt von II und Methyljodid (IV). Zu einer Lösung von 4,7 g (0,02 Mol) II in 25 ml Aceton wurden unter Kühlung eine Lösung von 6,3 g (0,04 Mol + 10 %) Methyljodid in 25 ml Aceton zugetropft. Nach 1 Stunde wurde die gebildete Kristallmasse abgesaugt und sorgfältig mit Aceton gewaschen. Ausbeute: 9,4 g Kristalle (IV) vom Schmp 191—192° (Zersetzung). Nach Umkristallisation aus Wasser + Aceton war der Schmp 192—193° (Zersetzung). (Gef.: C 27,7; H 6,2; N 5,4; I 49,1; Si 5,4. Ber. für $(CH_3)_2Si[O \cdot CH_2 \cdot CH_2 \cdot N(CH_3)_3J]_2 = C_{12}H_{32}O_2N_2J_2Si$ (518,3): C 27,8; H 6,2; N 5,4; J 49,0; Si 5,4).

Additionsprodukt von III und Äthyljodid (V). 5,8 g III in 25 ml Aceton und 7,0 g (0,04 Mol + 10 %) Äthyljodid in 25 ml Aceton wurden vermischt. Am nächsten Tage wurden 6,5 g Kristalle (VI) abgesaugt und aus Aceton umkristallisiert. Schmp >225° (Zersetzung). (Gef.: C 36,1; H 7,5; N 4,7; J 42,3; Si 4,6. Ber. für $(CH_3)_2Si[O \cdot CH_2 \cdot CH_2 \cdot N(C_2H_5)_3J]_2 = C_{18}H_{44}O_2N_2J_2Si$ (602,5): C 35,9; H 7,4; N 4,7; J 42,1; Si 4,7).

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* Die Äquivalentgewichte wurden hier und im folgenden durch Titration mit Salzsäure (Methylrot) in einem Gemisch von Wasser und Alkohol ermittelt.

ZUSAMMENFASSUNG

Es wurde die Darstellung von Dimethyl-di(β-aminoäthoxy)silan und einige seiner N-Alkylderivate beschrieben.

LITERATUR

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Fractional Precipitation of Serum Proteins by Means of Specific Anions

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This paper presents an investigation of the fractional precipitation of serum proteins with protein precipitating anions as described in a previous report [1]. The method constitutes a general application of procedures, which have been used occasionally by different authors in the purification of various proteins [2-8]. A selective precipitation could be produced by acidification of a protein solution containing such ions. By subsequent neutralisation and dialysis the protein compounds formed could be split again and the proteins regenerated. Serum from different species, as well as the effect of different anions and varying conditions of precipitation have been studied.

MATERIALS AND PROCEDURES

The anions tested here were sulfosalicylate, tungstate, molybdate, phosphomolybdate, ferrocyanide, silicofluoride and metaphosphate. Most of the investigations were performed with bovine serum obtained from recalcified oxalated ox plasma. Experiments were also made with human and horse serum and with samples of antidiphtheria or antitetanus horse serum (kindly supplied by *The State Serum Institute*, Copenhagen). Before precipitation the serum samples were diluted with approximately equal volumes of phosphate buffer $M/15$ pH 7.6 containing NaCl to $\mu = 0.2$.

Three different procedures were used in performing the precipitations. The first two of these methods were generally used in preliminary experiments. The third method became the method of choice when more detailed information was necessary.

Method I: Various amounts of precipitating anion were added in the form of solutions of the free acid, and the resulting pH values were recorded. *Method II:* A certain pH value was maintained by means of HCl during addition of the precipitating anion, which was used in the form of a solution of the sodium salt. *Method III:* An excess of the precipitating anion (in solution as sodium salt) was added between pH 7 and pH 9, and the precipitation was achieved by adding HCl. All precipitations were performed in the cold (ice bath). A cooled centrifuge was used for the separation of the precipitates, which were redissolved in an appropriate amount of 0.9 % sodium chloride by addition of small amounts of solid $NaHCO_3$. After thorough dialysis against phosphate — sodium chloride buffer all precipitates and supernatants were analysed by electrophoresis in our Tiselius apparatus [9]. None of the precipitates were washed because the presence of a

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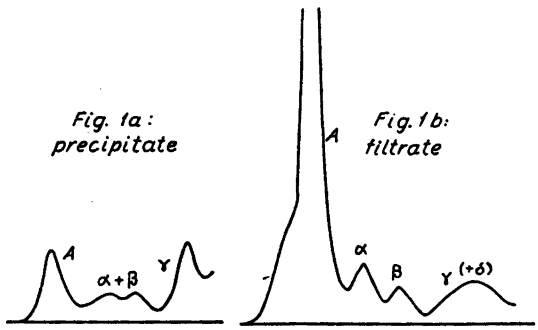


Fig. 1. Precipitate (a) and supernatant (b) from precipitation of bovine serum with tungstate. 20 ml serum + 20 ml NaCl-phosphate buffer pH 7.6 + 0.6 ml 1 M sodium tungstate brought to pH 6.4 with 1 N HCl. Electrophoresis: NaCl (μ = 0.15) — phosphate (μ = 0.05) buffer, pH 7.6; a. 150 min., 18.8 mA; b. 154 min. 19.0 mA. Ascending pattern.

small amount of albumin in the samples facilitated the comparison of the various electrophoresis diagrams. With a few exceptions the mobilities of the protein components remained completely unchanged by the fractionation procedures.

RESULTS

The experiments presented in the following are illustrative examples. They represent only a small number of the experiments performed.

Precipitation of bovine serum with tungstate. A solution of sodium tungstate (0.6 ml 1 *M* tungstate) was added to four 40 ml samples of diluted bovine serum and precipitated by addition of 1.20, 1.55, 1.70 and 2.05 ml 1 *N* HCl respectively (Method III). The resulting pH values obtained were 6.4, 6.0, 5.6 and 5.2 respectively. The composition of precipitate and supernatant in the first sample (pH 6.4) is shown in Fig. 1. The precipitate contained the bulk of the γ-globulin and only small amounts of albumin and other globulins. The electrophoresis patterns obtained from the precipitates and supernatants resulting from the other samples showed that the composition was only slightly changed by decreasing the pH-values to 5.2. At this point appreciable quantities of albumin and of α- and β-globulin appeared in the precipitate (Fig. 2).

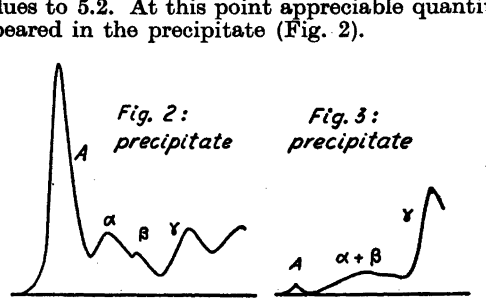


Fig. 2. Precipitate from bovine serum with tungstate. 20 ml serum + 20 ml NaCl-phosphate buffer pH 7.6 + 0.6 ml 1 M sodium tungstate brought to pH 5.2 with 1 N HCl. Electrophoresis: NaCl (μ = 0.15)-phosphate (μ = 0.05) buffer pH 7.6; 150 min., 190. mA. Ascending pattern.

Fig. 3. Precipitate from bovine serum with tungstate at pH 6.2, redissolved in NaCl — phosphate buffer and reprecipitated at pH 7.0. Procedure similar to that in Figs. 1 and 2. Electrophoresis: NaCl (μ = 0.15) — phosphate (μ = 0.05) buffer pH 7.6; 160 min., 19.0 mA. Ascending pattern.

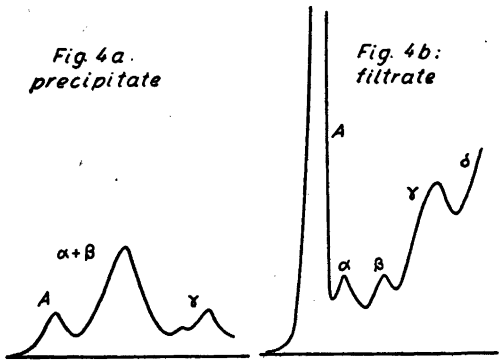


Fig. 4. Precipitate (a) and supernatant (b) from precipitation of bovine serum with sulfosalicylate. 20 ml serum + 20 ml NaCl — phosphate buffer pH 7.6 brought to pH 4.0 with 1 N HCl and precipitated with 3.8 ml 0.2 M sodium sulfosalicylate. Electrophoresis: NaCl (μ = 0.15) — phosphate (μ = 0.05) buffer pH 7.6; a. 150 min., 18.8 mA; b. 129 min., 18.7 mA. Ascending pattern. Concentration of precipitate relative to supernatant: 2.8.

A sample of bovine serum was precipitated with tungstate at pH 6.2 (Method III), redissolved in phosphate buffer and reprecipitated with tungstate at pH 7.0. The electrophoretic patterns showed that the precipitate after the second precipitation consisted of almost pure γ-globulin (Fig. 3).

In another experiment acid was added to the supernatant from the first tungstate precipitation. Bovine serum was first precipitated with tungstate at pH 6.8 and the precipitate removed. To the supernatant more acid was added in order to complete the precipitation of γ-globulin. However, the resulting precipitate contained only traces of γ-globulin thus indicating a maximal precipitation of γ-globulin at pH 6.8. Even after precipitation at pH 4.7 the supernatant still contains a certain amount of γ-globulin.

This observation prompted a further investigation of the action of tungstate on a solution of γ-globulin. A sample containing mainly γ-globulin and only small amounts of the other protein components was therefore prepared from 120 ml bovine serum by repeated precipitation with 40 % saturated ammonium sulfate. The sample (35 ml) was dialyzed against 0.9 % sodium chloride, mixed with 30 ml phosphate-sodium chloride buffer and 20 ml 0.1 *M* sodium tungstate, and precipitated by lowering the pH to 5.4 with 0.5 *N* HCl (Method III). Refractometric measurements showed that about 2/3 of the γ-globulin had been precipitated. The filtrate, containing the rest, was now concentrated *in vacuo* to 10 ml and precipitated with 8.5 ml buffer, 6 ml 0.1 *M* tungstate, and lowering the pH to 5.4 with HCl. Refractometric measurements showed that the precipitated γ-globulin represented again 2/3 of that originally present. This incomplete precipitation of γ-globulin by means of tungstate is possibly caused by the solubility of the protein compound formed and is probably not caused by a qualitative difference in the precipitation of the components of the γ-globulin.

Precipitation of bovine serum with sulfosalicylic acid. When sulfosalicylate was used as precipitating anion results different from those obtained with tungstate were found. The electrophoretic patterns of the precipitate and supernatant obtained after addition of 3.8 ml 0.2 *M* sodium sulfosalicylate to 40 ml diluted serum at pH 4.0 are presented in Fig. 4 (Method II). In contrast to the results obtained with tungstate the precipitate consisted chiefly of α- and β-globulin. At lower pH values increasing amounts of albumin were precipitated, while most of the γ-globulin remained in solution. It is remarkable in this experiment, that in the diagram of the precipitate no resolution was obtained between α- and β-globulin. They appear as a single peak with an intermediate mobility. This indicates that the described fractionation procedure involves changes in the protein molecule, which may not be completely reversed by subsequent dialysis.

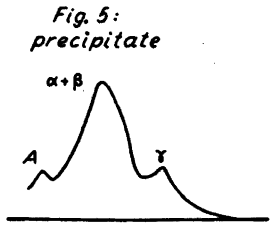


Fig. 5. Precipitate from bovine serum with sulfosalicylate. 19 ml serum + 19 ml NaCl — phosphate buffer pH 7.6 brought to pH 4.0 with 2.28 ml 10 per cent sulfosalicylic acid. Electrophoresis: NaCl (μ = 0.10) — phosphate (μ = 0.10) buffer pH 7.6; 75 min., 22 mA. Descending pattern.

Similar results were obtained when sulfosalicylic acid was added to the buffered serum according to Method I. The amounts used in such an experiment were 1.50, 1.70, 2.28, 3.10 and 3.95 ml 10 % sulfosalicylic acid to 38 ml diluted serum, and the corresponding pH values were 4.7, 4.3, 4.0, 3.5 and 3.0. The composition of the precipitate obtained at pH 4.0 is shown in Fig. 5.

Precipitation of human serum with tungstate. When sodium tungstate was added to diluted human serum (Method III) results as presented in Fig. 6 were obtained. It appears, that the β-globulin, and to a lesser degree the α-globulin, is precipitated first, followed at lower pH values by albumin and some γ-globulin. The range investigated was from pH 7.2 to pH 6.0. This result differs from that obtained by precipitation of bovine serum with tungstate but is similar to the results found when sulfosalicylic acid was used on bovine serum. This observation indicates a fundamental species difference in the properties of the serum proteins. It is interesting to note that the selective precipitation of α- and β-globulins also in this case is characterized by the appearance of one single peak with a mobility slightly greater than that of the β-globulin in the original sample. Neither the precipitate nor the supernatant showed the usual β-anomaly after the fractionation procedure.

Precipitation of bovine and human serum by other anions. Among the other precipitants, molybdate, phosphomolybdate, and ferrocyanide showed properties similar to those of tungstate though the selectivity of the precipitations was slightly less. Metaphosphate precipitated γ-globulin rather selectively. Fig. 7 illustrates the composition of the precipitate after adding 2.3 ml 10 % HPO_3 to 40 ml buffered bovine serum (pH 4.8). The precipitate consists almost exclusively of γ-globulin. Experiments with silicofluoride showed this substance to be a completely unspecific precipitant. One experiment on human serum is presented in Fig. 8. Precipitate and supernatant contain all the pro-

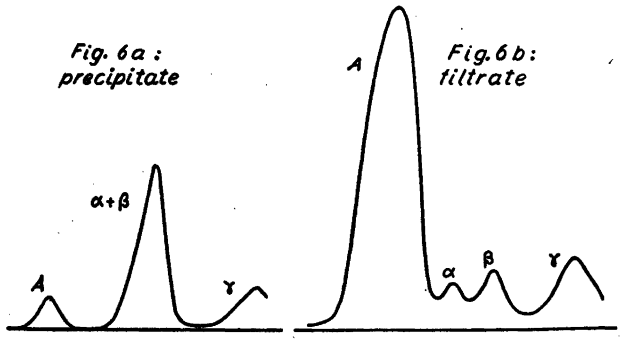
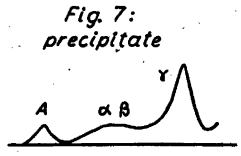


Fig. 6. Precipitate (a) and supernatant (b) from precipitation of human serum with tungstate. 10 ml serum + 14 ml NaCl — phosphate buffer + 6.2 ml 0.1 M Sodium tungstate brought to pH 6.35 with 0.2 N HCl. Electrophoresis: NaCl (μ = 0.025) — phosphate (μ = 0.075) buffer pH 7.55; 120 min., 18.0 mA. Descending pattern. Concentration of precipitate relative to supernatant: 3.1.

Fig. 7. Precipitate from bovine serum with metaphosphate. 20 ml serum + 20 ml NaCl — phosphate buffer brought to pH 4.8 with 2.3 ml 10 % metaphosphoric acid. Electrophoresis: NaCl (μ = 0.15) — phosphate (μ = 0.05) buffer; pH 7.5; 150 min., 18.5 mA. Ascending pattern.



tein components in about equal proportions. Similar results were obtained with bovine and horse serum. Silicofluoride may thus be considered unsuited for serum protein fractionation.

Precipitation of horse serum. When normal horse serum was precipitated with tungstate, results similar to those obtained with bovine serum were obtained. Thus in an experiment where normal horse serum was precipitated at pH 5.8 with tungstate according to Method III, the first precipitate consisted mainly of γ-globulin accompanied by small amounts of α- and β-globulins (Fig. 9). However, varying results were obtained when immune sera were used. Thus when antidiphteria or antitetanus serum were precipitated by tungstate the α- and β-globulins were precipitated first (at pH 5.6) together with some γ-globulin (Fig. 10). At lower pH values increasing amounts of γ-globulin and albumin were found in the precipitates.

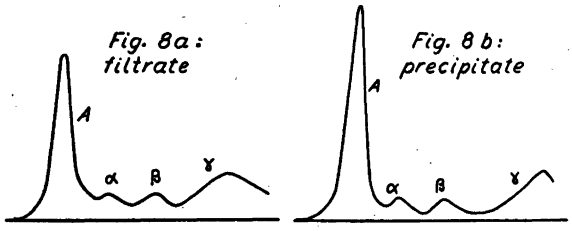
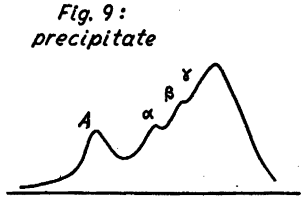


Fig. 8. Precipitate (a) and supernatant (b) from precipitation of human serum with silicofluoride. 20 ml serum + 40 ml saturated neutral Na_2SiF_6 brought to pH 6.5 with 0.5 N HCl. Electrophoresis: NaCl-phosphate buffer pH 7.6; a. 150 min., 18.2 mA; b. 150 min., 18.0 mA. Ascending pattern.

Fig. 9. Precipitate from normal horse serum with tungstate. 20 ml serum + 20 ml NaCl — phosphate buffer + 0.7 ml 1 M sodium tungstate brought to pH 5.8 with 1 N HCl. Electrophoresis: NaCl (μ = 0.15) — phosphate (μ = 0.05) buffer pH 7.5; 151 min., 18.0 mA. Descending pattern.



DISCUSSION

The fractional precipitation of serum proteins has always been a mattes of much importance, and in the course of time several different procedurer have been tried. In the methods mostly used the fractionation is achieved through the addition of various amounts of certain salts, such as ammonium sulfate, sodium sulfate and others. Large scale preparation of several serum protein components has also been accomplished by precipitation with alcohol in a procedure mainly developed by Cohn *et al.*[10]

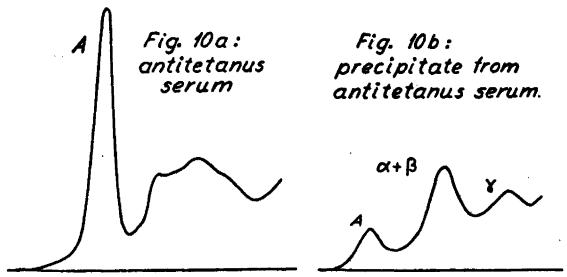


Fig. 10. Heat sterilized antitetanus serum from horse (a) and precipitate obtained with tungstate (b). 20 ml serum + 20 ml NaCl — phosphate buffer + 0.7 ml 1 M sodium tungstate brought to pH 5.6 with 1 N HCl. Electrophoresis: NaCl (μ = 0.15) — phosphate (μ = 0.05) buffer pH 7.5; a. 150 min., 18.2 mA; b. 154 min., 18.8 mA. Ascending pattern. Concentration of precipitate relative to original serum: 3.8.

The method of serum protein fractionation with specific anions differs from most other procedures of fractionation in that it is not based on the different solubilities of the native proteins. We may suppose that the added anions combine reversibly with the basic groups of the protein molecules. By addition of acid the carboxylic groups are deprived of their negative charge and the protein compounds will precipitate according to their solubilities, which will depend to a high degree on the character and number of bound anions.

In spite of the promising possibilities of this method it has so far only been used in a few cases, such as the purification of mare serum gonadotropin [2,3], precipitation of egg albumin [4], and the purification of renin [5,6], of cathepsin [7], and of pepsin inhibitor [8].

In the present paper an attempt has been made to achieve a fractionation of the serum proteins from different species. The various anions tested showed a considerable variability in selectivity. The best separations were obtained with tungstate, sulfosalicylate, and metaphosphate. From bovine serum γ-globulin was precipitated, when tungstate was added at pH of about 6.4, or metaphosphate at pH 4.8. In contrast to this the addition of sulfosalicylate at pH 4.0 caused a precipitation of α- and β-globulin. It is thus evident that the composition of the precipitated proteins as well as the appropriate pH depend on the anion used. Furthermore a distinct species difference could be demonstrated. The fractionation of human serum with tungstate caused a precipitation of α- and β-globulin very similar to that obtained from bovine serum with sulfosalicylate, and in contrast to the γ-globulin precipitated from bovine serum by tungstate. Similar variations were encountered with normal horse serum and heat treated horse immune sera, the main constituents of the tungstate precipitates being γ-globulin in the first case, α- and β-globulin in the second.

It was occasionally found that the separations were accompanied by changes in the β-globulin fraction. The β-anomaly of the human serum disappeared, and in several cases α- and β-globulin moved as a rather broad single peak, indicating some degree of complex formation. These effects are not necessarily due to real changes in the proteins such as denaturation, but

might be explained by the removal of certain components by the precipitation procedure.

When the precipitations were interrupted after only a small amount of protein had been precipitated, it was possible to obtain these first fractions with a high degree of purity. In this case, however, the filtrate contained an appreciable amount of the fraction in question. Attempts to achieve a more complete precipitation by lowering the pH were not successful, as this led to the precipitation of considerable amounts of other fractions as well. Some experiments indicate that the solubility of the anion compounds does not entail a quantitative precipitation, even at the optimal pH-value. These difficulties might be overcome by the addition of neutral salts or of a small amount of alcohol as used in the fractionation procedures of Cohn *et al.* [10,11] It is thus probable that the optimal conditions and the most suitable agents have as yet not been found and that suitable variations may lead to much more complete separations.

Three precipitation procedures were used. The first of these was considered to be of least value, as pH and concentration of the precipitating anion could not be varied independently. The second method, in which the precipitant was added at a constant pH value, should be preferred, if the differences between the protein compounds formed depended mainly on the affinity to the added anion. The third procedure, in which the precipitation was performed at different pH values in the presence of an excess of anion, should be preferred if the compounds differed in their solubility and in the position of their isoelectric points. In those instances where different precipitation procedures were used with the same anion, no significant differences in the composition of the precipitates could be noted. It must be admitted that only few such experiments were performed as it seemed most promising to work with an excess of anion as in procedure III, which was therefore generally preferred.

Cohn *et al.*[11] have described a fractionation procedure, which takes advantage of the specific combination of the proteins from human serum with various metals and of the characteristic solubilities of the compounds. In the present paper the possibility and value of using specific anions for similar fractionations has been demonstrated. The method may prove a valuable supplement to existing methods for protein fractionation.

SUMMARY

1. The low solubility of compounds between proteins and inorganic and organic anions has been used in the fractionation and isolation of serum proteins.

2. The course of the fractionation depends on the character of the anion as well as on the species from which the serum sample was obtained.

3. The investigation shows that the precipitation by means of specific anions deserves consideration as a method supplementing the generally applied methods for serum protein fractionation.

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On Attainment of the Stationary (Steady) State in Nucleation

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The steady state treatment of the nucleation process in precipitation reactions has been supplemented by a treatment which takes the time of attainment of the steady state into account. The results are only valid in a time interval in which the composition of the solution is effectively the original one.

Comparison with experiments from elsewhere indicates that the attainment of the steady rate of nucleation is nearly instantaneous.

Careful comparison of the author's [1] treatment (1951) of nucleation kinetics with Becker and Döring's [2] treatment (1935) shows exact agreement in principle. Both of them are incomplete in so far as only the steady state was considered.

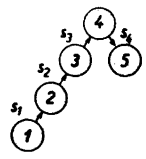
The basic idea in the author's paper was that the nucleation process begins with the successive formation of polymer clusters of increasing size. Polymer clusters have the property of being much more prone to lose a monomer than to capture an additional one.

In contrast to this a nucleus has the property of being much less prone to lose a monomer than to capture an additional one. In the flow sheet (I) below X_4 is the last cluster and X_5 the nucleus. If follows that the further growth of nuclei to visible crystallites is assumed to be effectively irreversible.

We shall now try to complete the treatment of the case of formation of polymers from monomers.

Let the sequence of reactions (1) and the corresponding flow-sheet (I) be

$$\begin{aligned} X_1 + X_1 &\rightleftharpoons X_2 \quad (\pm 1) \\ X_1 + X_2 &\rightleftharpoons X_3 \quad (\pm 2) \\ X_1 + X_3 &\rightleftharpoons X_4 \quad (\pm 3) \\ X_1 + X_4 &\rightarrow X_5 \quad (4) \end{aligned} \qquad (1)$$



(I)

The resulting rates of the reactions (s_i) are

$$\begin{aligned} x_1w_1 - x_2w_{-1} &= s_1 \\ x_2w_2 - x_3w_{-2} &= s_2 \\ x_3w_3 - x_4w_{-3} &= s_3 \\ x_4w_4 &= s_4 \end{aligned} \tag{2}$$

When this system is solved for x_1 we get

$$1 = s_1\varphi_1 + s_2\varphi_2 + s_3\varphi_3 + s_4\varphi_4 \equiv u_1 + u_2 + u_3 + u_4 \tag{3}$$

$$\varphi_1 = 1/x_1w_1;\ \varphi_2 = \varphi_1w_{-1}/w_2;\ \varphi_3 = \varphi_2w_{-2}/w_3;\ \varphi_4 = \varphi_3w_{-3}/w_4 \tag{4}$$

In (2) the w_{-i} are constants while the w_{+j} depend on the concentration x_1 of the monomer X_1 and through this on time. x_i and s_i may depend explicitly on time. We shall assume that during the nucleation x_1 is effectively equal to its initial value a. This must be true according to an estimate due to La Mer [3] and based on his comprehensive knowledge of the Tyndall effect: For a precipitate to be visible polymers must contain about 10^9 monomers each and there must be about 10^6 such individuals in one cubiccentimetre of the suspension. On the other hand if the initial concentration of the monomer is 0.01 molar it contains about $6 \cdot 10^{18}$ monomers in one cubiccentimetre.

But as a matter of course if the polymers are left to grow by diffusion they will soon collect a major part of the initial monomers, thereby stopping further nucleation. It may be therefore a permissible procedure as an approximation to treat the two phases, nucleation and growth of nuclei, separately although in principle they must evidently result from the integration of one and the same set of differential equations. After this digression we return to our calculations, considering from now on all the w's and therefore also all the φ's as constants. Multiplication of the equations (2) with $\varphi_1, \varphi_2 \ldots$, respectively, differentiation with respect to time (indicated by a dot) and use of (4) and (5)

$$\begin{aligned} x_1w_1\varphi_1 &= 1 \\ s_2 - s_1 &= -\dot{x}_2 \\ s_3 - s_2 &= -\dot{x}_3 \\ s_4 - s_3 &= -\dot{x}_4 \end{aligned} \tag{5}$$

finally leads to the following system of equations for the u's

$$\begin{aligned} u_1w_{-1} - u_2w_2 &= -\dot{u}_1 \\ -u_1w_{-1} + u_2(w_2 + w_{-2}) - u_3w_3 &= -\dot{u}_2 \\ - u_2w_{-2} + u_3(w_3 + w_{-3}) - u_4w_4 &= -\dot{u}_3 \\ - u_3w_{-3} + u_4w_4 &= -\dot{u}_4 \end{aligned} \tag{6}$$

One set of solutions of this system can readily be found by equating the right hand sides of the system (6) to zero. This yields successively

$$\begin{aligned} u_4 &= u_3w_{-3}/w_4 \\ u_3 &= u_2w_{-2}/w_3 \\ u_2 &= u_1w_{-1}/w_2 \end{aligned}$$

Assuming furthermore that $u_1 = \varphi_1/\varphi$, comparison with (4) shows that

$u_2 = \varphi_2/\varphi$; $u_3 = \varphi_3/\varphi$; $u_4 = \varphi_4/\varphi$

If therefore $\varphi = \varphi_1 + \varphi_2 + \varphi_3 + \varphi_4$ the set fulfills the condition (3). Next we look for a set of time dependent solutions v_1, v_2, v_3, v_4 which must have the property $v_1 + v_2 + v_3 + v_4 = 0$. To find this set (6) must be solved generally. Obviously (6) contains only three independent equations so that the last one may be replaced by $\Sigma v_i = 0$. The solution can be found by a well known method which of course requires the probabilities w to be known. The exact solution however becomes of a somewhat complicated from. To get an impression of the form of the result we shall make two rather drastic assumptions. The first and maybe the least objectionable one is that w_2, w_3 and w_4 are small enough to be neglected besides w_{-1}, w_{-2} and w_{-3}. This assumption agrees with the basic assumption that clusters are much less prone to capture a new monomer than to loose one. The second assumption is that $w_{-1} = w_{-2} = w_{-3}$. The assumption is most probably true so far as orders of magnitude are concerned. But the exact validity of this assumption is doubtful for the reason that a cluster which contains a greater number of monomers presumably will have a greater probability of loosing a monomer than one which contains a smaller number. However, any definite assumption concerning the relative value of w_{-1}, w_{-2} and w_{-3} will be open to some doubt and we may therefore in a first attempt to solve the problem assume them to be equal. There will be no difficulty in refining the treatment by using other perhaps more natural assumptions.

Putting now $w_{-1} = w_{-2} = w_{-3} = \lambda$ we arrive at the following system of equations:

$$\begin{aligned} \lambda v_1 &= -\dot{v}_1 \\ -\lambda v_1 + \lambda v_2 &= -\dot{v}_2 \\ -\lambda v_2 + \lambda v_3 &= -\dot{v}_3 \end{aligned} \qquad (6,1)$$

The boundary conditions are: at $t \to 0$, x_2, x_3 and x_4 all converge against zero, which by inspection of (2) and the first equation (5) is seen to mean that at $t = 0$, $u_2 = u_3 = u_4 = 0$.

Successive solution of these equations show that

$$\begin{aligned} v_1 &= \exp(-\lambda t) \\ v_2 &= \lambda t \exp(-\lambda t) \\ v_3 &= 1/2\ (\lambda t)^2 \exp(-\lambda t) \\ v_4 &= -(1 + \lambda t + 1/2\ (\lambda t)^2) \exp(-\lambda t) \equiv -e_2 \exp(-\lambda t) \equiv -L_2 \end{aligned} \qquad (7)$$

form a set of solutions which fulfills the condition $v_1 + v_2 + v_3 + v_4 = 0$. They may be multiplied by any common constant and will still represent a set of solutions with the same property. Adding the values for the stationary case to the time dependent ones we get

$$\begin{aligned} \varphi u_1 &= \varphi_1 + \varphi_4 \exp(-\lambda t) \\ \varphi u_2 &= \varphi_2 + \varphi_4 \lambda t \exp(-\lambda t) \\ \varphi u_3 &= \varphi_3 + \varphi_4 (\lambda t)^2 \exp(-\lambda t)/2 \\ \varphi u_4 &= \varphi_4 (1 - L_2) \end{aligned} \qquad (8)$$

These equations do not exactly conform with the boundary conditions at $t = 0$: $u_1 = 1$; $u_2 = 0$; $u_3 = 0$. The only boundary condition which is exactly fulfilled is $u_4 = 0$ at $t = 0$.

This discrepancy arises from the approximation that w_2, w_3 and w_4 are disappearingly small as compared to w_{-1}, w_{-2} and w_{-3}: Under this assumption $\varphi \to \varphi_4$, $\varphi_3/\varphi \to 0$; $\varphi_2/\varphi \to 0$; $\varphi_1/\varphi \to 0$. With these approximations we get in stead of (8)

$$\begin{aligned} u_1 &= \exp\ (-\lambda t) \\ u_2 &= \lambda t \exp\ (-\lambda t) \\ u_3 &= (\lambda t)^2 \exp\ (-\lambda t)\ /\ 2 \\ u_4 &= 1 - L_2 \end{aligned} \tag{8,1}$$

which fulfill the boundary conditions.

The only result of these calculations which may be compared with experiments is the expression for $u_4 = s_4\varphi_4$, where s_4 represents the rate of nucleation $\dot{m}$. We find

$$\int_0^m \varphi_4 \mathrm{d}m = \int_0^t (1 - L_2)\ \mathrm{d}t \equiv y_2 \tag{9}$$

We know from the estimate p. 910 that the precipitate becomes visible before any appreciable alteration of the concentration of monomers has taken place. We therefore treat φ_4 as a constant *i. e.* we write

$$\varphi_4 m = y_2 \tag{9,1}$$

and it must be added, that our system of differential equations (6) has been derived under the said assumption.

The integral y_2 and its first three derivatives are zero at $t \to 0$. Its fourth derivative at $t \to 0$ is λ^3. Let now $w_1 = w_2 = w_3 = w_4 = \varepsilon a$, where a is the initial concentration of X_1. Using $w_{-1} = w_{-2} = w_{-3} = \lambda$ we thus get

$$m\ /\ a = (t\ \varepsilon\ a)^4\ /\ 24 \tag{9,2}$$

If t is large as compared to $1/\lambda$, φ_4 still being effectively constant, integration of (9) yields

$$\varphi_4 m = t - 3/\lambda \tag{9,3}$$

that is we find $m \simeq \varepsilon^4 a^5 t/\lambda^3$.

If therefore the precipitate can be observed when m, the concentration of nuclei has reached a certain value, independent of a, we evidently arrive at a relation of the form found experimentally by Tovborg Jensen [4], Nielsen [5], La Mer [6] and other authors.

A more complete discussion requires the integration $\int L_2 \mathrm{d}t$ to be performed. Repeated use of partial integration yields $\int_0 L_2 \mathrm{d}t = 3 - (1 + e_1 + e_2) \exp$ $(-\lambda t)$ which inserted in (9) yields

$$m\lambda\varphi_4 = m\lambda^4/\varepsilon^4 a^5 = \lambda t - 3 + (1 + e_1 + e_2) \exp\ (-\lambda t) \tag{9,4}$$

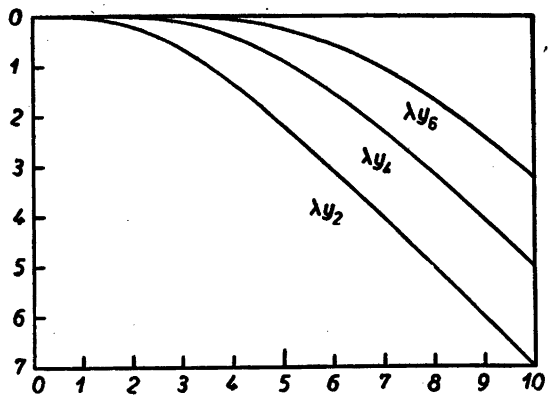


Fig. 1. Graph of the functions λy_2, λy_4 and λy_6. For definitions see text. Ordinates and abscissae are pure numbers.

The numerical values of the expression on the right hand side have been calculated for values of λt from zero to ten. Beyond that value the contributions from the last term disappears and the curve thus becomes a straight line. Fig. 1 shows its form. If the power a in (9,4) is greater than 5 the deviation of the curve from its horizontal tangent becomes appreciable at higher values of λt. Curves for

$$\lambda y_4 = \lambda t - 5 + (1+e_1+e_2+e_3+e_4) \exp(-\lambda t)$$

and

$$\lambda y_6 = \lambda t - 7 + (1+e_1+e_2+e_3+e_4+e_5+e_6) \exp(-\lambda t)$$

are also in Fig. 1.

e_n is defined as

$$e_n \equiv (\lambda t)^n/n! + (\lambda t)^{n-1}/(n-1)! + \ldots \lambda t/1 + 1.$$

If there are n steps in the sequence (2), that is if the nucleus contains $n+1$ monomers the integral in question becomes y_{n-2}.

DISCUSSION

The calculations above show that the numerical magnitude of λ determines the time of attainment of the stationary (steady) state. If for example $1/\lambda$ is a small fraction of a second the steady state will be reached immediately after the beginning of the process and the experiments by Nielsen [5] seem to confirm that it is so because, even at precipitation times so small as 10^{-2}—10^{-3} seconds, an equation of a form analogues to (9,3) without the addend $(n-1)/\lambda$ is fulfilled.

It seems therefore that there is no need to take the time of attainment of the steady state in consideration.

The great problem seems therefore still to be the interplay between nucleation and growth. This has been treated empirically by Tovborg Jensen [4].

Nielsen and the author [7] tried to integrate the equations numerically getting only partial agreement with Tovborg Jensens results.

There are also older contributions from Ono [8], and new experimental material has been procured *inter alia* by Turnbull [9] and by Johnson and O'Rourke [10]. Turnbull's idea is that a number of nuclei are formed at once which then grow by diffusion according to the laws given for the first time by Smoluchowski [11] and revised by Frisch and Collins [12]. Obviously the difficulties arising from the initial (nucleation) period are avoided by this procedure, but on the other hand it is evident that the results obtained cannot agree with the experiments in the initial period if nucleation is not instantaneous and then stops.

That the number of nuclei which have been formed at a certain not too small time has a limiting value can be seen by numerical integration of the equation

$$\frac{\mathrm{d}m}{\mathrm{d}t} = Ka^p\,(1-\alpha)^p$$

where m is the number of nuclei at time t and α is the experimentally determined fraction of monomers which has disappeared from the solution.

It seems therefore to be a sound procedure to do as Johnson and O'Rourke[10] have done, to consider the two phases, nucleation and growth, separately. Anyhow there can hardly be any doubt that in the treatment by Frisch and Collins [12] the assumption m = constant is fulfilled for the later part of the curves found by Turnbull and others.

In a forthcoming paper, A. E. Nielsen will present some new results and a collection of data from elsewhere.

The author's thanks are due to A. E. Nielsen for aid in preparing the graph and the numerical calculations.

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Enzymatic Hydrolysis of Organophosphorus Compounds

III. Effect of Cholinesterase Inhibitors and Inhibition of Cholinesterase in the Presence of Tabunase

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The existence of an enzyme (phosphorylphosphatase," tabunase") catalysing the hydrolysis of dimethylamido-ethoxy-phosphoryl cyanide (tabun) has been reported recently [1]. The properties of the enzyme and the analysis of the reaction products have also been described [2]. In the present paper the effect of reversible cholinesterase inhibitors (physostigmine, prostigmine) on tabunase is described and the significance of considering the presence of tabunase in studies on tabun as a cholinesterase inhibitor is demonstrated.

METHODS AND MATERIAL

The enzymatic hydrolysis of tabun was measured by the Warburg manometric technique [1]. Tabun and DFP (di*iso*propoxyphosphoryl fluoride) of high purity were used as substrates. The influence of the following compounds on the tabunase activity was studied: acetylcholine chloride (ACh), choline chloride, physostigmine sulphate, and prostigmine bromide.

Human and rabbit plasma were employed as enzyme preparations. In the determination of the influence of tabunase on the inhibiting effect of tabun on human serum cholinesterase, Fraction IV—1 (tabunase) and Fraction IV—6 (cholinesterase) were used, prepared by the method of Cohn [1].

RESULTS

Effect of acetylcholine and choline on the enzymatic hydrolysis of tabun. In these experiments human plasma was used as enzyme source for tabunase. The results from one series of experiments are shown in Fig. 1. Neither choline nor acetylcholine has any influence on the tabunase activity. In the experiment with acetylcholine which, in the absence of tabun, is hydrolysed at a high rate by the plasma used, tabun has two functions, *i.e.*, to inactivate cholinesterase completely and to act as a substrate for tabunase. In the system "tabun + ACh" (Fig.1) therefore the cholinesterase activity is not involved. The concentration of tabun in that system was 2.66×10^{-3} *M*; the concentration

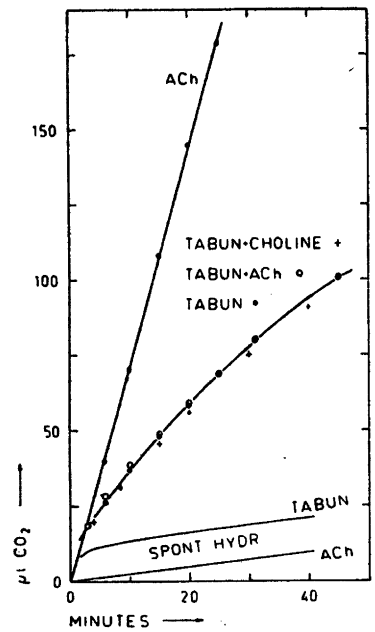

Fig. 1. The effect of acetylcholine (ACh) and choline on the enzymatic hydrolysis of tabun by human serum. Tabun, 2.66 × 10^{-3} M; ACh-chloride, 1.65 × 10^{-3} M; choline chloride, 1.00 × 10^{-3} M.

necessary to give complete inhibition of cholinesterase is about 10^{-8} *M*. From these experiments we conclude firstly that tabunase is a separate enzyme from cholinesterase, and secondly that acetylcholine or its hydrolysis products do not have any influence on the former enzyme.

Effect of physostigmine and prostigmine on the enzymatic hydrolysis of tabun and DFP. The effect of the two best-known reversible inhibitors of cholinesterase, physostigmine and prostigmine, was studied for the ezymatic hydrolysis of tabun by rabbit plasma. This plasma differs from the human plasma in that tabun is hydrolysed at a higher rate than is acetylcholine; the reverse holds for human plasma. Those concentrations of physostigmine as well as of prostigmine, which are sufficiently high to give complete inhibition of the plasma cholinesterase present (acetylcholine as substrate), have no effect on the tabunase activity. These results are demonstrated in Fig. 2. In all these experiments the enzyme preparation was incubated for about 45 minutes with the inhibitor before the substrate (an organophosphorus compound) was added.

Similar experiments were performed with DFP as a substrate and with rabbit plasma as an enzyme source. As seen in Fig. 3 physostigmine has no influence on the enzymatic reaction in this case either. When tabun and DFP are present at the same time the results obtained are in agreement with the suggestion [1] that both these compounds are split by the same enzyme. In a summation experiments, such as that shown in Fig. 3, the initial reaction velocity is somewhat higher than in the system in which the substrates were present alone. This higher reaction velocity, however, is by no means as high as it should be if two enzymatic reactions were going on simultaneously; it is

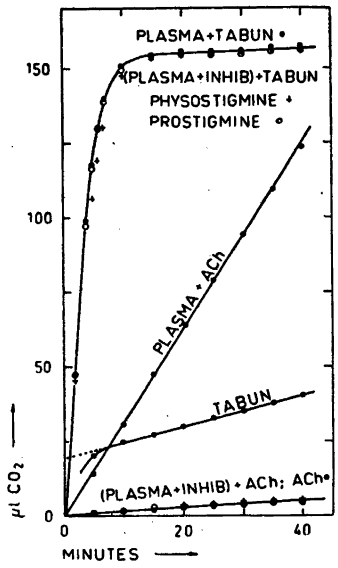

Fig. 2. The effect of physostigmine and prostigmine on the enzymatic hydrolysis of tabun and acetylcholine (ACh) by rabbit plasma. Tabun, 5.3 × 10⁻³ M; ACh-chloride, 1.10 × 10⁻² M; inhibitor (physostigmine sulphate or prostigmine bromide), 1.00 × 10⁻³ M; concentration values refer to the reaction mixture (2.00 ml). Amount of plasma present, 0.4 ml.

due to the higher (two-fold) concentration of the two substrates (taken together as a single entity). The same increase in reaction velocity was obtained when the concentration of each substrate (tabun or DFP) was doubled. Further results of this kind obtained with a variety of organophosphorus compounds will be published later.

The inhibition of cholinesterase by tabun mixtures containing tabunase. One of the present authors has recently [3,4] reported that in experiments with crude enzyme preparations the inhibition of plasma (butyryl-) cholinesterase by tabun is less than that of the acetylcholinesterase (erythrocytes, electric tissue). It is now known that this difference is not due to a true selective difference in inhibition, but is the result of the interference by tabunase present in most animal blood plasma. It is obvious that the simultaneous presence of enzymes which split organophosphorus compounds has to be taken into consideration when these compounds are studied as cholinesterase inhibitors. In Fig. 4 the inhibiting effect of tabun on the cholinesterase of human serum is demonstrated. This effect obtained with the original crude serum was compared with the effects observed with a purified preparation (Fraction IV—6) of cholinesterase and with a mixture containing this preparation and tabunase (Fraction IV —1); both these fractions were prepared from the serum used for comparison. The pI_{50} value (negative log of the molar concentration of tabun which gives 50 % inhibition) obtained for the original serum is much lower than that for a cholinesterase preparation free of tabunase. It is also demonstrated in Fig. 4 that when the tabunase preparation is added to the fraction containing cholinesterase the pI_{50} value is lowered, *i.e.*, a higher concentration of tabun is necessary to give 50 % inhibition. The results show that tabun actually inhibits butyrylcholinesterase and acetylcholinesterase of human blood to

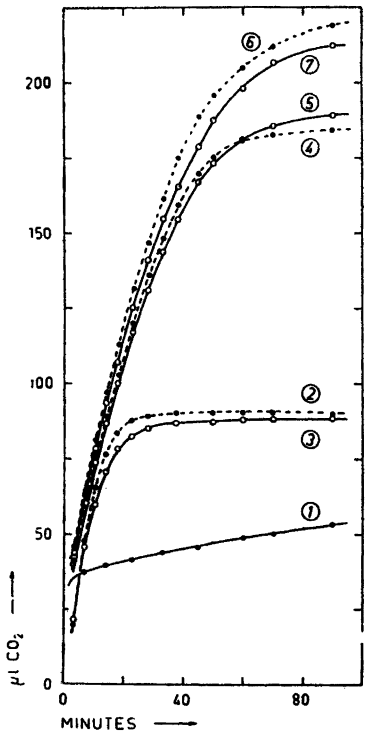

Fig. 3. The effect of physostigmine on the enzymatic hydrolysis of tabun and DFP by rabbit plasma. Tabun and DFP, 2.65 × 10^{-3} M; physostigmine sulphate, 1.00 × 10^{-3} M; concentration values refer to the reaction mixture (2.00 ml). Amount of plasma present, 0.1 ml. 1. Tabun + DFP + physostigmine; 2. Tabun + plasma; 3. Tabun + (plasma + physostigmine); 4. DFP + plasma; 5 DFP + (plasma + physostigmine); 6. (Tabun + DFP) + plasma; 7. (Tabun + DFP) + (plasma + physostigmine).

about the same extent. These results are in agreement with the fact that in crude serum there are at least two factors which combine with tabun: cholinesterase which is inactivated, and tabunase which splits tabun into two molecules each being inactive as a cholinesterase inhibitor. In addition to the catalytic effect on the hydrolysis of tabun, tabunase also reactivates the inhibited cholinesterase, as was recently demonstrated in this laboratory; a special report on this enzymatic reactivation of tabun-inhibited cholinesterase will be published elsewhere.

DISCUSSION

It was demonstrated in previous papers [1,2] and in the present report that tabun is enzymatically hydrolysed by a variety of animal tissues and that the enzyme (tabunase, phosphorylphosphatase) responsible for this reaction is a separate enzyme with no relation to the group of cholinesterases or phosphatases. In any experiment carried out with crude extracts or untreated blood plasma in order to determine the inhibiting effect of tabun or another organophosphorus compound on the cholinesterase activity, the possible presence of a phosphorylphosphatase must be considered. The presence of such an enzyme will always give a distorted picture as regards the cholinesterase inhibiting effect of an organophosphorus compound. The conflicting

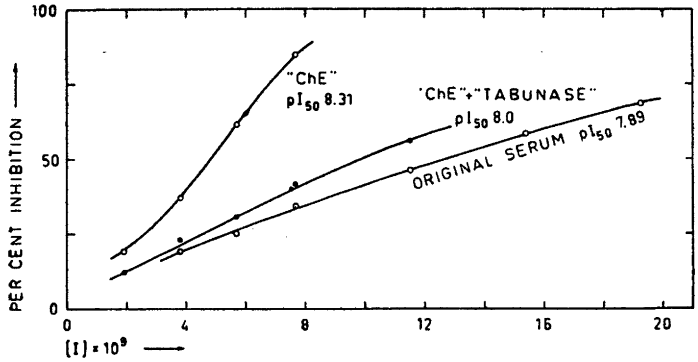

Fig. 4. The inhibiting effect of tabun on the cholinesterase of human serum in the absence and presence of tabunase. [I], molar concentration of tabun during enzyme incubation; pI_{50}, the concentration which gives 50 % inhibition. "ChE": Fraction IV—5+6; "tabunase": Fraction IV—1; the same amount (10 mg) of each fraction was used; the same result was obtained with 1·0 mg of Fraction IV—6. Substrate, 1.10 × 10^{-2} M acetylcholine.

results obtained in this respect by various groups of workers with different methods may be explained by this observation. The suggestion of other authors that the organophosphorus compounds react with proteins other than cholinesterases *in vivo* is confirmed by the demonstration of the presence of the phosphorylphosphatases. These latter enzymes, however, may not be the only representatives of such proteins.

Little is known about the degradation and detoxication mechanism of the organophosphorus compounds. The phosphorylphosphatases may play an important part in that mechanism. The reaction products formed from tabun by enzymatic degradation are biologically inactive in the concentrations to be considered. The toxic effects on the organism may therefore be reduced when these enzymes are present. In addition, it has been demonstrated that these enzymes also reactivate cholinesterases which are completely inactivated by tabun *in vitro*. This latter reaction may be one of the factors which is responsible for the restoration of normal cholinesterase activity in a living organism intoxicated by a phosphorus compound.

SUMMARY

Acetylcholine and choline do not interfere with the tabunase activity of human serum. Reversible cholinesterase inhibitors, such as physostigmine and prostigmine, do not inhibit the enzymatic hydrolysis of tabun and di*iso*-propoxyphosphoryl fluoride (DFP) by rabbit plasma; tabun and DFP are most probably split by the same enzyme in this case.

A much higher concentration of tabun is necessary to inhibit the cholinesterase of untreated human serum and isolated serum cholinesterase when mixed with tabunase, than a cholinesterase preparation free of tabunase.

Tabun and DFP were prepared at the Research Institute of National Defence, Department 1, Chief Professor Gustaf Ljunggren, to whom we are indebted for his continual interest. The authors wish to express their sincere thanks to Ing. Henrik Björling of Kabi, AB, Stockholm, for the preparation of the various serum fractions, and to Dr. J. A. Aeschlimann, Hoffmann-La Roche, Inc., Nutley, New Jersey, for the gift of prostigmine bromide.

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Kinetics of Complex Formation

I *. The Chromium(III)-thiocyanate System

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A study of the chromium(III)-thiocyanate complexes has been made in a controlled salt medium. The equilibrium constants for the uptake of the 1st and 2nd thiocyanate ion, together with the rate constant for the uptake of the 1st thiocyanate ion have been estimated at 25, 50 and 75° C by means of an argentometric determination of the concentration of the free thiocyanate ion. The rate constant for the dissociation of the 1st thiocyanate ion from the hexathiocyanate complex has been estimated at 15 and 25° C by a spectrophotometric method. From the temperature dependence of the constants, the activation energies and frequency factors as well as the changes in enthalpy and entropy for the reactions in question are calculated.

The chromium(III) ion exists in aqueous solution as a hexaquo ion [2], and it is characteristic that chromium(III) complexes are usually formed by slow time reactions. The thiocyanate system was investigated by Niels Bjerrum [3], who showed that all six possible complexes were formed. N. Bjerrum worked out elegant methods to determine the concentration of the individual complexes, and estimated all the 6 equilibrium constants at 50° C. He also gave approximate values for the rate of aquation of all the complexes with the exception of the dithiocyanato complex.

Let us for the bimolecular formation reaction

$$MA_{n-1}aq_{7-n} + A \rightarrow MA_naq_{6-n} + aq$$

denote the velocity contant, frequency factor and activation energy by k_n, Z_n and E_n, and for the corresponding pseudo-unimolecular dissociation reaction

$$MA_naq_{6-n} + aq \rightarrow MA_{n-1}aq_{7-n} + A$$

* The numerical values given in the present paper supercede those presented in a preliminary communication by J. Bjerrum at the 7th »Nordiska Kemistmötet», Helsinki 1950 (*cf.* ref. 1).

by k_{-n}, Z'_{-n} ($= Z_{-n} \cdot C_{aq}$) and E_{-n}, respectively. According to the Arrhenius equation we then have for the uptake of the n'th ligand

$$\log k_n = \log Z_n - \frac{E_n}{2.3\,RT}; \tag{1}$$

for the dissociation of the n'th ligand

$$\log k_{-n} = \log Z'_{-n} - \frac{E_{-n}}{2.3\,RT}; \tag{2}$$

and for the equilibrium constant

$$\log K_n = \log \frac{k_n}{k_{-n}} = \log \frac{Z_n}{Z'_{-n}} + \frac{E_{-n} - E_n}{2.3\,RT}. \tag{3}$$

Denoting the heat and entropy for the uptake of the n'th ligand by ΔH_n and ΔS_n, respectively, the consecutive formation constant is also given by the formula:

$$\log K_n = \frac{\Delta S_n}{2.3\,R} - \frac{\Delta H_n}{2.3\,RT} \tag{4}$$

where

$$\Delta H_n = E_n - E_{-n} \tag{5}$$

and

$$\Delta S_n = 2.3\,R \log \frac{Z_n}{Z'_{-n}} \tag{6}$$

In the present paper some of the reactions in the system are studied in controlled neutral salt medium and at different temperatures. Data for the examined reactions are given in Table 1. The complex formation was followed by determination of the free thiocyanate by titration with silver nitrate. Using this method the formation velocity constant k_1 and the consecutive complexity constants K_1 and K_2 could be obtained. The two first mentioned of these constants indirectly also determine k_{-1}, the dissociation velocity constant of the monothiocyanate complex. The dissociation reactions of the thiocyanate chromium complexes are with the exception of the hexathiocyanato complex

*Table 1. Comparison of thermodynamic and kinetic results *.*

n	$\log K_n$ (25°)	$-\Delta H_n$(kcal)	$\Delta S_n/2.3\,R$	Medium
1	1.87 ± 0.01	1.4 ± 0.2	0.8 ± 0.2	1 *M* $NaClO_4$
2	1.11 ± 0.07			»
	$\log k_n$ (25°)	E_n(kcal)	$\log Z_n$	
1	− 5.21 ± 0.03	25.0 ± 0.3	13.2 ± 0.2	»
1	− 4.5 (calc.)	21.4 ± 0.3	11.2 ± 0.2	$\mu = 0.1$
	$\log k_{-n}$(25°)	E_{-n}(kcal)	$\log Z'_{-n}$	
1	− 7.08 ± 0.04	26.5 ± 0.4	12.4 ± 0.3	1 *M* $NaClO_4$
6	− 4.662 ± 0.004	25.4 ± 0.5	14.0 ± 0.3	»
6	− 4.634 ± 0.002	25.7 ± 0.3	14.3 ± 0.2	$\mu = 0.1$

* Concentrations in moles per liter and time in seconds.

strongly catalyzed by hydroxyl ions as shown by N. Bjerrum [3], but as k_1 is independent of the hydrogen ion concentration, the computed value for k_{-1} must be the dissociation velocity constant for the pentaaquo-monothiocyanato chromium ion. The aquation velocity constant for the hexathiocyanato ion (k_{-6}) was determined by spectrophotometric estimation of the free thiocyanate after addition of ferric salt.

For a reaction between a trivalent and a monovalent ion of opposite charges one should expect a frequency factor essentially higher than the normal value for a bimolecular reaction (log $Z \sim 11$). It will be seen that the value found for log Z_1 only in 1 M $NaClO_4$ is higher, but not nearly as high as assumed by some authors. Moelwyn-Hughes [4] and Frost and Pearson [5] use N. Bjerrum's values for k_1 (at two temperatures), and estimate log $Z_1 = 19.5$, but Bjerrum's values are only rough estimates and were never meant to be used for such a calculation. On the other hand, for the aquation of the thiocyanate complexes one should expect a normal frequency factor. The value found for log Z'_{-6} seems rather high, but taking into consideration that $C_{H_2O} = 55$, one estimates log $Z_{-1} = 10.7$ and log $Z_{-6} = 12.3$. A considerable salt effect is to be expected on k_1 in agreement with the experimental results. E_1 and log Z_1 are seen both to decrease with increasing salt concentration. Conversely, there should be no salt effect on the aquation reactions as experimentally verified for k_{-6}.

Our values for the complexity constants are smaller than those determined by N. Bjerrum in solutions of varying salt concentration, but this correlates well with the kinetic salt effect found on k_1 (see Table 2, in which Bjerrum's [6] roughly approximated values to $\mu = 0$ are also given).

Table 2. Comparison of complexity constants at 50° C.

n	This paper log K_n (1 M $NaClO_4$)	N. Bjerrum [6] log K_n	Salt-Normality	log K_n ($\mu = 0$)
1	1.79 ± 0.01	2.51	0.04—0.06	3.1
2	1.0 ± 0.05	1.24	0.06—0.2	1.7
3		0.66	0.1 —2.0	1.0
4		0.29	0.7 —4.5	0.3
5		— 0.09	3.0 —4.5	— 0.7
6		— 0.39	4.5	— 1.6

Assuming log $K_6 \sim -0.4$ in molar solution at 25° we estimate log $k_6 \sim -5.0$. This shows that the trend in the complexity constants, which N. Bjerrum [6] accounted for on statistical and electrostatic grounds, is mainly refound in the velocity constants for the aquation reactions, and here again it is especially the activation entropy, *i.e.* log Z, which increases with the number of complex-bound thiocyanate ions.

EXPERIMENTAL

A stock solution of sodium perchlorate was made by neutralizing perchloric acid solution (Bakers A. R.) with sodium hydroxide solution.

Sodium thiocyanate solutions were made from recrystallized sodium thiocyanate (Merck purum).

Chromium(III) perchlorate was made by dissolving chromium hydroxide (Riedel de Haën puriss.) in perchloric acid solution. The 0.2 molar stock solution was weakly acidic (pH = 1.45). By spectrophotometric comparison with an acid chromium(III) nitrate solution made from crystallized chromic nitrate it was shown to be practically free of latently basic chromium compounds [7]. After three years at room temperature this was shown still to be the case while the originally soluble chromic hydroxide in the meantime had been converted into an insoluble latently basic form.

Potassium hexathiocyanato chromate(III) was made according to Roesler [8] by boiling chromic alum with an excess of potassium thiocyanate in concentrated solution. After completion of the reaction K_2SO_4 was precipitated by mixing with ethanol, and filtered off. By staying at room temperature, crystals of $K_3Cr(SCN)_6$, 4 H_2O separated from the filtrate. After 3 recrystallisations from water-ethanol, a very pure salt was obtained — no free thiocyanate was observed by the iodide-azide reaction (this seems to indicate, incidentally, that the ligands are attached to the chromium atom by the sulphur atom). Analysis gave results corresponding very well to the theoretical formula.

Chromium concentrations were determined by oxidizing the sample with ammonium persulphate in approx. 1 *N* sulphuric acid solution in the presence of Ag^+, boiling off excess of persulphate, and after cooling, titrating the chromium(VI) with ferrous ammonium sulphate solution using diphenylamine-sulphonate as redoxindicator. Phosphoric acid was added to eliminate the colour of ferric ions formed.

Thiocyanate concentrations were determined by titration with standard silver nitrate solution using ferric sulphate as indicator.

All experiments were carried out in a thermostated bath, closed from light to avoid possible photochemical reactions. The temperature could be kept constant within ± 0.02° C.

ESTIMATION OF EQUILIBRIUM CONSTANTS.

N. Bjerrum [3] examined solutions of chromic nitrate and potassium thiocyanate to which no excess of free acid was added. The chromic salt, therefore, was partly hydrolyzed and considerable amounts of polynuclear basic compounds were formed during the equilibrium adjustment [7]. To escape N. Bjerrum's cumbersome corrections for latently basic compounds we have examined solutions which were 0.02 molar in free perchloric acid, paying due regard to the fact that thiocyanate ions are not stable in strongly acid solution. To show that the slow hydrolysis is negligible at this acid concentration a solution of the composition $C_{Cr(ClO_4)_3} = 0.02$, $C_{HClO_4} = 0.02$ was heated to 75° C in 24 hours. After cooling it was found that the conductance had risen about 1 % and that — log $[H^+]$ had changed from 1.70 to 1.69. Heating to 75° C for three more days had very little influence on the acidity. At 50° C latently basic compounds are formed to a much smaller extent (*cf.* ref. 7, p. 126).

The chromium thiocyanate solutions used in the equilibrium experiments were mixed from the stock solutions and placed in the thermostat in 100 ml glass-stoppered bottles covered with rubber sheets. The equilibrium was reached after approx. 2 weeks at 25° C and some hours at 75° C.

From the equilibrium solutions samples were pipetted into ice-cooled Erlenmeyer flasks containing an excess of silver nitrate solution. It this way — by freezing the complex reactions in the system — it was possible to estimate the free thiocyanate concentration $[SCN^-]$ by titration of the excess silver nitrate with standard sodium thiocyanate solution using a semi-micro burette.

The total chromium concentration (C_{Cr}) was determined from another sample, which was boiled for some minutes with a small excess of ammonium hydroxide [3]. The precipitated basic chromium compounds were filtered off

and washed, and the chromium determined by the method described above. In the bulk of the filtrate and washing water the total thiocyanate concentration (C_{SCN^-}) was determined. The experimental data, therefore, give directly the formation function:

$$\bar{n} = \frac{C_{SCN^-} - [SCN^-]}{C_{Cr}} \tag{7}$$

In the solutions with greater complex formation, detection of the endpoint in the Volhard-titration became difficult, because the reddish colour of the complexes covered the indicator system [3]. Due to this and also because we cannot consider the salt medium as constant when $C_{SCN^-} \lesssim 0.1$, only the lst and 2nd formation constants could be determined with reasonable certainty.

The constants were estimated by a graphical procedure similar to that described by Olerup [9]. To this purpose we introduce the function

$$G_1([A]) = \frac{\bar{n}}{[A]} = K_1(1-\bar{n}) + \sum_{n=2}^{n=N} (n-\bar{n})\beta_n[A]^{n-1} \tag{8}$$

where $\beta_n = K_1K_2 \ldots K_n$, and in this case N = 6 and [A] = [SCN^-]. Graphical representation of $G_1([SCN^-])$ versus the free ligand concentration and extrapolation to [SCN^-] = 0 gives K_1 directly. Table 3 presents the results obtained

Table 3. Data for equilibrium solutions in 1 M sodium perchlorate.

Temperature	No.	C_{Cr} × 10^3	C_{SCN^-} × 10^3	[SCN^-] × 10^3	$\bar{n}$	$\frac{\bar{n}}{[SCN^-]}$
25°	1	12.25	4.187	2.321	0.152	65.6
25°	2	15.05	5.899	3.027	0.191	63.1
25°	3	16.06	7.299	3.754	0.223	60.0
25°	4	15.79	8.467	4.415	0.257	58.3
25°	5	16.81	9.939	5.170	0.284	55.9
50°	6	15.19	1.571	0.825	0.049	59.3
50°	7	18.32	3.057	1.561	0.082	56.7
50°	8	18.04	4.739	2.388	0.131	54.7
50°	9	18.20	6.202	3.142	0.165	52.6
50°	10	19.44	7.774	3.904	0.199	51.0
50°	11	20.45	10.677	5.384	0.259	48.0
75°	12	22.04	6.404	3.241	0.147	45.5
75°	13	22.78	7.405	3.729	0.163	43.5
75°	14	23.70	9.184	4.643	0.193	41.7
75°	15	25.39	10.014	5.044	0.196	38.9
75°	16	24.61	10.773	5.425	0.217	40.0

at 25°, 50° and 75° for concentrations of free ligand up to ~ 0.005 M, and in Fig. 1 their graphical representation is seen. From the intersection of the curves and the ordinate axis are found

$$K_1(25°) = 74 \pm 1,\ K_1(50°) = 61.5 \pm 1,\ K_1(75°) = 52 \pm 2.$$

It is also possible to estimate the value of K_2 from the curves. Differentiation of (8) with respect to [A] gives for the slope at the origin

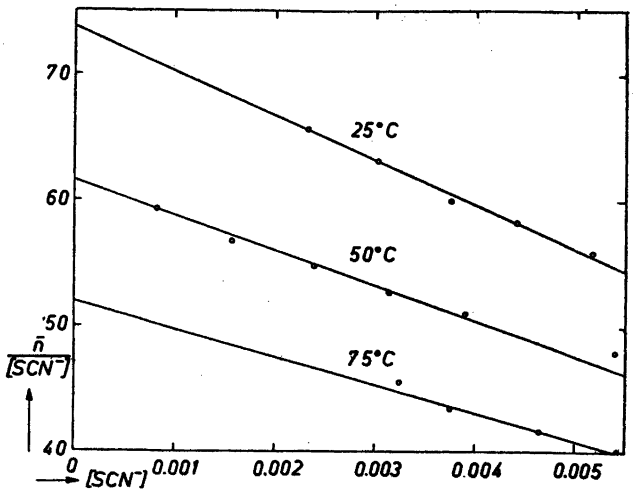

Fig. 1. Graphical estimation of K_1 as the value of $\frac{\bar{n}}{[SCN^-]}$ for $[SCN^-] = 0$.

$$\left(\frac{dG_1[A]}{d[A]}\right)_{[A]=0} = K_1(2K_2 - K_1) \qquad (9)$$

from which the following values were computed

$$K_2(25°) = 13 \pm 2,\ K_2(50°) = 9 \pm 2,\ K_2(75°) = 4 \pm 2.5.$$

K_2 was also obtained from data at higher ligand concentrations by rearranging equation (8) and plotting a new function G_2 ([A]) versus the free ligand concentration

$$G_2([A]) = \frac{G_1([A]) - K_1(1-\bar{n})}{[A]} = 2\beta_2\left(1 - \frac{\bar{n}}{2}\right) + \sum_{n=3}^{n=N}(n-\bar{n})\beta^n[A]^{n-2} \qquad (10)$$

The intersection of the curve with the ordinate axis gives in this case: $2\beta_2 = 2K_1K_2$. Table 4 and Fig. 2 give some experiments at 50° with concentrations of free ligand from ~ 0.01—0.06 *M*. Unfortunately the colour of the solutions here made the titrations more uncertain. The value estimated for β_2 (= 700

Table 4. Data for estimation of K_2 in 1 M sodium perchlorate solution at 50° C.

No.	$C_{Cr} \times 10^3$	$C_{SCN^-} \times 10^3$	$[SCN^-] \times 10^3$	$\bar{n}$	$\frac{\bar{n}}{[SCN^-]}$	$G_2([SCN^-])$
1	27.58	29.75	13.8	0.576	42.7	1 190
2	30.59	39.68	18.1	0.710	39.2	1 170
3	34.38	49.60	22.2	0.797	35.9	1 050
4	46.05	89.4	39.6	1.082	27.3	816
5	49.12	99.2	43.1	1.142	26.5	820
6	52.07	109.2	48.2	1.172	24.3	726
7	57.74	128.8	60.3	1.186	19.7	517

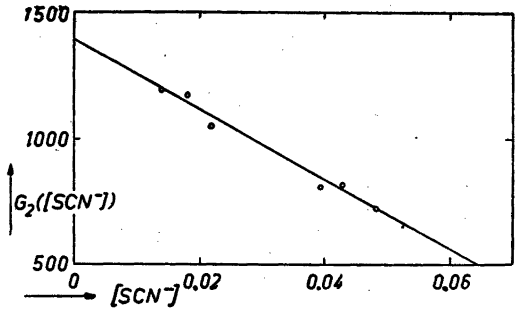

Fig. 2. Graphical estimation of $\beta_2 = K_1K_2$ *as the value of* $\frac{1}{2} G_2$ *(*$[SCN^-]$*) for* $[SCN^-] = 0$.

$\pm$ 50) indicates $K_2 = 11 \pm 1$ to be compared with the value 9 ± 2 obtained from Fig. 1.

From the temperature-dependence of K_1 and K_2 the following values for the heats of formation in kcal. were estimated:

$$-\Delta H_n = \frac{RT'T''}{T'-T''} \ln \frac{K'_n}{K''_n} \quad (T = \text{abs. temperature})$$

Temperature range	$-\Delta H_1$	$-\Delta H_2$
25°—50° C	1.4 ± 0.2	2 ± 2
50°—75° C	1.5 ± 0.5	8 ± 6

ESTIMATION OF VELOCITY CONSTANTS

The rate constant for the uptake of the 1st thiocyanate ion was determined by the same analysis-technique as used in the equilibrium experiments. Thermostated solutions containing the ions to react — and neutral salt, when this was used — were mixed in a 500 ml bottle also in the bath. Samples taken at different times were analyzed and the concentrations of free and total thiocyanate and total chromium determined. Only the first part of the reaction was followed where the reciprocal reaction and further transformations could be neglected, and the velocity constants were in all cases determined by means of the expression for a bimolecular reaction

$$k_1 = \frac{1}{t} \cdot \frac{2.303}{C_{Cr}-C_{SCN^-}} \log \frac{C_{Cr}[SCN^-]}{[Cr^{+++}]C_{SCN^-}} \qquad (11)$$

Some preliminary experiments showed that the initial velocities were independent of the hydrogen ion concentration in the range 0.02—0.10 molar. Some experiments at different temperatures in 1 M $NaClO_4$, and in solutions without sodium perchlorate ($\mu \sim 0.1$) are given in Table 5. For each experiment is given the equilibrium value for the formation function $\bar{n}_{eq.}$, and the data show that k_1 from the technique used can be determined with reasonable certainty from the very beginning of the reaction, where $\bar{n} \ll \bar{n}_{eq}$.

Table 5. Estimation of rate constants for uptake of the 1st thiocyanate ion. $C_{HClO_4} \sim 0.02$, $C_{NaClO_4} = 1.00$

Temp. °C	Time (min.)	$C_{Cr} \times 10^3$	$C_{SCN^-} \times 10^3$	$[SCN^-] \times 10^3$	$[Cr^{+++}] \times 10^3$	$k_1 \times 10^3$ ($C^{-1} \cdot sec^{-1}$)	n
25°	0	23.39	21.16	21.16	23.39		0
25°	1 260	23.39	21.16	20.95	23.18	0.00569	0.009
25°	2 880	23.39	21.16	20.56	22.79	0.00720	0.026
25°	4 320	23.39	21.16	20.32	22.55	0.00680	0.036
25°	5 760	23.39	21.16	20.18	22.41	0.00598	0.042
25°	8 640	23.39	21.16	19.92	22.15	0.00518	0.053
					Mean	0.0062 ± 0.0004	0.494 $\bar{n}_{eq.}$
50°	0	20.46	19.17	19.17	20.46		0
50°	30	20.46	19.17	19.04	20.33	0.176	0.006
50°	60	20.46	19.17	18.92	20.21	0.175	0.012
50°	103	20.46	19.17	18.76	20.05	0.171	0.020
50°	189	20.46	19.17	18.52	19.81	0.152	0.032
					Mean	0.169 ± 0.006	0.445 $\bar{n}_{eq.}$
75°	0	23.46	18.49	18.49	23.46		0
75°	11	23.46	18.49	17.73	22.69	2.79	0.032
75°	20	23.46	18.49	17.11	22.07	2.85	0.059
75°	40	23.46	18.49	15.97	20.93	2.77	0.108
75°	60	23.46	18.49	14.91	19.87	2.78	0.153
75°	90	23.46	18.49	13.87	18.83	2.55	0.197
					Mean	2.75 ± 0.05	0.363 $\bar{n}_{eq.}$
		Without sodium perchlorate ($\mu \sim 0.1$)					
50°	0	13.80	16.00	16.00	13.80		0
50°	27.5	13.80	16.00	15.92	13.72	(0.421)	0.005
50°	38.5	13.80	16.00	15.83	13.62	0.481	0.011
50°	78.5	13.80	16.00	15.56	13.35	0.508	0.028
50°	124	13.80	16.00	15.29	13.08	0.500	0.044
50°	178	13.80	16.00	15.01	12.80	0.482	0.062
50°	199	13.80	16.00	14.81	12.60	0.519	0.074
					Mean	0.498 ± 0.006	~0.64 $\bar{n}_{eq.}$
75°	0	13.50	12.28	12.28	13.50		0
75°	14.7	13.50	12.28	11.56	12.78	5.15	0.053
75°	28.5	13.50	12.28	10.85	12.07	5.59	0.106
75°	39.8	13.50	12.28	10.40	11.62	5.67	0.139
75°	49.6	13.50	12.28	9.94	11.16	5.54	0.173
75°	58.1	13.50	12.28	9.81	11.03	5.31	0.183
					Mean	5.5 ± 0.1	~0.43 $\bar{n}_{eq.}$

The values for the parameters in the Arrhenius equation E_1 and log Z_1 in 1 *M* $NaClO_4$ and in solutions with $\mu \sim 0.1$ given in Table 1 are computed from the mean values for k_1 given in Table 5. The corresponding parameters for the reciprocal reaction E_{-1} and log Z_{-1} in 1 *M* $NaClO_4$ are computed by combination with the known values for the equilibrium constant K_1 in this medium using the relationships (3)—(6).

SPECTROPHOTOMETRIC ESTIMATION OF THE FREE THIOCYANATE AFTER ADDITION OF FERRIC SALT

In the presence of thiocyanate-richer complexes the silver nitrate titration fails, and N. Bjerrum [3] has used in such cases colorimetric estimation of the free thiocyanate concentration after addition of ferric nitrate. The method is due to the very rapid formation and strong absorption of the iron(III) thiocyanate complexes. It has been developed here to an accurate spectrophotometric method, and used to study the velocity of the aquation of the hexathiocyanato complex.

The complexity constant of the monothiocyanato iron(III) complex is according to M. Møller [10] as high as 197. Therefore even in rather dilute ferric salt solutions the main part of added thiocyanate is bound as monocomplex when $C_{Fe} \gg C_{SCN^-}$. The light absorption of the monocomplex is not much lower than that of the other thiocyanato iron(III) complexes. It has maximum absorption at $\sim$ 480 mμ ($\varepsilon^{max}_{FeSCN^{++}} = 3800$). At 480 m$\mu$ the molar extinction coefficient of the hexathiocyanato chromium(III) is as low as 3.25 while the absorption of the ferric ions is negligible. We should therefore expect that the optical density D_{480} of such solutions eventually corrected for the absorption of added chromium salts should be very nearly proportional to the free thiocyanate concentration. That this is so is shown by the data given in Table 6. D_{480}(calc.) given in the last column of the table is calculated from the formulae:

$$D_{480} = 2220\, C_{NaSCN} + 0.003 \quad (12)$$

$$D_{480} = 2500\, C_{NaSCN} + 0.003 \quad (13)$$

for solutions with $C_{NaClO_4} = 1.00$ and without sodium perchlorate, respectively.

The experiments on the hydrolysis of the hexathiocyanato complex were made as follows: Thermostated 0.02 *M* $HClO_4$ (1 *M* as for $NaClO_4$ in one series) was added to a weighed amount of the very pure complex salt $K_3Cr(SCN)_6$, 4 H_2O (see p. 924); the reaction time was reckoned from the moment of shaking. Samples taken from time to time by a Krogh syringe pipette were diluted 10.09 times in a volumetric flask and mixed with the adequate volumes of ferric nitrate and perchloric acid corresponding to the conditions in Table 6, and measured as quickly as possible in a Beckman DU spectrophotometer. To get reproducible results it was necessary to have not only the reaction flask but also the samples closed from light until measurement. The concentrations of free thiocyanate were computed by means of the relations (12) and

Table 6. Optical densities referring to a 1 cm cell at the wavelength 480 mμ for some ferric thiocyanate solutions at room temperature.

C_{NaClO_4}	C_{HClO_4}	$C_{Fe(NO_3)_3} \times 10^3$	$C_{NaSCN} \times 10^3$	D_{480}(exp.)	D_{480}(calc.)
	0.100	5.86	0.1501	0.378	0.378
	0.100	5.86	0.2996	0.752	0.752
	0.100	5.86	0.4990	1.246	1.250
1.00	0.100	5.86	0.1499	0.337	0.336
1.00	0.100	5.86	0.2994	0.668	0.668
1.00	0.100	5.86	0.4991	1.106	1.111

Table 7. Estimation of rate constants for the dissociation of the 1st thiocyanate ion from the hexathiocyanato complex.

$C_{HClO_4} = 0.02$, $C_{NaClO_4} = 1.00$

Temp. °C	Time (min.)	$C_{Cr} \times 10^3$	D^{1cm}_{480} corr.	$[SCN^-] \times 10^3$	$k_{-6} \times 10^5$ (sec^{-1})	$\bar{n}$
15.1	0	20.02	0			
15.1	29	20.02	0.035	0.145	(0.418)	5.99
15.1	125.5	20.02	0.158	0.704	0.477	5.97
15.1	181	20.02	0.241	1.082	0.511	5.95
15.1	243	20.02	0.305	1.372	0.487	5.93
15.1	333.5	20.02	0.438	1.977	0.520	5.90
				Mean	0.50 ± 0.01	
25°	0	19.96	0			
25°	39	19.96	0.213	0.954	2.10	5.95
25°	58.5	19.96	0.317	1.427	2.12	5.93
25°	81.5	19.96	0.447	2.018	2.18	5.90
25°	103	19.96	0.560	2.532	2.20	5.87
25°	127	19.96	0.680	3.077	2.19	5.85
25°	149	19.96	0.793	3.591	2.23	5.82
25°	175	19.96	0.914	4.140	2.22	5.79
25°	210.5	19.96	1.082	4.904	2.22	5.75
				Mean	2.18 ± 0.02	
			Without sodium perchlorate ($\mu \sim 0.1$)			
15.1	0	20.06	0	0		
15.1	55.5	20.06	0.094	0.367	(0.555)	5.98
15.1	113.5	20.06	0.187	0.743	0.555	5.96
15.1	163.5	20.06	0.253	1.009	0.534	5.95
15.1	229.0	20.06	0.336	1.344	0.507	5.93
15.1	274.5	20.06	0.403	1.614	0.509	5.92
15.1	310.0	20.06	0.454	1.820	0.511	5.91
15.1	339.0	20.06	0.493	1.978	0.510	5.90
				Mean	0.521 ± 0.008	
25°	0	19.97	0	0		
25°	14.5	19.97	0.101	0.396	2.30	5.98
25°	48	19.97	0.326	1.304	2.35	5.94
25°	76	19.97	0.497	1.993	2.23	5.90
25°	101	19.97	0.648	2.603	2.31	5.87
25°	122	19.97	0.780	3.136	2.34	5.84
25°	140.5	19.97	0.886	3.564	2.33	5.82
25°	159	19.97	0.993	3.996	2.34	5.80
25°	180	19.97	1.107	4.456	2.34	5.78
				Mean	2.32 ± 0.01	

(13). The optical densities referring to a 1 cm cell — besides being corrected for the absorption of the chromium salt — were also corrected for the change occurring in the time interval between the sample being removed and measured. To this purpose an experiment was carried out with the ferric nitrate added to the reaction flask from the beginning. Ferric salt catalyzes the reaction to some extent — at the concentration used here ($C_{Fe(NO_3)_3} = 0.00586\ M$) it increased the rate of the hydrolysis by about 70 %.

The hydrolysis reaction was only followed until $\bar{n} \sim 5.8$, and the first order rate constants obtained from the expression

$$k_{-6} = -\frac{2.303}{t} \log \frac{C_{Cr}-[SCN^-]}{C_{Cr}} \quad (14)$$

are given in Table 7. Measurements were made at 15° and 25° C. The values for the parameters in the Arrhenius equation E_{-6} and log Z_{-6} in 1 M $NaClO_4$ and in solutions with $\mu \sim 0.1$ given in Table 1 are computed from the mean values for k_{-6} given in the table.

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On the Existence of Ternary Transition Metal Oxides

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Among the transition metals Mo, W, Ti — Cu binary oxides with a metallic character are formed by Ti, V, Cr, Mo, W. The TiO and VO phases of the NaCl structure have broad homogeneity ranges, *viz.* $TiO_{0.60-1.25}$ [1] and $VO_{0.80-1.20}$ [2]. The atomic arrangement of the tetragonal $VO_{0.15-0.25}$ [3] phase is closely related to that of the cubic body-centered vanadium metal. The oxides Cr_3O, Mo_3O and W_3O are not isomorphous. The Cr_3O [2] and W_3O [4] phases are of the "ordered" and "disordered" A15 type respectively. Mo_3O [2] has a BiF_3 structure where only three quarters of the 4 Bi and the 12 F positions of the unit cell of that structure are occupied. The MnO, FeO, CoO, and NiO phases of the B1 type cannot, for several reasons, be characterized as metallic oxides.

The ternary Me′—Me″—O systems where Me′ = Ti, V, Cr, Mn, Fe, Co, Ni, Cu and Me″ = Mo and W have been examined in the present study.

EXPERIMENTAL

All the starting materials used in the present investigation were of fairly high purity, about 99.5 %. The oxides were prepared in two different ways.

A. Finely powdered metals were mixed in different ratios, pressed into small brickettes and sintered in zirconia crucibles in a high frequency vacuum induction furnace at a temperature of about 1 600° C. The alloys were then powdered and carefully oxidized by means of steam in the presence of hydrogen in excess at various temperatures between 600° C and 800° C.

B. Mixtures of a metal oxide $Me''O_2$ (M″ = Mo, W) and the metals Me′ (Me′ = Ti—Fe) and Me″, or metal oxide Me′O (Me′ = Mn—Ni) and Me′, Me″ were heated in evacuated and sealed silica tubes at various temperatures between 800° C and 1 100° C for up to two weeks. For Me′ = Cu the Cu_2O oxide was used. Some of the samples were heated in zirconia crucibles in a vacuum induction furnace at about 1 500° C.

The phase analysis was performed by means of powder photographs taken in focussing cameras of the Guinier type, using monochromatized Cu*Ka* and Cr*Ka* radiation.

SURVEY OF THE RESULTS

The Ti—Mo—O and Ti—W—O systems

No ternary metallic oxides were found to exist in these two systems. Because of the great stability of the binary Ti oxides these were formed during the oxidation process. After all the Ti present in the TiMe″ alloys had been

converted into TiO_2, Me″ oxides were also formed. The lattice constant of the TiO phase was found to vary between $a = 4.16$ Å and $a = 4.23$ Å in the presence of both Mo and W. The solubility of Me″ in TiO could not be determined but is most likely very small.

The V—Mo—O and V—W—O systems

No ternary metallic oxides could be prepared. The oxidation process was of the same nature as for Me″ = Ti. The lattice constants of the V_4O and VO phases were found to vary between the same limits as obtained in the investigation of the V—V_2O_3 system [2].

The Cr—Mo—O and Cr—W—O systems

No true ternary metallic oxides were found to exist. But non-metallic oxides of the composition (Cr, Me″)$_2O_3$ were obtained with the Cr/Me″ ratio probably somewhat greater than unity. The structure of these compounds is related to that of Cr_2O_3 [5,6] of the D51 type. For Cr_2O_3 the rhombohedral lattice constants are $a = 5.38$ Å, $\alpha = 56.80°$ and $Z = 2$. If hexagonal crystal axes are chosen for the Cr_2O_3 and (Cr, Me″)$_2O_3$ phases the following relation is valid: $c \sim c_{Cr_2O_3}/2$ and $a \sim a_{Cr_2O_3}$. The lattice constants of (Cr, Me″)$_2O_3$ are:
Hexagonal axes:

(Cr, Mo)$_2O_3$ $a = 4.937$ Å, $c = 6.763$ Å, $c/a = 1.370$, $V = 142.7$ Å^3, $Z = 3$
(Cr, W)$_2O_3$ $a = 4.938$ Å, $c = 6.787$ Å, $c/a = 1.374$, $V = 143.3$ Å^3, $Z = 3$

Rhombohedral axes:

(Cr, Mo)$_2O_3$ $a = 3.643$ Å, $\alpha = 85.59°$, $V = 47.58$ Å^3, $Z = 1$
(Cr, W)$_2O_3$ $a = 3.640$ Å, $\alpha = 85.43°$, $V = 47.76$ Å^3, $Z = 1$

According to a preliminary investigation the space-group is D_3^7—$R32$ with the atoms located in the positions: 2 Me distributed at random in 2 (*c*) x,x,x; $\bar{x},\bar{x},\bar{x}$ with $x \sim 0.17$ and 3 O in 3 (*d*) $0,x,\bar{x}$; $\bar{x},0,x$; $x,\bar{x},0$ with $x \sim 0.47$. It was not possible to determine the values of the x parameters with any accuracy because of the unknown deviation of the Cr/Me″ ratio from unity. The oxygen atoms in Cr_2O_3 and (Cr, Me″)$_2O_3$ are arranged in a deformed hexagonal and strongly deformed cubical close-packing respectively.

No similar oxides were found to exist in the cases of Me′ = Cr and Me″ = Ti or V.

The Mn—Mo—O and Mn—W—O systems

In these two systems ternary oxides with the ideal formula $Mn_3Me''_3O$ were obtained, although the easy formation of MnO prevented their preparation in a pure state. Oxides isomorphous with the high speed steel carbide (η carbide) Fe_3W_3C [7,8] have earlier been reported by this author, *viz.* in the case of $Me_3'Ti_3O$ [9] Where Me′ = Mn, Fe, Co, Ni or Cu *.

The values of the axial lengths measured for the η oxides were $a = 11.07$—11.11 Å for Mn_3Mo_3O and $a = 11.10$—11.13 Å for Mn_3W_3O, indicating

* η nitrides $Me_3'Me_3''N$, where Me′ = Mn, Fe, Co, Ni and Me″ = Mo or W, have also been prepared by the present author. A detailed report will shortly appear elsewhere.

comparatively small homogeneity ranges. For the isomorphous carbides Mn_3Mo_3C and Mn_3W_3C Kuo [10] reports $a = 11.13$ Å in both cases. When the different radius values of C and O, 0.77 Å and 0.60 Å respectively, are considered, it seems remarkable that the lattice constants of the η oxides and the η carbides are almost the same.

The Me′—Me″—O systems with Me′ = Fe or Co and Me″ = Mo or W

In these four systems two types of ternary metallic oxides occur, *viz.* of the η carbide structure and the L′3 type (two metal atoms distributed at random in a hexagonal close-packed unit cell). The latter oxides with the ideal formula Me′Me″O have been described earlier [2]. The following lattice constants were obtained for the η oxides: Fe_3Mo_3O $a = 10.92$—10.95 Å, Fe_3W_3O $a = 10.95$—10.97 Å, Co_3Mo_3O $a = 10.86$—10.88 Å, Co_3W_3O $a = 10.87$—10.90 Å. The existence of the analogous carbides Fe_3Mo_3C [11], Fe_3W_3C [7,8], Co_3Mo_3C [12], Co_3W_3C [11] is well established, and the observed lengths of the cube edges are about 1 % greater than for the corresponding η oxides.

No metallic oxides other than those mentioned above could be prepared, but a hexagonal oxide (Fe, W)O_2 with a structure closely related to that of the monoclinic WO_2 was obtained. The Fe/W ratio could not be determined but appears to lie between $\frac{1}{4}$ and $\frac{1}{2}$. The unit cell dimensions of WO_2 [13] are: $a = 5.560$ Å, $b = 4.884$ Å, $c = 5.546$ Å, $\beta = 118.93°$, $V = 131.8$ Å^3, $Z = 4$ and of (Fe, W)O_2: $a = 11.46$ Å, $c = 4.749$ Å, $c/a = 0.415$, $V = 450.0$ Å^3. The unit cell of (Fe, W)O_2 is derived from the cell of WO_2 if the symmetry is raised to hexagonal ($\beta = 120°$ so that the monoclinic b axis turns into the hexagonal c axis) and the monoclinic a and c axes are doubled (each turning into a hexagonal a axis). For this reason the probable cell content of (Fe, W)O_2 is 16 formula units. No systematic absence of reflections were found in the powder photograph.

The Ni—Mo—O and Ni—W—O systems

Only metallic η oxides were found to exist in these two systems as in the corresponding ternary systems with Me = Mn. The following lattice constants were measured: Ni_3Mo_3O $a = 10.85$—10.86 Å, Ni_3W_3O $a = 10.87$ Å. Kuo [10] has recently reported the existence of the η carbide Ni_3Mo_3C, while the corresponding Ni_3W_3C [11] carbide was known earlier.

The Cu—Mo—O and Cu—W—O systems

No ternary oxides could be prepared. A careful oxidation only caused the formation of Mo and W oxides.

DISCUSSION

According to the observations of the author the affinity of the transition element used for oxygen under the above mentioned reaction conditions decreases in the sequence Ti—V—Cr—Mn—Fe—Mo—W—Co—Ni—Cu. There is no doubt about the position of Mo before W but the mutual order between

Fe and the pair Mo—W is not quite clear. In any case the differences between the affinities of these three metals for oxygen seem to be small. But it is a remarkable step between Mn and Fe on one side and between W and Co on the other. It is of interest to note that Mo and W only form metallic ternary phases with Mn, Fe, Co, and Ni, *i. e.* metals which are situated close to Mo and W in the above series. η oxides are formed with Mn, Fe, Co, Ni and L'3 oxides with Fe and Co, but evidently not with Mn or Ni. In the non-metallic black compounds $(Cr, Me'')_2O_3$ the metals Mo and W seem to have the valence number 3. The degree of affinity for oxygen cannot be a dominating factor for the formation of η oxides, however, as Ti forms such oxides with the metals Mn, Fe, Co, Ni, Cu which have a much smaller affinity for oxygen than Ti. It has even proved to be easier to prepare Cu_3Ti_3O than for instance Mn_3Mo_3O.

The oxygen positions in the Cu_3Ti_3O phase could be determined from X—ray data (to be published) confirming the suggestion made by Westgren [8] that the non-metal atoms in Fe_3W_3C occupy the centres of slightly deformed Me'' octahedra. For isostructural reasons this grouping will, in all probability, include all η carbides and analogous oxides and nitrides. Thus no Me'—O contacts occur. In all metallic oxides hitherto prepared — with the exception of Cr_3O, Mo_3O and W_3O — the oxygen atoms are located at the centres of metal atom octahedra. The metals forming metallic Me—O bonds are found among the transition metals in groups IV—VI.

The ratio of the atomic radii of Me'' to Me' lies within the narrow ranges 1.09—1.13 for Me'' = Mo, W. However, the attainment of this ratio of atomic radii is not itself a sufficient condition for the formation of such carbides [10] and oxides [2] since there are metals with this ratio which fail to form such η compounds.

In order to find the mutual solubility of η oxides with Me' = Mn—Co and Me'' = Mo or W, different ternary and quarternary alloys with the gross ratio Me'/Me'' = 1 were prepared and carefully oxidized with steam. It proved difficult to obtain equilibrium, but the obtained results indicated that a homogeneous phase $(Mn, Fe, Co, Ni)_3 (Mo, W)_3 O$ most likely exists, where the different kinds of metals can be mixed in all proportions. Attempts to partially replace the nonmetals in Co_3W_3C and Co_3W_3N with O failed and other phases were formed. The question is therefore open if a total mutual solid solubility exists between the η carbides, nitrides and oxides of the metals Mo and W.

SUMMARY

Oxide phases in the systems Me'—Me''—O, where Me' = Ti—Cu and Me'' = Mo and W, have been prepared in different ways and investigated by X-ray methods.

Metallic ternary oxide phases of the η carbide structure — $Me'_3Me''_3O$ — and of the L'3 type (the metal atoms distributed at random in a hexagonal close-packed unit cell) — Me'Me''O — have been found to exist with the following combination of atoms: $Me'_3Me''_3O$, Me' = Mn, Fe, Co, Ni and Me'' = Mo, W; Me' Me''O, Me' = Fe, Co and Me'' = Mo, W.

Besides these metallic oxides ternary non-metallic oxides have been prepared, *viz.* a $(Cr, Me'')_2O_3$ phase (Me'' = Mo, W) with a rhombohedral structure

related to that of Cr_2O_3, and a (Fe, W)O_2 phase with a hexagonal structure closely related to the monoclinic one of WO_2.

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Hyaluronic Acid

V. On the Molecular Shape of Potassium Hyaluronate *

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Corresponding values of specific viscosities of solutions containing 1 mg potassium hyaluronate in 1 ml are plotted against experimentally determined molecular weights. The graph is in agreement with Staudinger's relation which is valid for flexible linear molecules.

It was realized rather early that a relation must exist between the symmetry of suspended macromolecules and the viscosity of a colloid colution [1,2]. The macromolecules are turned and twisted in Brownian motions with the consequence that the molecules appear to occupy a larger volume than they actually do. The streamlines of flow are interrupted on account of the twisting of the particles, and an additional expenditure of energy is needed to maintain a given velocity of flow, which is manifested as an increase in the viscosity.

The relation between particle symmetry and viscosity is complicated and has been treated theoretically by a number of workers [3–8]. Staudinger [9] investigated the problem from an experimental point of view using a series of long-chain hydrocarbons dissolved in inert solvents. He found that for dilute solutions, the specific viscosity, η_{sp}

$$\left(\eta_{sp} = \frac{\eta_{sol}}{\eta_{solv}} - 1\right)$$

is a function of the number of submolecules ("Grundmoleküle", *e.g.* CH_2 groups in a paraffin chain) contained in the chain — the degree of polymerisation. According to Staudinger the equation $\eta_{sp} = K \cdot P \cdot c$ is valid for randomly coiled flexible long-chain molecules. In this formula K is a constant for a given series of compounds, P is the degree of polymerisation, and c is the concentration by weight.

Part I: *Acta Chem. Scand.* **3** (1949) 584; Part II: *Ibid.* **7** (1953) 603; Part III: *Ibid.* **8** (1954) 292; Part IV: *Acta Pharmacol. Toxicol.* **10** (1954) 83.

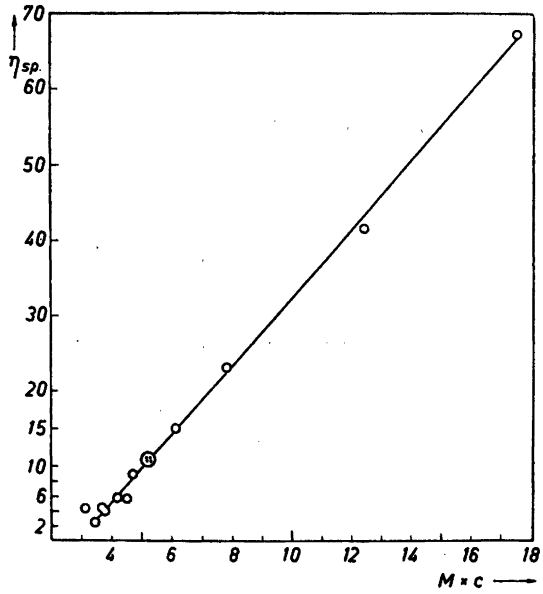

Fig. 1. The graph shows the relation between the specific viscosity (ordinates), and the product of molecular weight and concentration by weight (abscissae).

In the experiments to be reported here the degree of polymerisation (P) is replaced by the molecular weight, and the Staudinger equation is thus converted to: $\eta_{sp} = K \cdot M \cdot c$. The chemical characteristics of most specimens of potassium hyaluronate are given in recent papers by the author [10–13]. The preparations of potassium hyaluronate are of different origin: human umbilical cords, bovine synovial fluid, human pseudomucinous ovarian cysts, and the endolymph of the labyrinths of sharks. The methods of isolation have also been varied. The relative viscosities at a concentration of 1 g/l, and at the temperature of 20.0° C are determined by a Kvorning and Dalgaard-Mikkelsen [14] viscosimeter, and the molecular weights by an inverted microosmometer recently constructed by J. A. Christiansen and the author [15]. As seen from Fig. 1 the experimental data fit in with the Staudinger formula, and thus it is reasonable to conclude that the molecular shape of hyaluronic acid is of the flexible long-chain type.

Attention is drawn to the fact, that opinions are still at variance as regards the validity of Staudinger's viscosity rule [16], but it is of special interest to the present investigation that a great number of cellulose derivatives conform closely to this empiric rule [17]. On account of the great constitutive similarity between hyaluronic acid and various cellulose derivatives we may believe in the validity of Staudinger's rule also as regards hyaluronic acid.

Another observation which favours the assumption of a long-chain shape for the molecules of hyaluronic acid is the fact that our experimental data do not fit into the well-known expression of Einstein: $\eta = \eta_0 (1 + 2.5 \Phi)$, which

Table 1. Relation between specific viscosity and molecular weight of different preparations of potassium hyaluronate.

Relative viscosity	Specific viscosity	Molecular weight × 10^{-5}
3.61 [2]	2.61	3.42
4.93 [1]	3.93	3.75
5.46 [1]	4.46	3.16
5.75 [2]	4.75	3.67
6.99 [2]	5.99	4.12
6.40 [3]	5.40	4.53
10.04 [3]	9.04	4.61
11.80 [1]	10.80	5.17
11.91 [1]	10.91	5.30
12.12 [1]	11.12	5.27
12.19 [1]	11.19	5.16
16.10 [3]	15.10	6.11
24.24 [4]	23.24	7.89
42.81 [3]	41.81	12.42
68.46 [1]	67.46	17.50

1) Preparations from human umbilical cords
2) » » bovine synovial fluid
3) » » human ovarian cysts
4) » » endolymph of sharks.

is based upon the assumption of spherical rigid particles (Φ is the volume fraction occupied by the dispersed particles). We get according to Einstein

$$\eta/\eta_0 - 1 = K_{\text{Einstein}} \cdot c$$

and according to Staudinger

$$\eta/\eta_0 - 1 = K_{\text{Staudinger}} \cdot M \cdot c$$

The fundamental difference between these expressions is evident.

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Paper Chromatography of Podophyllin

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A paper-chromatographic technique has been developed for the separation and recognition of previously recorded natural constituents of *Podophyllum peltatum* L. and *Podophyllum emodi* Wall. as well as of some artifacts.

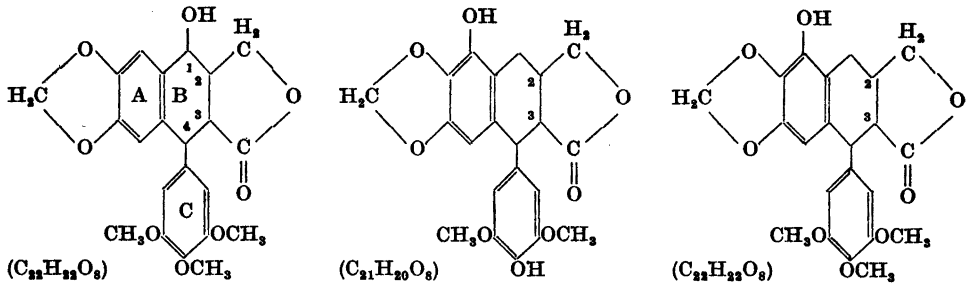
At least two spots in chromatograms of *Podophyllum peltatum* could not be accounted for on the basis of constituents at present on record. Their possible origin is discussed.

The influence of various factors on flow rates in the system formamide (sorbed stationary phase) / benzene (mobile phase) has been examined.

Podophyllin is an old cathartic, which in recent times has become of considerable interest, because it is a mitotic poison of a selective action against certain types of tumor tissue and hence is believed to be a promising point of attack in the search for a chemotherapy of cancer.

The drug podophyllin of most European pharmacopoeias and of the National Formulary USA is the alcohol-soluble resin from rhizome and roots of *Podophyllum peltatum* L. The natural antimitotic constituents so far discovered are podophyllotoxin, first isolated and named in 1881 by Podwyssotzki [1], and α- and β-peltatin, recently discovered by Hartwell and Detty [2]. The extensive structure studies by Hartwell and collaborators [2,3,6–9] lead to a revision of the original podophyllotoxin formula proposed independently by Borsche and Niemann [4] and by Späth *et al.*[5] in 1932. The latest formulations suggested by Hartwell *et al.*[6,7] are given in Fig. 1.

There are three asymmetric carbon atoms in the peltatins and four in podophyllotoxin. Hartwell and Detty [9] have prepared two optical isomers of each of the peltatins. They are denoted A and B and are shown to be C_3-epimers (Fig.1). The naturally occurring and tumor-active isomers A are readily and apparently irreversibly inverted to the tumor-inactive B-compounds when treated with basic reagents and reprecipitated with acid (opening of the lactone ring, followed by relactonization in the alternative configuration). Individual series of derivatives (esters, ethers) are formed by the four peltatins. The A-series is levorotatory, the B series is dextrorotatory or at least less levorotatory than the A series. The inversion of peltatin-A to peltatin-B has



Podophyllotoxin (PT)
trans-1:2-*trans*-2:3-*cis* 3:4

Picropodophyllin (PP)
trans-1:2-*cis*-2:3-*trans*-3:4

Epipodophyllotoxin (EPT)
cis-1:2-*trans*-2:3-*cis*-3:4

Epipicropodophyllin (EPP)
cis-1:2-*cis*-2:3-*trans*-3:4

α-Peltatin-A
trans-2:3-*cis*-3:4

α-Peltatin-B
cis-2:3-*trans*-3:4

β-Peltatin-A
trans-2:3-*cis*-3:4

β-Peltatin-B
cis-2:3-*trans*-3:4

Fig. 1. Natural constituents of Podophyllum peltatum L. *and their epimers. (Hartwell et al.)* *

been accomplished also for a great number of derivatives. Infra-red spectroscopy indicates less strain in the B-isomers. On this basis and from inversion and pyrolysis studies Hartwell and collaborators suggested that the A-series is *trans*, the B-series *cis* around the carbon atoms 2 and 3, and the complete configurations [7] are as indicated in Fig. 1.

Picropodophyllin (PP) has formerly been regarded as a structural isomer of podophyllotoxin (PT) with different positions of attachment of the lactone ring. Hartwell and Schrecker [6,9] have demonstrated that the substances are in fact C_3-epimers, analogous to the peltatins, podophyllotoxin belonging to the A-series, picropodophyllin to the B-series. They both contain one asymmetric carbon atom (C_1) more than the peltatins. The above authors have actually prepared, by a Walden inversion *via* the C_1-halides, two substances

* In a quite recent paper by J. Press and R. Brun (*Helv. Chim. Acta* **37** (1954) 190) these formulæ have been subjected to doubt. The alternative structures proposed by these investigators, mainly on the basis of elementary analyses and UV-spectra, differ fundamentally from those of Hartwell *et al.* even in the empirical formulæ. The "A-series" is claimed to be structurally different from the corresponding members of the "B-series" and not epimers as advocated by Hartwell.

The experimental results reported in the present paper are consistent with the formulations of Hartwell *et al.* At least one observation is incompatible with the Press-Brun formulæ. β-Peltatin-A and -B both give intense orange colourations with diazo-reagent in accordance with Hartwell's formula containing a phenolic hydroxy-group with a free *p*-position. The Press-Brun formula on the other hand cannot explain this reaction since there are no possibilities of coupling. The relative positions of the various substances on the paper chromatograms are equally well understood on the basis of any of the two sets of formulæ, both showing an increasing number of hydroxy-groups with decreasing R_F (Table 1).

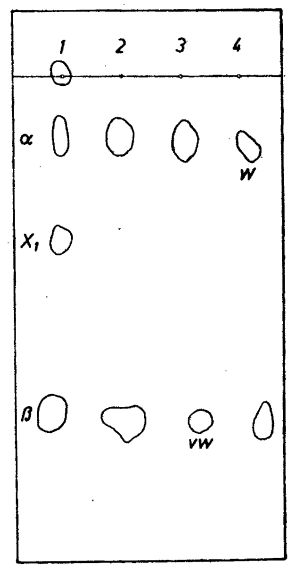


Fig. 2. Chromatogram of natural peltatins (diazo-reagent).

1. *Podophyllin ND I dep. (e peltatum).*
2. *Artificial mixture of natural podophyllotoxin, α-peltatin and β-peltatin.*
3. *Natural α-peltatin*
4. *Natural β-peltatin*

w = weak vw = very weak.

named epipodophyllotoxin (EPT) and epipicropodophyllin (EPP) (*cf.* Fig. 1) which are claimed to be C_1-epimers of the naturally occurring compounds.

For some research in another department of this institute pure podophyllotoxin and peltatins were desired. Whereas the former compound is easily prepared in a pure state by the chromatographic procedure of Hartwell and Detty [9] (activated alumina, benzene-ethanol) their method gave an imperfect separation of the peltatins. The criteria of identity and purity used by these authors were melting points and methoxyl determinations (α-peltatin contains two, β-peltatin three methoxy-groups).

In the search for a simpler and more sensitive purity- and identity test and an effective method for separation of the peltatins paper chromatography was tried. Since the substances are almost completely insoluble in water, the normal procedure involving a distribution between adsorbed water as the stationary phase and a suitable organic solvent as the mobile phase was impracticable. It was found, however, that a perfect separation could be obtained, when the paper was impregnated with formamide and benzene was used as the mobile phase. This technique has been succesfully employed in glucoside work by Zaffaroni [11] and later by Reichstein [12]. Spraying with diazobenzene-sulphonic acid solution was used for detection of the spots. The peltatins, containing one and two phenolic hydroxy-groups respectively, gave intensely coloured coupling products. Podophyllotoxin, being a secondary alcohol, did not respond to the reagent.

Fig. 2 shows a one-dimensional descending papyrogram of podophyllin alongside with the isolated components. No. 1 is a commercial podophyllin (*e peltatum*), further purified according to Hartwell and Detty [8] by precipitation of indifferent resins from an ethanolic solution on addition of benzene.

In the following it will be named "Podophyllin ND I, dep.". There are three distinct spots visible after spraying. Two of these (marked α and β) are readily identified as being due to the natural α- and β-peltatins, *cf.* Nos. 3 and 4, representing authentic products of these substances prepared according to the method of Hartwell and Detty [9]. It is seen that neither α-, nor β-peltatin is pure. The α-peltatin preparation is contaminated with minute amounts of β-peltatin and the β-peltatin contains considerable amounts of α-peltatin. There is no doubt, however, about the identity of the spots and the separation is excellent. The method therefore constitutes a simple and adequate new means of analyzing these mixtures. In No. 1 and in several runs in the following figures there is a spot left at the starting point. It was visible *before* spraying, had a brownish colour, which altered somewhat upon spraying and represents some indifferent resin still left in the purified podophyllin, probably quercetin and related flavone derivatives, which on account of several phenolic hydroxy-groups (five in quercetin) are strongly retained by the formamide-phase and do not travel on the paper.

The spot X_1 in Fig. 2 has not so far been identified. It cannot possibly be podophyllotoxin, because this substance is non-phenolic and hence should not couple with the diazo-reagent. A decisive proof is given in Fig. 2, run No. 2 in which an artificial mixture of podophyllotoxin and the peltatins (Nos. 3 and 4) has been chromatographed. Only the peltatin spots are visible. The relative position of podophyllotoxin has been established by means of a different reagent, *vide infra*. The location of X_1 in Fig. 2 differs somewhat from that in the following chromatograms, the reason being that conditions in this exploratory experiment differed from the standard procedure adopted in the following work. Fig. 2 clearly demonstrates that the position of one compound is not influenced by the presence of another constituent in the moving spot. This was a general experience in all the work described in this paper.

Next it was investigated whether X_1 might represent an epimer of one of the peltatins. Samples of natural α- and β-peltatin were inverted to the corresponding B-compounds as described by Hartwell and Detty [2] by boiling with dilute sodium hydroxide and reprecipitating with acid. The inverted products were chromatographed as Nos. 4 and 5 in Fig. 3 together with the natural peltatin-A's, Nos. 3 and 6, and the podophyllin sample containing the unknown X_1 (No. 1). It is obvious from the relative positions and the colours of the spots, *cf.* Table 1, that the unknown X_1 is not identical with α-peltatin-B or β-peltatin-B. This was confirmed in a number of experiments from which data are included in Table 1. It may be noticed that the chromatograms, Figs. 3—6, corroborate the hypothesis of Hartwell *et al.* that the inversion of A-compounds to B-compounds is complete and not a racemization, since the chromatograms of the inverted peltatins-B are absolutely devoid of A-spots.

For general analytical purposes it was desirable to be able to detect also podophyllotoxin and picropodophyllin on the chromatograms. Antimony pentachloride was found fairly suitable for this purpose. This reagent forms coloured complexes with aromatic ring systems and in this manner podophyllotoxin (PT in Fig. 4) could be located between β-peltatin-A and β-peltatin-B. Picropodophyllin (PP) has a slightly smaller R_F, *cf.* Table 1 and Fig 6. The

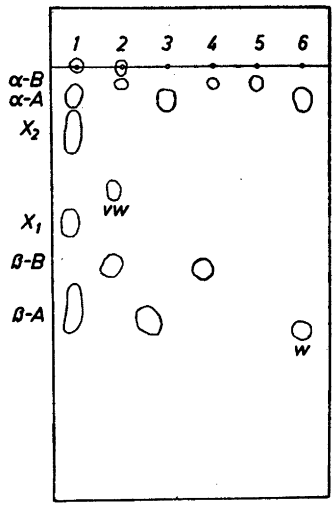


Fig. 3. Chromatogram of C_3-epimers of the peltatins (B-series of Hartwell et al., inverted according to the method of these authors [9]). Sprayed with diazo-reagent.

1. *Podophyllin ND I dep.*
2. *Podophyllin ND II dep., inverted*
3. *Natural β-peltatin*
4. *Inverted β-peltatin*
5. *Inverted α-peltatin*
6. *Natural α-peltatin.*

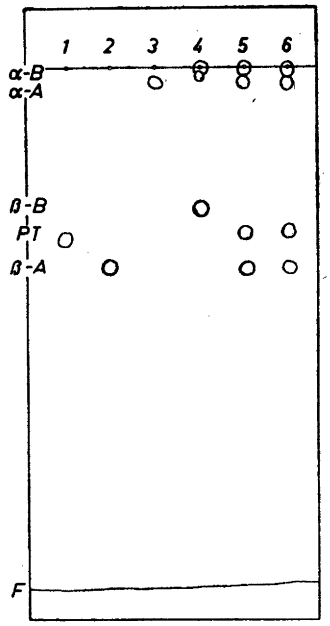


Fig. 4. Chromatogram showing the position of podophyllotoxin. Sprayed with antimony-reagent.

1. *Natural podophyllotoxin*
2. *Natural β-peltatin*
3. *Natural α-peltatin*
4. *Podophyllin ND II dep., inverted. The spot β—B covers the picropodophyllin spot, cf. Fig. 6.*
5. *Podophyllin ND I dep.*
6. *Podophyllin ND II dep.*

reaction is not very sensitive and rather large quantities of the unpleasant reagent are needed, but we have not so far succeeded in finding a more satisfactory colour-reaction, which was suitable for both the peltatins and podophyllotoxin and being at the same time indifferent to formamide. The conventional hydroxamic acid test for esters produced a distinct colour with the lactone ring, common to all the relevant compounds, but unfortunately formamide gives the same reaction and therefore had to be removed prior to spraying. On extraction of the formamide with water the spots were dissolved as well. Most of the formamide could be removed by heating the strip to 100° C for 24 hours, which did not affect the spots. The last traces of formamide were, however, difficult to get rid of and resulted in a strongly coloured background upon spraying.

Formamide can be replaced by propylene glycol, which does not interfere in the hydroxamic acid test. It is, however, rather expensive.

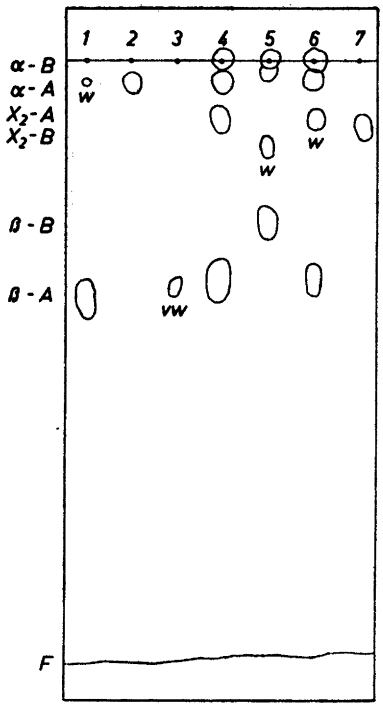


Fig. 5. Chromatogram II, Table 1.

1. *Natural β-peltatin*
2. *Natural α-peltatin*
3. *Natural podophyllotoxin*
4. *Podophyllin ND II dep.*
5. *Podophyllin ND II dep., inverted.*
6. *Podophyllum peltatum L., freshly prepared ethanol extract of fresh roots and rhizomes.*
7. *Fraction from column chromatography silicagel-formamide / benzene) containing the unknown X_2.*

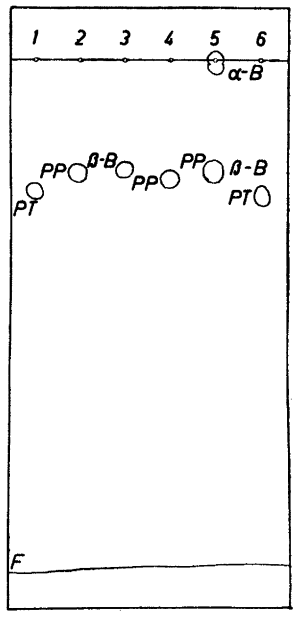


Fig. 6. Chromatogram (VIII, Table 1) showing the location of picropodophyllin.

1. *Natural podophyllotoxin*
2. *Natural podophyllotoxin, inverted*
3. *Natural β-peltatin, inverted*
4. *As No. 2*
5. *Podophyllin ND II dep., inverted*
6. *As No. 1.*

Examination of the chromatograms in ultra-violet radiation disclosed the well known fluorescence fronts due to impurities in the paper, but the spots could not be located in this manner.

A number of commercial samples of podophyllinum Ph. Dan. 48 have been investigated for the occurrence of the unknown X_1. It did not, however, appear in any of them save the one first examined (marked ND I).

During these experiments which are mainly of pharmaceutical interest (they will be published elsewhere) chromatograms were run with somewhat larger quantities of podophyllin on the paper, up to 50 μg. This resulted in the discovery of still another spot provisionally named X_2 (Fig. 5, *cf.* Table 1) in chromatograms of nearly all the podophyllin samples investigated. A

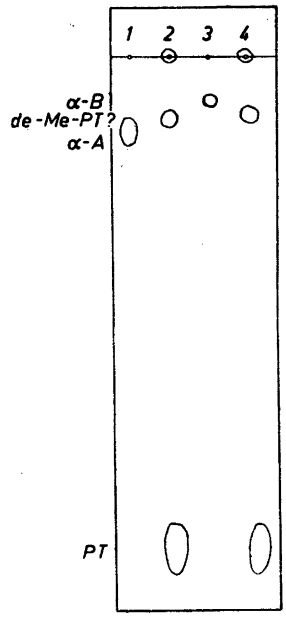


Fig. 7. Chromatogram showing the location of a constituent of Podophyllum emodi Wall., probably demethyl-podophyllotoxin.

1. *Natural α-peltatin*
2. *Emodi-podophyllin*
3. *Natural α-peltatin, inverted*
4. *As No. 2*

chromatogram of fresh roots and rhizomes of *P. peltatum* (freshly prepared ethanol extract), Fig. 5, No. 6, also showed the spot X_2, which is therefore to be regarded as a genuine constituent of this species. When podophyllin samples containing X_2 were boiled with alkali and reprecipitated with acid under conditions which convert "A-compounds" to "B-compounds" the R_F-value of X_2 was somewhat increased. Chromatograms before and after the treatment are shown in Fig. 5, Nos. 4 and 5 respectively (*cf.* Table 1). It is possible that X_2 is related to the compounds in Fig. 1 and analogously undergoes C_3-inversion. The lower spot X_2-B would then represent an epimer of the natural compound X_2-A. This obviously requires further investigation, particularly because the "inversion" causes a shift of the spot in the opposite direction to that found for the peltatins and for podophyllotoxin.

Fresh plant material of *Podophyllum peltatum* was found devoid of X_1. Since this spot has been found in one podophyllin sample only, it may be due to an artifact or to adulteration. Commercial podophyllin *e peltatum* may well be confused with resins of *Podophyllum emodi* Wall. (both are official in the British pharmacopoia). Alternatively it was not impossible that *P. peltatum* in fact contained small amounts of the inherent constituents of *P. emodi*, and one of these might be responsible for the spot X_2 found in all *peltatum* preparations. It was therefore decided to extend the paper-chromatographic work to *Podophyllum emodi*, in which Hartwell and collaborators [13] have discovered small amounts of demethylpodophyllotoxin and 1-O-(β-D-glucopyranosyl)-picropodophyllin besides the main constituent, podophyllotoxin.

Table 1. Approximate R_F-values of podophyllum constituents. Solvent system, sorbed formamide/ Sb = antimony

Chromatogram	I 12/12/2	II 15/12/1 fig. 5	III 14/12/1 fig. 4	IV 14/12/2	V 19/1/1	VI 20/1/2	VII 26/1/2
Temperature °C	20—23°	20—23°	20—23°	20—23°	21—22°	21—22°	20.8°
Spray-reagent	Diaz.	Diaz.	Sb	Diaz.	Diaz.	Diaz.	Sb
φ / Substance	0.33	0.44	0.46	0.46	0.51	0.54	0.55
α-Peltatin-B (α—B)	0.02_4	0.01_8	0.01_7		0.02_3	0.01_6	
Demethylpodophyllotoxin? (de-Me-PT)							
α-Peltatin-A (α-A)	0.04_6 0.05_1 0.04_9 0.05_1 0.04_9 } 0.05	0.03_1 0.03_6 0.03_8 0.03_8 } 0.04	0.03_2 0.03_2 0.02_9 } 0.03	0.04_5	0.03_6 0.03_9	0.03_0 0.03_0	0.02_2
Unknown (X_2)		0.11 0.10 0.10 } 0.10		0.09_6	0.11	0.09_0 0.09_0 } 0.09	
Epimer of X_2? (X_2—B)	0.15	0.15			0.15	0.12	
Unknown (X_1)	0.31						
β-Peltatin-B (β—B)	0.33	0.27	0.27		0.27	0.23	
Picropodophyllin (PP)							0.22 0.23
Podophyllotoxin (PT)			0.32 0.33 0.32 } 0.32				0.29 0.28 0.26
β-Peltatin-A (β—A)	0.41 0.41 0.41 0.41 } 0.41	0.37 0.37 0.37 0.39 } 0.38	0.38 0.39 0.38 } 0.38	0.41	0.39 0.40	0.35 0.35	0.35
β-Naphthole (N)							

Fresh roots of *P. emodi* were worked up to emodi-podophyllin by precipitating an ethanolic extract with 10 volumes 0.3 *N* hydrochloric acid, washing with water and air-drying. A paper-chromatogram of this product is given in Fig 7, Nos. 2 and 4. Emodi-podophyllin gives two spots detectable with

benzene. φ = gram formamide per gram paper. Diaz. = diazobenzene sulphonic acid reagent. pentachloride reagent.

VIII 27/1/1 fig. 6	IX 20/1/1	X 16/12/1	XI 16/12/2	XII 27/1/2	XIII 17/1/1	XIV 19/1/2	XV 21/1/1	Colour of spot	
25.0	21—22°	20—23°	20—23°	25.0±0.2	9.5°	21—22°	20.8°		
Sb	Sb	Diaz.	Diaz.	Sb	Sb	Sb	Sb		
0.57	0.58	0.59	0.59	0.59	0.60	0.61	0.67	diazo-reagent	antimony reagent
	0.01_7				0.01_7			red	violet
	0.02_4				0.02_3			no reaction	violet
	0.03_1, 0.03_4 } 0.03				0.02_3, 0.02_9	0.02_9, 0.02_9	0.02_1	brownish red	violet
			0.11					yellow	
								yellow	
								bright yellow	
(0.22) 0.22	0.23	0.24, 0.24 } 0.24	0.24	(0.18) 0.19	0.23	0.24		orange	brownish yellow
0.23 0.22				0.19 0.19			0.19 0.18	no reaction	brownish yellow
0.26 0.27	0.29, 0.30, 0.31, 0.30, 0.29 } 0.30			0.24 0.23	0.27 0.28	0.28, 0.29, 0.28, 0.28, 0.27 } 0.28	0.22 0.22	no reaction	brownish yellow
	0.35, 0.36, 0.34 } 0.35	0.34, 0.35, 0.36 } 0.35	0.34, 0.34, 0.34 } 0.34		0.35	0.33 0.34	0.26	orange	brownish yellow
		0.46	0.43					red	

antimony pentachloride, neither responds to diazo-reagent in the concentrations in which the peltatins produce intense colouration. The lower spot is due to the main constituent, podophyllotoxin. The upper spot may well represent demethylpodophyllotoxin. It is located above α-peltatin, where

one might expect to find it, for the following reasons. Demethylpodophyllotoxin has a phenolic hydroxy-group in stead of a methoxy group in 4-position in the ring C (Fig. 1). It follows that α-peltatin and demethylpodophyllotoxin bear the same relationship to eachother as do β-peltatin and podophyllotoxin. Since in the latter pair the shift of a hydroxy-group from ring A (phenolic function) to ring B (alcoholic function) causes a decrease in R_F, demethylpodophyllotoxin should have a lower R_F-value than α-peltatin. The apparent rule that an increasing number of phenolic hydroxy-groups decreases the R_F value is supported by the observation that the indifferent resins, mainly quercetin (pentahydroxy-flavone) do not travel at all on the formamide paper (*cf.* p. 944).

The fact that the upper spot does not respond to the diazo-reagent, although this is by far the more sensitive, is also believed to support the hypothesis that it be due to demethylpodophyllotoxin. Coupling with the diazonium group never occurs in meta position to a hydroxy-group, so it is fairly certain that the positive reaction of the peltatins only involves ring A, and that the diazonium group enters the *p*-position to its phenolic hydroxy-group. As a consequence the spot, if due to demethylpodophyllotoxin, should not couple, as indeed we find. However, Hartwell and collaborators [13] report that demethylpodophyllotoxin gives an orange-red colour with diazotized *p*-nitroaniline. The spot under discussion does not respond to this reagent either. A rigorous proof of its identity by matching it with an authentic sample is therefore most needed. This is on our program. It is less probable that the picropodophyllin-glucoside, discovered in emodi-podophyllin is responsible for the upper spot in Fig. 7, Nos. 2 and 4, although this possibility cannot be quite excluded.

Some experiments were made on the reproducibility of the R_F-values. They were found to be strongly dependent on the amount of formamide present in the paper. Some data are collected in Table 1. φ is the formamide content in g per g paper. On some chromatograms several spots occurred of the same substance. Individual R_F-values as well as the average are given in the table. The deviation between any two individual R_F's on the same strip was less than 5 % and usually much smaller. The mobile phase was benzene throughout the experiments and the temperature was 20—23° C. No significant variation of R_F with temperature was observed, *cf.* chromatogram XIII run at 9.5° C. When the formamide content was increased from about 0.3 to 1.3 g per gram paper, the R_F-values gradually decreased. High formamide contents diminished the rate of flow of the solvent, and the formamide phase was imcompletely sorbed by the paper tissue, which was noticeable as a slow downward drift of the initial spots during the period of equilibration. With long irrigation periods good separation, but large, diffuse spots were obtained. When the formamide content was decreased below 0.2, development went too rapidly, the R_F-values varied with φ in the opposite way, probably due to imperfect equilibrium, separation was poor and there was a marked tailing. Optimal conditions were at 0.4 to 0.7 g formamide per g paper (3 to 6 mg/cm^2). Although a certain desired amount of formamide could fairly easily be reproduced by the method given in the experimental section, and the R_F-values in periods were quite reproducible, other factors, difficult to control, seem to influence the

flow rates, for instance moisture variations *etc.* in the formamide phase. Fluctuations up to 10 % were noticed in experiments carried out under supposedly identical conditions. The use of absolute R_F-values for identification purposes therefore had to be abandoned, and reliable identification of individual spots was accomplished by running authentic samples simultaneously on the paper. The sequence of the various substances in the chromatograms was invariably that given in Table 1, but all R_F-values should be regarded as relative values, strictly comparable only within the individual paper strip (vertical columns). This still presupposes an even distribution of formamide on the strip. Experience showed that this could be secured by the impregnation method described in the experimental section.

The original aim at a more effective preparative separation of the constituents was now conveniently combined with a search for the unknowns X_1 and X_2 in quantity. Column partition chromatography on the principle outlined in this paper has been performed on a larger quantity of podophyllin. The paper-chromatographic technique described in this publication was used as an indicator for cutting the fractions. In Fig. 4, No. 7 is reproduced a chromatogram of the fraction containing X_2. Attempts to isolate and identify this substance — or these substances — are in progress, and will be reported in a following publication.

EXPERIMENTAL

Materials. A commercial formamide Merck was used without further purification. It had n_D^{20} 1.4472 and the density D_4^{22} 1.130 (lit. 1.4472 [14] and 1.1313 [15], respectively). Ordinary thiophene-free benzene was used. A chromatographic test proved that it was sufficiently pure for the present purpose.

Podophyllotoxin was prepared according to the method of Hartwell and Detty [2]. Recrystallized from benzene and air-dried it had a melting point 111.5—112° C. This product, which contained liquid of crystallization, was used in the experiments. Borsche and Niemann [16] report a melting point of 112—115° for a product of the composition 2 $C_{22}H_{22}O_8$, 2 H_2O,C_6H_6. Our preparation, after drying at 110° C and 0.01 mm Hg for 18 h had a m. p. 188—89° (lit. 183—84°).

The peltatins were also prepared according to the procedure of Hartwell and Detty [2]. α-Peltatin melted at 227—228° (lit. 230.5—232.5), β-peltatin at 214—228° (lit. 231—238). Each of them contained some of the other peltatin, as shown by the chromatograms.

Picropodophyllin was prepared by refluxing 0.1 g podophyllotoxin for 6 hours with 2 ml absolute ethanol and 0.02 g sodium acetate, precipitating the product with cold water and recrystallizing from ethanol. M. p. 230—231.5° C (lit. 224.5—226.5 [13]).

Podophyllin ND I, dep. and Podophyllin ND II dep. Two commercial samples meeting the requirements of Pharmacopoea Danica, ed. 1948, further purified by precipitation of indifferent resins from an ethanolic solution on addition of benzene.

Emodi-podophyllin. Roots and rhizome of *Podophyllum emodi* Wall, grown in Denmark were worked up to podophyllin immediately after having been digged up. The material was washed, ground in a laboratory mill with 2 parts of 96 % ethanol, and macerated for 2 days, then for 8 days with 2 parts of absolute ethanol and finally for 14 days with two parts of a mixture of equal amounts of benzene and ethanol. The extracts were mixed, evaporated to one third at a temperature not exceeding 40° C and poured into 10 volumes of 0.3 *N* hydrochloric acid. The yellow, crystalline precipitate was washed with water and dried.

Apparatus. The chromatography chamber was made from an all-glass battery case, 21 by 30 cm and 60 cm high. It was closed with a heavy glass-plate, sufficiently tightened by means of a thin velvet tape, glued to the plane-ground upper edge of the case. In the uppermost part of the chamber was fastened a stainless-steel rack, supporting the usual

glass-trough for the descending technique. During the runs the glass chamber was placed in a closed wooden cabinet, provided with glass windows for inspection of the strips. This arrangement gave a sufficient temperature-constancy for the present purpose; Chromatograms were run at 20—23° C.

Paper strips. Whatman paper No. 1 was used. The strips were usually 22 × 55 cm (net weight 10 g), requiring approximately 5 g formamide. A desired and evenly distributed formamide content could fairly easily be obtained by spraying the previously weighed paper strip as uniformly as possible with 100 % excess of formamide, leaving the strip between filter paper sheets for at least 18 hours and until the formamide content was reduced to the desired value.

Procedure. The substance was applied to the impregnated paper strip as circular spots along a marked starting line in the usual way. When a single substance was chromatographed, normally a 1 % ethanolic solution was used; of this 1—2 μl was required, *i. e.* the quantity in each spot was approximately 10—20 μg; 1 μg still gave a detectible spot with the diazo-reagent, whereas the lower limit with antimony reagent was *ca.* 10 μg. When natural or artificial mixtures were chromatographed the quantity applied to the paper was increased *pro rata* (up to 400 μg). After evaporation of the solvent the strips (two were run simultaneously) were suspended in the chamber, which contained a layer of formamide-saturated benzene on the bottom. Two opposite walls of the chamber were lined with filter paper, soaked in and dipping into the bottom liquid. The solvent vapour pressure in the chamber was essentially that of benzene, since at 20° C formamide has a very small vapour pressure and is practically insoluble in benzene. Equilibration of the strips in the solvent atmosphere were extended to 18 h prior to development. The solvent used for development was benzene saturated with formamide. The normal time of development on paper containing 0.5 g formamide per gram was *ca.* 4 hours (travelling distance 40 cm from starting line to solvent front). The strips then should be removed from the chamber and the solvent-front quickly marked. In this connection the volatility of benzene was a great nuisance. Some investigators have proposed to develop to a previously marked pencil-line, which means continuous watching of the chromatograms towards the end of the development period, and limits the number of chromatograms, run simultaneously, to one only. An automatic and accurate location of the solvent-front was obtained in the following way. Before equilibration and development 4—6 narrow, equidistant and vertical bands of "sudan blue-G" were applied to the paper as a 1 % solution in benzene and beginning approximately 5 cm from the lower edge of the strip and extending right to the edge. The development could then be terminated at any convenient time after the benzene front had reached the indicator-bands, and the front would be clearly and permanently indicated by a concentration of the dye. Although sudan blue-G (1-methylamino-4-(*p*-toluidino)antraquinone) is completely insoluble in benzene-saturated formamide and very soluble in the benzene phase, the substance usually moves slightly behind the actual front, probably due to adsorption on the paper. For this reason vertical bands *in situ* were preferred to spots travelling with the solvent.

After development the strips were air-dried for some minutes and then sprayed in a fume-cupboard with either antimony pentachloride (3.5 ml dissolved in 10 ml tetrachloromethane) or with an alkaline diazo-reagent prepared in the following manner. 0.5 g sulfanilic acid was dissolved in 12.5 ml concentrated hydrochloric acid and 75 ml water. Immediately before use 4 ml of this stock-solution was mixed with 1 ml 0.1 *N* aqueous sodium nitrite and 5 ml 2 *N* sodium hydroxide. The boundaries of the spots were traced with a pencil, because the colour gradually faded.

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Studies on Lignin Models

The Reaction of Phenyl-Coumaran Structures with Thioglycolic Acid

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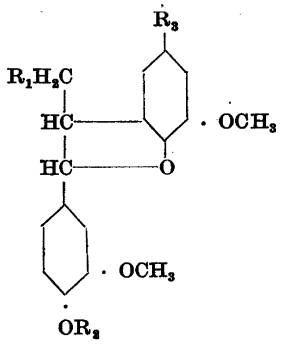
By heating with thioglycolic acid the lignin model substances (III—VI), which contain a 2-phenyl-coumaran structure, are converted into the substances (IX—XIV).

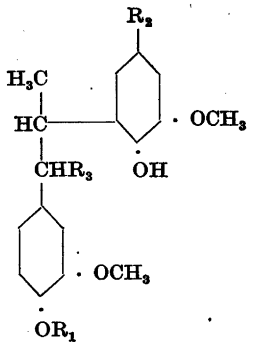
As assumed by Freudenberg and his co-workers the biosynthesis of gymnosperm lignin involves dehydrogenative polymerization of coniferyl alcohol[1], producing three intermediate compounds arising from two coniferyl alcohol molecules being linked together in different ways [2]. These dimeric compounds contain benzyl alcohol and benzyl ether groups; structures which have previously been assumed to be responsible for the sulphonation of lignin [3].

One of the dimeric compounds, dehydro-diconiferyl alcohol (I), contains a benzylaryl ether group constituting part of a phenyl-coumaran system. Previously, Freudenberg [4] had assumed that such structures are building elements of the lignin molecules, and his recent findings support this assumption. That the acid (II)[5], which contains the phenyl-coumaran structure, is transformed by acid sulphite into the sulphonic acid (VII) by rupture of the benzylaryl ether link [6] is further support for Freudenberg's assumption. The acid (II) reacts with thioglycolic acid in a similar manner forming compound (VIII)[6].

The acid (II) contains a carboxyl group which may influence the stability of the benzylaryl ether link, and we have therefore studied the reaction of thioglycolic acid with model substances containing the coumaran structure but no carboxyl group: namely dehydro-di*iso*eugenol (III), dihydro-dehydro-di*iso*eugenol (IV) and their methyl ethers (V and VI).

Heating these substances for 1.5—2 hours with thioglycolic acid containing hydrochloric acid afforded in practically quantitative yield thioglycolic acid adducts which were difficult to purify as they were syrups from which crystalline derivatives could not be obtained. For this reason the analytical values obtai-





	R_1	R_2	R_3
I	OH	H	$CH{:}CH{\cdot}CH_2OH$
II	H	CH_3	COOH
III	H	H	$CH{:}CH{\cdot}CH_3$
IV	H	H	$CH_2{\cdot}CH_2{\cdot}CH_3$
V	H	CH_3	$CH{:}CH{\cdot}CH_3$
VI	H	CH_3	$CH_2{\cdot}CH_2{\cdot}CH_3$

	R_1	R_2	R_3
VII	CH_3	COOH	SO_3H
VIII	CH_3	COOH	SCH_2COOH
IX	H	$CH_2{\cdot}CH_2{\cdot}CH_3$	»
X	H	$CH(SCH_2COOH){\cdot}CH_2{\cdot}CH_3$	»
XI	H	$CH_2{\cdot}CH(SCH_2COOH){\cdot}CH_3$	»
XII	CH_3	$CH(SCH_2COOH){\cdot}CH_2{\cdot}CH_3$	»
XIII	CH_3	$CH_2{\cdot}CH(SCH_2COOH){\cdot}CH_3$	»
XIV	CH_3	$CH_2{\cdot}CH_2{\cdot}CH_3$	»

ned on powdery derivatives (benzylthiuronium salts or acetates) did not agree well with those calculated. However, they showed that one molecule of the hydrogenated substances (IV) and (VI) reacted with about one molecule of the thioglycolic acid, and that the other, (III) and (V), reacted with about two molecules of the acid.

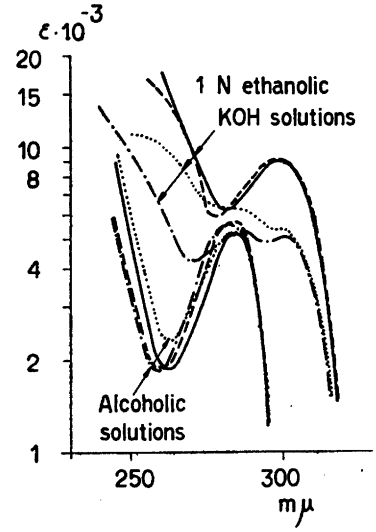


Fig. 1. The absorption curves for the thioglycolic acid adducts.
The adduct from (III) ———
(IV) — — — —
(V) .....
(VI) —·—·—

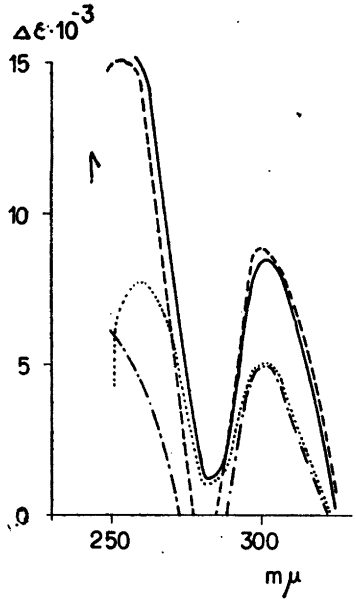


Fig. 2. The Δε-curves for the thioglycolic acid adducts.
The adducts from (III) ————
(IV) – – – –
(V) .....
(VI) —·—·—

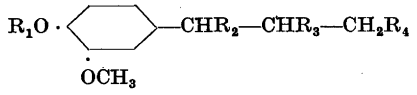
The structures of the adducts were elucidated by a spectrophotometric examination. In alcohol the model substance (III) has λ_{max} 274 mμ, ε_{max} 20 000 [7]. The hydrogenated derivative (IV) has λ_{max} at about the same wave-length, 281 mμ; ε_{max} for that derivative, however, is 5 400, which is much lower than the value for (III) [7]. The difference in ε_{max} may be attributed to the fact that only (III) has an aliphatic double bond conjugated with a benzene nucleus. The spectra of all the thioglycolic acid adducts have λ_{max} at 282—284 mμ and ε_{max} about 5 500 in alcoholic solution (Fig. 1). Thus, the curves of the products are very similar to the curves of the hydrogenated derivative (IV) but diverge from that of (III). It was concluded, therefore, that the aliphatic double bonds of (III) and (V) had disappeared during the reaction by addition of thioglycolic acid.

If the benzylaryl ether group in the model substances reacted as that in the acid (II) the adducts should contain a new phenolic group. The number of phenolic groups in the adducts has been estimated by the spectrophotometric method introduced by Aulin-Erdtman [8]. The absorption spectra of neutral and alkaline solutions of the adducts are shown in Fig. 1, and in Fig. 2, the so-called Δε-curves. All the Δε-curves have a maximum at about 300 mμ, which is characteristic for phenols which do not contain a double bond conjugated with the benzene ring. The value of Δε at this maximum is for the adducts from the methylated model substances (V) and (VI), about 5 000. This value is about the same as that found by Aulin-Erdtman [8] for monophenols and therefore these adducts contain one phenolic group in the molecule. The corresponding values for the adducts from the other model substances (III and IV) is about 9 000 showing that they contain two phenolic groups.

Thus, this evidence shows that during the reaction with thioglycolic acid a new phenolic group is formed.

To sum up, the thioglycolic acid most probably reacted with the benzyl-aryl ether links of the four compounds and with the aliphatic double bonds of (III) and its methyl ether (V). The products obtained may, therefore, have the structures (IX—XIV).

When thioglycolic acid is added to the aliphatic double bond in (III) or its methyl ether (V) two isomers can be formed: (X) and (XI) from (III), and (XII) and (XIII) from (V). Holmberg [9] has shown that when styrene reacts with thioglycolic acid the isomer containing the $S \cdot CH_2 \cdot COOH$ group in the β-position to the phenyl group resulted. We have found that when thioglycolic acid was added to *iso*eugenol two isomers, the benzylthiuronium salt of one having m.p. 120—1° and that of the other having m.p. 130—1°, were formed. The first of these compounds was also obtained from 1-(3-methoxy-4-hydroxyphenyl) propanol (XV) by heating with thioglycolic acid, and as it is known that a hydroxyl group in a benzyl alcohol can be substituted by a $S \cdot CH_2 \cdot COOH$ group in this manner [9,10] it is reasonable to assume the compound is the α-derivative (XVII), and the other isomer the β-derivative (XIX). Only one compound was obtained from the methyl ether of *iso*eugenol, and as it was not identical with the product from 1-(3,4-dimethoxyphenyl) propanol (XVI), the α-derivative (XVIII), it must therefore be the β-derivative (XX). It appears possible from the above evidence that the thioglycolic acid adducts obtained from (III) and its methyl ether (V) are mixtures of (X) and (XI), and of (XII) and (XIII) respectively.



	R_1	R_2	R_3	R_4
XV	H	OH	H	H
XVI	CH_3	OH	H	H
XVII	H	SCH_2COOH	H	H
XVIII	CH_3	SCH_2COOH	H	H
XIX	H	H	SCH_2COOH	H
XX	CH_3	H	SCH_2COOH	H
XXI	H	H	H	SCH_2COOH

Thioglycolic acid was also added to the double bond of eugenol but the reaction was slow in comparison with that of *iso*eugenol. The acid obtained was not identical with the α-derivative (XVII) or the β-derivative (XIX). This evidence and the fact that the adduct contained no C-methyl group show that it was the γ-derivative (XXI).

If lignin contains phenyl-coumaran structures of the type examined in this investigation, it is probable that they will react with thioglycolic acid during the preparation of lignothioglycolic acid from lignin. As mentioned above

phenolic elements are formed in this reaction. Thus it is possible to estimate the frequency of such coumaran structures in lignin from the number of phenolic groups formed during the preparation of thioglycolic acid, *e. g.* the difference in the number of phenolic groups in lignothioglycolic acid and that of lignin.

According to Aulin-Erdtman [11] about one seventh of the phenylpropane monomers in lignothioglycolic acid contains a phenolic group. It is known that lignin in the native state also contains phenolic elements, but their frequency is not known. For that reason the number of phenolic groups formed cannot be estimated, but, the low frequency in lignothioglycolic acid shows that the number produced during the preparation cannot be high. It is probable, therefore, that the frequency of phenyl-coumaran structures of the above-mentioned type is rather low in lignin.

EXPERIMENTAL

A mixture of the model compound (m g), thioglycolic acid (t g), water (w ml) and 2N HCl (c ml) was heated at 100° for h hours. A large amount of water was added to the product and the resultant precipitate washed well with water, dissolved in ether and separated into acidic (a g), and phenolic or neutral material (b g) by bicarbonate. The following compounds were examined.

1-(3-Methoxy-4-hydroxyphenyl) propanol (XV). m 0.6, t 1.5, w 1.5, c 0.1, h 1.5, b 0.02. The acidic reaction product was neutralized with 2 *N* NaOH and a solution of benzylthiuronium chloride (800 mg) in water (1.2 ml) was added. The oily precipitate, which solidified overnight, was dissolved in hot alcohol. The insoluble benzylthiuronium salt (20 mg) of thioglycolic acid was collected and hexane was added to the filtrate. The benzylthiuronium salt (860 mg) of S-α-guaiacylpropyl-thioglycolic acid (XVII) crystallized in needles, m.p. 120–1°. Yield 63 %. (Found: OCH_3 7.3; S 14.9; C 57.0; H 6.19; N 6.2. Calc. for $C_{20}H_{26}O_4S_2N_2$ (422): OCH_3 7.3; S 15.2; C 56.8; H 6.16; N 6.6.)

This salt (500 mg) was covered with ether and decomposed by addition of dilute HCl. The free acid (250 mg), m.p. 52–53.5°, crystallized very slowly. The acid was converted to its sodium salt and crystallized from alcohol by addition of ether. (Found: Na 8.28. Calc. for $C_{12}H_{15}O_4SNa$ (278): Na 8.14.)

1-(3,4-Dimethoxyphenyl) propanol (XVI). m 0.42, t 1.0, w 1.0, c 0.1, h 1.5, b 0.02. The acidic product was converted to the benzylthiuronium salt. The oily precipitate, which solidified immediately, crystallized from alcohol. The benzylthiuronium salt (550 mg) of S-α-veratrylpropyl-thioglycolic acid (XVIII) crystallized in fine needles, m.p. 149.5–150°. Yield 59 %. (Found: OCH_3 14.2; S 14.3; C 58.0; H 6.65; N 7.0. Calc. for $C_{21}H_{28}O_4S_2N_2$ (438): OCH_3 14.2; S 14.6; C 57.5; H 6.39; N 6.4.)

Isoeugenol. m 15, t 17, w 15, c 1, h 1.5, a 16, b 2. The acidic product was neutralized with 2 *N* NaOH and kept in a refrigerator overnight. The crude Na-salt (4.8 g) of S-α-guaiacylpropyl-thioglycolic acid (XVII) separated. On concentrating the mother liquor to half its volume, a further quantity (0.25 g) of the same material was obtained. The sodium salt was crystallized from alcohol giving the analytically pure salt (3.25 g), from which the benzylthiuronium salt was prepared. No m.p. depression was observed on admixture with the product obtained from 1-(3-methoxy-4-hydroxyphenyl) propanol. The free acid regenerated from this salt also showed the same m.p. and mixed m.p. with that obtained from the propanol.

The mother liquor was diluted with an equal amount of water and a solution of benzylthiuronium hydrochloride (15 g) in water (50 ml) added. The oily salt which separated was washed with water, dissolved in chloroform (100 ml). The solution was dried and kept in a refrigerator. The benzylthiuronium salt (6.6 g) of S-α-guaiacylpropyl-thioglycolic acid separated from the solution and after washing with chloroform the salt was obtained in pure state.

The chloroform solution was concentrated to dryness, the residual syrup dissolved in octyl alcohol (70 ml) and was kept in the refrigerator. The crude benzylthiuronium salt (4.39 g) of S-β-guaiacyl*iso*propyl-thioglycolic acid (XIX) precipitated and after crystallization from acetone-ether gave the pure salt (3.9 g) in thin plates, m.p. 130—1°. It showed marked depression on admixture with the benzylthiuronium salt of S-α-guaiacylpropyl-thioglycolic acid. (Found: OCH_3 7.1; S 15.1; C 56.7; H 6.27; C-CH_3 3.84, 3.62. Calc. for $C_{20}H_{26}O_4S_2N_2$ (422): OCH_3 7.3; S 15.2; C 56.8; H 6.16; C-CH_3 6.16.)

A large amount of petroleum ether was added to the mother liquor and the oil which separated was dissolved in acetone, a small amount of petroleum ether added and the solution kept in a refrigerator for two weeks. The crystals which separated were crystallized from alcohol-petroleum ether giving a further quantity (450 mg) of benzylthiuronium salt of S-α-guaiacylpropyl-thioglycolic acid. Yields: α 36, β 12 %.

Isoeugenol methyl ether. m 4.5, t 4.8, w 3.8, c 0.3, h 3, a 5.4, b 0.25. The acidic product was converted to its benzylthiuronium salt in the usual way. The salt obtained directly as a crystalline mass was dissolved in alcohol and precipitated by hexane. The precipitate (4.4 g), m.p. 133—4°, was crystallized from alcohol. The benzylthiuronium salt of S-β-veratryl*iso*propyl-thioglycolic acid (XX) was obtained in plates, m.p. 135—6°. It showed marked depression on admixture with the benzylthiuronium salt of the α-acid (XVIII). Yield 42 %. (Found: OCH_3 13.9; S 14.5; C 57.7; H 6.72; N 6.5. Calc. for $C_{21}H_{28}O_4S_2N_2$ (438): OCH_3 14.2; S 14.6; C 57.5; H 6.39; N 6.4). None of the α-isomer could be detected.

Eugenol. m 11, t 13, w 15, c 3, h 7, a 5, b 6.5. The acidic product was converted in the usual way to the benzylthiuronium salt, which was dissolved in hot alcohol. On cooling, the benzylthiuronium salt (2 g) of S-γ-guaiacylpropylthioglycolic acid (XXI), m.p. 144.5—145.5°, crystallized. Ether was added to the mother liquor giving a further quantity (2.5 g) of the same material having the same m.p. Yield 40 %. (Found: OCH_3 7.8; S 14.9; C 56.6; H 6.39; C-CH_3 0.8. Calc. for $C_{20}H_{26}O_4S_2N_2$ (422): OCH_3 7.3; S 15.2; C 56.8; H 6.16; C-CH_3 0.0. None of the salt of the β-isomer (XIX) could be detected.

S-γ-guaiacylpropyl-thioglycolic acid (0.28 g) (XXI) of m.p. 69—70° was obtained from this salt (0.5 g) in the manner described above for (XV). (Found: S 12.3. Calc. for $C_{12}H_{16}O_4S$ (256): S 12.5.)

Dihydro-dehydro-diisoeugenol (IV). m 5, t 15, w 10, c 1, h 2, a 7.26, b 0.11. The acidic product was acetylated with pyridine and acetic anhydride. The acetylated derivative of (IX) was obtained as a white amorphous powder, m.p. 60—64°. (Found: OCH_3 11.7; S 6.7. Calc. for $C_{26}H_{32}O_6S$ (504): OCH_3 12.3; S 6.4).

Dehydro-diisoeugenol methyl ether (V). m 5, t 20, w 25, c 3.5, h 1.5, a 6.8, b 0.08. The acidic product was converted in the usual way to its benzylthiuronium salt, which was purified by precipitation from a chloroform solution by addition of petroleum ether. The salt begins to sinter at about 70° and melts at 100—110°. (Found: OCH_3 10.4; S 14.1. Calc. for $C_{41}H_{52}O_8S_4N_4$ (856): OCH_3 10.9; S 14.9.)

Dehydro-diisoeugenol (III). m 5, t 20, w 25, c 3.5, h 1.5, a 6.1, b 0.05. The acidic product was converted to its benzylthiuronium salt, which was purified by precipitation from an abs. alcoholic solution by petroleum ether and washed with ether. M.p. 69—80°. (Found: OCH_3 7.9; S 14.3. Calc. for $C_{40}H_{50}O_8S_4N_4$ (842): OCH_3 7.4; S 15.2).

Dihydro-dehydro-diisoeugenol methyl ether (VI). m 0.7, t 2.1, w 1.4, c 0.14, h 1.5, a 0.86, b 0.02. The acidic product was analyzed directly. (Found: OCH_3 21.6; S 7.3. Calc. for $C_{23}H_{30}O_6S$ (434): OCH_3 21.4; S 7.4.)

The authors wish to acknowledge their indebtedness to Dr. G. Aulin-Erdtman for valuable discussions on the spectrophotometric part of this work, and to Professor E. Adler for the gift of the alcohols (XV) and (XVI.)

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The Potential Determining Process of the Exchange of Cadmium between Amalgan and Cadmium Cyanide Solutions

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The cadmium exchange between amalgam and solutions containing the free cadmium ion or cadmium complexes of moderate strength, *e.g.*, bromide, iodide, and ammonia complexes, was investigated by one of the present authors in two previous works [1,2]. Then it was proved that at the cadmium concentrations chosen and at equilibrium between the two phases the slowest partial process of the exchange was the diffusion of cadmium ions and complexes in the solution phase up to the interface.

Accordingly, the expression for the rate h of the exchange was of the form

$$h = k \cdot C \tag{1}$$

where C denotes the total cadmium concentration of the solution. The rate constant k was of about the same magnitude for different complexes, and this was to be expected for a diffusion-controlled process.

On the other hand, for very strong cadmium complexes it can be presupposed the the loosening of the ligands, which must precede the electron transfer, requires such a great free energy of activation that this partial process will be the slowest one in the whole cadmium exchange. In view of this, some preliminary measurements of the exchange with cadmium cyanide solutions, containing sodium cyanide in excess, were carried out using the experimental procedure described before [1]. Then it was found that the value of the ratio h/C was less than one tenth of the values of k in the previous investigations. Such a result could not possibly be explained by differences in diffusion coefficients, and thus these introductory measurements indicated that with cyanide solutions of such composition that the saturated complex predominates, the cadmium exchange is not controlled by diffusion.

For that reason it seems possible to derive the kinetic law of the ligand loosening and electron transfer by means of a radioactive tracer. Furthermore, from the kinetic law it should be possible to decide which of the complexes predominates in the exchange process under the existing conditions and thus

causes the attainment of a rather well defined equilibrium potential difference between the phases. The aim of the present investigation is to give an answer to these questions.

THEORY OF THE KINETIC INVESTIGATION

In order to obtain equations as general as possible we must presuppose that each complex of the cadmium cyanide system can take part in the cadmium exchange at the interface. According to an investigation by Leden [3] the system is composed of mononuclear complexes and the saturated one is $Cd(CN)_4^{2-}$. Thus, we have a set of reversible electrode processes of the type:

$$Cd(CN)_j^{2-j} + 2\ e^- \rightleftharpoons Cd + j\ CN^- \qquad (2)$$

with $j = 0, 1, \ldots 4$. Here the water ligands, present in the lower complexes with $j < 4$, have been omitted for the sake of brevity. When an equilibrium potential difference between the phases has been attained, the processes in both directions in (2) proceed at constant rates, and the sum of the rates of the forward processes for different complexes is equal to the sum of the rates of the reverse processes and constitutes the total rate h of the cadmium exchange.

The discharge process of a certain complex can be presupposed to involve two stages (*cf.* Glasstone, Laidler, and Eyring [4]). In the first stage all the ligands, including water molecules, are loosened, and the "naked" cadmium ion is transferred to the amalgam surface. In the following stage, which we may call the electron transfer, the cadmium ion at the surface is taken up in the amalgam. For strong complexes it is plausible that the first-mentioned stage is the slowest one and thus is rate-determining.

It is characteristic of electrode processes of this kind that the electric potential difference between electrode and solution influences the free energy of activation. In Fig. 1 the electrochemical potential (designation according to Guggenheim [5]) of the cadmium ion is schematically represented as a function of the distance r from the amalgam surface. At first we presuppose that the electrode potential has a fixed value ε'. Then the value of $\mu_{Cd^{2+}}$ in the potential pocket at A is that of a naked cadmium ion adsorbed on the amalgam surface, and when r increases, the $\mu_{Cd^{2+}}$-value follows the curve AB (*cf.* Fowler [6]).

On the other hand, when the cadmium ion is bound in the complex $Cd(CN)_j^{2-j}$ in the solution the $\mu_{Cd^{2+}}$-value is that at the point C. For decreasing values of r the complex is deformed, and $\mu_{Cd^{2+}}$ increases along the curve CB. Thus we obtain an energy barrier ABC before the amalgam surface, and at B we have the activated state.

Now we presuppose that the electric potential difference between amalgam and solution is changed to $\varepsilon(< \varepsilon')$. This means that the potential energy curve AB is moved down in relation to the curve BC and takes the position A'B', the transfer being equal to $(\varepsilon'—\varepsilon)2F$, where F is the faraday. It is easy to deduce from Fig. 1 the following approximate expression for the decrease d in the $\mu_{Cd^{2+}}$-value at the crossing of the potential-energy curves.

$$d = \frac{\delta_2}{\delta_2—\delta_1}(\varepsilon'—\varepsilon)2F \qquad (3)$$

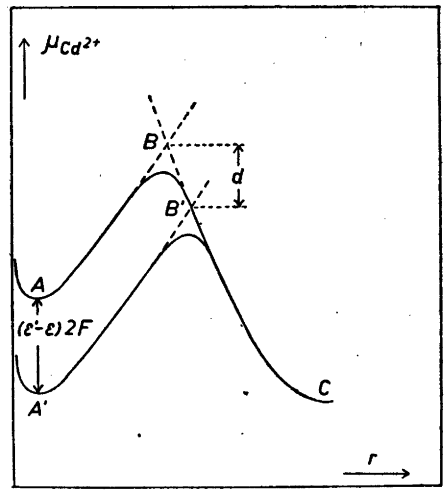

Fig. 1. The electrochemical potential $\mu_{Cd^{2+}}$ *as a function of the distance r from the amalgam surface.*

Here δ_1 and δ_2 denote the slopes of the curves A'B' and BC at the point of intersection B'. For the sake of brevity we put $\alpha = \delta_2/(\delta_2 - \delta_1)$. It is evident that the validity of the relation (3) presupposes that ε is not very different from ε', which means that the variations in ε must not be very great. Under these conditions α can be treated as a constant, and we have $0 < \alpha < 1$.

In passing it can be mentioned here that because of the existence of the quantum mechanical exchange, or resonance, energy (Ref.[4], p. 136) in the activated state the resulting energy barrier should be represented somewhat rounded off at B and B' as indicated in Fig. 1.

From the theory above it is obvious that when the electrode potential is changed from ε' to ε the free energy of activation of the forward process of (2) decreases by the quantity $\alpha(\varepsilon' - \varepsilon)2F$, and that of the reverse process increases by the quantity $(1-\alpha)(\varepsilon' - \varepsilon)2F$. Then for the reaction rate h_j of the discharge of $Cd(CN)_j^{2-j}$ at the electrode potential ε we get the relation:

$$h_j = k_j'[Cd(CN)_j^{2-j}] \cdot e^{\alpha(\varepsilon'-\varepsilon)\,2F/RT} \tag{4}$$

Here k_j' is the specific reaction rate at the potential ε'. Now we assume that ε is the equilibrium electrode potential, and then we have:

$$\varepsilon = \varepsilon_j^0 + \frac{RT}{2F} \ln \frac{[Cd(CN)_j^{2-j}]}{q[CN^-]^j} \tag{5}$$

where q denotes the cadmium concentration in the amalgam. The very dilute amalgam is an approximately ideal solution (*cf.* Fronæus [1], p. 772), and if the activity coefficients in the water solution can be treated as constants, when a neutral salt medium of constant ionic strength is used, then k_j' and ε_j^0 are constants.

When ε is eliminated by combination of the equations (4) and (5) the following relation is obtained:

$$h_j = \text{constant} \cdot q^{\alpha}[\text{Cd(CN)}_j^{2-j}]^{1-\alpha}[\text{CN}^-]^{j\alpha} \quad . \tag{6}$$

If the complex solutions contain sodium cyanide in excess, Cd(CN)_4^{2-} predominates in the solutions, and we have approximately $[\text{Cd(CN)}_4^{2-}] = C$. Then introducing the complexity constants β_j we get:

$$[\text{Cd(CN)}_j^{2-j}] = \beta_j\beta_4^{-1}\, C\, [\text{CN}^-]^{j-4} \tag{7}$$

Finally, the equations (6) and (7) are combined, and the different constant factors are replaced by a single quantity k_j, that may be called the rate constant of the discharge of Cd(CN)_j^{2-j}. For the total rate $h = \sum_{j=0}^{4} h_j$ of the cadmium exchange at equilibrium electrode potential we then obtain the expression:

$$h = \sum_{j=0}^{4} k_j\, q^{\alpha}\, C^{1-\alpha}\, [\text{CN}^-]^{j-4\,(1-\alpha)} \tag{8}$$

It should be mentioned that α can have different values for different complexes. However, it is probable that within a limited concentration range of the free cyanide ion practically only one of the complexes is responsible for the exchange. Then the corresponding term in the right member of eq. (8) predominates, and from the dependence of the measured exchange rate h upon q, C, and $[\text{CN}^-]$ the values of α and j can be determined from the following set of relations:

$$\left(\frac{\Delta \log h}{\Delta \log q}\right)_{C,[\text{CN}^-]} = \alpha; \quad \left(\frac{\Delta \log h}{\Delta \log C}\right)_{q,[\text{CN}^-]} = 1-\alpha \tag{9a—b}$$

$$\left(\frac{\Delta \log h}{\Delta \log [\text{CN}^-]}\right)_{q,C} = j-4\,(1-\alpha) \tag{9c}$$

Thus the value of α can be obtained in two independent manners. This gives us a check on the validity of the relation (8) which is especially valuable.

For α-values between 0.25 and 0.75 and constant values of q and C it is evident that h_0 and h_1 decrease while h_3 and h_4 increase for increasing values of $[\text{CN}^-]$, and thus a relative minimum in h may appear.

EXPERIMENTAL

Chemicals. Stock solutions of sodium cyanide were prepared, and their concentrations were checked by titrations with silver nitrate according to Liebig [7]. The solutions are not stable.

The other chemicals used were prepared as before [1].

Measurements. The apparatus previously [1] described was not suited for the exchange measurements in the present investigation, because the times of contact between the phases were too short to give accurately measurable exchanges with the radioactive cadmium perchlorate available. Furthermore, for lower rates of discharge a longer time of contact should be chosen to secure the attainment of the equilibrium electrode potential after a small fraction of the time of contact.

In the present case the following experimental procedure was applicable. V (= 15.0) ml of a radioactive solution of the composition

$$\begin{cases} C \text{ mC } Cd(ClO_4)_2 \text{ containing some } Cd^{115} \\ C_{CN^-} \text{ mC NaCN} \\ (1\,000 - C_{CN^-}) \text{ mC } NaClO_4 \end{cases}$$

were introduced into a glass-stoppered bottle. Then the bottle and the solution were freed from oxygen by a stream of nitrogen. From a microburette a fixed amount of inactive cadmium amalgam was added rapidly, after which the bottle was shaken in a thermostat for a time τ varying between 2 and 9 minutes at a temperature of 20.0° C. In the calculations below τ was taken as the time of contact. In order that the average area of the amalgam surface should be constant in all the exchange experiments one and the same bottle was used, and the shaking was always performed in exactly the same way.

After the shaking the amalgam was separated from the solution as rapidly as possible and washed with water. The amalgam was then treated with dilute nitric acid, which dissolved the cadmium and some mercury. By addition of sodium chloride the mercury was removed, and the cadmium was precipitated as hydroxide. This was redissolved in a small amount of hydrochloric acid, and a solution of sodium chloride was added, so that a medium suitable for a polarographic cadmium analysis was obtained. This was carried out with a Radiometer polarograph (model PO 3h), and from the amount of cadmium per unit volume of solution the corresponding amount m of amalgam was calculated.

On a fixed volume of the solution the beta radioactivity was measured with the apparatus used before [1], and the concentration C_a' of radioactive cadmium was expressed in the number of counts per minute (cpm) obtained. Furthermore, the concentration C_a of radioactive cadmium in the complex solution was measured in the same way before as well as after the shaking with the amalgam, and the average value was computed. Corrections for coincidence losses and recalculations of corresponding values of C_a' and C_a to a common time were made by using an active cadmium perchlorate solution with a radioactivity lower than C_a as the reference solution.

Then the concentration q_a of radioactive cadmium in the amalgam after the time of contact τ was obtained from the relation: $q_a \cdot m = C_a'$.

DERIVATION OF THE KINETIC LAW FROM THE MEASUREMENTS

In the equations below the additional quantities q_i and C_i are used, representing the concentrations of inactive cadmium in the amalgam and in the solution. The differential equation for the radioactive exchange was deduced in an earlier paper [1], and it has the following general form:

$$m_0 \frac{dq_a}{dt} = \frac{s_0 h}{C q} (C_a q_i - C_i q_a) \qquad (10)$$

m_0 is the weight of the amalgam phase and s_0 the average area of the interface. In the present case with good stirring in both phases q_a certainly had the same value throughout the whole amalgam. Because of the experimental conditions mentioned above s_0/m_0 is a constant, which may be included in the rate constant. Furthermore, q_i and C_i are practically equal to the total concentrations q and C and thus independent of the time t. Then, if the relation: $m_0 q_a =$

Table 1. Measurements of the exchange of radioactive cadmium between cyanide solutions and amalgams.

q per cent by weight	C mC	[CN⁻] mC	τ min	$\frac{q_a(\tau)}{C_a(\Theta\tau)}$ ml . g⁻¹
1.00	8.9	26.8	3.0	0.27
			4.5	0.40
			6.0	0.55
			7.5	0.65
			9.0	0.84
1.00	17.8	26.8	3.0	0.22
			4.5	0.31
			6.0	0.45
			8.0	0.57
			9.0	0.65
1.00	8.9	62.5	3.0	0.19
			4.5	0.30
			6.0	0.37
			7.5	0.46
			9.0	0.55
1.00	17.8	62.5	3.0	0.16
			4.5	0.23
			6.0	0.31
			7.5	0.36
			9.0	0.45
2.00	8.9	26.8	2.0	0.21
			3.0	0.32
			4.0	0.47
			5.0	0.57
			6.0	0.77
2.00	17.8	62.5	3.0	0.20
			4.5	0.24
			6.0	0.35
			7.5	0.41
			9.0	0.45

$V\{C_a(0)—C_a(t)\}$ is taken into consideration, it is evident that eq. (10) can easily be solved. But since the exchange is followed for short times and not until radioactive equilibrium between the phases, we prefer to treat eq. (10) in the following way:

$$q_a(\tau) = h \int_0^\tau \left(\frac{C_a(t)}{C} - \frac{q_a(t)}{q} \right) \mathrm{d}t \qquad (11)$$

putting $q_i = q$ and $C_i = C$. Now for low t-values q_a/q is negligible in comparison with C_a/C, and thus we get

$$q_a(\tau) = \frac{h}{C} \cdot C_a(\Theta\tau) \cdot \tau \qquad (12)$$

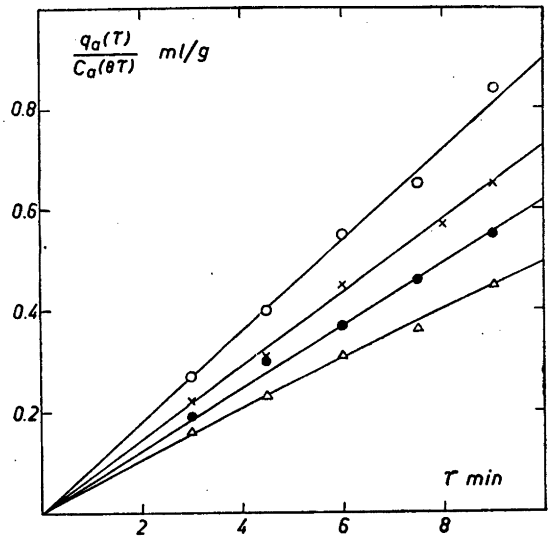

Fig. 2. The function $q_a(\tau)/C_a(\Theta\tau)$ for q = 1.00 per cent and for different values of C and [CN⁻]. *1. C = 8.9 mC,* [CN⁻] = *26.8 mC* (○); *2. C = 17.8* mC, [CN⁻] = *26.8* mC (×) *3. C = 8.9* mC, [CN⁻] = *62.5* mC (●); *4. C = 17.8* mC, [CN⁻] = *62.5* mC (△).

with $0<\Theta<1$. As the decrease in C_a, caused by the exchange, was rather small for the times of contact used, we can put with sufficient accuracy $C_a(\Theta\tau) = 0.5 \{C_a(0) + C_a(\tau)\}$. Then, if $q_a(\tau)/C_a(\Theta\tau)$ at constant values of q, C, and [CN⁻] is graphically represented as a function of τ, a straight line should be obtained, the slope of which gives the value of h/C.

The data from the exchange measurements are collected in Table 1. As mentioned above practically all of the cadmium in the solution is bound in the fourth complex at the surplus of sodium cyanide used (*cf.* Leden [3]), and thus the values of [CN⁻] were calculated from the relation [CN⁻] = $C_{CN^-} - 4C$, where C_{CN^-} denotes the total cyanide concentration.

If the q-values are expressed in mmoles per gram, it is found that the quantity q_aC/qC_a has values lying between 0.01 and 0.1, and thus the condition for the applicability of eq. (12) is fulfilled. From Figs. 2 and 3 it is evident that when $q_a(\tau)/C_a(\Theta\tau)$ is plotted against τ for constant values of the concentration parameters, straight lines are obtained for the first five measurement series. This indicates that s_0, now included in the rate constant, really has a constant value within one and the same series. The last series in Table 1 gives a slightly curved line, but for decreasing τ-values the slope tends to that, valid for the third series, and this is in accordance with the equation (8). Thus it is very probable that s_0 has the same value also within different series, and the conditions for a determination of α and j from the measurements seem to be very well fulfilled.

From the slopes of the curves in Figs. 2 and 3 the six h-values recorded in Table 2 have been obtained. Then from the first and fifth values, put into eq.

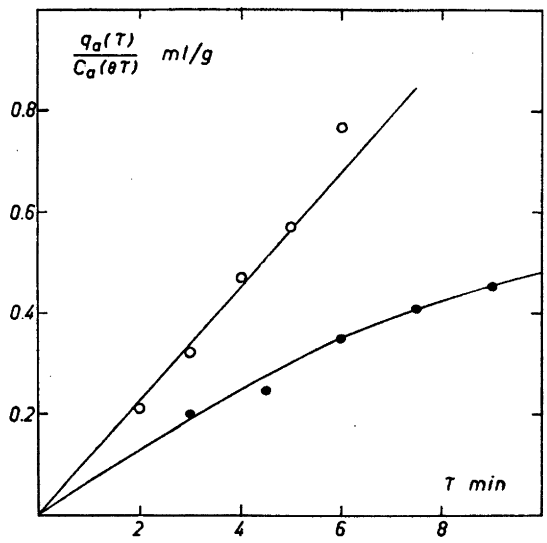

Fig. 3. The function $q_a(\tau)/C_a(\Theta\tau)$ for q = 2.00 per cent and for different values of C and [CN⁻]. *1. C = 8.9* mC, [CN⁻] = *26.8* mC (○); *2. C = 17.8* mC, [CN⁻] = *62.5* mC (●).

(9a), we get $\alpha = 0.34$ and from the fourth and sixth ones $\alpha = 0.36$. Furthermore, applying eq. (9b) to the first and second h-values on one side and to the third and fourth ones on the other, we find $\alpha = 0.30$ and 0.34 respectively. Thus the q-dependence and the C-dependence of h give the same α-values within the limits of experimental random errors, and this is a very good check on the validity of eq. (8).

Finally, eq. (9c) is used on the first and third h-values, giving j—4(1—α) = —0.44, and on the second and fourth h-values with the result —0.47. Then combining the average values 0.34 ± 0.02 of α and —0.46 ± 0.02 of j—4(1—α) we get $j = 2.2 \pm 0.1$.

Table 2. The exchange rate h for different values of the concentration parameters.

No.	q per cent by weight	C mC	[CN⁻] mC	$h \cdot 10^7$ mole.g^{-1} min^{-1}
1	1.00	8.9	26.8	8.0
2	1.00	17.8	26.8	13.0
3	1.00	8.9	62.5	5.5
4	1.00	17.8	62.5	8.7
5	2.00	8.9	26.8	10.1
6	2.00	17.8	62.5	11.2

DISCUSSION

The result of the present investigation shows that within the concentration range used of the cyanide ion surplus the total rate of the cadmium exchange is practically completely controlled by the discharge process of $Cd(CN)_2$. Thus this complex causes the attainment of the equilibrium electrode potential, though its concentration is very small in comparison with the concentrations of the third and fourth complexes. Obviously the cause is that for the electrode potentials existing at the cyanide concentrations used the simultaneous loosening of three or four cyanide ligands requires very high energies of activation. On the other hand, the hydrated cadmium ion and the first complex, which certainly require lower energies of activation for discharge, cannot influence the exchange rate noticeably, as their concentrations are too low.

After the present investigation had been carried out an investigation of the same electrode process by Gerischer [8] has been reported in the literature. The experimental procedure involved impedance measurements on the amalgam electrode, a method developed and used by several authors.

It is of great interest to compare the results obtained by the two quite different methods, especially as it seems as if radioactive tracers have not been previously used for the study of the kinetics of such electrode processes. At the cyanide ion concentrations used by us Gerischer finds that the rate of exchange at equilibrium electrode potential is controlled by the discharge of the second complex, and in this respect the accordance between the results is complete. On the other hand, from the q-dependence of the rate he gets the value 0.25 ± 0.02 of α (denoted $1-\alpha$ by him), and thus there is some difference from our value. However, it should be mentioned that, judging from what is reported, he has not used the C-dependence of the rate for a check on the α-value determined.

According to Gerischer the exchange is controlled by the discharge of the third complex at higher cyanide ion concentrations (and thus lower ε-values). We did not extend our measurements to high concentrations, as we found that under such conditions there is some dissolution of cadmium from the amalgam.

SUMMARY

By using a radioactive tracer the exchange of cadmium at equilibrium potential between liquid amalgams and cadmium cyanide solutions, containing sodium cyanide in surplus, is investigated.

Preliminary measurements according to an experimental procedure described before [1] show clearly that the rate of exchange is not diffusion-controlled in the present case but is determined by the discharge process at the interface. From the closer investigation, giving more accurate values of the radioactive exchange, the kinetic law of the discharge process is derived.

By comparing this kinetic law with the expression theoretically derived, it can be established that for the cyanide ion concentrations used (between 25 and 65 mC), when practically all of the cadmium in the solution is bound in

the complex $Cd(CN)_4^{2-}$, the rate of exchange is controlled by the discharge of $Cd(CN)_2$, present in very small concentrations. Thus this complex is the "potential determining" one under the conditions given.

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A Comparative Study of Cytochrome c from Beef, Chicken and Salmon

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In 1941 Theorell and Åkeson [1] published the first extensive chemical analysis of cytochrome c. The enzyme was in this case prepared from beef heart; horse heart cytochrome c was reported to be identical as regards molecular weight, nitrogen and sulphur content. Moreover, the titration curves of the two cytochromes were identical between pH 1.6 and 11. Three histidine groups were found in the molecule and it was suggested that two of them participated in the linkage of the iron-porphyrin to the protein. In 1951 Paul [2] showed that at least one histidine residue is linked to the iron atom; he could not exclude the possibility that a carboxyl group of some other amino acid residue as well as the imidazole group was linked to the iron atom.

The main purpose of our experiments to be reported was to correlate the histidine content of the cytochromes from beef, chicken and salmon with their titration curves. In addition, the pepsin degradation method of Tsou [3] was applied to these cytochrome studies and the peptides obtained analyzed for histidine.

Since the time when Theorell and Åkeson's investigation was reported new methods of preparation and analysis have been introduced, which make it possible to carry out amino acid analysis on only a few milligrams of protein. Thus paper chromatography as well as spectrophotometric measurements and acid-base titrations could be employed in the present work.

METHODS

Histidine determinations. Since Pauly's reaction is not specific for histidine in a mixture of amino acids (Kapeller-Adler [4]) and only small amounts of cytochrome were available (in some cases only a few milligrams) Henderson and Snell's microbiological method[5] which permits determination of as little as 3 micrograms of histidine was used *.

The cytochrome (either before or after pepsin treatment) was hydrolyzed under reflux with 20 % HCl, usually for 20 hrs. It was checked in some experiments that histidine

* The microbiological determinations were kindly performed by Dr. A. Wretlind at the Pharmacological Department, Karolinska Institutet, Stockholm.

was not destroyed during the hydrolysis by adding a known amount of L-histidine to the cytochrome c preparation to be hydrolyzed and comparing it with a hydrolysate without any addition. As long as the temperature never exceeds 108° there is no danger of racemization (Fränkel [6], Neuberger [7]) which would interfere with the microbiological determination. The hydrolysate was freed from insoluble humin-substances * by filtration through a sintered funnel and evaporated *in vacuo* to remove the hydrochloric acid. The pH was adjusted with NaOH to 6.8 and the solution diluted to 5 or 10 ml depending on the amount of hydrolyzed sample. A suitable concentration of histidine for the assay is 3—10 μg per ml. The experimental error of the method is given as ± 5 % by Henderson and Snell [5], but in control experiments the author has found that the error is usually smaller.

Tryptophane determination. Samples of 0.4—1.5 mg protein were hydrolyzed in 2 ml 5 *N* NaOH at 56° for 15.5 hrs in sealed tubes and tested for tryptophane according to the method of Bates [9], which permits determination of as little as 50 μg of tryptophane.

Paper chromatography. The amino acids of the hydrolysates were chromatographed according to the method of Consden, Gordon and Martin [10]. The same solvents and paper were used as by Maehly and Paléus [11]. Usually 300—400 μg of amino acids were applied to the paper in each experiment.

Paper electrophoresis. A method described by the author [12] was used. Paper "Munktell 20, 150 g" was found most useful for analysing samples of 1—2 mg protein. The electrophoresis was performed at pH 9.87 and 7.0 and developed with 0.2 % ninhydrin in 95 % water saturated butanol and 5 % 2 *N* acetic acid.

Titrations. The measurements were performed in a glass beaker of about 10 ml volume into which fitted a plexiglas chamber with compartments for: a) a glass electrode (Radiometer G 100A), b) a burette capable of adding 0.2 μl at a time **, c) a salt bridge (3 % agar in saturated potassium chloride solution) and d) gas inlet and outlet tubes. An electronic potentiometer *** was used for measuring the potentials. Magnetic stirring was employed. The glass-electrode was checked against three buffers: 0.05 potassium biphthalate, pH 4.00, Sörensen phosphate buffer, pH 6.8, and a Beckman standard buffer pH 9.22. The temperature was kept at 20°.

The titrations of ferricytochrome were performed in an atmosphere of nitrogen freed from carbon dioxide by passing through sodium hydroxide. Ferrocytochrome was prepared according to Theorell [1] by adding 10—15 mg platinum black to the sample at neutral reaction and letting first N_2 and then H_2 pass through until the potential was stable. The platinum black was prepared according to O. Loew [13] and its reducing capacity checked by reducing an aliquot of the cytochrome that was used during the titration and measuring its absorption at 550 mμ before and after the addition of $Na_2S_2O_4$. The result showed that the reduction by platinum black + H_2 was 90 % complete. The hydrogen used was washed free from O_2 by passing it through a vanadous sulphate solution (Meites and Meites [14]) before entering the titration vessel.

An amount of cytochrome containing 1.3—1.5 × 10^{-6} gram atoms of iron dissolved in a volume of 2.0 ml glass distilled water was used. Prior to titration with NaOH (1.43 *N*) the solutions were acidified to about pH 3.5 with HCl (2.7 *N*) in order to remove CO_2. During the titration the ionic strength never exceeded 0.02. According to Paul [15] cytochrome c is stable between pH 1.6 and 12.3 but in these experiments the titration range was limited to pH 3.5—9.2, as beyond these limits too many groups will interfere with the evaluation of the curves.

Spectrophotometry. The measurements were made at room temperature in a Beckman spectrophotometer Model DU using, as a rule, cells of 1 cm optical path length.

The spectrophotometric data were obtained in 0.1 *M* acetate buffer, pH 6.0 (Tint and Reiss [16]). It was verified that the spectrophotometric values did not vary within the pH-range 5.5—6.5. The reducing agent was solid sodium dithionite. The cuvettes were covered with glass plates.

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* It has been emphasized by Horn *et al.*[8] that this step is important for the subsequent microbiological assay.

** In this case an "Agla" micrometer syringe manufactured by Burroughs Welcome & Co., London.

*** Radiometer, Copenhagen, type PHM 3 f.

MATERIAL

1) Preparations. As the source of cytochrome c, hearts from beef, chicken* and salmon were used. Chicken and salmon hearts were frozen after killing and collected until about 10 kg had accumulated, when they were thawed and treated like fresh beef hearts. The first steps of the cytochrome c preparation followed Keilin and Hartree [17]. The mince was thus extracted with 2.5 % trichloroacetic acid, except in the case of salmon, where the yield was much better when the trichloroacetic acid was replaced by 5 % H_2SO_4 to a pH of 4.0, fractionated with $(NH_4)_2SO_4$ after neutralization and the cytochrome precipitated with 20 % trichloroacetic acid. This precipitate was centrifuged down, suspended in the smallest possible volume of 0.05 *N* ammonia and dialyzed against the same solution. After lyophilization the enzyme was purified according to a modification of the method of Paléus and Neilands [18]. The precipitation of the eluate from the ion exchanger by trichloroacetic acid was avoided by using lyophilization. This has also been recommended by Margoliash [19] and by Neilands [20]. The fraction with the highest iron content (Fraction II) was used.

The cytochromes used for titration were beef cytochrome with an iron content of 0.42 %, chicken cytochrome with 0.40 % iron and salmon cytochrome with 0.43 % iron.

2) Digestion with pepsin. The cytochrome preparations described above were degraded by Tsou's [3] method. Crystallized pepsin "Bios Laboratories" was used and its activity tested (Anson [21]). Currie and Bull [22] have shown the pH to be an important factor determining the size of the peptide produced. In all digestion experiments the pH was therefore carefully adjusted to 1.50 against a glass electrode. The same number of Hb-units of pepsin per milligrams of cytochrome were used in all experiments.

RESULTS

The results of the Fe and N determinations are summarized in Table 1.

From the above figures it is seen that the iron contents of the beef and salmon preparations are lower than those found by the author in 1950 (0.466 % and 0.461 %, resp.). The chicken preparations, however, contain a little more iron than is usually reported. The author found an iron content of 0.42 % (unpublished experiments) in agreement with Tint and Reiss [16].

Table 1. Nitrogen and iron determinations of the different cytochromes.

Preparation	N %	Fe-content %	Molecular weight based on Fe-content
Beef	15.01	0.449	12 400
Chicken			
Prep. 1	—	0.434	12 900
Prep. 2	15.33	0.436	12 800
Salmon			
Prep. 1	—	0.434	12 800
Prep. 2	15.57	0.452	12 350

* The chicken hearts were kindly supplied by courtesy of AB Findus, Hälsingborg.

Table 2. Results of the histidine determination of the different cytochromes.

Source	Histidine (%)	Histidine (moles) found	probable	Earlier results found	probable
Beef	3.39	2.70	3	2.91 (Theorell and Åkeson [1])	3
				2.97 (Paul [2])	3
Chicken	3.38	2.82	3		
Salmon	2.16	1.76	2		

The histidine analysis of the above preparations gave the results recorded in Table 2.

The experimentally determined histidine values lie somewhat below the theoretical numbers but this is to be expected. During the handling of the preparation losses occur especially during the filtration from humin substances. There is also some error inherent in the assay method which, however, is diminished by taking the mean value of several hydrolysates.

The amino acid composition of the different cytochromes was studied by paper chromatography on samples of up to 400—500 μg protein in order to detect amino acids which occur only in small amounts (Table 3).

*Table 3. Amino acid composition of the different cytochromes according to paper chromatography. Weak spots are indicated by brackets *.*

	phe	ala.	gly.	ser.	thr.	pro.	opr	his.	arg.	lys.	cys.	tyr.	try.	glu.	asp.	leuc.	val.	met
Beef	(+)	+	(+)	+	+	+	—	(+)	+	+	+	+	+	+	+	+	+	(+)
Chicken	(+)	+	(+)	+	+	+	—	(+)	+	+	+	+	+	+	+	+	+	(+)
Salmon	(+)	+	(+)	+	+	+	—	(+)	+	+	+	+	+	+	+	+	+	(+)

* Abbreviations of the amino acids according to Brand and Edsall [40], cys. = cysteic acid.

Methionine gave only a very weak spot but the iodoplatinate test of Winegard and Toennies [23] (performed on the hydrolysate) gave a positive reaction in all samples.

Table 3 shows the amino acid distribution of the cytochrome of beef, chicken and salmon to be very similar. Only quantitative differences in the participating amino acids seem to exist. This is confirmed by the nitrogen content and, to a certain degree, by spectrophotometric and titrimetric data given below.

From Table 4 it is seen that the extinction coefficients ($E^{1\%}_{1cm}$) of the different species vary more than the molecular extinction coefficients (ε). The difference could mean that the protein part of the molecules have different compositions. This is also indicated by the ratios in Table 5, where, especially

Table 4.

Cyto-chrome (form)	Wave-length (mμ)	$E^{1\%}_{1cm}$ Beef	Salmon	Chicken	ε * Beef	Salmon	Chicken	M.W.
oxidized	280	19.15	20.19	18.46	22 940	26 250	23 630	Beef = 11 980
	520	12.91	11.56	11.62	15 466	15 028	14 870	Salmon = 13 000
reduced	535	5.87	5.40	5.67	7 035	7 020	7 260	Chicken = 12 800
	550	22.41	20.19	21.02	26 882	26 247	26 905	

* Molar extinctions per gram atom of iron.

Table 5. Some ratios between the molar extinction coefficients at different wavelengths.

$\frac{\varepsilon_{550}}{\varepsilon_{280}}$ Beef	Salmon	Chicken	$\frac{\varepsilon_{550}}{\varepsilon_{535}}$ Beef	Salmon	Chicken	$\frac{\varepsilon_{520}}{\varepsilon_{535}}$ Beef	Salmon	Chicken
1.17	1.00	1.13	3.82	3.70	3.70	2.20	2.13	2.05

in the case of salmon $\frac{\varepsilon_{550}}{\varepsilon_{280}}$ differs from the others, possibly due to a rather high content of the aromatic amino acids in this particular cytochrome. The alkaline hydrolysate of salmon cytochrome gives an especially strong tryptophane reaction.

Table 6. Results of the titrations. The first three columns show the titration values for the ferricytochromes. The last column shows the titration values of beef ferrocytochrome.

pH	Equivalents *			
	chicken [a]	salmon [a]	beef [a]	beef [b]
4.5	6.40	5.25	6.20	5.90
5.0	7.85	7.30	7.65	7.18
5.5	8.65	8.75	8.65	8.36
6.0	9.30	9.50	9.35	9.16
6.5	9.75	9.85	9.75	9.66
7.0	10.10	10.10	10.15	10.10
7.5	10.35	10.35	10.40	10.44
8.0	10.60	10.65	10.65	10.72
8.5	10.95	11.15	10.90	10.94
9.0	11.65	12.20	11.50	11.21

* The equivalent scale has an arbitrary origin and is arranged so that 10 equivalents are used at pH 6.8.

[a] oxidized.

[b] reduced.

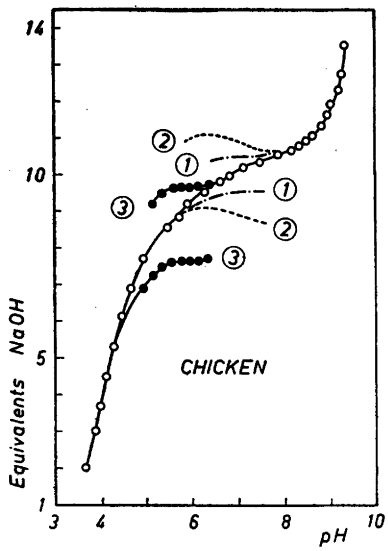

Fig. 1. The titration curve of chicken cytochrome c.

Abscissa: pH, ordinate: equivalents of NaOH. The origin of the ordinate is arranged so that ten equivalents are reached at pH 6.8. —O—O—O—O experimental curve.

(1) The curve obtained by subtracting the theoretical dissociation curve for one equivalent with pK 6.8.

(2) The curve obtained by subtracting the theoretical dissociation curve for two equivalents.

(3) The curve obtained by subtracting the theoretical dissociation curve for two equivalents with a pK of 5.5 from curve (1).

Titrations; Table 6 and Figs. 1—3 give the values from the titrations of beef, chicken and salmon cytochromes. There is a striking similarity between the titration values of ferricytochromes from beef and chicken. The difference in shape between the curves for ferri- and ferrocytochrome from beef is very small (Table 6), especially in the region pH 5.5—8.5. In the case of salmon more equivalents are titrated below pH 5.0 than in ferricytochromes of beef and chicken. Between pH 5 and 8.5 the difference between the three ferricytochromes is small, but becomes greater again at higher pH.

The titration curve of a complex protein molecule is composed of the dissociation curves of individual dissociating groups. The number of equivalents titrated and the positions of the dissociation curves of the individual groups along the pH axis can vary with the protein studied. Nevertheless it seems possible by a process of trial and error to obtain the pH values corresponding to the inflections of the individual curves, *i.e.* the pK's of the groups *.

It is known from earlier work of Greenstein [24] and Wyman [25] that histidine residues are usually titrated in the region of about 5 to 8.5. In cytochrome c from beef and chicken (Figs. 1 and 3) roughly three equivalents and in salmon

* Personal communication from Dr. A. C. Maehly (paper in preparation).

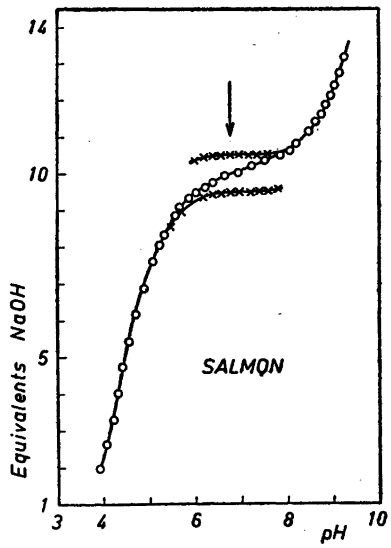

Fig. 2. The titration curve of salmon cytochrome.

The coordinates are the same as those in Fig. 1. The experimental curve (—O—O—O—O) is corrected by subtracting one equivalent with pK 6.5. (✱✱✱✱✱).

cytochrome four are titrated in this range, but it cannot be excluded that other groups than histidine are titrated within this range, as will be discussed later.

A comparison of the experimental titration curves with the theoretical dissociation curve of a single dissociating group immediately shows a great similarity in the range between 6 and 7. A subtraction from the titration curve of the theoretical curve for one acid group with a pK 6.8 (for beef and chicken), and a pK 6.5 (for salmon) gives a resultant curve with a horizontal plateau around pH 7. If other positions are tried, the resultant curve shows a maximum, which is absurd, or rises slowly, implying that other amino acid residues are still being titrated in that range (Fig. 4). Since the resulting curve has almost perfectly horizontal portions it is evident that the two pK-values (6.5 and 6.8) found represent the upper limits (Figs. 1—3).

In the relevant range (pH 5.0—8.5) there are now still two equivalents (for beef and chicken) and three (for salmon) to account for, but in the curve corrected for one equivalent, no position for a further single group can be located in the manner described above. If it is assumed that the two groups titrated in this range have equal affinity for hydrogen ions and do not interact, it is permissible to double the ordinates of the theoretical curve for one equivalent (von Muralt [26], Klotz [27]) and try whether this curve for 2 equivalents can be fitted into the corresponding experimental curve. In beef and chicken cytochrome a satisfactory plateau (Figs. 1 and 3) is obtained by subtracting two equivalents with pK 5.5 and 5.4 respectively. In salmon, no dissociating groups could be located (Fig. 2) with a pK between 4.75 and 6.5. The possibility that the tail of the titration curves of carboxyl groups in the pK region 3.0—4.0 might interfere with the plotting was excluded since a subtraction of 8 equivalents at pK 3.7 did not appreciably influence the shape of the curve around pH 5.4.

DIGESTION OF CYTOCHROME C

Enzymatic breakdown of the cytochromes was performed with pepsin (Tsou [3]). In the case of chicken and salmon the available material was not sufficient for a complete analysis but was only used for the histidine determination and the spectrophotometric and chromatographic investigations. In Table 7 the iron content, molecular weights and histidine contents of the different cytochrome peptides are listed, and in Table 8 the chromatographic results are presented.

Table 7. Iron and histidine content of the different cytochromes degraded by the method of Tsou.

	Fe-content %	Molecular weight	Histidine %	Histidine (moles) found	probable
Beef	2.45	2 280	6.12	0.90	1
Chicken	2.27	2 460	5.35	0.85	1
Salmon	2.35	2 370	7.35	1.05	1
Beef (Tsou [41])	2.21	2 520			

Table 8.

Table. 8. Amino acid composition of the different degraded cytochromes according to paper chromatography.

	Ala.	Arg.	Asp.	Cys.	Glu.	Gly.	His.	Leu.	Lys.	Met.	Phe.	Pro.	Thr.	Try.	Tyr.	Val.	Ser.
Beef:	+	−	+	+	+	+	+	+	+	−	+	+	+	−	−	+	+
Chicken:	+	−	+	+	+	+	+	+	+	−	+	+	+	−	−	+	+
Salmon:	+	−	+	+	+	+	+	+	+	−	+	+	+	−	−	+	+

It follows from these tables that there is only one mole of histidine per mole of peptide. In the case of chicken the value is rather low which could indicate that the preparation is not quite homogeneous. During the course of the digestion different breakdown products are formed. The possibility exists that during the dialysis other peptides could remain inside the dialyzing bag. Therefore paper electrophoresis was used for checking the homogeneity of the pepsin-treated cytochromes. In all these cases only one spot moving towards the anode was found both at pH 7.0 and 9.9.

Table 7 shows that only one mole of histidine is present per mole of digested cytochrome. A striking similarity between the different species is shown as in the case of the intact cytochromes. In all the peptides the same amino acids are absent after peptic digestion, namely arginine, methionine, tryptophane and tyrosine.

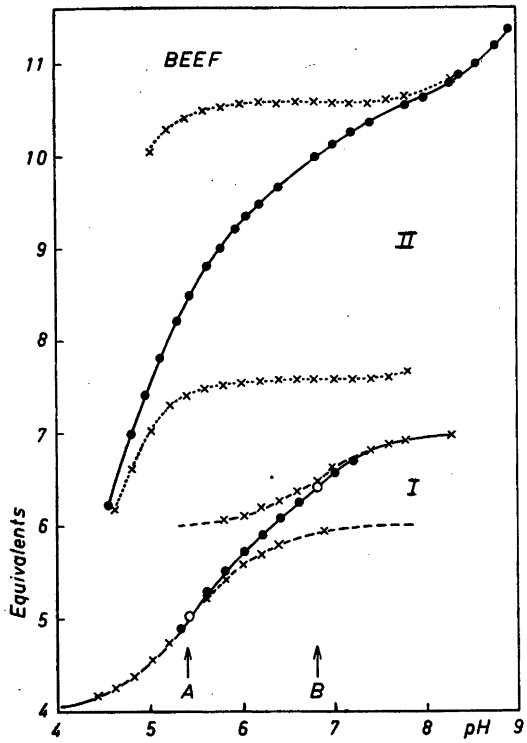

Fig. 3. The titration curve of beef cytochrome showing the theoretical dissociation curve (I) and the experimental curve (II).

I. The theoretical dissociation curve calculated from the theoretical curve for one equivalent with pK 6.8 (B) and two equivalents with pK 5.4 (A).
II. The experimental curve (—●—●—●—●—) and the curve obtained by subtracting curve (I) from the experimental values (···×···×—×—).
The coordinates are the same as in Figs. 1 and 2.

DISCUSSION

Chemical analysis. The work of Theorell [28,29] and Paul [30] has shown that two of the porphyrin-protein linkages are formed between two cysteine residues and the α-carbon atoms of the side chains 2 and 4 of the porphyrin. The nature of the iron protein linkages on the other hand, is not so certain and the information available about the protein structure of cytochrome c is still incomplete.

Using different methods of preparation it has been possible to obtain cytochrome c with different iron contents. Even cytochrome c prepared according to the same method can show different iron content in different batches. This could mean that the molecule could have been partly decomposed by the preparation procedure. The most common methods used for preparation of cytochrome c from animal tissues are listed in Table 9.

Table 9. Different preparation methods for cytochrome c from animal sources.

	Fe-content	Molecular weight	Method	Author	Year
1.	0.34	16 400	A,B,C	Keilin and Hartree [17]	1937
2.	0.43	13 000	A,B,C,D	Theorell and Åkeson [31]	1939
3.	0.34, 0.43	16 400, 13 000	A,B,C	Keilin and Hartree [32]	1945
4.	0.466	11 980	A,B,C,F	Paléus and Neilands [18]	1950
5.	0.45	12 400	A,B,C,E	Paléus [12]	1952
6.	0.43	13 000	A,B,C,F	Margoliash [19]	1950

A = Extraction with 2.5 % trichloroacetic acid
B = $(NH_4)_2SO_4$ fractionation
C = Precipitation with 20 % trichloroacetic acid
D = Electrophoresis
E = Paperelectrophoresis
F = Ion-exchanger

This table shows a variation of 11 980 to 16 400 in the molecular weights of cytochrome c from beef, assuming one iron atom per molecule. Considering the variations of the iron content due to the preparation method used, it was felt that a comparative study of cytochromes from different sources would give valuable information about cytochrome c, especially the iron protein bonds, only if the same method of preparation was employed. The method of preparation will affect the iron protein bonds to a much less degree than the rest of the protein structure, as shown by the fact that the enzymatic activity is not lost in any of the methods of preparations listed above.

It can be seen from Table 1 that there is a slight variation in the iron content between the three cytochromes studied. The values for beef and chicken cytochrome agrees rather well with those of Tint and Reiss [16], 0.453 % and 0.421 %, respectively. Salmon cytochrome has been previously investigated only by Paléus and Neilands [18], who found an iron content of 0.461 %.

The present data as well as those of Theorell [1] and Paul [2] (Table 2) clearly show that beef cytochrome contains three residues of histidine per mole. The same number of histidine groups is found in chicken cytochrome, while salmon cytochrome was found to contain only two. The analytical results for horse cytochrome c published by de Barbieri and Zamboni [33] are hard to explain in the light of these findings. These authors found less than one mole of histidine per mole of cytochrome but do not give any of the very important details of the microbiological and chemical methods used for determining the histidine.

Table 4 shows that the molecular absorption-coefficients of beef and chicken cytochrome at 280 mμ are very similar but that the corresponding coefficient of salmon cytochrome shows a marked deviation. This result, together with the data from paper chromatography indicates that the different cytochromes contain the same varieties of amino acids but not the same number of each.

Titrations. From the titration curves (Figs. 1—3, Table 6) it is seen that three equivalents are titrated in the range pH 5.0—8.5 for beef and chicken cytochrome. One equivalent in each is titrated with pK 6.8 and the other two

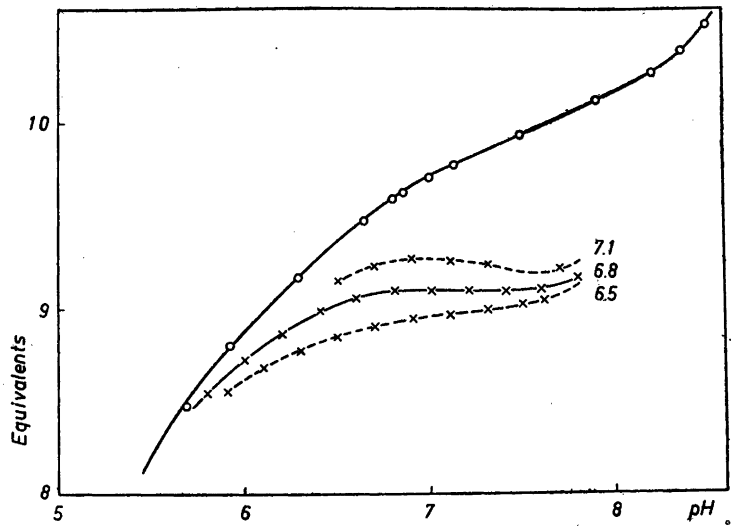

Fig. 4. The sensitivity of the plotting method.

The theoretical dissociation curves for one equivalent with the pK-values 6.5, 6.8 and 7.1 respectively are subtracted from the experimental acid-base titration curve of beef cytochrome and the resulting curves are plotted. It can be seen that the position of the appropriate pK value can be determined to within 0.3 units with certainty. The correct value is obtained when the resulting curve shows a horisontal plateau (6.8).

equivalents with pK 5.4 for beef and 5.5 for chicken. In the case of salmon cytochrome one equivalent with a pK 6.5 was titrated, but it was impossible with the method used above to find another pK above pH 4.75. The three remaining equivalents titrated in this range must belong to groups which dissociate below pH 4.75.

An attempt was made to relate the histidine groups obtained by microbiological assay with the groups titrated in the region pH 5.0—8.5. The pK 6.8 (beef and chicken) lies in the region where the imidazole group is usually titrated and it is therefore believed to belong to histidine. Wyman [34] has shown by potentiometry that oxyhemoglobin has a heme-linked group with a pK at 6.8, which he attributes to an imidazole group, since the apparent heat of dissociation of oxyhemoglobin in this pH range was found to be +6 500 calories per equivalent. Wyman's data on horse oxyhemoglobin gave very good agreement between the number of histidine groups calculated from the apparent heat of dissociation and from chemical analysis respectively.

Theorell and Åkeson [1] found two pK's at 9.35 and $\sim$ 9.85 respectively. Since these pK's shift upon reduction they probably correspond to heme-linked groups. The authors propose that it is the imino groups of the imidazole rings linked to the iron which are titrated there. Boeri *et al.*[35] recently studied the magnetic and spectrophotometric properties of different compounds of ferricytochrome of beef appearing in acid solution and concluded that the

heme was linked to two nitrogen atoms in the protein moiety. These findings fit well into the hypothesis that the hemin is linked to two imidazole groups in cytochrome c.

The fact that the reduction of cytochrome c does not shift the pK 6.8 (beef and chicken) can be regarded as a further proof that the third imidazole group is not heme-linked. The two other residues of histidine in beef and chicken found by microbiological assay are presumably bound to iron, as suggested by Theorell and collaborators (see above).

Amino acid analysis of salmon cytochrome reveals only two molecules of histidine. One of them is titrated with a pK 6.5 and by analogy with beef cytochrome it may be supposed that this group is not linked to the iron. The other histidine group is available for the linkage to the iron. The second heme-linked group in this cytochrome would thus not be an imidazole group.

In measurements of the oxidation-reduction potentials of beef cytochrome Paul [36] found a dissociation constant in ferricytochrome c with a pK of 6.86, while Rodkey and Ball [37] found one with a pK of 7.8. The discrepancy between these two values, as well as their incompatibility with our present results, which indicate a non heme-linked group at pK 6.8, (not shifting on reduction) still remains to be explained.

Let us now consider the nature of the groups titrated with pK 5.4 and 5.5 respectively. Theorell and Åkeson [1] concluded from their experiments on the apparent heat of dissociation of cytochrome c that groups other than histidine were titrated in the range pH 5.5—8.5. They could not observe any shift in the pK's within this range upon reduction, as is confirmed by the present experiments. This means that no heme-linked groups were titrated in the pH range 5.5—8.5. Of the carboxyl groups known to occur in proteins those belonging to aspartic acid show the highest values, 3.0—4.7 (Edsall [38]). Thus it is hard to see how carboxyl groups of the protein moiety could give rise to a pK of 5.4 and 5.5. On the other hand it may be expected that the two carboxyl groups of the propionic acid residues in the porphyrin are titrated in the neighbourhood of pH 5. Theorell [29] has titrated "porphyrin c" and found a pK of 5.63 for its two carboxyl groups.

The digestion experiments. Arginine, methionine, tryptophane and tyrosine could not be detected on the paper chromatograms, which might be due to the small amounts of hydrolysates available. The experiments of Tsou [3] (see his Fig. 2) indicated a rapid initial drop and a slow subsequent rise in the total cytochrome concentration as measured spechophotometrically during the digestion with pepsin. This could indicate a rearrangement of the molecule in one way or the other. Only one histidine group per mole of peptide is found by chemical analysis and hence the peptide must contain at least one heme linked group other than histidine. Noteworthy is that the peptide is autoxidizable and gives a CO compound Tsou [39]).

In the case of the peptide from beef cytochrome the total nitrogen content was 12.07 % corresponding to 20 N-atoms per mole. This is the number of N-atoms corresponding to one mole of each of the amino acids found in the peptide plus the four N-atoms belonging to the porphyrin nucleus. When all amino acids and the molecular weight of hemin (652) is added up, a minimum molecular weight of 2 170 is arrived at. The minimum molecular

weight according to the iron content is 2 280. The peptide molecule thus remarkably enough seems to contain scarcely more than one mole of each of the amino acids found to be present.

SUMMARY

1. Three histidine groups per mole were found by microbiological assay in beef and chicken cytochrome, two in salmon cytochrome.

2. Titration experiments showed one equivalent with a pK of 6.8 and two equivalents with a pK of 5.4 and 5.5 respectively for beef and chicken cytochrome. These pK-values were not shifted upon reduction of the cytochromes. Between pH 5—8.5 one equivalent with a pK of 6.5 was titrated in salmon cytochrome. The single equivalent with a pK of 6.8 and 6.5 is attributed to an imidazole group, not linked to the iron, the two equivalents with pK at 5.4 and 5.5 are attributed to the carboxyl groups from the porphyrin.

3. The amino acid composition of the three different cytochromes is rather similar but the number of each amino acid varies with the species.

4. Pepsin digestion of the three cytochromes yielded peptides containing only one histidine group. The amino acid composition was different from the original material but very similar in all three cytochromes studied. The heme linkages in the peptides must be different from that of ordinary cytochrome.

5. The results obtained here were correlated to those of Theorell and coworkers and have clarified to some degree our knowledge of the structure of cytochrome c.

Acknowledgements. Throughout this work the author has had the great benefit and encouragement of frequent discussions with Professor Hugo Theorell. He has also leant much on the ready advice of Laborator K. G. Paul, particularly in theoretical matters. Dr. A. C. Maehly from the University of Pennsylvania, Philadelphia, and Mr. A. Ehrenberg have made many valuable suggestions concerning the interpretation of the results.

It is a pleasure to thank Miss B. Rolander who performed many careful micro-analyses.

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Studies on the Chemistry of Lichens

VI *. The Structure of Pustulan

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The occurrence of a galactofuranoside, umbilicin (Part V) in the lichen *Umbilicaria pustulata* suggested the presence of polysaccharides with galactofuranoside units. Total hydrolysis of the methanol-extracted lichen revealed the presence of galactose, glucose and mannose. When the hydrolysis was performed with 0.01 *N* hydrochloric acid overnight, only galactose was liberated, although in small amounts indicating that part of the galactose in the polysaccharides is furanosidic. Attempts to isolate a polysaccharide containing these units, however, were not successful, but during these experiments another polysaccharide, pustulan, was isolated in quantity. This polysaccharide was isolated from the same source by Drake [1], who proved that it was a glucan and assumed that the units were connected with β-1,6-linkages. (Drake called the polysaccharide pustulin, but following the suggestion of Whistler and Smart [2], the name pustulan is adopted.) Pustulan, therefore, should be related to the glucan lutean (original name, luteose), the malonic ester of which is produced by a *Penicillum* mould [3].

In order to study the structure of pustulan a sample was partially hydrolysed and fractionated on a charcoal column, using the gradient elution technique. Glucose, a di-, a tri- and a tetrasaccharide were isolated in this manner. The disaccharide was identified as gentiobiose. The β-acetates of the tri- and tetrasaccharide had the same values for m.p. and rotation as those of 6-β-gentiobiosido-glucose [4] and 6-β-gentiobiosido-gentiobiose [4]. For these saccharides we suggest the names gentiotriose and gentiotetraose. Not even traces of other di-, tri- or tetra-saccharides were observed, indicating that the pustulan molecule is linear, containing exclusively β-1,6-linkages.

The sugars were obtained as amorphous powders, chromatographically pure but containing small amounts of water. The purity of the samples and the concentration of solutions were determined by hypoiodite titrations. The data for the sugars and their acetates are given in Table 1.

* Part V, *Acta Chem. Scand.* 8 (1954) 821.

Table 1. Properties of the sugars obtained by hydrolysis of pustulan (19.7 g).

Sugar	Yield, g	$[\alpha]_D$ water ($c=2$)	β-acetate, m.p. Observed (uncorr.)	Lit.	β-acetate $[\alpha]_D$ chloroform ($c=2$) Observed	Lit.
Glucose	4.5	52.7				
Gentiobiose	3.25	9.5	191—192	196	— 5.3	—5.4
Gentiotriose	2.08	—6.5	211—211.5	219—220	—7.0	—8.0
Gentiotetraose	1.0	—14.5	134—135	135, 207—209	—10.9	—11.1

When, for the sugars or their acetates $[M]_D/n$ is plotted against $n—1/n$, where $[M]_D$ is the molecular rotation and n the number of glucose residues per molecule, a straight line is obtained (Fig. 1) in agreement with the Freudenberg-Blomquist relationship [5]. Glucose and β-glucose pentaacetate fall on the curve, although this has no significance. The other extreme, pustulan itself, is close to the curve. The observed specific rotation for pustulan is —46°, and from the values for the dextrins a value of —43° can be calculated. For the pustulan acetate the calculated rotation —17.4°, does not correspond at all with that observed, + 9.1°. The yield of chloroform soluble acetate by triple acetylation of pustulan with acetic anhydride in pyridine, after swelling in formamide, was only about 25 %, and the obvious conclusion is not that the Freudenberg-Blomquist relationship does not hold, but that the pustulan acetate does not go into true solution but is dissolved as large aggregates, the rotation of which should not be compared with that of the low-molecular acetates.

The R_F-values of glucose, gentiobiose, -triose and -tetraose in the solvent ethyl acetate-acetic acid-water, 12 : 5 : 6, were 0.32, 0.167, 0.091 and 0.047

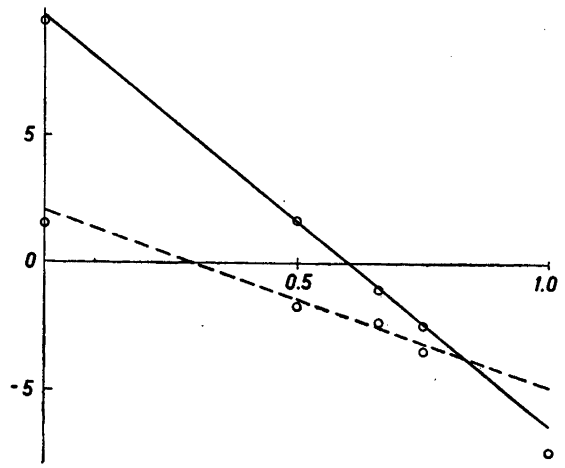

Fig. 1. Application of the Freudenberg-Blomquist relationship to the oligosaccharides (————) and their acetates (— — — — —). Ordinate: $10^{-3}[\alpha]_D M/n$. Abscissa: $n-1/n$.

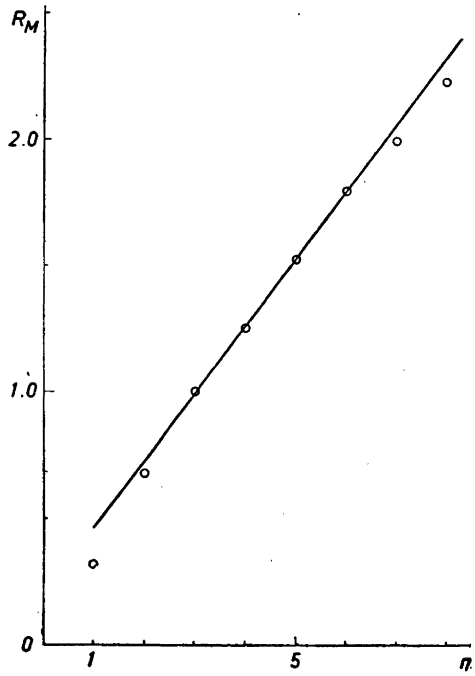

Fig. 2. Relation between R_M-values and chain length.

respectively. The fractions, which should contain the pentaose, hexaose, heptaose and octaose were not characterised, but their R_F-values were determined to be 0.029, 0.016, 0.011 and 0.006. When the R_M-values [6,7] are plotted against n, the number of glucose units in the oligosaccharide, a straight line is obtained (Fig. 2), strongly indicating that all the oligosaccharides belong to the same series.

These results indicate that pustulan is a linear glucan with β-1,6-linkages between the units, in agreement with Drake's original suggestion[1].

EXPERIMENTAL

Extraction of pustulan. The lichen *Umbilicaria pustulata* (200 g), after continuous extraction with ether for three days and with methanol for fourteen days, was given a pre-extraction with sodium carbonate solution (2 %, 2 × 2 l) for fourteen days, filtered and washed with cold water. The lichen was then extracted four times with boiling water (2 l) for a period of four hours. The hot solutions were filtered through linen and frozen solid overnight. Next morning the product was allowed to thaw. The crude polysaccharide was collected by filtration, drained well and the process of dissolution in hot water, freezing and thawing repeated a further five times. Before the last freezing small amounts of undissolved material were removed by centrifugation. The pustulan finally was washed with ethanol and ether and dried in a desicator over sulphuric acid to yield light buff flakes (14 g). Ash content: 0.52 %. Moisture 11.6 %. (Determined by keeping a sample at 110° overnight). The presence of glucose only in the hydrolysate of the purified pustulan was shown by paper chromatography.

Partial hydrolysis. After some preliminary experiments a larger amount of pustulan (19.7 g) was refluxed with *N* sulphuric acid (1 l) for 135 minutes, cooled and neutralised with barium carbonate. The mixture was filtered and concentrated under reduced pressure to yield a clear syrup. In order to remove higher dextrins, the syrup was dissolved in 10 % ethanol (200 ml) and filtered through a short column of carbon-Celite, which was then washed with 50 % ethanol (2 l) and the solutions concentrated. The syrup obtained, dissolved in 1 % ethanol (200 ml), was added to the top of a carbon-Celite column (50 × 6.5 cm) and subjected to gradient elution with 1 % to 40 % ethanol (8 l). The eluate was divided into fractions, which were investigated by paper chromatography. (Solvents: ethyl acetate-acetic acid-water, 3:1:1, and for the higher members 12:5:6). Similar fractions were combined and concentrated under reduced pressure. The separation was satisfactory, with empty fractions between all the sugars.

Each oligosaccharide was dried thoroughly and the chromatographic purity established. The percentage of oligosaccharide in the amorphous powder was determined to be 95, 93, and 94 for the biose, triose and tetraose by hypoiodite oxidation [8]. In addition to the sugars listed in Table 1, the pentaose to the octaose were isolated in yields of 0.78, 0.48, 0.33, and 0.25 g. They were investigated only by paper chromatography and found to be fairly pure.

Acetylation of oligosaccharides. Each oligosaccharide was acetylated with acetic anhydride in the presence of anhydrous sodium acetate by heating in a glycerol bath at 130° until the solid went into solution. The mixture crystallised from ice water and was recrystallised from ethanol to constant m.p.

Acetylation of pustulan. Pustulan (2 g) was given two treatments with sodium chlorite-acetic acid according to the method of Wise *et al.*[9] (Yield 1.6 g) $[\alpha]_D = -46°$ (water $c = 0.5$ or NaOH, $c = 0.5$). The bleached pustulan (1.1 g) was then acetylated three times following the method of Carson and Maclay [10]. The grey amorphous residue (1.6 g) was dried throughly and extracted with chloroform in a continuous extractor for 2 hours. The chloroform was evaporated under reduced pressure, affording a strong, clear film (0.41 g). The value for the specific rotation of this material, +9.1° (chloroform, $c = 0.3$), agreed well with that obtained by Drake [1], +9°. Acetyl content, 43.4 %, required for the completely acetylated polysaccharide, 44.78 %.

SUMMARY

The structure of pustulan as a linear glucan with β-1,6-linkages has been established by the method of partial hydrolysis and isolation and characterisation of oligosaccharides formed.

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Studies on Sphagnum Peat

III *. A Quantitative Study on the Carbohydrate Constituents of Sphagnum Mosses and Sphagnum Peat

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The chemical composition of the chief peat-forming plants, the *Sphagnum* mosses, and of the *Sphagnum* peat is still rather incompletely known [1]. The aim of the present investigation has been to study the variation in the quantity of the various sugars, from which the polysaccharides in *Sphagnum* mosses and *Sphagnum* peat are built up, with huminosity and age.

BOTANICAL AND GEOLOGICAL DATA

Two living mosses and five peat samples have been investigated. One moss, *Sph. fuscum*, W. Klinggr., collected from Bälinge, Uppland, is one of the predominating peat-forming mosses in Sweden. The other moss, *Sph. imbricatum*, Russ., supplied by Professor Einar du Rietz and collected from Kanarp, Småland, is the predominating moss in the peat samples A—E. The peat samples were collected from the central part of Ageröd Mosse, Skåne. The age estimations were made by Laborator Tage Nilsson, and are based upon earlier pollen analytical investigations of the same section of the bog. The botanical investigation and the determination of the huminosity was carried out by Professor Carl Malmström. The data are summarized in Table 1.

FRACTIONATION

The samples were subject to the following subsequent treatments:

1. Continuous extraction with benzene for 2 days.
2. Continuous extraction with methanol for 5 days.
3. Extraction with water at 60—65° C, 3 × 3 hours.
4. Bleaching with sodium chlorite and acetic acid at 60—65° C, 1 h in 2—4 steps.
5. Swelling in liquid ammonia.
6. Extraction with water at 40—45° C, 3 × 3 hours.
7. Extraction with 5 % potassium hydroxide at room temperature, 2 × 4 h.

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Table 1. Botanical and geological data of Sphagnum fuscum, Sphagnum imbricatum and the peat samples A—E.

Sample	Middle depth in centimeters	Age in years	Huminosity *	Details
Sph. fuscum	5	—	0	Collected in September 1951
Sph. imbricatum	5	—	0	Collected in August 1952
A	17	200—400	3	Abundant leaf- and stem remains of *Sph. imbricatum*. Traces of *Cyperaceae*
B	47	700—1 000	4	Abundant remains of *Sph. tenellum*. Traces of *Sph. recurvum*, *Sph. cuspidatum*, *Sph. imbricatum*, *Sph.* of *Acutifolia* type and *Cyperaceae*
C	57	900—1 200	6	Abundant remains of *Sph. imbricatum*. Traces of *Sph. recurvum* and *Cyperaceae*. Some mud without organized structure, with greatest probability from *Sph.*
D	113	1 900—2 200	3	Abundant leaf- and stem remains of *Sph. imbricatum*. Traces of *Sph. recurvum* and *Cyperaceae*.
E	295	4 300—4 800	6—7	Most of the sample is mud, most probably coming from *Sph.*, as a continuous change of *Sph.* remains at various stages of destruction could be seen in it. Some of the remains could be identified as *Sph.* of *Acutifolia* type, *Sph. imbricatum*, *Sph. recurvum* and traces of *Cyperaceae*.

* Estimated by the microscopical investigations, as a measure of the physical destruction on a scale from 0 to 10.

The moisture and ash contents were determined on the original sample and after steps 3 and 4 above. The amount of unbleached holocellulose is estimated as the difference between the weight of the original sample and the weights of the extracts 1, 2 and 3, and the losses by the bleaching are also calculated as the weight difference. Summation of the fraction yields will thus be 100 %, and the accuracy of the recorded holocellulose content is thus artificial. The real values may differ about $\pm$ 1 % from those given in Table 2.

The pH of the press-water from the peat samples was about 4.5. The ash contents of the mosses and the peat do not differ very much. The ash contents of the peat samples appears to vary directly with the huminosity and is probably due to a loss of organic material. On the other hand the living mosses

Table 2. Fractions and ash contents of Sphagnum fuscum, Sphagnum imbricatum and the peat samples A—E. (All the values are given as a percentage of the weight of the total organic material in moss or peat.)

	Sphagnum fuscum	*Sphagnum imbricatum*	A	B	C	D	E
Ash (%)	1.5	1.8	1.0	1.1	1.7	0.7	1.7
Main fractions							
Benzene extract	1.7	1.7	4.0	3.1	5.9	3.3	6.7
Methanol extract	6.3	4.5	3.6	4.5	4.6	3.9	4.9
Water extract	4.6	3.6	1.5	2.0	1.6	1.0	1.1
Losses by bleaching*	3.7	3.7	26.3	24.7	34.5 (43.9)	22.1	36.2 (57.1)
Holocellulose *	83.7	86.5	64.6	65.7	53.4 (44.0)	69.7	51.1 (30.2)
Holocellulose fractions							
Water extract	5.6	6.9	8.0	8.5	8.8	8.3	8.8
Alkali extract	3.7	3.6	5.6	5.5	6.5	5.1	5.2
Residue **	72.5	73.4	48.5	47.8	31.5 (27.8)	53.8	31.7 (20.4)
Losses by the holocellulose fractionation	1.9	2.6	2.5	3.9	6.6	2.5	5.4

* To minimize the loss of carbohydrates the holocellulose fractionation and all quantitative sugar analyses, except that of uronic acids in C and E, were made on the samples bleached in 2 steps only. The samples C and E were bleached a further 2 steps (the values in brackets), which was necessary to get excess of chlorine dioxide in the solution after the bleaching. These values therefore represent the true amounts of bleaching losses and holocellulose.

** The values in brackets represent the amount of residual holocellulose after a further 2 steps bleaching before the analysis of uronic acids.

have greater ash contents than peat of low huminosity, which is natural as they grow on, and get their minerals from the peat.

The quantity of the very complex mixture, bitumen, extracted by benzene, appears to increase with the huminosity. The amounts extracted by both methanol and water do not vary much, but the losses by bleaching increase considerably with the huminosity, in other words the holocellulose content decreases with increasing degree of huminosity. The bleaching losses of the mosses, 3,7 %, agree fairly well with the value of Klason lignin, about 5 %, obtained for some *Sph. mosses* (Part II[2]). The losses by the holocellulose fractionation are partly manipulative, and partly those of non-carbohydrate material during the alkali treatment. The value of the alkali extract in Table 2, represents that part of the extract, precipitated by 75 % ethanol. Especially for C and E, in which the holocelluloses were not bleached sufficiently, a fairly large part of organic material was certainly left in the mother liquors.

THE CARBOHYDRATES IN THE METHANOL AND WATER EXTRACTS

The quantitative estimation of the constituent sugars in the methanol and water extracts after hydrolysis is represented in Table 3.

Table 3. The constituent sugars in the methanol and water extracts of Sphagnum fuscum, Sphagnum imbricatum and the peat samples A—E. (All values are given as a percentage of the weight of the total organic material in moss or peat. + = Traces. — = Not observed.)

Sugars	*Sphagnum fuscum*	*Sphagnum imbricatum*	A	B	C	D	E
Methanol extract							
Uronic acids *	+	+	+	+	+	+	+
Galactose	0.3	0.2	+	0.1	0.1	0.1	+
Glucose	0.8	0.5	+	+	+	+	+
Mannose	0.1	0.1	+	+	+	+	+
Fructose	1.7	1.0	—	—	—	—	—
Arabinose	0.2	0.1	+	+	+	+	+
Xylose	0.3	0.2	+	0.1	0.1	+	+
Rhamnose	+	+	+	+	+	+	+
Water extract							
Uronic acids *	0.4	0.3	0.2	0.1	0.1	0.1	+
Galactose	0.4	0.3	0.2	0.1	0.2	0.1	0.1
Glucose	0.2	0.2	0.2	0.2	0.2	0.1	0.1
Mannose	0.2	0.2	0.1	0.1	0.1	0.1	+
Fructose	1.3	1.1	—	—	—	—	—
Arabinose	0.3	0.2	+	+	+	+	+
Xylose	0.3	0.3	0.2	0.1	0.1	0.1	0.1
Rhamnose	0.1	0.1	—	+	0.1	+	+

* Approximative values visually estimated by paper chromatography.

It may be seen from Table 3 that fructose and glucose predominate in the methanol extracts, and fructose in the aqueous extracts of the mosses, and that fructose is absent in the peat. The methanol and water extracts of the mosses were investigated before the hydrolysis.

In the methanol extract glucose, fructose and sucrose were identified together with fructose containing substances with R_F-values lower than that of sucrose. On the chromatograms the presence of two substances with higher R_F-values and fructose reactions (the red colouration with resorcinol-hydrochloric acid) were obtained. However, these were shown to be artefacts (methyl-fructosides), as the same spots appeared in chromatograms of fructose treated with methanol containing traces of acetic acid. Sucrose was also isolated from the methanol extract of *Sph. balticum* Russow in a pure state in a yield of 1.0 %.

Chromatograms of the water extract, sprayed with the resorcinol reagent, only showed faint spots with R_F-values lower than that of sucrose and obviously identical with the corresponding spots in the methanol extract, in addition to a very strong spot with $R_F = 0$. This indicates the presence of a fructan and its oligosaccharides, which are probably formed by enzymic transglycosidation, as frequently observed in other plants (compare *e.g.* Bacon and Edelman [3]).

The small amounts of the sugars, which build up the holocellulose, present in the hydrolysates of the methanol and water extracts of the mosses and peat samples may come from fragments of polysaccharides formed during

the peat formation. The relatively large amount of these sugars in the moss extracts may be indicative of initial decomposition in the lower part of these plants. A similar investigation was therefore performed with the tops (0.5 cm) of *Sph. fuscum*. Except for traces of other sugars the hydrolysate of the methanol extract contained only fructose and glucose and the hydrolysate of the water extract only fructose, lending further support to the above assumption.

The presence of fucose (in traces only) in the methanol and water extracts of the mosses and peat samples, and also in the holocellulose fractions, was indicated by means of paper chromatography.

THE CARBOHYDRATES IN THE HOLOCELLULOSE FRACTIONS

The analytical data of the holocellulose fractions are given in Table 4.

Losses. It may be seen from Table 4 that about 30 % of the holocellulose is still unaccounted for by the sugar analysis or as losses in connection with the fractionation. There are three possible reasons for this difference: decomposition of sugars during the hydrolysis (compensated for by applying empirical factors), incomplete hydrolysis, by which sugars (with exception of the uronic acids, estimated directly by decarboxylation) remain undetected in the analytical determination, and the occurrence of non-carbohydrate material.

In order to study these possibilities and to obtain information about the acidic sugars, a larger amount of holocellulose from *Sph. fuscum* was hydrolysed. The following data were obtained (all figures are percentages of the holocellulose).

Non-dissolved (a dark-brown material with fragments of pollen)	2 %
Recovered from the solution by concentration to dryness (calculated as polysaccharides)	76 %
Difference	22 %
Acids, precipitated from the hydrolysate as strontium salts in 90 % ethanol	23 %
Total amount of uronic acids in the hydrolysate (Of the uronic acids about 2.5 % were monomeric and 1 % was not precipitated as strontium salts in 90 % ethanol)	14 %

As the decomposition losses of non-acidic sugars, calculated applying empirical factors, plus the analytically estimated losses of uronic acids were about 12 %, which are only partly volatile, at least 10 % of the difference observed, 22 %, must be due to other causes. It has been demonstrated in other experiments that sparingly soluble strontium salts of acidic sugars, formed during the neutralization of the hydrolysate, are very difficult to wash out from the strontium sulphate precipitate. It is, therefore, very probable that at least about 5 % non-acidic sugars, bound to uronic acids, are lost in this way.

From the difference between the precipitated acidic sugars, 23 %, and the uronic acids in this fraction, 13 %, it may be concluded that about a further 10 % non-acidic sugars, bound as aldobiuronides or higher complexes are present in the hydrolysate.

Table 4. The constituent sugars in the holocellulose calculated as polysaccharides. (All values are given as a percentage of the weight of the total organic material in moss and peat. w.h. = water soluble holocellulose, a.h. = alkali soluble holocellulose and r.h. = residue holocellulose).

Sugar component		*Sphagnum fuscum*	*Sphagnum imbricatum*	A	B	C	D	E
Uronic acids	w.h.) a.h.)	3.3	3.4	3.3	3.8	2.5	3.1	1.8
	r.h.	15.6	14.5	7.8	7.3	3.7	8.7	1.1
Galactose	w.h.	0.3	0.3	0.3	0.3	0.3	0.3	0.2
	a.h.	0.2	0.2	0.2	0.2	0.1	0.2	0.1
	r.h.	5.2	5.3	2.6	3.0	1.1	3.4	0.9
Glucose	w.h.	0.3	0.3	0.7	0.6	1.0	0.5	0.5
	a.h.	0.2	0.2	0.4	0.3	0.4	0.3	0.4
	r.h.	26.8	28.4	21.8	18.9	13.6	22.3	11.4
Mannose	w.h.	0.1	0.1	0.2	0.2	0.2	0.2	0.2
	a.h.	0.1	0.1	0.2	0.2	0.2	0.1	0.1
	r.h.	1.3	1.3	2.2	3.0	1.1	2.4	0.6
Arabinose	w.h.	0.1	0.1	0.1	0.1	+	+	−
	a.h.	0.1	0.1	+	+	+	+	−
	r.h.	−	−	−	−	−	−	−
Xylose	w.h.	0.3	0.3	0.4	0.4	0.3	0.4	0.2
	a.h.	0.2	0.2	0.3	0.4	0.3	0.3	0.2
	r.h.	3.9	2.6	2.2	3.0	0.9	2.9	0.3
Rhamnose	w.h.	0.1	0.1	0.1	0.2	0.1	0.1	+
	a.h.	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	r.h.	+	+	+	+	+	+	+
Polysaccharides		58.2	57.6	42.9	42.0	25.9	45.3	18.1
Total amount of holocellulose		83.7	86.5	64.6	65.7	53.4	69.7	51.1
Losses by the fractionation of the holocellulose and by the extra bleaching of the holocellulose residues of C and E		1.9	2.6	2.5	3.9	10.3	2.5	16.7
Holocellulose unaccounted for. (The figures in the brackets are percentage of total holocellulose		23.6 (28)	26.3 (30)	19.2 (30)	19.8 (30)	17.2 (32)	21.9 (31)	16.3 (32)

The non-acidic part of the hydrolysate could be calculated at 52 % and the non-acidic sugars found by analysis amounted to 44 %. The difference between these values, 8 %, represents the upper limit of incompletely hydrolysed non-acidic sugars. The presence of oligosaccharides in the non-acidic part of the hydrolysate was demonstrated by paper chromatography, but the value 8 % is certainly too high, as it also includes non-carbohydrate material.

To sum up, the main losses are due to incomplete hydrolysis of acidic sugar complexes and as the percentages of uronic acids decrease to a greater extent with the huminosity than the total amount of holocellulose, the losses of non-

acidic sugars should also decrease with the huminosity. The fact that the amount of holocellulose unaccounted for is fairly constant may therefore be due to an increase of non-carbohydrate material in the holocellulose.

The analytical determination of the carbohydrate constituents is thus subject to several systematic errors, which probably cannot be reduced with the methods available. The data given in Table 4, for the different samples, however, are obtained under strictly identical experimental conditions, and for a comparison between the samples they are certainly significant.

The acidic sugars. The polymeric acidic sugars in the above mentioned hydrolysate from *Sphagnum fuscum*, isolated from a carbon-Celite column, were further hydrolysed, and qualitative examination of the hydrolysis products by paper chromatography showed uronic acids, galactose, xylose, rhamnose and material with lower R_F-values and sugar reactions. The predominating role of rhamnose is of interest.

In the main part of the neutralized hydrolysate, the acidic components were separated from the neutral by ion exchange and investigated by paper chromatography. About a dozen spots plus some trailing were observed. The spots could be divided into two groups, one with uronic acid reactions (bright red with anisidine reagent) and the other with aldose reactions (yellow, orange or brown).

In the uronic acid group, galacturonic acid was one of the predominating constituents and was isolated in a pure state. The other chief constituent gave a spot somewhat faster than glucuronic acid in ethyl acetate-acetic acid-water (Solvent A), and in butanol-ethanol-water (Solvent B) it gave a spot a little faster than glucose. The substance was isolated by chromatography on thick paper in an impure state and contained 11 % methoxyl, and hence is probably a monomethyl uronic acid. Besides these chief components there were spots with lower R_F-values in which digalacturonic acid seemed to predominate. This and the higher polymers are probably formed from a pectin like polysaccharide built up from galacturonic acid units. There were also two minor spots with higher R_F-values, the first with the same R_F-values as the lactone of glucuronic acid and the second with about the same R_F-values as xylose in the two solvents.

From the other group two crude fractions, which may be aldobiuronic acids, were isolated by chromatography on thick paper. The faster showed the anisidine reaction typical for pentoses (orange) and the slower that of hexoses (brown). After further hydrolysis, paper chromatograms of these hydrolysates showed the presence of xylose and galactose, together with the above mentioned unknown methyl-uronic acid and the lactone with the same R_F-value as glucuronic acid.

A paper chromatographic investigation of the acidic sugars of the peat sample A also showed the above-mentioned components.

In order to demonstrate that the acidic polysaccharides were not of microbial origin (*e.g.* as the polysaccharides in soil studied by Forsyth and Webley [4]) formed by an initial decomposition, the upper parts (0.5 cm) of *Sph. fuscum* were studied. The acidic components in a hydrolysate of the holocellulose showed the same picture as the main moss sample (also the amount of holocellulose and the total amount of uronic acids were equal).

The quantity of uronic acids decreases at a faster rate than the holocellulose with the huminosity. The spot with the R_F-value of glucuronolactone was observed in the hydrolysates of all peat samples. The amount of the uronic acids extracted from the holocellulose by water and alkali is fairly constant for all the samples. The same thing is found with galactose, xylose and rhamnose, the sugars which are linked to uronic acids as mentioned above. It may be mentioned that treatment of the holocellulose with pectinase (Pectinol 10) was practically without effect. By treatment with ammonium oxalate solution (0.5 % at 60° C) or dilute hydrochloric acid (0.5 *N* at room temperature) only small amounts of uronic acid material was extracted and it had no effect on the subsequent extractions with water and alkali.

The neutral sugar constituents. Galactose decreases with the huminosity at about the same rate as the uronic acids.

Glucose, the predominating constituent, is probably present to a greater extent as cellulose, as demonstrated in earlier investigations [5,6]. The decrease of glucose with the huminosity follows that of the holocellulose. The amount of glucose in the water and alkalisoluble holocellulose is small and increases with the huminosity.

Mannose shows a maximum in peat of low huminosity. On the other side the amount of mannose in the methanol and water extracts (Table 3) is greater in the mosses than in the peat.

The amount of xylose shows only a small decrease from mosses to peat of low huminosity but a rapid decrease and a great accumulation in the holocellulose extracts with higher huminosity.

Arabinose is a minor component, which is present in an easily extracted form, and shows a rapid decrease with the huminosity. By the above mentioned comparison between holocellulose hydrolysates from the main sample of *Sphagnum fuscum* and the tops of this moss, the amount of arabinose, which was greater in the tops, was the only significant difference.

Rhamnose seems to be bound to uronic acids in a very difficultly hydrolysable complex and no great decrease of it can be seen with increasing huminosity.

General conclusions. Two general conclusions may be drawn from Table 4. Firstly, no significant difference is noticed between the two mosses and between the samples A and B, formed from *Sph. imbricatum* Russ. and *Sph. tenellum* Pers., respectively. The difficulties in estimating the physical huminosity must be emphasized, and a comparison between the physical huminosity and the chemical composition consequently cannot be stressed too strongly. Secondly, the age of the peat has practically no effect on the composition of the carbohydrates, which seems to depend chiefly upon the huminosity. This fact shows that the chief changes, due to chemical and microbiological processes, take place at the surface of the bog and obviously have already begun in the lower parts of the mosses.

A diagram, describing the changes in composition of moss, peat of low huminosity (A, B and D) and peat of higher huminosity (C and E) is given in Fig. 1.

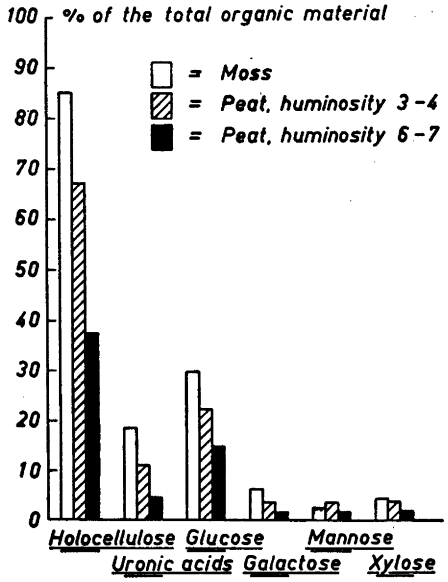

Fig. 1. The holocellulose and the total amount of sugars found, calculated as polysaccharides in Sphagnaœ, Sphagnum peat of huminosity 3—4 and Sphagnum peat of huminosity 6—7.

EXPERIMENTAL

Evaporations were carried out under reduced pressure at 30—40° and all neutralizations were made at room temperature. The melting points are uncorrected.

Fractionation. The samples were dried at room temperature in thin layers, the moss samples after washing with water. Traces of other plants were removed. The dried samples (about 20 g) were continuously extracted with benzene for 2 days and with methanol for 5 × 1 days. The dried residue was then treated with water at 60—65° (25 ml/1 g) with stirring, 3 × 3 h, filtered and washed. For *Sph. imbricatum* the ratio of dissolved material in the 3 steps was 75 : 24 : 1. The extracted samples were bleached according to the method of Wise *et al.*[7] with sodium chlorite and acetic acid. The temperature, however, was only 60—65°. Generally 2 treatments of one hour each were sufficient, but for some samples 4 steps were needed.

Part of the holocellulose (about 1 g) was carefully dried and swollen in liquid ammonia. This treatment followed with some modifications according to the small scale, the procedure of Bishop and Adams [8]. The swollen ammonia-free holocellulose was successively treated with water of 40—45° C, 3 × 3 h, and 5 % potassium hydroxide (in nitrogen atmosphere) at room temperature, 2 × 4 h., followed by washing with water. The solid-solvent ratio in these treatments was about 1:20. The holocellulose residue was carefully washed with water, aqueous acetic acid and acetone before drying. The aqueous extract was directly concentrated to dryness. The alkaline extract was neutralized with hydrochloric acid to pH 6, concentrated, adjusted to pH 4, and ethanol added to a concentration of 75 %. The precipitate was collected by centrifugation and purified by reprecipitation. Practically no carbohydrates were found in the mother liquors.

Hydrolysis. All fractions were hydrolysed with 1 *N* sulphuric acid at 100 ° (oil bath). The holocellulose residue was first swollen in 72 % sulphuric acid at room temperature for 2 hours according to standard methods. The optimal time of hydrolysis, in order to obtain as high a yield of monosaccharides as possible, was determined with the aid of semiquantitative paper chromatography to be 2 hours for the methanol and water extracts and 16 hours for the holocellulose fractions.

The sample, about 20 mg, was hydrolysed with the acid, 2 ml for the methanol and water extracts and the water and alkali soluble parts of the holocellulose, 4 ml for the

holocellulose residue, in centrifuge tubes provided with cold fingers. After hydrolysis the cooled solution was neutralized with barium carbonate, centrifuged and washed three times with water. The combined solutions were adjusted to 5 and 10 ml, respectively.

To ascertain the amounts of sugars destroyed during the hydrolysis, model experiments with pure sugars were performed. The sugars were dissolved in 1 *N* sulphuric acid and heated to 100° for 2 and 16 hours. The amount of sugar in the solutions was then determined by measuring the optical rotation. For the galacturonic acid the amount was estimated by quantitative paper chromatography as below (no traces of arabinose could be observed). The following figures for the losses were found:

Fructose (2 hours)	27 %
Galactose, Glucose, Mannose (16 hours)	6
Xylose, Arabinose, Rhamnose (16 hours)	11
Galacturonic acid (16 hours)	48

The analytical data obtained were corrected for the figures above, except for the uronic acids, which were determined by a separate analysis on unhydrolysed material. These corrections, of course, involve an approximation, as the sugars do not occur as monomers during the whole treatment time with the hydrolysing acid. There might also be some decomposition during the treatment with 72 % sulphuric acid.

Analysis of monomers. The uronic acid content of the holocellulose and holocellulose residue was determined by the semimicro decarboxylation method of Johansson *et al.*[9]

The non-acidic sugars in the hydrolysates were determined by quantitative paper chromatography, according to Hirst and Jones [10]. On a Whatman No 1 paper (50 × 26 cm) a known volume of the solution to be analysed was applied on a line with a micro pipette (Agla) and concentrated if necessary on the paper (the amount of each sugar generally was 0.080—0.300 mg). On each side of this line a reference spot of the same concentration was applied. For the separation of the components, the nature of which had been determined by ordinary paper chromatography different solvent systems had to be used. Generally, multiple development (2 × 18 h at 25°) with butanol-pyridine-water (3 : 1 : 1.5) in which solvent the uronic acids have low R_F-values and do not interfere with the non-acidic sugars, was satisfactory. Mannose and arabinose, however, do not separate well in this solvent, and for solutions with higher amounts of both these sugars, they were determined in a separate analysis, using the solvent system ethyl acetate-acetic acid-water (3 : 1 : 1) (18 h at 25° C). After development the sugars were located on the reference strips with suitable spraying reagents. The strips with the different components plus blank strips were cut out and extracted with water (24 h at 25°) in an ordinary chromatography jar into large test tubes. The eluate was then adjusted to about 5 ml, oxidized and titrated. The recovery by the extraction of the strips was quantitative, and the empirical factors for formic acid formation by the oxidation about the same as those given by Hirst and Jones.

For synthetical mixtures of galactose, glucose and fructose or galactose, glucose, mannose, xylose, arabinose and rhamnose (0.080—0.300 mg of each), which were "hydrolysed", neutralized and analysed just as with the moss and peat fractions, and corrected for decomposition during the "hydrolysis", good separations and recoveries of 92 ± 5 % were observed. The losses, 8 %, are probably mechanical and to a greater extent caused by incomplete washing following the neutralization; the empirical factor 0.92 has been used in all the determinations.

The amount of minor constituents was estimated by visual comparison of the intensity of a spot with those of standard solutions of different concentrations. In addition to the solvent mixtures mentioned above, *n*-butanol-ethanol-water (5 : 1 : 4), alone or combined with the others by successive developments, was used for the qualitative investigations, especially of the acid sugars.

The following spray reagents were used: Silver nitrate-sodium ethoxide, aniline hydrogen phthalate, *p*-anisidine phosphate and resorcinol-hydrochloric acid.

Isolation of sucrose. When the water-soluble part of the methanol extract from *Sphagnum balticum* was fractionated on a hydrocellulose column, sucrose $[\alpha]_D^{20}$ + 64.2 (water, c = 2.2), m.p. 183—184° undepressed on admixture with an authentic sample was isolated in a yield corresponding to 1.0 % of the organic part of the moss.

Investigation of the uronic acids and acid sugars. A sample of *Sphagum fuscum* (30 g) was treated with 1 *N* sulphuric acid at 100° for 16 hours after treatment with 72 % sulphuric acid. The cooled hydrolysate was neutralized with strontium carbonate, the precipitate filtered off, thorougly washed with hot water and the combined solutions after adjustment to pH 5—6 were concentrated to a smaller volume. To part of the filtrate, ethanol was added to a concentration of 90 %, the precipitate centrifuged and washed three times with 90 % ethanol. The strontium salts were dissolved in water and converted to the acids by ion exchange (Amberlite IR 120). After concentration to dryness they were dissolved in 1 % ethanol and added to the top of a carbon-Celite column. Monomers were eluted with 1 % ethanol and compounds of higher molecular weight with 25 % ethanol. The latter eluate after concentration to dryness was hydrolysed with 1 *N* sulphuric acid at 100° for 16 hours, and the hydrolysate studied by paper chromatography.

The main part of the filtrate was treated with the ion exchange resin IR—120, and the total amount of material in the hydrolysate was determined by concentrating part of the solution to dryness. The rest of the hydrolysate after concentration, was neutralized with sodium hydroxide and added to the top of a column of the ion exchange resin Dowex 2 in the acetate form.

The column was eluted with 1 *N* acetic acid, and the eluate, divided into fractions, was investigated by paper chromatography using the solvents discussed above. Only a partial separation was obtained. In this way pure galacturonic acid was isolated by separation on thick paper (Whatman 3 MM) and characterized as its strontium salt $[\alpha]_D^{20}$ + 26.0, authentic: $[\alpha]_D^{20}$ + 29.1 (water $c = 1.3$), as the free acid, $[\alpha]_D^{20}$ + 49.0, authentic: $[\alpha]_D^{20}$ + 51.3 (water $c = 2$) and further by bromine oxidation to mucic acid m.p. 214° (decomp.). The uronic acid also gave the characteristic brick-red precipitate with basic lead acetate [11].

With the aid of thick paper another uronic acid, with higher R_F-values than galacturonic acid in various solvents and a methoxyl value of 11 %, was isolated in an impure state. Two impure aldobiuronic acids were also isolated by the same method. A further investigation of these and other acidic substances, demonstrated by paper chromatography, however, is outside the scope of the present investigation.

SUMMARY

A qualitative and quantitative investigation of the carbohydrates in two *Sphagnum* mosses and five samples of *Sphagnum* peat of different age and degree of huminosity has been performed.

The two mosses investigated showed no significant differences. Samples of very different age but with the same degree of physical huminosity were very similar, indicating that the chief changes occur at the top of the bog and/or are determined by the conditions at the start of the humification. The total amount of carbohydrates was about 90 % of the organic material in the mosses and about 65 % and 35 % in peats with a degree of huminosity of 3—4 and 6—7, respectively.

Of the constituent sugars, fructose which occurred in the mosses, was completely absent in the peat. Another sugar, which occurs in nature as a furanoside, arabinose, disappeared almost completely during the humification.

The uronic acids and galactose decreased faster, while xylose and glucose decreased at about the same rate as the total carbohydrates. Mannose and probably also rhamnose are the most stable components and accumulate during the humification.

The polysaccharides in mosses and peat seem to constitute a very complex mixture. The presence of a fructan in the living moss, of a polyuronide (pectin) and a large amount of more complex polysaccharides built up of galactose,

xylose, rhamnose and uronic acids is indicated. The glucose, the most important constituent, probably occurs chiefly as cellulose, the presence of which has been demonstrated by other workers. Finally the behaviour of mannose during the humification indicates the presence of a stable mannan. There is no evidence of polysaccharides formed by microorganisms in the peat.

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Biosynthesis of ^{14}C-labeled Linoleic Acid and Its Fate in Normal and Fat-deficient Rats

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The effects of linoleic acid and of other "essential" fatty acids as curatives for the fat-deficiency syndrome have been rather extensively studied, but far less data are available concerning absorption, distribution, and intermediary metabolism of these compounds. Attempts have been made to study these problems by means of certain easily demonstrable derivatives of the "essential" acids, such as conjugated fatty acids, but whether these are physiologically equivalent to the original compounds seems rather doubtful. Certainly, a more promising method would be the use of isotope-labeled "essential" fatty acids, but as far as we are aware no reports of such work have up to now been published.

In this paper we describe the biosynthetic production of ^{14}C-labeled linoleic acid and report observations on its absorption, distribution, and metabolism after administration to a normal and a fat-deficient rat.

The biosynthetic incorporation of ^{14}C from carbon dioxide into linoleic acid was accomplished in two steps: (1) the photosynthesis of ^{14}C-labeled sugars by green leaves and (2) the production of ^{14}C-labeled linoleic acid from these sugars by means of a yeast-like organism.

EXPERIMENTAL

Radioactivity determinations. All samples were counted with a thin mica end-window Geiger-Müller counter. Two different mounting techniques were used. Barium carbonate mounts were prepared according to a method essentially identical with that described by Henriques, Kistiakowsky, Margnetti and Schneider [1]. Lipids and other organic materials were mounted using a modification of the method of Entenman, Lerner, Chaikoff and Dauben [2]. To facilitate the measurement of samples of low activity it was desirable to get more material mounted than a lens paper alone could hold. Therefore we used aluminum dishes (surface: 19.6 cm^2) with 2 mm edges in which the lens paper was placed. As a rule 10 000 counts were collected, but samples with low activity were not counted for more than 32 minutes. All counts were corrected for coincidence, background, self-absorption, and variations in geometry. In the case of lipids and other organic materials, empiric correction factors were used to convert the obtained counts into corresponding

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barium carbonate counts. The statistical evaluation of the counting data was done by means of the generally used formulas based on the Poisson relation.

Photosynthesis of ^{14}C-labeled sugars. The photosynthesis was carried out by means of *Canna indica* leaves according to the method of Hassid [3]. As the sugars in the alcoholic leaf extract were to be used only as a substrate in the following microbial synthesis, no efforts were made to isolate them in pure form. The extract was, however, converted into an aqueous solution by gently heating it until all alcohol had evaporated and by simultaneously adding water to keep the volume constant.

Three runs of photosynthesis were carried out with 260 to 400 microcuries of $^{14}CO_2$ in each *. The total amount of $^{14}CO_2$ used was 990 microcuries corresponding to 34 million counts per minute under our counting conditions. The utilization of carbon dioxide by the leaf was practically quantitative, and the total yield of radioactivity in the alcoholic leaf extract was 31 million counts per minute or 91 % of the amount given to the leaves.

Microbial synthesis of linoleic acid. The organism used for the production of linoleic acid was *Trichosporon pullulans*, a yeast-like fungus belonging to the group *Mycotoruloideae* **. In earlier work by one of us [4] this organism had given good yields of lipids, nearly half of which consisted of linoleic acid.

The yeast was grown as a surface culture in a 1 000-ml Roux flask. The volume of the medium was 300 ml, and its composition was as follows: glucose 4 %, diammonium hydrogen phosphate 0.3 %, KH_2/K_2HPO_4 to give the concentration 0.3 *M* and the initial pH 6.0, and inorganic salts to give the following quantities per liter of the medium: magnesium sulfate 400 mg, sodium chloride 160 mg, calcium chloride 80 mg, ferrous sulfate 40 mg, manganous sulfate 40 mg, and zinc sulfate 16 mg. Aeration was started immediately after inoculation and carried on at a rate of 400 ml per hour.

The sugar consumption of the yeast was followed during the experiment by determining the reducing substances in the medium every other day according to the method of Somogyi [5].

To avoid the loss of ^{14}C in the metabolism of the yeast during the period of the most intensive growth, the extract containing the labeled sugars was not added to the culture until on the fifth day, when the sugar concentration of the medium had fallen to about one-half of the initial. The added extract contained about 300 mg of sugars, and its total radioactivity was 29 million counts per minute. Traps containing 2.4 *N* sodium hydroxide for carbon dioxide collection were connected to the culture flask immediately after addition of the radioactive material. On the tenth day, when about 5 % of the initial amount of sugar remained in the medium, the experiment was stopped.

The yeast mycelium was separated from the medium, transferred into a 250 ml flask, and hydrolyzed with boiling 5 *N* hydrochloric acid during 20 minutes. The mixture was kept under nitrogen during this process and during further manipulations. The lipids were extracted from the hydrolysate with three 250 ml portions of ether. The combined ether extracts were evaporated, and 4 ml of inactive ethyl linoleate were added as a carrier to the separated lipids. The lipids were saponified with 15 ml boiling 1.8 *N* ethanolic potassium hydroxide for one hour. The ethanol was evaporated, and the residue dissolved in water. The unsaponifiable material was removed with three successive 50 ml portions of ether. The fatty acids were then liberated by adding 5 ml concentrated hydrochloric acid and extracted with three (50 – 25 – 25 ml) portions of low-boiling petroleum ether. The petroleum ether solution was washed with 10 % sodium chloride until neutrality and dried with anhydrous sodium sulfate.

The fatty acids dissolved in petroleum ether were brominated by adding bromine drop by drop under constant stirring at a temperature below –13° C. The insoluble bromination product was filtered on a small Buchner filter and washed with cold petroleum ether. At this stage it weighed 1 350 mg. After recrystallization from ethylene dichloride, the obtained snow-white crystals melted at 115.7° C (corrected). The bromine content of 13.3 mg of the crystals determined according to the method of Willard and Thompson [6] was found to be 52.5 % (calculated for tetrabromostearic acid 53.3 %).

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* The carbon 14 used in this investigation was supplied by the *Oak Ridge National Laboratory, Oak Ridge, Tenn.*, on authorization from the *Isotopes Division, U.S. Atomic Energy Commission.*

** This species was supplied by the "*Centraalbureau voor Schimmelcultures*", *Baarn, Netherlands.*

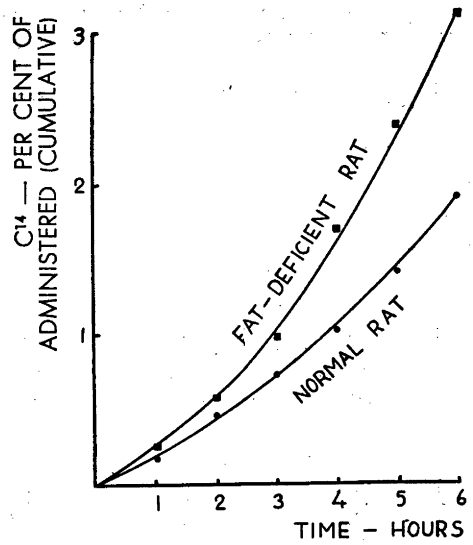

Fig. 1. Excretion of ^{14}C activity in the form of carbon dioxide by a normal and a fat-deficient rat after administration of ^{14}C-labeled ethyl linoleate.

920 mg of the purified tetrabromostearic acid was debrominated with zinc and hydrogen chloride in absolute ethanol. The crude ethyl linoleate was purified by distillation under vacuum (2.5—6 mm Hg). The final yield of the purified ester was 403 mg and its specific activity was 232 counts per minute per mg of carbon, corresponding to 73 100 counts per minute for the total sample.

Fate of ^{14}C-labeled linoleic acid in the normal and the fat-deficient rat. The radioactive ethyl linoleate was fed to two female rats, one of which had been maintained on our stock diet and the other on a fat-deficient diet * until it had plateaued in weight. At the beginning of the experiment the normal rat weighed 191 g and the fat-deficient rat 163 g. The age of the former was 146 days and of the latter 183 days.

The ^{14}C-labeled ethyl linoleate was given with a carefully calibrated syringe into the pharynx of the animals. The dose per rat was 176 mg corresponding to 31 800 counts per minute or to about 0.9 microcuries. Each of the animals was then immediately placed into a small metabolism apparatus, which was connected with sodium hydroxide traps for the collection of expired carbon dioxide. Carbon dioxide-free air was drawn through the apparatus at a rate of 6—7 liters per hour.

The animals were kept in the metabolism apparatus for six hours and then killed with ether. The following parts were separated from the carcass: liver, kidneys, heart, brain, subcutaneous tissue, perirenal adipose tissue, mesentery and omentum, genital adipose tissue, and gastrointestinal tract with its contents combined with the excreted feces. These parts were thoroughly homogenized by means of a scalpel, scissors and finally an all-glass homogenizer of the Potter-Elvehjem type. The rest of the carcass was also homogenized, and an aliquot was treated in the same way as the parts mentioned above. The lipids were separated from the homogenized samples by extracting them three or four times with boiling alcohol and thereafter with ether essentially according to the

* Sucrose 72 %, casein, fat-low, 24 %, salt mixture 4 %. Synthetic vitamins were incorporated in the diet to give the following quantities per kilogram of the diet: thiamine 6 mg, riboflavin 8 mg, pyridoxin 4 mg, calcium pantothenate 20 mg, nicotinic acid 20 mg, inositol 500 mg, folic acid 0.3 mg. Vitamin A and alpha-tocopherol were given twice weekly in amounts corresponding to daily doses of 140 I.U. vitamin A and 1 mg alpha-tocopherol per rat.

Table 1. Recovery of ^{14}C activity in the tissues and excretions of a normal and a fat-deficient rat six hours after administration of ^{14}C-labeled ethyl linoleate.

	Normal rat		Fat-deficient rat	
Total recovered activity, % of the administered	89.6		79.6	
Activity remaining in the gastrointestinal tract and its contents, % of the administered	65.4		46.5	
	Recovered activity, % of the absorbed *	Specific activity of lipids, c.p.m./mg	Recovered activity, % of the absorbed *	Specific activity of lipids, c.p.m./mg
Carcass				
Lipids				
Organ				
Liver	10.3	2.4	26.7	7.3
Kidneys	0.5	0.8	1.4	1.7
Heart	0.5	0.8	1.0	3.4
Brain	(0.2) **	(0.1) **	0.4	0.3
Depot				
Subcutaneous	12.9	0.3	4.9	0.2
Perirenal	(0.3) **	(0.02) **	1.3	0.2
Mesenterial and omental	2.6	0.3	5.7	0.6
Genital	(0.9) **	(0.03) **	(1.8) **	(0.1) **
The rest of the carcass	62.8	0.7	45.2	0.8
Non-lipid material	traces	traces	traces	traces
Excretions				
Expired CO_2	7.8	—	9.4	—
Urine	1.2	—	2.2	—
	100.0		100.0	

* Absorbed = total recovered activity minus the activity recovered in the gastro-intestinal tract and its contents.

** These figures represent low counts which do not significantly differ from the background ($P > 0.05$).

methods described by Bloor [7]. Finally the extracted lipids were transferred into petroleum ether and mounted for radioactivity determinations. The lipid-free tissues remaining after extraction and the small alcohol-extracted non-lipid fractions were also tested for radioactivity. The urine was mounted in the same way as the lipids and counted.

The excretion of ^{14}C-labeled carbon dioxide by the two animals during the experiment is shown in Fig. 1. The total excretion of carbon dioxide, indicating the general rate of metabolism, was 24.1 mg CO_2 per hour in the normal rat and 36.4 mg CO_2 per hour in the fat-deficient animal.

The distribution of the ^{14}C-activity at the end of the experiment is given in Table 1.

DISCUSSION

In view of the well-known difficulties in the synthesis of linoleic acid by chemical methods, the biosynthetic preparation of this compound seems to be the only practical procedure available at present. But even the latter

method is not without difficulties. The yields obtained by us cannot be regarded as very satisfactory. The total yield of ^{14}C in the final ethyl linoleate was only about 0.2 % of the amount in the carbon dioxide used as the initial ^{14}C source. The first phase of the biosynthesis could be carried out quite effectively, as over 90 % of the initial radioactivity was recovered in the alcoholic leaf extract. This accords well with many earlier reports on the efficiency of photosynthetic procedures [8,9]. On the other hand, considerable losses occurred in the second phase. These were partly unexpected as calculations based on previous information of the linoleic acid production by *Trichosporon pullulans* [4] and also on a number of preliminary experiments with inactive sugar indicated that about 1 % of the initial ^{14}C of sugar should be recoverable in the form of purified linoleic acid. The unduly low yield of linoleic acid may have been caused by an excessively strong alkali treatment to which the lipids were inadvertently subjected during the evaporation of alcohol from the saponification mixture.

The small yield of radioactive ethyl linoleate, unfortunately, permitted animal experiments on a very limited scale only. Therefore, the conclusions based on them are necessarily of a preliminary character. The rate of intestinal absorption of ethyl linoleate was greater in the fat-deficient rat than in the normal one (53 % of the dose given in the former *versus* 35 % in the latter). Thus the need of the body for linoleic acid seems to be one of the factors determining the rate of its absorption. As is known, a similar principle governs the absorption of certain other nutrients, *e.g.* iron [10,11].

Less than one-tenth of ^{14}C of the absorbed ethyl linoleate was excreted by the animals as carbon dioxide during the six-hour experiment. The excretion was a little faster in the fat-deficient rat. On the other hand, the general metabolic rate of this animal was considerably higher than that of the normal rat.

At the end of the experiment only traces of radioactivity were found outside the lipid fractions of the carcass. The distribution pattern of the ^{14}C of linoleate showed distinct differences in the two animals. In the fat-deficient rat a proportionally much greater part of the absorbed radioactivity was deposited in the liver (26.7 % *versus* 10.3 % in the normal rat). A similar difference between the two animals was also found in regard to the kidneys and the heart. In the normal rat, on the other hand, the subcutaneous adipose tissue took up a larger percentage of the absorbed radioactivity (12.9 % *versus* 4.9 % in the fat-deficient rat), and such was also the case with the "rest of the carcass" consisting mainly of the muscles and the skeleton (62.8 % *versus* 45.2 % in the fat-deficient rat). These differences seem rather plausible and are obviously explainable by assuming the existence of a vital need for essential fatty acids in organs such as liver, kidneys, and heart. The great avidity with which these organs in the fat-starved animal take up the ^{14}C of linoleate is also reflected by the high specific activities attained by their lipids. Especially the activity of the heart lipids is notable as being over four times that of the normal rat.

In view of the generally known role of the liver in fat metabolism, it is hardly unexpected that a considerable portion of radioactivity would be found retained in this organ — at least a relatively short time after the administration. It is perhaps of more interest to note the high uptake of ^{14}C of

linoleate by the lipids of the heart and of the kidneys, a fact which may be indicative of some specific function of the essential fatty acids in the physiology of these organs.

SUMMARY

^{14}C-labeled linoleic acid was produced biosynthetically in two steps: (1) the photosynthesis of ^{14}C-labeled sugars by *Canna indica* leaves and (2) the synthesis of linoleic acid from these sugars by means of the yeast *Trichosporon pullulans*.

The radioactive linoleic acid was administered in the form of ethyl linoleate to one fat-deficient and one normal rat, and the animals were sacrificed after six hours. In the fat-deficient rat the intestinal absorption of linoleate was more rapid, and a proportionally greater part of the absorbed activity was deposited in the liver, the kidneys, and the heart. In the normal rat the subcutaneous adipose tissue took up relatively more of the absorbed ^{14}C. Less than one-tenth of the activity was excreted as carbon dioxide, and no marked difference was found between the animals in this respect.

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Pyrazole Studies

VII *. The Kinetics of Oxidation by Air of 4-Alkylsubstituted Pyrazol-5-ones to 4-Alkyl-4-hydroxysubstituted Pyrazol-5-ones

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The oxidation of some 4-alkylsubstituted pyrazol-5-ones by oxygen has been investigated. With methanol as solvent and triethylamine present in excess the oxidation follows a first order scheme when the concentration of oxygen is kept constant. This means that the velocity of oxidation for a series of pyrazolones may be compared by comparing the time necessary for 50 % oxidation, 1 mole of oxygen being consumed per mole of pyrazolone.

The reaction products, isolated in almost quantitative yield, are the 4-hydroxysubstituted pyrazolone and triethylamine oxide. It has been shown that a substance composed of 2 moles of 4-hydroxysubstituted pyrazolone and 1 mole of triethylamine oxide is formed as a preliminary product which, when treated with water or dilute hydrochloric acid, is decomposed into the hydroxysubstituted pyrazolone and triethylamine oxide.

Veibel and Westöö [1] showed that 4-alkylsubstituted pyrazol-5-ones are spontaneously oxidised by air and that both the corresponding bis-pyrazolone and the 4-hydroxysubstituted pyrazolone could be isolated from the oxidationproducts. The reaction was found catalysed by cupric ions (*cf.* Smith [2]) but no attempt to determine the kinetics of the reaction was made.

By a closer investigation of the reactions we found that they may proceed with measurable velocity also in the absence of cupric ions, the product obtained being dependent on the reaction of the solution. In alkaline solution the 4-hydroxysubstituted pyrazol-5-one may be the only isolable reaction product whereas in neutral solution the bis-pyrazol-5-one is the main product and often the only isolable reaction product.

The reaction seems to proceed most regularly in organic solvents, organic bases being present in great excess, and in this paper only such reactions are investigated. The following technique was applied:

0.01 mole of the pyrazolone was dissolved in methanol. 0.05 moles of triethylamine were added and the solution made up to 25 ml with methanol

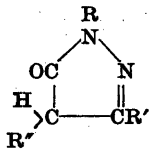
* *Acta Chem. Scand.* 8 (1954) 768.

in a 100 ml flask. The air in the flask was expelled by oxygen and the flask was connected with an oxygen-reservoir, formed as a gas-burette, allowing the direct reading of the oxygen absorbed. The flask was mechanically stirred during the experiment in order to secure constant saturation of the solution with oxygen, the concentration of which therefore may be regarded as constant. The reaction proceeds at room temperature but no measures for keeping the temperature strictly constant were taken so that deviations of $\pm$ 3° may have occurred.

The absorption of oxygen was observed. It proceeds regularly, approaching asymptotically 240 ml or 0.01 mole of oxygen per mole of pyrazolone Straight lines are obtained when log (240 — x) is plotted against time (x being ml of oxygen absorbed), showing that the reaction follows a first order scheme (*i.e.* the velocity is proportional to the concentration of pyrazolone, the concentration of oxygen being regarded as constant), so that the velocity of the reaction may be given as the time required for 50 % oxidation.

In Table 1 a summary of the pyrazolones investigated and their velocity of oxidation is recorded.

Table 1. Oxidation of different pyrazolones with oxygen.



R	R′	R″	pK_B	Time for 50 % oxidation	Yield %	M.p. of the hydroxy-pyrazolone
I C_6H_5	CH_3	CH_3	11.3	26 h	95—100	105°
II C_6H_5	CH_3	C_2H_5	11.4	20 h	95—100	130°
III C_6H_5	CH_3	C_3H_7 *n*	11.4	26 h	95—100	116°
IV C_6H_5	CH_3	C_4H_9 *n*	11.4	32 h	95—100	95°
V C_6H_5	CH_3	C_3H_7 *iso*	11.6	22.5 h	95—100	115°
VI C_6H_5	CH_3	C_6H_{11}	11.6	9 h	95—100	110°
VII C_6H_5	CH_3	$C_6H_5CH_2$	11.8	72 h	95—100	145°
VIII CH_3	CH_3	C_2H_5	10.5	7 h	65	38—39°
IX CH_3	CH_3	$C_6H_5CH_2$	10.9	23.5 h	95—100	156°
X C_6H_5	*Cyclo*hexano		11.4	13.5 h	90	145°

When the reaction is finished the solvent is removed by evaporation, leaving an oil which, on treatment with water, is converted into a crystalline 4-hydroxy derivative of the pyrazolone examined. From the aqueous layer trimethylamine oxide may be isolated, most conveniently as the hydrochloride.

In one case, X (Table 1), the oil left on evaporation of the reaction mixture crystallised on standing to a mass of colourless crystals, contaminated by a small amount of oil which could be removed (see below), and the pure substance XI was obtained as colourless leaves of m. p. 120°, *i. e.* another sub-

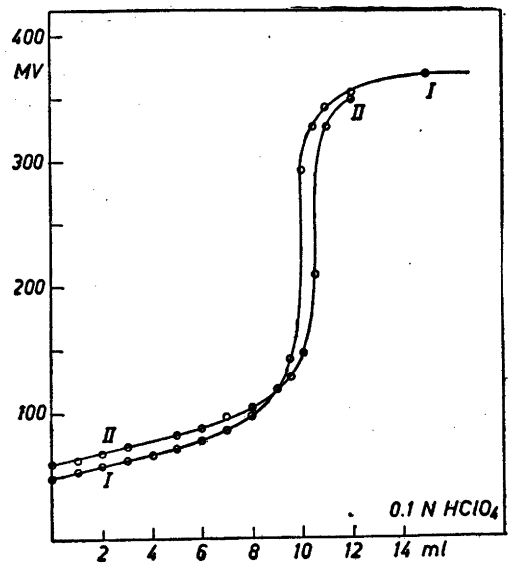
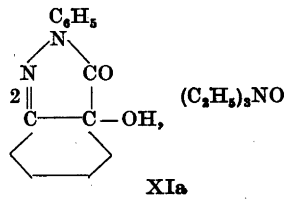


Fig. 1. Titration of XI (curve I) and of triethylamine oxide (curve II) with 0.1 N *perchloric acid. I: 0.5770 g; M = 577.7. II: 0.1237 g; M = 117.2.*

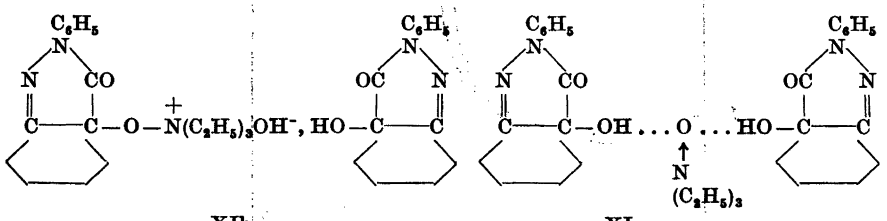
stance than the hydroxypyrazolone. The composition of this substance corresponds to the empirical formula $C_{32}H_{43}O_5N_5$, M = 577.7 (see elementary analysis below). Titrated with perchloric acid in glacial acetic acid (Veibel *et al.*[3]) the molecular weight was found to be 582, the form of the titration curve (Fig. 1, I) showing that the substance is a rather strong base ($pK_B < 9$). Treated with water the crystalline compound behaves as do the oils isolated from the other pyrazolones investigated, *i. e.* the crystals are decomposed into a 4-hydroxy derivative of X and triethylamine oxide, the latter being most conveniently isolated as its hydrochloride when an equivalent amount of hydrochloric acid is added to the substance with M = 577 together with the water. The hydroxy compound separates out and from the filtrate the triethylamine oxide hydrochloride is obtained on evaporation *in vacuo*. The ratio of hydroxy compound to triethylamine oxide isolated is approximately 2 : 1. From this is deduced that the composition of the substance first isolated is:



XIa

The substance is readily soluble in non-polar solvents and in alcohols but is, as mentioned, decomposed by water. The titration curve mentioned above is very similar to the titration curve of triethylamine oxide (Fig. 1, II), and possibly the substance is decomposed by glacial acetic acid into the hydroxy compound and triethylamine oxide.

The m. p. of the substance (120°), its solubility in non-polar solvents and its hydrolysis by water to a substance, the acidic properties of which cannot be detected, and a base, seems to indicate that the substance is not a salt. The following formulations may be discussed, but XI b must be rejected, being too strongly polar to be reconcilable with the properties of the substance.



XIb XIc

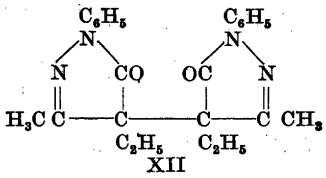
The triethylamine used should be freshly distilled. Incidentally, it was discovered that a sample of triethylamine which had been kept in a glass-stoppered bottle for a long time (1—2 years), thereby being slightly coloured, caused a much faster oxidation than the same sample freshly distilled. With VI as the pyrazolone investigated the time for 50 % oxidation was with the freshly distilled amine 9 hours, with the not purified sample 1.5 hours. In both cases a first order reaction was found by plotting log (240—x) against time, and the 4-hydroxy substituted pyrazolone could be isolated in a nearly quantitative yield.

It was examined whether a sample of distilled triethylamine, kept for one and a half month in a glass-stoppered flask, during which time it had assumed a slight colour and had deposited a trace of an oily substance, would cause a more rapid oxidation than a freshly distilled sample. In this experiment the pyrazolone I was oxidised. The time for 50 % oxidation was with freshly distilled triethylamine 26 hours, with the other sample of triethylamine 27 hours, this difference not exceeding the limit of error in the determination.

The nature of the impurity causing the strong acceleration of the velocity of oxidation when very old samples of triethylamine are used has not been further investigated so far.

Cupric ions accelerate the oxidation very strongly but the result of the oxidation is different from that obtained in absence of cupric ions. To a standard experiment with oxidation of the pyrazolone II (time for 50 % oxidation 20 hours) was added one drop of a 0.1 N solution of cupric sulphate, *i. e.* $c_{Pyrazolone} = 0.4\ M$, $c_{Cu^{++}} \leqq 0.0004\ N$. No appreciable absorption of oxygen occurred during the first half hour, but then suddenly absorption of oxygen started, accompanied by a recognisable evolution of heat. After 10 minutes the reaction was over, 100 ml of oxygen having been absorbed against the calculated 240 ml. The reaction products were dark coloured and obviously

more than one substance had been formed. The only substance which we succeeded in isolating in a state of purity was the bis-pyrazolone XII:



XII

4-Substituted pyrazolones may be oxidised to 4-substituted 4-hydroxy-pyrazolones by tert. butylhydroperoxide too. The stoichiometry of this reaction will be described in a subsequent paper. The result may, however, indicate that peroxides are taking part in the oxidation investigated here, a possible course of the reaction being the following where PyRH means a 4-monoalkylsubstituted pyrazol-5-one, possibly alkyl- or arylsubstituted at N_1 and at C_3.

$$PyRH + (C_2H_5)_3N \rightleftharpoons PyR^- + (C_2H_5)_3\overset{+}{N}H \quad (1)$$

$$PyR^- + O_2 \rightarrow PyROO^- \quad (2)$$

$$2\ PyROO^- + 2\ (C_2H_5)_3\overset{+}{N}H \rightarrow PyROH\ldots\underset{\displaystyle N(C_2H_5)_3}{\underset{|}{O}}\ldots HORPy + (C_2H_5)_3NO \quad (3)$$

$$PyROH\ldots\underset{\displaystyle N(C_2H_5)_3}{\underset{|}{O}}\ldots HORPy + H_2O \rightarrow 2\ PyROH + (C_2H_5)_3N(OH)_2 \quad (4)$$

This sequence of reactions covers the stoichiometry of the reaction. Examples of oxygen atoms acting as receptors for two hydrogen bonds are given by Pauling [4], who mentions substances where a carbonyl oxygen with only two lone electron pairs acts as receptor for two hydrogen bonds, whereas the oxygen atom in the amine oxide presumed in the present paper has three lone electron pairs and therefore should be still more liable to act as receptor for hydrogen bonds than the carbonyl oxygen.

As to the kinetics of the reaction it has to be presumed that (2) is the velocity determining step as the reaction follows a first order scheme when the concentration of oxygen is kept constant.

The velocity of the reaction is dependent on substituents present both at N_1 and at C_4. Table 1 gives a record of the time necessary for 50 % oxidation of the pyrazolones investigated. It is seen that an aryl group at N_1 decreases the velocity as compared with 1-alkylsubstituted pyrazolones.

The 4-alkylsubstituted pyrazolones examined are oxidised at nearly the same rate when the substituent at N_1 is kept constant. When a *cyclo*hexyl-radical is substituted for an alkyl-radical the velocity is considerably increased whereas the substitution of an alkyl group with a benzyl group decreases the velocity sensibly. For the pyrazolones investigated the following expressions hold good:

$$\left[\frac{t^{C_2H_5(C_4)}_{50\ \%}}{t^{C_6H_5CH_2(C_4)}_{50\ \%}}\right]_{C_6H_5(N)_1} = \left[\frac{t^{C_2H_5(C_4)}_{50\ \%}}{t^{C_6H_5CH_2(C_4)}_{50\ \%}}\right]_{CH_3(N_1)} = 0.30$$

$$\left[\frac{t^{CH_3(N_1)}_{50\ \%}}{t^{C_6H_5(N_1)}_{50\ \%}}\right]_{C_6H_5(C_4)} = \left[\frac{t^{CH_3(N_1)}_{50\ \%}}{t^{C_6H_5(N_1)}_{50\ \%}}\right]_{C_6H_5CH_2(C_4)} = 0.34$$

or: *the relation between the time for 50 % oxidation of pyrazolones with different substituents at C_4 is independent of the substituent at N_1*, and *the relation between the time for 50 % oxidation of pyrazolones with different substituents at N_1 is independent of the substituent at C_4.*

Table 1 records the pK_B-values of the pyrazolones too, most of them taken from a preceding paper (Veibel *et al.*[5]). It is seen that there is no direct dependency of the rate of oxidation on the pK_B of the pyrazolone.

The investigation on the oxidation of pyrazolones is continued.

EXPERIMENTAL PART *

The pyrazolones investigated were prepared by current methods. For I—III, V—VI, and VIII see Veibel *et al.*[5], for IV, VII, and X see [6-8], respectively. The pK_B values recorded in Table 1 were determined as indicated by Veibel *et al.*[5].

1,3-Dimethyl-4-benzylpyrazol-5-one (IX). A mixture of 66 g (0.3 mole) of ethyl α-benzylacetoacetate, 100 ml of methanol and 300 ml of *N* methylhydrazine solution (Veibel *et al.*[5]) are stirred mechanically at 70° for 8 hours. Methanol and most of the water are then removed *in vacuo* (bath temperature 50°), the pyrazolone crystallising from the residue on cooling. It may be recrystallised from water, aqueous ethanol or ethyl acetate. Yield 70 %; m.p. 155°. (Found: C 71.17; H 7.03; N 13.83. Calc. for $C_{12}H_{14}ON_2$ (202.2): C 71.27; H 6.98; N 13.86.)

Oxidation of pyrazolones. The technique applied has been described above. The experiment is interrupted when oxygen is not absorbed longer. The solvent is removed *in vacuo* (bath temperature ca. 40°). The oily residue is dissolved in a minimum amount of methanol, water is added and the mixture shaken mechanically for half an hour during which an oil separates out. The oil is extracted with ether and the ethereal layer is extracted twice with water. The ether is removed at room temperature leaving a solid colourless or slightly coloured substance usually in a nearly quantitative yield. The substance may be recrystallised from ligroin (b.p. 100—140°) or purified by treatment of its methanolic solution with absorption carbon, removing the solvent *in vacuo* after filtration.

From the aqueous layer triethylamine oxide may be isolated as its hydrochloride after addition of the equivalent amount of hydrochloric acid and evaporation on the steam bath.

As an example the isolation and purification of the hydroxy compound in an experiment with 5 times the standard amount of pyrazolone shall be described.

7 g (0.05 mole) of 1,3-dimethyl-4-ethylpyrazol-5-one and 35 ml (0.25 mole) of triethylamine were diluted with methanol to 125 ml in a flask. The flask was connected with an oxygen-reservoir and shaken mechanically for 24 hours during which 1 100 ml of oxygen were absorbed (calculated 1 200 ml of oxygen). The solvents were removed *in vacuo* (bath temperature 40—50°), leaving a viscous oil which was shaken for 1 hour with 15—20 ml of water and 0.05 mole of concentrated hydrochloric acid. Then 200 ml of ether were

* All m.p.s uncorrected; microanalyses by Mr. W. Egger, Department of Organic Chemistry, University of Copenhagen.

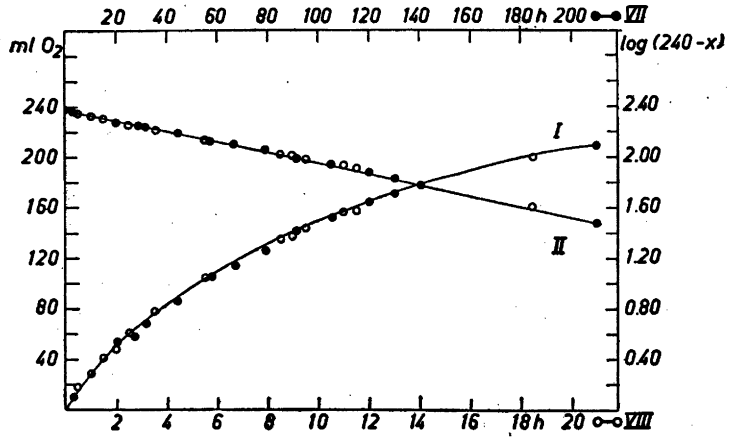


Fig. 2. Uptake of oxygen (curve I) and log (240−x) (curve II) for the pyrazolones VII (○——○) and VIII (●——●).

added and sufficient anhydrous calcium chloride to absorb the water. After 5—6 hours the solution was filtered and the ether removed from the filtrate *in vacuo*, leaving a viscous oil which eventually crystallised. M.p. *ca.* 30°, yield 7 g, calculated for 1,3-dimethyl-4-ethyl-4-hydroxypyrazol-5-one 7.8 g, *i. e.* a yield of 90 %. The hydroxy compound may be distilled *in vacuo*, b.p. 140°$_{15\ mm}$. It may be purified by recrystallisation from ethyl acetate-petroleum ether, using absorption carbon to decolourise the solution. The filtrate is chilled to −40°i n order to improve the yield which was 5 g or 65 % with m.p. 38—39°. (Found: C 53.93; H 7.81; N 18.05. Calc. for $C_7H_{12}O_2N_2$ (156.2): C 53.84; H 7.70; N 17.94.)

When 1-phenyl-3,4-*cyclo*hexanopyrazol-5-one (X) is oxidised it is possible to isolate an intermediate compound in a crystalline state:

10.7 g of X and 35 ml of triethylamine were dissolved in methanol to 125 ml, the flask connected with the oxygen-reservoir and shaken mechanically for 48 hours, 1 150 ml of oxygen being absorbed during this time (calculated 1 200 ml). The solvents were removed *in vacuo*, leaving a viscous oil which crystallised when left for some time (or during the evaporation of the solvents). Yield 16.95 g, m.p. 95—100°. The crystals include some oil which, however, is insoluble in warm hexane in which the crystals are soluble. The solution is decolourised with absorption carbon and filtered, the filtrate depositing colourless crystal leaves on cooling. M. p. 120°.

Another way of removing the oil is by dissolving the crystals in ethyl acetate and slowly adding petroleum ether or low boiling ligroin. The oil is first precipitated; the supernatant solution is decanted from the oil and on further addition of petroleum ether the pure substance XI is obtained. Yield of pure substance 12 g = 80 %.

This substance is readily soluble in acetone, chloroform, methanol and other alcohols, benzene and ethyl acetate, somewhat soluble in ether from which solvent it may be recrystallised. It is slightly soluble in petroleum ether. Its composition corresponds to the formula $C_{32}H_{43}O_5N_5$ or two moles of the hydroxy compound and one mole of triethylamine oxide. (Found: C 66.68; H 7.60; N 12.29. Calc. for $C_{32}H_{43}O_5N_5$ (XI) (577.7): C 66.53; H 7.50; N 12.12).

2.9 g of this substance (0.005 mole) was dissolved in 25 ml 0.2 *N* hydrochloric acid (0.005 mole) and heated to boiling for a few minutes. On cooling 2.2 g of a substance (XIII) with m.p. 145° was precipitated. The filtrate was evaporated to dryness *in vacuo*, leaving a substance XIV with m. p. ca. 150°, containing ionogenic chlorine.

The same two substances were obtained by shaking 2.9 g of XI mechanically with 6 ml *N* hydrochloric acid (20 % excess) for half an hour. The crystal leaves of the original compound were converted into a finegrained crystal powder which was filtered off, washed

Table 2. Absorption of oxygen.

Pyrazolone																			
I		II		III		IV		V		VI		VII		VIII		IX		X	
Time h	O_2 ml	Time h	O_2 ml	Time h	O_2 ml	Time h	O_2 ml	Time h	O_2 ml	Time h	O_2 ml	Time h	O_2 ml	Time h	O_2 ml	Time h	O_2 ml	Time h	O_2 ml
1	7	1	7	0.5	4	1	5	1	10	0.25	4	3	10	0.5	19	0.5	3	0.5	5
2	14	2	13	1	6	2	8	2	14	0.5	10	6	21	1	29	1	5	1.75	11
3	21	2.5	16	7.5	44	2.5	12	2.5	18	0.75	15	8	23	1.5	42	8	45	3	30
4	26	3	20	9	47	3	13	3.5	22	1	20	9	25	2	54	10.5	56	4	39
8	46	4	26	19.5	88	3.5	14	4.5	30	1.5	30	20	48	2.5	62	11.5	63	5	50
9	52	8	53	21.5	95	7	31	5.5	38	2	38	27	58	3.5	77	12.5	69	9	85
21	101	10	67	24	105	13	57	6	43	3	56	29	63	5.5	104	15.5	86	12	111
26	118	11	73	30	131	23.5	87	6.5	45	3.5	64	31.5	66	8.5	134	16.5	92	13	117
31	138	13.5	93	32	137	27.5	100	7	49	4	71	44	85	9	138	17.5	98	14	123
45	178	23	127	44	168	29	104	10	70	5.5	90	57	105	9.5	143	33.5	154	23	170
		27	140	49	180	35	127	11	75	6	94	66.5	113	10.5	151	35.5	156	25	178
		31.5	156	53	191	47	152	13	85	12.5	147	79	126	11	154	39.5	164	26	183
		37.5	175	69	220	51.5	162	24	123	20	180	91	139	11.5	157	57	190	28	187
		47.5	195			57.5	177	26	130			105	152	18.5	199	61	193	29	190
		52	204			59.5	181	28	135			120	164					46	221
								30	143			130	170					49	223
								33	154			140	178					51	226
								35	158			210	210						
								37	163										
								47.5	185										
								60.5	211										
								70	220										

with water and dried in a vacuum-desiccator. Yield 2.20 g with m. p. 140° which could be raised to 145° by recrystallisation from a minimum amount of ethyl acetate or an ethyl acetate-petroleum ether mixture. The filtrate was extracted with ether. The colourless aqueous layer was evaporated to dryness *in vacuo*, the residue dissolved in methanol and precipitated with ether, yielding 0.7 g of a compound with m. p. 152—153° (dec.). For the hydrochloride of triethylamine oxide is indicated m. p. 154° (dec.), for the hydrochloride of triethylamine m. p. 253—254°, and the m. p. of XIV was not depressed by admixture of an authentic sample of triethylamine oxide hydrochloride.

$$\underset{\text{XI}}{C_{32}H_{43}O_5N_5} + HCl \rightarrow 2\ \underset{\text{XIII}}{C_{13}H_{14}N_2O_2} + \underset{\text{XIV}}{(C_2H_5)_3N\langle^{OH}_{Cl}}$$

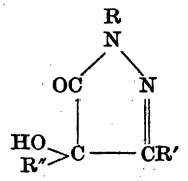
(Found: C 68.08; H 6.03; N 12.26. Calc. for $C_{13}H_{14}N_2O_2$ (XIII) (230.2): C 67.81; H 6.13; N 12.17.)

Further investigation of XI showed that it may be titrated with perchloric acid in glacial acetic acid and that the titration curve is practically identical with that of triethylamine oxide. In Fig. 1, curve I is the titration curve of XI, curve II that of triethylamine oxide.

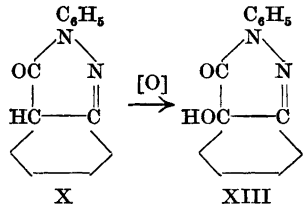
If XI was a salt of triethylamine oxide (which is hardly possible when its behavior towards solvents is taken into consideration) triethylamine oxide picrate should be precipitated on addition of a methanolic solution of XI to a saturated solution of picric acid in methanol. No such precipitation takes place.

Evidently XI is decomposed by hydrochloric acid into two new compounds, one of which is triethylamine oxide hydrochloride. The other one is a hydroxy derivative of the pyrazolone X, a hydroxy group being substituted for a hydrogen atom at C_4:

Table 3. Analyses of some pyrazolones



	R	R′	R″	Formula		% C	% H	% N
I ox	C_6H_5	CH_3	CH_3	$C_{11}H_{12}O_2N_2$	Calc.	64.68	5.93	13.72
					Found	64.72	5.86	13.89
II ox	C_6H_5	CH_3	C_2H_5	$C_{12}H_{14}O_2N_2$	Calc.	66.04	6.47	12.84
					Found	66.17	6.59	12.77
III ox	C_6H_5	CH_3	C_3H_7 *n*	$C_{13}H_{16}O_2N_2$	Calc.	67.20	6.94	12.06
					Found	67.32	7.21	11.96
IV ox	C_6H_5	CH_3	C_4H_9 *n*	$C_{14}H_{18}O_2N_2$	Calc.	68.27	7.37	11.38
					Found	68.34	7.18	11.50
V ox	C_6H_5	CH_3	C_3H_7*iso*	$C_{13}H_{16}O_2N_2$	Calc.	67.20	6.94	12.06
					Found	67.42	6.96	12.28
VI ox	C_6H_5	CH_3	C_6H_{11}	$C_{16}H_{20}O_2N_2$	Calc.	70.57	7.40	10.29
					Found	70.70	7.23	10.41
VII ox	C_6H_5	CH_3	$C_6H_5CH_2$	$C_{17}H_{16}O_2N_2$	Calc.	72.85	5.75	10.00
					Found	72.81	5.64	10.23
VIII ox	CH_3	CH_3	C_2H_5	$C_7H_{12}O_2N_2$	Calc.	53.84	7.70	17.94
					Found	53.93	7.81	18.05
IX ox	CH_3	CH_3	$C_6H_5CH_2$	$C_{12}H_{14}O_2N_2$	Calc.	66.02	6.47	12.84
					Found	66.24	6.32	12.89
X ox	C_6H_5	*Cyclo*hexano		$C_{13}H_{14}O_2N_2$	Calc.	67.81	6.13	12.17
					Found	68.08	6.03	12.26



X XIII

By the titration of XI with perchloric acid the molecular weight of XI was found to be 582 (0.5770 g of substance require 9.9 ml of 0.1 *N* perchloric acid in glacial acetic acid). This and the amounts of XIII and XIV isolated indicate that XI is composed of two moles of XIII and one mole of XIV. For XI the structure indicated above (XI c, p. 1010) is proposed.

When other pyrazolones were oxidised we did not succeed in isolating the primary oxidation product in a crystalline form but only as oils which upon treatment with hydrochloric acid were decomposed into the corresponding hydroxy-substituted pyrazolone and triethylamine oxide or its hydrochloride. In Table 1 is indicated the yield and the m.p. of the different hydroxy-substituted pyrazolones. In Fig. 2 the absorption of oxygen is plotted against time for two typical pyrazolones and in Table 2 the oxygen uptake of all the pyrazolones investigated is recorded. Finally, Table 3 is a summary of the analyses of all the 4-hydroxy-substituted pyrazolones prepared.

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The Hydrolysis of Some Choline Esters

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Knowledge of the rate of spontaneous hydrolysis of choline esters is important both in the determination of their stability in aqueous solutions when used as drugs, and in the study of their enzymatic hydrolysis. The only choline esters which have been investigated in this respect are acetylcholine and succinylcholine (Celocurin). The hydrolysis of acetylcholine at various pH levels was studied by Hofmann [1]; its dependence on the ionic concentration by Nilsson [2]; and, lastly, a complete investigation of its kinetics was conducted by Butterworth *et al.*[3]. The hydrolysis of succinylcholine at various pH levels and temperatures was studied by Tammelin [4]. The object of the present investigation was to obtain kinetic data on the hydrolysis of the iodides of acetyl-β-methylcholine (Betacholyl), propionylcholine and butyrylcholine.

EXPERIMENTAL

The hydrolysis of the choline esters, synthesized by Tammelin [5], was measured in Clark and Lub's standard buffer solutions (Kolthoff [6]) at pH 1, 2, 3, 6, 7, and 8 and at 60.0 °, 75.0°, and 90.0° C. The pH levels of the solutions at those temperatures were measured with a Radiometer pH-meter, Type 24; a phosphate buffer solution and a phthalate buffer solution with known pH levels at these temperatures [7] were used as references. The pH of the solutions was determined at the beginning and end of hydrolysis, and was found to remain constant. The ionic products of water at the various temperatures were calculated with the formula reported by Harned and Hamer [8]. The temperature variation in the thermostat was less than ±0.2° C.

Procedure. Forty milliliters of the buffer solution was brought to the thermostat temperature in a vessel fitted with a stoppered condenser. At zero time, 0.030 g of the choline ester was added and the vessel shaken, so that the compound rapidly dissolved. At intervals, 1.00 ml samples were withdrawn and analysed. The concentration of unhydrolysed choline ester was determined colorimetrically according to the method reported by Hestrin [9]. The principle of this method is to convert the choline ester with hydroxylamine into hydroxamic acid, which forms a coloured complex with ferric ions. This complex exhibits absorption maximum at 5 200 Å. The colour intensities of the samples were measured in a 10 mm cell with a Beckmann B spectrophotometer. Because Lambert-Beer's law is obeyed, the concentration of the unhydrolysed choline ester is direct proportional to the optical density.

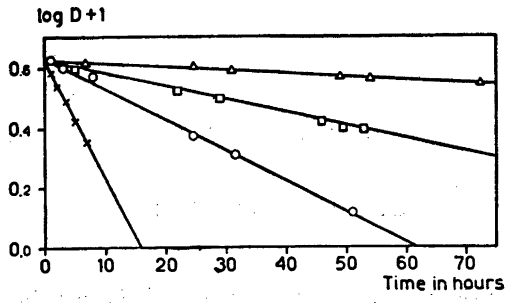

Fig. 1. Hydrolysis of acetyl-β-methylcholine at 75.0° C. ○ = *pH 2.11;* △ = *pH 3.13;* □ *pH 5.95;* × = *pH 6.91.*

RESULTS AND DISCUSSION

The hydrolysis of choline esters is catalyzed by both hydronium and hydroxyl ions as ordinary, non-ionic esters. Since the reaction with water molecules is of subordinate importance compared with the reaction with hydronium and hydroxyl ions, the rate constant of the hydrolysis may be equated as

$$k = k_{H+}\,[H_3O^+] + k_{OH-}[OH^-] \tag{1}$$

In this equation k_{H+} and k_{OH^-} are the catalytic constants for hydronium and hydroxyl ions respectively. By making use of the ionic product of water, K_w, elimination of $[OH^-]$ gives

$$k = k_{H+}[H_3O^+] + \frac{k_{OH^-}K_w}{[H_3O^+]} \tag{2}$$

Since the hydronium ion concentration in the buffer solution remains constant during the reaction, the hydrolysis can be treated as a first order reaction, and as Lambert-Beer's law is obeyed, the rate constant at each pH was calculated from the first order equation

$$k = -\frac{1}{t}\ln\frac{D}{D_\circ} \tag{3}$$

where $D_\circ$ and D are the optical densities at zero time and t time, respectively. This is illustrated in Fig. 1 by the hydrolysis of acetyl-β-methylcholine, where a straight line is obtained when the logarithm of the optical density is plotted against the time.

In acid solutions the second term in equation (2) will be negligible compared with the first one, so that equation (2) can be expressed as

$$\log k = \log k_{H+} - \mathrm{pH} \tag{4}$$

In neutral and alkaline solutions equation (2) can be transformed into

$$\log k = \log k_{OH^-} - \mathrm{p}K_w + \mathrm{pH} \tag{5}$$

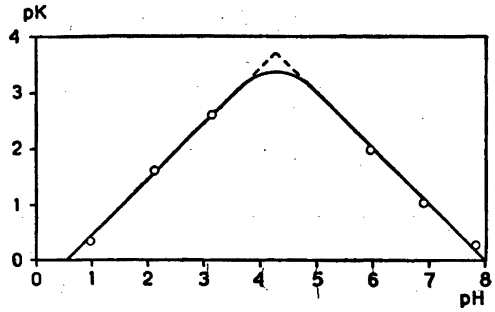

Fig. 2. Rate constants of the hydrolysis of acetyl-β-methylcholine at 75.0° C, plotted against the pH.

because k_{H+} is much less than k_{OH^-}. Fig. 2 shows this to be valid within the experimental accuracy. From equations (4) and (5) the catalytic constants k_{H+} and k_{OH^-} respectively were calculated in respect of the choline esters studied, and their values are given in Table 1.

Table 1. Catalytic constants k_{H+} and k_{OH^-} for the hydrolysis of acetyl-β-methylcholine, propionylcholine, and butyrylcholine.

t° C	Acetyl-β-methylcholine		Propionylcholine		Butyrylcholine	
	k_{H+} 10^3	k_{OH^-}	k_{H+} 10^3	k_{OH^-}	k_{H+} 10^3	k_{OH^-}
	$l \cdot mole^{-1} \cdot sec.^{-1}$		$l \cdot mole^{-1} \cdot sec.^{-1}$		$l \cdot mole^{-1} \cdot sec.^{-1}$	
60.0	0.319	6.42	0.600	18.7	0.392	10.9
75.0	0.973	13.5	1.90	35.3	1.18	22.8
90.0	2.45	20.4	5.47	69.2	3.36	35.8

To obtain the activation energies of the acid catalyzed reactions the values of log k_{H+} were plotted against $1/T$, as shown in Fig. 3. The lines were drawn according to the method of least squares, and the constants in Arrhenius' equation

$$\log k_{H+} = A_{H+} - B_{H+}/T \qquad (6)$$

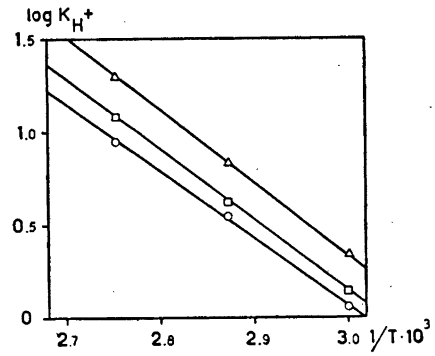

Fig. 3. Catalytic constants of the hydronium-ion catalyzed hydrolysis of acetyl-β-methylcholine (○), propionylcholine (△), and butyrylcholine (□) plotted against 1/T.

were determined from the slope of the lines and from the intercepts. The corresponding constants for the base catalyzed reactions were determined in the same manner. The values of these constants and the activation energies, E, are given in Table 2.

Table 2. Values of the constants A and B in Arrhenius' equation and the activation energies, E, for the hydrolysis of acetyl-β-methyl-choline, propionylcholine, and butyrylcholine.

	A_{H+}	B_{H+}	E_{H+} cal	A_{OH^-}	B_{OH^-}	E_{OH^-} cal
Acetyl-β-methylcholine	7.377	3 621	16 600	7.266	2 150	9 800
Propionylcholine	8.541	3 920	17 900	7.843	2 190	10 000
Butyrylcholine	7.933	3 780	17 300	7.653	2 203	10 100

In the acid hydrolysis of choline esters the reaction proceeds at approximately the same rate as in the acid hydrolysis of non-ionic esters. The length of the carbon-chain of the acid component and the branching of the alcohol component seem, however, to influence the rate of hydrolysis of choline esters less than that of ordinary esters. In alkaline hydrolysis minor changes in the structure of the alcohol component and acid component produce a greater effect on the reaction rate than in acid hydrolysis, which parallels that observed in ordinary ester hydrolysis. The rate of hydrolysis, however, is more rapid for choline esters than for non-ionic esters, which fact may be attributable to the greater affinity of the hydroxyl ion to the positively charged choline ester than to the uncharged ester.

SUMMARY

The kinetics of hydrolysis of the iodides of acetyl-β-methylcholine, propionylcholine, and buturylcholine were studied in acid and alkaline buffer solutions at 60°, 75°, and 90° C; and the constants in Arrhenius' equation and the activation energies for these compounds were calculated.

My thanks are due to the Director of the Research Institute of National Defence, Dept. 1, Professor G. Ljunggren, for the kind interest he has shown in this work.

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The Crystal Structure of the Double Lithium Antimony(V) oxide $LiSbO_3$

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In the review of antimonates given by Wells [1] it is pointed out that all antimonates, which do not contain water, are best described as complex oxides all based on octahedral SbO_6 coordination. When one of us in trying to prepare a double lithium antimony(III) oxide halogenide found that this gave crystals evidently of $LiSbO_3$, it was therefore decided to continue the investigation on lithium antimonate by Schrewelius [2]. Schrewelius had to base his investigation on powder photographs from which he could find probable positions of the antimony atoms but had to leave the question of the oxygen and lithium positions open. It thus seemed of interest to see if $LiSbO_3$ would contain isolated antimonate groups or rather be described as a double oxide.

PREPARATION

Schrewelius [2] prepared $LiSbO_3$ by heating $LiSb(OH)_6$ and by heating lithium carbonate and antimony(III)oxide in various proportions at various temperatures. This did not give crystals sufficiently large for single crystal photographs.

In this investigation $LiSbO_3$ was prepared by heating a mixture of Sb_2O_3 and a large amount of LiBr or LiCl in a porcelain boat in a tube heated at temperatures between 550° and 800° C and in a stream of air or oxygen for about three hours. The melt was then extracted with water, and the residue of $LiSbO_3$ filtered and dried. The best way to prepare crystals of sufficient size was to heat a mixture of Sb_2O_3 and a large amount of LiCl in an open porcelain crucible at 750°—800° C with a stream of oxygen passing over the surface of the melt. Portions of LiCl were poured in now and then as LiCl evaporated fairly rapidly. The time was about 24 hours. The residue of $LiSbO_3$ was then made free from LiCl by dissolving the latter in water. The crystals were needle-shaped.

ANALYSES

$LiSbO_3$ is very difficult to dissolve in acids, so it was dissolved and pentavalent antimony determined in the following way. Approximately 200 mg of the sample was mixed with 100 ml concentrated hydrochloric acid and 2 g potassium iodide in a distilling flask through which was lead a stream of carbon dioxide. The distilling flask was heated and the liberated iodine received in a flask containing potassium iodide. The time of distillation was 5—9 hours. The iodine was titrated with sodium thiosulfate. In the distilling flask, the remaining trivalent antimony was precipitated as sulfide, which as a control

was dissolved in hydrochloric acid and titrated with potassium bromate according to Smith and May [3] with naphthol blue-black as indicator. The thiosulfate and the bromate titration gave practically the same result. To free the filtrate from iodide it was run through a cation exchange column containing Dowex 50. Lithium and potassium were then extracted with hydrochloric acid. From this mixture lithium chloride was extracted with absolute alcohol and ether according to Palkin [4] and then converted to the sulfate and weighed as Li_2SO_4.

	Antimony %	Lithium %
Calc. for $LiSbO_3$:	68.91	3.93
Found:	68.5, 68.7, 68.9	3.94, 3.90, 3.88

UNIT CELL AND SPACE GROUP

Single crystals were selected and set and rotation and Weissenberg photographs were taken round two of the axes with Cu-radiation. The following Weissenberg photographs were taken: *hk*0, *hk*1, *hk*2, 0*kl*, 1*kl*, and 2*kl*. The crystals proved to be orthorhombic with the *c*-axis coinciding with the needle axis. The cell dimensions were more accurately determined from powder photographs of which a section is given in Table 1. The values from the present investigation are compared with those by Schrewelius [2].

Present investigation	Schrewelius
$a = 4.883$ kX $= 4.893$ Å	$a = 4.89$ kX
$b = 8.474$ kX $= 8.491$ Å	$b = 8.46$ kX
$c = 5.172$ kX $= 5.183$ Å	$c = 5.18$ kX
$V = 214.010$ kX^3 $= 215.335$ Å^3	

with an accuracy of about $\pm$ 0.05 %.

If 4 formula units are assumed per unit cell the density would be $d_{calc} = 5.45$. $d_{obs} = 5.25, 5.26, 5.35$. (The density of the crystals was determined from the loss of weight in benzene).

In the photographs the following reflections are systematically absent

$$\begin{aligned} 0kl &\text{ with } k + l = 2n + 1 \\ h0l &\text{ with } l = 2n + 1 \\ hk0 &\text{ with } h + k = 2n + 1 \end{aligned}$$

This is characteristic of the space group No. 52, *Pncn*—D_{2h}^{6}. (Space group number according to International Tables [5].)

PATTERSON SYNTHESES TO FIND THE ANTIMONY POSITIONS

Schrewelius [2] has, from powder data, placed the four antimony atoms of the cell in the fourfold position 4 (*c*): $0, y, \frac{1}{4}; 0, \bar{y}, \frac{3}{4}; \frac{1}{2}, \frac{1}{2} + y, \frac{3}{4}; \frac{1}{2}, \frac{1}{2} - y, \frac{1}{4}$ with $y = 0.20$.

In this investigation it seemed best to start with Patterson syntheses to find the positions of the antimony atoms. The values of the intensities were estimated visually from the Weissenberg photographs (double films) and then corrected for the Lorentz and polarization factors using the curves given by Kaan and Cole [6]. These corrected intensities were used instead of the F^2 values.

Table 1. A section of a powder photograph of $LiSbO_3$. CrKa-radiation. $\lambda = 2.2863$ kX = 2.2909 Å. For lines, where α_1 and α_2 reflections are separated the given $\sin^2\Theta_{obs}$ are the weighted averages. Notations: v st = very strong, st = strong, m = medium, w = weak, v w = very weak.

hkl	$10^4\sin^2\Theta$ calc	$10^4\sin^2\Theta$ obs	*I* obs
123	5 673	5 681	m
232	5 784		
033	6 035	6 043	m
321	6 149	6 158	st
060	6 551	6 557	m
330	6 570	6 579	v w
133	6 583		
213	6 771	6 769	w
302	6 886		
152	7 051	7 070	v st
242	7 058		
331	7 058		
312	7 068		
251	7 230		
223	7 317		
161	7 587	7 589	w
322	7 614		
004	7 817		
143	7 857	7 862	w
233	8 227	8 222	m
341	8 332	8 335	m++
104	8 365		
062	8 505	8 506	m
332	8 524	8 538	m
024	8 545		
114	8 547		
252	8 696		
260	8 743	8 741	w+
400	8 768	8 768	m++
053	8 946		
162	9 053	9 044	v w
261	9 231		
071	9 405	9 403	m+
411	9 439		
170	9 464	9 461	w
350	9 481	9 475	m++
153	9 494		
420	9 496		
243	9 501		
313	9 511		

In space group No. 52, *Pncn*-D_{2h}^6 we have the following equivalent positions:

8 (e) x,y,z; $\bar{x},y,\frac{1}{2}-z$; $\frac{1}{2}-x,\frac{1}{2}-y,z$; $\frac{1}{2}+x,\frac{1}{2}-y,\frac{1}{2}-z$;
$\bar{x},\bar{y},\bar{z}$; $x,\bar{y},\frac{1}{2}+z$; $\frac{1}{2}+x,\frac{1}{2}+y,\bar{z}$; $\frac{1}{2}-x,\frac{1}{2}+y,\frac{1}{2}+z$

4 (d) $\frac{1}{4},\frac{1}{4},z$; $\frac{3}{4},\frac{3}{4},\bar{z}$; $\frac{1}{4},\frac{3}{4},\frac{1}{2}+z$; $\frac{3}{4},\frac{1}{4},\frac{1}{2}-z$

4 (c) $0,y,\frac{1}{4}$; $0,\bar{y},\frac{3}{4}$; $\frac{1}{2},\frac{1}{2}+y,\frac{3}{4}$; $\frac{1}{2},\frac{1}{2}-y,\frac{1}{4}$

4 (b) $0,\frac{1}{2},0$; $0,\frac{1}{2},\frac{1}{2}$; $\frac{1}{2},0,0$; $\frac{1}{2},0,\frac{1}{2}$

4 (a) $0,0,0$; $0,0,\frac{1}{2}$; $\frac{1}{2},\frac{1}{2},0$; $\frac{1}{2},\frac{1}{2},\frac{1}{2}$

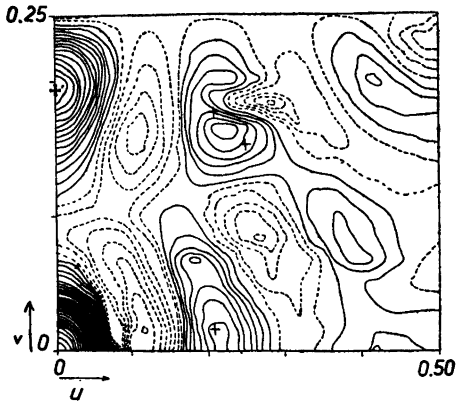

Fig. 1. P(uvp). Contours at an interval of 100 arbitrary units. Negative values dotted. + Sb—Sb vectors and + Sb—O vectors calculated with the final parameters.

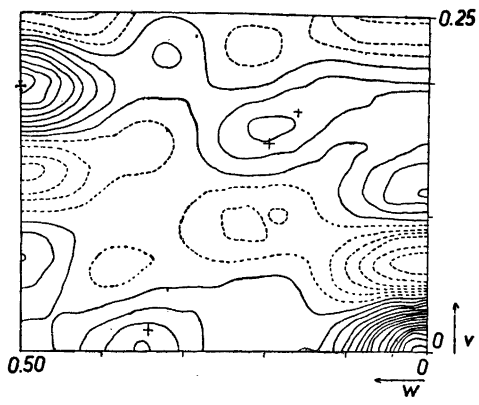

Fig. 2. P(pvw). Contours at an interval of 100 arbitrary units. Negative values dotted. + Sb—Sb vectors and + Sb—O vectors calculated with the final parameters.

In the Patterson space $P(uvw)$ the fourfold positions give the following vectors

I	within 4 (*a*) and 4 (*b*) of weight 4		$(0,0,\frac{1}{2})$; $(\frac{1}{2},\frac{1}{2},0)$; $(\frac{1}{2},\frac{1}{2},\frac{1}{2})$
II	within 4 (*c*)	A of weight 2	$\pm\ (0,2y,\frac{1}{2})$; $\pm\ (\frac{1}{2},\frac{1}{2}+2y,0)$
		B of weight 4	$(\frac{1}{2},\frac{1}{2},\frac{1}{2})$
III	within 4 (*d*)	A of weight 2	$\pm\ (\frac{1}{2},\frac{1}{2},2z)$; $\pm\ (\frac{1}{2},0,\frac{1}{2}+2z)$
		B of weight 4	$(0,\frac{1}{2},\frac{1}{2})$

The calculated $P(uvp)$-projection is given in Fig. 1 and the $P(pvw)$-projection in Fig. 2. The positions 4 (*a*) and 4 (*b*) would require a maximum in $P(pvw)$ at $v = 0$ $w = \frac{1}{2}$, which does not exist, so that these positions can be excluded. As the maximum next in height to that at the origin and of approximately half of its height is situated at $u = 0$ for $P(uvp)$ and at $w = \frac{1}{2}$ for $P(pvw)$ it seems probable that antimony is placed in 4 (*c*).

From $P(uvp)$ we thus have $2y = v = 0.1950$, 0.8050, 1,1950 .... which gives $y = 0.0975$, 0.4025, 0.5975 ....

From $P(pvw)$ we have $2y = v = 0.1938$, 0.8062, 1.1938 ... which gives $y = 0.0969$, 0.4031, 0.5969 ....

The mean value is $y = 0.0972$, 0.4028, 0.5972 ... where we choose $y = 0.0972$. We can see that $y = 0.20$ given by Schrewelius [2] does not fall in the series of possible y-values.

The lower maxima in the Patterson projections might indicate Sb-O vectors, but it seems safer not to make any suggestions of the oxygen positions at this stage. (The subsequently calculated interatomic vectors are marked in Figs. 1 and 2 and we see that they coincide with some of the lower maxima.)

FOURIER SYNTHESES

The best way to refine the antimony parameter and try to find the oxygen positions seemed to be by Fourier syntheses. These syntheses have been calculated with $F_{obs} = \sqrt{\frac{I}{Lp}}$ where F is the structure factor, I is the estimated intensity, Lp are the combined Lorentz and polarization factors. In all syntheses the value of F (000) has been omitted.

The ϱ (XYp)- and ϱ (pYZ)-projections were calculated. Minor maxima were obtained that seemed to indicate quite probable oxygen positions. In order to refine the oxygen positions we applied the bounded projection method (Booth [7]). When the difference between the bounding planes is a half only the zero zone and the odd zones are included. We must, however, be careful that the bounding planes do not pass too near the atoms we want to locate. For this reason we calculated $B_{Z=-\frac{1}{4}}^{Z=+\frac{1}{4}}$ and $B_{X=0}^{X=\frac{1}{2}}$ as ϱ (XYp) and ϱ (pYZ) indicating that the twelve oxygen atoms are situated in 8 (e) with approximately $x = 0.21$, $y = 0.08$, $z = -0.09$ and in 4 (d) with approximately $z = 0.03$. To include as many reflections as possible in the determinations of the antimony parameter we also calculated $B_{Z=0}^{Z=\frac{1}{2}}$. In space group No. 52, $Pncn$-D_{2h}^6 we have the following expressions for the bounded projections:

$$B_{Z=-\frac{1}{4}}^{Z=+\frac{1}{4}} = \frac{2}{A}\sum_{h=0}^{h+k=2n}\sum_{k=0}^{\infty} F(hk0)\cos 2\pi hX\cos 2\pi kY \pm \frac{2}{A}\sum_{h=0}^{\infty}\sum_{k=0}^{\infty}\sum_{l=1}^{l=2n+1,\ h+k=2n}\frac{4}{\pi l}F(hkl)\sin 2\pi hX\sin 2\pi kY$$

where the signs are — for $l = 4n + 1$ and + for $l = 4n + 3$.

$$B_{Z=0}^{Z=\frac{1}{2}} = \frac{2}{A}\sum_{h=0}^{h+k=2n}\sum_{k=0}^{\infty} F(hk0)\cos 2\pi hX\cos 2\pi kY - \frac{2}{A}\sum_{h=0}^{\infty}\sum_{k=0}^{\infty}\sum_{l=1}^{l=2n+1,\ h+k=2n+1}\frac{4}{\pi l}F(hkl)\cos 2\pi hX\sin 2\pi kY$$

$$B_{X=0}^{X=\frac{1}{2}} = \frac{2}{A}\left\{\sum_{k=0}^{k=2n,\ l=2n}\sum_{l=0}^{\infty} F(0kl)\cos 2\pi kY\cos 2\pi lZ - \sum_{k=0}^{k=2n+1,\ l=2n+1}\sum_{l=0}^{\infty} F(0kl)\sin 2\pi kY\sin 2\pi lZ\right\}$$

$$- \frac{2}{A}\sum_{h=1}^{h=2n+1,\ k=2n,\ l=2n}\sum_{k=0}^{\infty}\sum_{l=0}^{\infty}\frac{4}{\pi h}F(hkl)\cos 2\pi kY\sin 2\pi lZ - \frac{2}{A}\sum_{h=1}^{h=2n+1,\ k=2n+1,\ l=2n+1}\sum_{k=0}^{\infty}\sum_{l=0}^{\infty}\frac{4}{\pi h}F(hkl)\sin 2\pi kY\cos 2\pi lZ$$

For $B_{Z=-\frac{1}{4}}^{Z=+\frac{1}{4}}$ and $B_{Z=0}^{Z=\frac{1}{2}}$ only the Weissenberg photographs $hk0$ and $hk1$ were used and were correlated after correction for the Lorentz and polarization factors by the aid of the Weissenberg photographs $0kl$, $1kl$ and $2kl$. For $B_{X=0}^{X=\frac{1}{2}}$ the Weissenberg photographs $0kl$ and $1kl$ were used and similarly correlated



Fig. 3. The bounded projection $B^{z=+\frac{1}{2}}_{z=-\frac{1}{2}}$. *Contours at an interval of 50 arbitrary units. Negative values dotted. The final O positions are marked + and the Li positions +. The bounding planes pass through the centers of the Sb and Li atoms.*

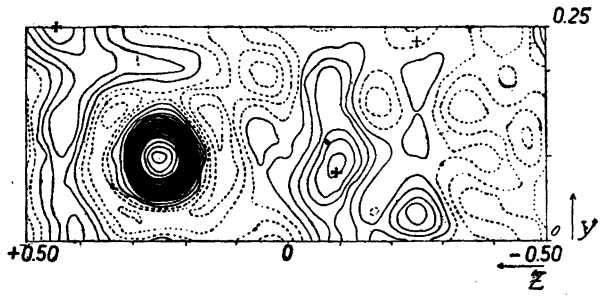

Fig. 4. The bounded projection $B^{x=\frac{1}{2}}_{x=0}$. *Contours at an interval of 50 arbitrary units. Negative values dotted. The final O positions are marked + and the Li positions +. The bounding planes pass through the centers of the Sb and Li atoms.*

by means of the Weissenberg photographs *hk*0, *hk*1 and *hk*2. For $B^{z=+\frac{1}{2}}_{z=-\frac{1}{2}}$ (Fig. 3) and $B^{x=\frac{1}{2}}_{x=0}$ (Fig. 4) only very few reflections were included from the higher zones and to these reflections only oxygen atoms contribute.

We arrived at the following set of parameters:

Sb in 4(*c*) with $y = 0.0971$ from $B^{z=\frac{1}{2}}_{z=0}$

O_1 in 4(*d*) with $z = 0.444$ from $B^{x=\frac{1}{2}}_{x=0}$

O_2 in 8(*e*) with $x = 0.209$, $y = 0.0728$ from $B^{z=+\frac{1}{2}}_{z=-\frac{1}{2}}$

$y = 0.0895$, $z = 0.907$ from $B^{x=\frac{1}{2}}_{x=0}$

mean value of $y = 0.081 \pm 0.009$

The two y-values for O_2 give an approximate estimation of the accuracy of the oxygen parameters, which is perhaps not larger than $\pm$ 0.02 and equivalent to about $\pm$ 0.15 Å.

These oxygen positions give a distorted octahedral coordination around antimony with Sb-O distances of about 2.0 Å, indicating that they seem to be quite probable.

THE LITHIUM POSITIONS

As the contribution from the lithium atoms to the $F(hkl)$ values is very low, we had to locate them from space considerations. The sum of the ionic radii are Li-O 2.10 kX (Goldschmidt) 2.00 kX (Pauling). The minimum distance Li-Sb was assumed to be the sum of the atomic radii, which are 3.08 kX when the coordination number is 8 (Goldschmidt). (Values of radii are taken from Internationale Tabellen [8].)

Spheres of appropriate radii, inside of which no lithium centres could occur, were drawn around the oxygen and antimony centres. Cuts were made parallel with the xy-plane for $0 \leq z \leq 0.5$. As the four lithium atoms ought to be situated in one of the fourfold positions, it was easily found that it must be 4(c) with approximately $y = 0.75$. In this position lithium is surrounded by six oxygen atoms 2 from O_1 and 4 from O_2 in a distorted octahedron. The more exact y-parameter was calculated assuming lithium equidistant from the four nearest O_2 atoms. This gave $y = 0.730$.

The atomic coordinates of $LiSbO_3$ are (the equivalent positions are given on p. 1023):

Sb in 4(c)	$y = 0.097_1$		
Li in 4(c)	$y = 0.73_0$		
O_1 in 4(d)	$z = 0.44_4$		
O_2 in 8(e)	$x = 0.20_9$	$y = 0.08_1$	$z = 0.90_7$

Table 2. Calculated and observed F-values of hk0 for a Weissenberg photograph of $LiSbO_3$. CuKa-radiation.

k	0k0		1k0		2k0		3k0		4k0		5k0		6k0	
	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o
0			0		104	111	0		185	187	0		143	133
1	0		134	87	0		104	105	0		159	186	0	
2	45	62	0		47	48	0		39	57	0		57	97
3	0		−35	28	0		−36	33	0		−35	30		
4	−105	125	0		−111	108	0		−101	114	0			
5	0		−162	154	0		−138	124	0		−173	152		
6	−174	168	0		−86	88	0		−150	176				
7	0		−66	68	0		−44	67						
8	17		0		19		0							
9	0		99	84	0		102	106						
10	143	117	0		150	117								

Table 3. Calculated and observed F-values of hk1 for a Weissenberg photograph of $LiSbO_3$. CuKa-radiation.

k	0k1		1k1		2k1		3k1		4k1		5k1		6k1	
	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o
0			0		0		0		0		0		0	
1			2		−63	48	−4		−62	56	6		−59	76
2	0		−98	103	−10		−108	154	12		−93	113	−12	
3	−82	107	−35	39	−121	103	25		−95	106	−12			
4	0		−66	63	−9		−77	77	12		−62	78		
5	−6	14	0		−12		−3		−10		5			
6	0		54	32	−1		53	28	1		55	39		
7	95	76	−3		97	81	4		98	103				
8	0		104	82	5		111	124						
9	64	54	21		87	100								
10	0		17	17										

Table 4. Calculated and observed F-values of 0kl for a Weissenberg photograph of $LiSbO_3$. CuKa-radiation.

k	0k0		0k1		0k2		0k3		0k4		0k5		0k6	
	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o
0			0		−71	95	0		77	92	0		−100	98
1	0		−41	62	0		45	54	0		−50	44	0	
2	26	35	0		−28	33	0		18	36	0		−29	28
3	0		−71	66	0		110	96	0		−74	69	0	
4	−59	70	0		64	55	0		−54	55	0		64	49
5	0		−3		0		6		0		−10			
6	−97	109	0		58	57	0		−62	53	0			
7	0		72	72	0		−73	60	0		79	70		
8	14		0		−14		0		23					
9	0		51	49	0		−79	71						
10	−79	69	0		−84	65								

Table 5. Calculated and observed F-values of 1kl for a Weissenberg photograph of $LiSbO_3$. CuKa-radiation.

k	1k0		1k1		1k2		1k3		1k4		1k5		1k6	
	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o
0					23	37	0		17	22	0		1	
1					−58	63	6		57	66	4		−61	58
2	0		−65	76	3		71	78	−1		−67	64	−6	
3	−18	10	−24	28	18	14	−1		−18	14	12		18	
4	0		−45	46	−1		50	43	2		−46	50	6	
5	−76	84	1		70	61	5		−70	66	5			
6	0		36	32	−17		−36	20	−14		36	32		
7	−34	43	−2		30	32	−4		−30	38				
8	0		70	76	−3		−73	59						
9	50	47	14		−50	40	1							

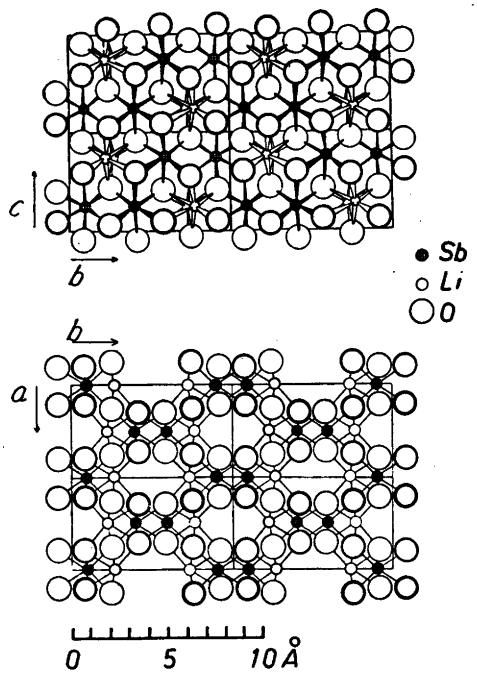

Fig. 5. Projections of the structure of $LiSbO_3$. In each projection four adjacent unit cells. The lines between the circles represent metal-oxygen contacts. The difference in thickness of the circles indicate the difference in height above the plane of projection.

with an accuracy of about ± 0.001 for Sb and ± 0.02 for O. As the Li-parameter is dependent of the O-parameters, its accuracy apparently is approximately the same as for O.

F-values were then calculated as

$$F_{calc} = \text{const} \left[A_{Sb} + \frac{f_O}{f_{Sb}} \sum_{1}^{2} A_{O_i} + \frac{f_{Li}}{f_{Sb}} A_{Li} \right]$$

where f_{Sb}, f_O and f_{Li} are the atomic scattering factors for Sb, O and Li with due consideration taken to their variation with Θ. The calculated and observed F-values are given in tables 2 (for $hk0$), 3 (for $hk1$), 4 (for $0kl$) and 5 (for $1kl$). The reliability factor $R = \frac{\sum \big| |F_o| - |F_c| \big|}{\sum |F_o|}$ varies for these zones between 0.13 and 0.19 when only the observed reflections have been included and between 0.15 and 0.28 when the absent reflections have been included assuming F_o zero.

DISCUSSION OF THE STRUCTURE

Projections of the structure of $LiSbO_3$ are given in Fig. 5, where four adjacent unit cells are given, so that the building principle of the structure will be more obvious. The interatomic distances are given in Table 6.

We can see that the oxygen atoms form a distorted hexagonal close packing, with antimony and lithium occupying some of the octahedral holes. The

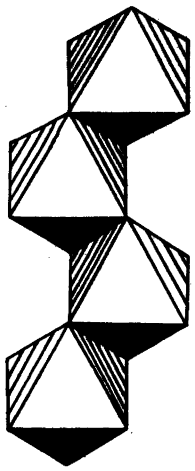

Fig. 6. A string of staggered octahedra with shared edges, representing the antimony oxygen configuration in $LiSbO_3$. The octahedra are idealized as regular polyhedra.

shortest O—O distance is 2.6_1 Å, which seems quite reasonable. The distances between antimony and the six nearest oxygen atoms vary between 2.0_0 and 2.0_5 Å, which is in agreement with distances reported from other antimonates. In this investigation, however, these distances are probably not accurate to more than ± 0.2—0.3 Å. Each oxygen octahedron around antimony shares two edges with two other antimony octahedra, thus forming a string of staggered octahedra (Fig.6) along the *c*-axis, which coincides with the needle axis of the crystal. This type of string is also found in $MnSb_2O_6$ (Brandt [9]), which is isomorphous with the mineral columbite (Strudivant [10]). The strings are coupled together in a way different from columbite by sharing corners. The

Table 6. Interatomic distances in $LiSbO_3$. Å units. The numerals 2 × and 4 × before a distance means that it occurs two or four times.

	Distorted Sb-O octahedron			
Sb-6O	2 × 2.0_0,	2 × 2.0_4,	2 × 2.0_5	
O—O edges	2.6_1,	2 × 2.6_4,	2 × 2.7_7,	2 × 2.8_3
	2 × 2.9_3,	2 × 3.1_3,	3.1_6	
O—O diagonals	2 × 4.0_3,	4.0_9		
	Distorted Li-O octahedron			
Li-6O	2 × 2.0_1,	4 × 2.0_7		
O—O edges	2.6_1,	2 × 2.7_7,	4 × 2.8_0,	2 × 2.8_9
	2 × 3.1_0,	3.2_8		
O—O diagonals	4.0_0,	4.1_0,	4.1_1	
O_1-2Sb (Sb-2O_1)	2 × 2.0_4			
O_1-2Li (Li-2O_1)	2 × 2.0_1			
O_3-2Sb (Sb-4O_3)	2.0_0,	2.0_5	(2 × 2.0_0,	2 × 2.0_5)
O_3-2Li (Li-4O_3)	2 × 2.0_7	(4 × 2.0_7)		

Sb-2Sb within a string of octahedra 3.06
Sb-2Sb (4Sb) between two strings 2 × 3.56 (2 × 4.89)
Li-2Li within a string of octahedra 2.4_7
Li-Li (4Li) between two strings 4.6_3 (2 × 5.1_3, 5.2_3)
Sb-3Li (9Li) [Li-3Sb(9Sb)] 2 × 2.9_7, 3.1_1 (2 × 3.6_9, 4 × 3.7_3)

oxygen O_2 forms the edges, which are shared within the string and the oxygen O_1 forms the corners, which are shared amongst the strings.

The distances between lithium and the six nearest oxygen atoms vary between 2.0_1 and 2.0_7 Å, which is in agreement with the sum of the ionic radii. One oxygen octahedron around lithium shares faces with two other lithium octahedra, thus forming a string along the *a*-axis. The Li—Li distance of 2.4_7 Å within the string seems very short compared with the atomic diameter of 3.04 Å, but is in good agreement with the Li—Li distance of 2.48 Å in $LiOH \cdot H_2O$ (Pepinsky [11]). The lithium octahedron also shares one edge with an adjacent antimony octahedron and two corners with other antimony octahedra. It therefore seems more appropriate to describe $LiSbO_3$ as a double lithium antimony(V) oxide than a lithium antimonate. The structure of $LiSbO_3$ seems to be different form other known double oxides of the type M^ISbO_3.

SUMMARY

$LiSbO_3$ has been investigated by X-ray crystallographic methods. It is orthorhombic, space group No. 52, $Pncn$-D_{2h}^6 with 4 formula units in the unit cell. The cell dimensions are given on p. 1022, the atomic coordinates on p. 1027, and the interatomic distances in Table 6. Antimony and oxygen positions have been determined from bounded projections of the electron density. Lithium positions have been determined from space considerations.

Projections of the structure are given in Fig. 5. The oxygen atoms form a distorted hexagonal close packing with antimony and lithium in some of the octahedral holes. Antimony and oxygen from a staggered chain of octahedra (Fig.6) along the *c*-axis, which coincides with the needle axis of the crystal. The structure of $LiSbO_3$ seems to be different from other known double oxides of the type M^ISbO_3.

It seems more appropriate to describe $LiSbO_3$ as a double lithium antimony (V)oxide than a lithium antimonate.

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The Crystal Structure of Tellurium Dimethanethiosulphonate

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The methanetiosulphonates, $S(S_2O_2CH_3)_2$, $Se(S_2O_2CH_3)_2$ and $Te(S_2O_2CH_3)_2$, were first prepared in 1950 [1]. They are derived from pentathionic, selenopentathionic and telluropentathionic acid, respectively, by substitution of methyl for the hydroxyl groups of the acids. The crystals of the methanethiosulphonates are isomorphous [1,2].

In the present article, the structure of tellurium dimethanethiosulphonate is described. A following article [3] reports the crystal structure of the ammonium salt of telluropentathionic acid. It will be shown that the S—S—Te—S—S chains in the two structures are closely analogous.

The structure of tellurium dimethanethiosulphonate, and subsequently of the isomorphous sulphur and selenium analogues, was solved by use of the heavy atom technique. The tellurium atom, in presence of four sulphur atoms, four oxygen atoms and two methyl groups, was found to dominate the signs of the reflections to a sufficient degree.

The structure is the first to be reported for a compound containing divalent tellurium bonded to sulphur.

X-RAY DATA

The crystals of tellurium dimethanethiosulphonate, $Te(S_2O_2CH_3)_2$, are monoclinic prismatic. They appear as needles or prisms, elongated along the b axis and in most cases flattened along the a axis.

The dimensions of the unit cell [1] are: $a = 11.43$ Å, $b = 5.29$ Å, $c = 16.32$ Å, $\beta = 91°$. There are four molecules per unit cell; density, calc. 2.36, found 2.35 g/cm^3. Absent reflections, $h0l$ when $h + l$ is odd, $0k0$ when k is odd. The space group is thus $C_{2h}{}^5$—$P2_1/n$.

For intensity measurements, zero layer Weissenberg photographs were taken about the b and a axes, using Cu$K\alpha$ radiation. The crystals employed were small, having cross-sections of about 0.05 × 0.05 mm and 0.1 × 0.1 mm, respectively, in the case of the b and a axis photographs. 145 of the 246 $h0l$

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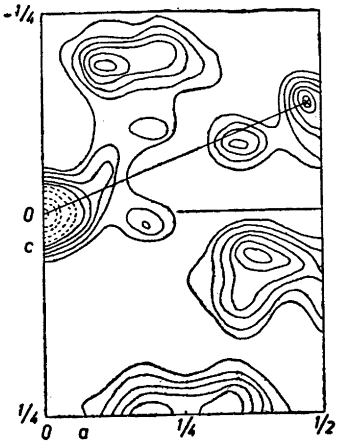

Fig. 1. Patterson projection along the b *axis. Contours at arbitrary but equal intervals, except at the origin peak, where the dotted contours represent intervals four times as large. The straight line is the Te-Te vector.*

reflections and 97 of the 114 0*kl* reflections theoretically observable with Cu$K\alpha$ radiation were recorded on the films with measurable intensities.

The intensities were estimated visually, and corrected for Lorentz and polarization factors. No correction for absorption was applied. The structure factors were eventually placed on an approximately absolute scale by comparison with the calculated values.

The calculated structure factors were based on the Hartree atomic scattering curves for sulphur and oxygen, and the Thomas-Fermi curve for tellurium. The same curve was used for all sulphur atoms, and the oxygen curve was used for the carbon atoms of the methyl groups, the hydrogen atoms otherwise being ignored. A temperature factor exp $(-B \sin^2\Theta/\lambda^2)$ with $B = 5.1$ Å^2 for the *h*0*l* reflections and $B = 3.1$ Å^2 for the 0*kl* reflections was applied to the calculated structure factors. These values of B were found to give the best agreement with the observed structure factors.

The Patterson and Fourier summations were made at 6° intervals along all three axes, using Beevers-Lipson strips. Peaks were located by Booth's method [4].

DETERMINATION OF THE STRUCTURE

The Patterson projection along the *b* axis is shown in Fig. 1. In the asymmetric unit, comprising a quarter of the unit cell, one Te—Te vector should occur. There are, however, four high peaks in the map, of approximately the same height. The Te—Te vector was identified by making a Fourier synthesis for each of the possible positions, using signs corresponding to the tellurium contributions alone. One of the Fourier maps, based on the tellurium coordinates, $x = 0.234$ and $z = -0.066$, showed four well resolved sulphur atoms. The three other high peaks in the Patterson map were subsequently found to be due to overlapping Te—S vectors.

The *z* coordinates were later confirmed through Patterson and Fourier projections along the *a* axis. Also, the 0*kl* data showed that the origin first

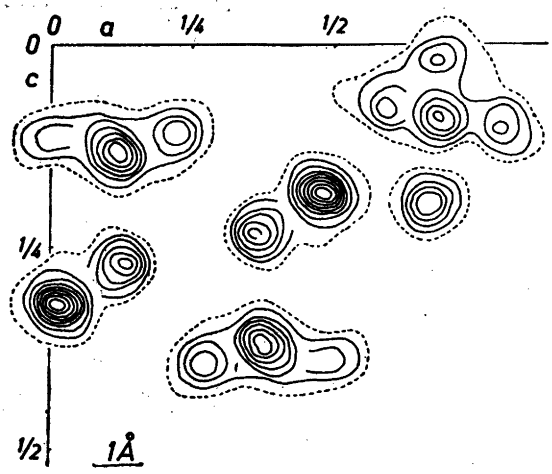

Fig. 2. Projection of Te $(S_2O_2CH_2)_2$ along the b *axis,* ϱ (xz). *The 2-electron line is dotted, and the first fully drawn line represents 6 e.Å^{-2} for tellurium and 4 e.Å^{-2} for the other atoms. Contour intervals thereafter: 8 e.Å^{-2} for tellurium, 4 e.Å^{-2} for sulphur, and 2 e.Å^{-2} for oxygen and carbon atoms.*

chosen in the case of the *b* axis projection, as reported in the preliminary note [2], corresponded to a twofold screw axis, located at $x = \frac{1}{4}$, $z = \frac{1}{4}$ from a centre of symmetry.

Refinement of the *h0l* projection was made by double Fourier series methods in the usual way. Peaks due to oxygen and carbon atoms appeared in the second Fourier map, after inclusion of the sulphur contributions in the calculated structure factors. The final electron density map, $\varrho(xz)$, and an explanatory diagram, are reproduced in Figs. 2 and 3.

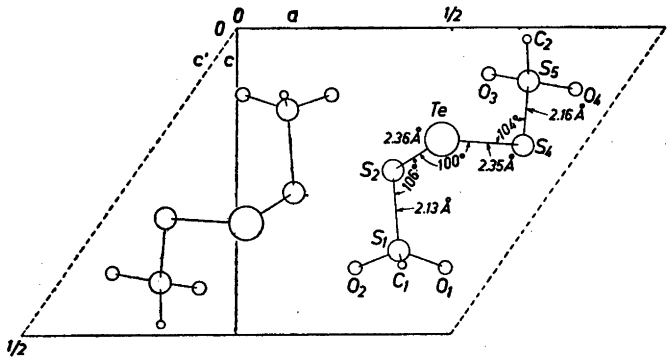

Fig. 3. Arrangement of the molecules as viewed along the b *axis. The dotted line,* c', *shows the direction of the* c *axis in the setting* $P2_1/c$. *The molecules are seen to be extended along the direction of translation of the glide plane.*

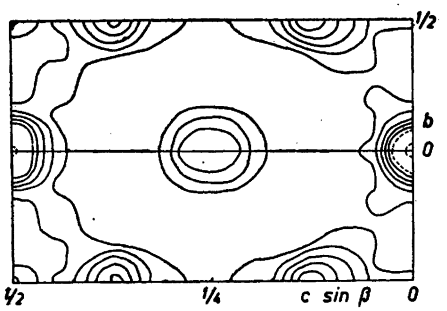

Fig. 4. Patterson projection along the a *axis. Contours at arbitrary but equal intervals, except for the dotted contours which represent intervals four times as large.*

The 0*kl* data were used to evaluate the y coordinates. Fig. 4 shows the Patterson projection along the a axis. The Fourier and Patterson symmetries being *pgg* and *pmm*, respectively, equivalent vectors, of double weight, occur in the Patterson map at $v = \frac{1}{2} + 2y$, $w = \frac{1}{2}$ and $v = \frac{1}{2}$, $w = \frac{1}{2} + 2z$, and equivalent vectors, of single weight, at $v = 2y$, $w = 2z$ and $v = -2y$, $w = 2z$. The high peak at $v = 0$, $w = \frac{1}{2}$ in Fig. 4 shows that the tellurium atom lies near the glide plane at $y = \frac{1}{4}$. Correspondingly, the vector at $v = 2y$, $w = 2z$, of single weight, occurs at $v = \frac{1}{2}$ and coincides with the vector at $v = -2y$, $w = 2z$, thus acquiring double weight. This peak, at $w = 0.372$, and that at $v = \frac{1}{2}$, $w = \frac{1}{2} + 2z$ both give the z coordinate of tellurium as 0.186, whereas the value derived from the b axis Patterson map, referred to a centre of symmetry as origin, is 0.184, and the final value, from both projections, 0.182.

In the plane group, *pgg*, atoms at $y = \frac{1}{4}$ do not contribute to 0*kl* reflections when l is odd, whereas for reflections with l even the contribution is large. Signs were therefore calculated for the 0*kl* reflections with l even on the basis of $y = \frac{1}{4}$ for the tellurium atom, and a Fourier synthesis was made using these even l terms only. Such a projection contains peaks corresponding to the atomic positions, and images of these atoms produced by the operation of a spurious centre of symmetry. The true peaks were identified partly from considerations of possible tellurium-sulphur and sulphur-sulphur bond lengths and angles, and ultimately by the use of Bragg-Lipson charts for some reflections the signs of which were difficult to determine. The last electron density map, $\varrho(yz)$, and a line diagram, are shown in Figs. 5 and 6.

The atomic coordinates are listed in Table 1. The z coordinates of the oxygen and carbon atoms are those determined from $\varrho(xz)$, since in $\varrho(yz)$ overlapping occurs for these atoms. Their y coordinates, except for C_2, were arrived at through trial and error calculations. For other atoms, the following z values, from $\varrho(xz)$ and $\varrho(yz)$, respectively, differed in the two projections: S_1, 0.363 and 0.361; S_2, 0.233 and 0.231; S_5, 0.086 and 0.088. These figures were used for the calculation of structure factors for the respective zones, whereas bond lengths and angles are based on the average z values.

In Table 4, observed and calculated values of structure factors are compared for all *h*0*l* and 0*kl* reflections within the range of Cu$K\alpha$ radiation. The reliability factor, $R = \Sigma \, || F_{obs} | - | F_{calc} || / \Sigma \, | F_{obs} |$, is 0.15 for the *h*0*l* reflections and 0.18 for the 0*kl* reflections, with an overall value of 0.16 for the two zones.

Table 1. Atomic coordinates, in fractions of cell edges. Origin at centre of symmetry.

	x	y	z		x	y	z
S_1	0.383	−0.031	0.362	O_1	0.487	−0.140	0.393
S_2	0.371	−0.058	0.232	O_2	0.279	−0.142	0.393
Te	0.484	0.279	0.182	C_1	0.384	0.301	0.393
S_4	0.672	0.101	0.195	O_3	0.592	−0.317	0.078
S_5	0.686	−0.134	0.087	O_4	0.792	−0.271	0.099
				C_2	0.680	0.119	0.015

The unobserved reflections are included in R, as the difference between $|F_{calc}|$ and the lowest observable value of $|F_{obs}|$, only when $|F_{calc}|$ is greater than the smallest value of $|F_{obs}|$. They are not included in $\Sigma|F_{obs}|$.

DESCRIPTION OF THE MOLECULE

The S—S—Te—S—S chain of tellurium dimethanethiosulphonate is unbranched and non-planar. The terminal sulphur atoms are rotated out of the plane of the middle atoms of the chain, to opposite sides of the plane. This is the *trans* form of a pentathionic compound [5].

Bond lengths and angles, calculated on the basis of the coordinates of Table 1, are given below. The numbering of atoms is shown in Figs. 3 and 6. The data are estimated as reliable to within ± 0.03 Å for the average Te—S and S—S bond lengths, and ± 0.06 Å for S—O and S—C bond lengths. Angles involving tellurium and sulphur atoms only are probably correct to within ± 3°.

Table 2. Sulphur-sulphur and tellurium-sulphur bond lengths and bond angles.

$S_1-S_2 = 2.13$ Å	$\angle S_1S_2Te = 106°$	$S_4-S_5 = 2.16$ Å
$S_2-Te = 2.36$	$\angle TeS_4S_5 = 104°$	$Te-S_4 = 2.35$
	$\angle S_2TeS_4 = 100°$	

Non-bonded distances

$S_1-Te = 3.57$ Å	$S_2-S_4 = 3.60$ Å	$Te-S_5 = 3.55$ Å
$S_1-S_4 = 4.38$	$S_1-S_5 = 5.74$	$S_2-S_5 = 4.36$

The dihedral angles, S_1S_2Te/S_2TeS_4 and S_2TeS_4/TeS_4S_5, are both 81°. These are the angles between not adjacent S—S and Te—S bonds, as seen projected on a plane normal to the intermediate S—Te bond.

The average S—SO_2CH_3 bond length, 2.15 Å, compares with corresponding bond lengths in analogous compounds of known structure, as follows: S—SO_2CH_3 = 2.10 Å in dimethanesulphonyl disulphide [6], S—SO_3 = 2.14 Å in barium pentathionate dihydrate [7], 2.12 Å in barium tetrathionate dihydrate [8], and 2.15 Å in potassium trithionate [9].

No Te—S bond lengths, directly measured, have hitherto been reported in literature. The average length, 2.36 Å, found in the present structure, is a little shorter than the Te—S single bond length, 2.41 Å, calculated on the basis of Pauling's covalent radii [10]. The difference, if real, is in accordance with the

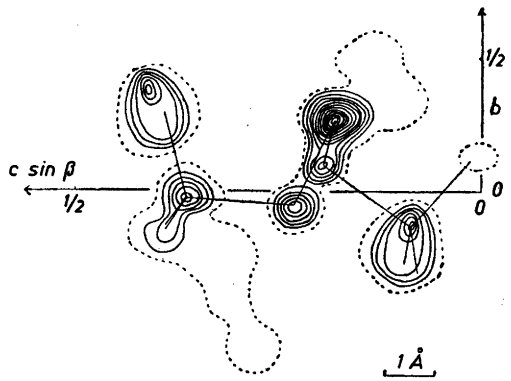

Fig. 5. Projection of Te $(S_2O_2CH_3)_2$ along the a axis, ϱ (yz). The 6-electron line is dotted. Contour intervals: Three times 4 e.Å^{-2} and thereafter 8 e.Å^{-2} for the tellurium atom, and 4 e.Å^{-2} for the other atoms.

shortening of the middle S—S bonds in barium pentathionate dihydrate [7], as compared with the external bonds.

The S—Te—S angle, 100°, is in fair agreement with the values, 102° and 98°, respectively, found in hexagonal tellurium [11] and tellurium dibromide [12].

Two oxygen atoms and a methyl group surround each of the terminal sulphur atoms, in approximately tetrahedral arrangement. The pertinent data are listed in Table 3. The average sulphur-oxygen bond length, 1.43 Å, lies in the range which is usual for such bonds [13,14], and the sulphur-carbon bond lengths, 1.83 Å and 1.78 Å, are close to the sum of the single radii [10], 1.81 Å.

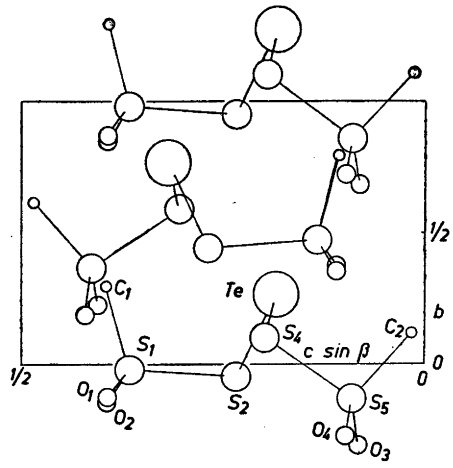

Fig. 6. Arrangement of the molecules as seen along the a axis.

Table 3. Dimensions of the sulphonyl groups. The right column represents non-bonded distances.

S_1-O_1	= 1.41 Å	$\angle S_2S_1O_1$	= 112°	O_1-O_2	= 2.33 Å
S_1-O_2	= 1.43	$\angle S_2S_1O_2$	= 107°	O_1-C_1	= 2.61
S_1-C_1	= 1.83	$\angle S_2S_1C_1$	= 110°	O_2-C_1	= 2.63
S_5-O_3	= 1.45	$\angle S_4S_5O_3$	= 114°	O_3-O_4	= 2.32
S_5-O_4	= 1.43	$\angle S_4S_5O_4$	= 105°	O_3-C_2	= 2.73
S_5-C_2	= 1.78	$\angle S_4S_5C_2$	= 96°	O_4-C_2	= 2.78

It was pointed out earlier [5] that a *trans* form of a pentathionic compound possesses a twofold axis of symmetry, provided that bond lengths and angles are the same in both halves of the molecule. In tellurium dimethanethiosulphonate, no molecular symmetry is crystallographically required. As appears from Table 2, however, the S—S—Te—S—S chain possesses, within the probable errors, a twofold axis of symmetry. The relative positions of the oxygen atoms and the methyl groups do not quite conform to the requirements of a twofold axis.

There appears to be no particularly short approaches between atoms belonging to different molecules. The shortest intermolecular S—S distance, 3.65 Å, is between S_4 and its equivalent along a screw axis.

SUMMARY

The crystal structure of tellurium dimethanethiosulphonate has been determined from X-ray data, by two-dimensional Patterson and Fourier methods.

The molecule consists of an unbranched and non-planar S—S—Te—S—S chain, with two oxygen atoms and a methyl group attached to each of the terminal sulphur atoms. The chain occurs in a *trans* form, the terminal sulphur atoms being rotated an angle of 81° out of the plane of the S—Te—S atoms, to opposite sides of the plane.

The average Te—S and S—S bond lengths are 2.36 Å and 2.15 Å, respectively. The S—Te—S bond angle is 100°, and an average value of 105° was found for the Te—S—S bond angles.

The crystal structures of sulphur and selenium dimethanethiosulphonate, which are isomorphous with tellurium dimethanethiosulphonate, will be published later.

The work has been aided by grants from *Norges Almenvitenskapelige Forskningsråd.* The support is gratefully acknowledged. One of us (O. F.) also wishes to express his thanks to *Nansenfondet og de dermed forbundne fond* for a grant.

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Table 4. Observed and calculated values of structure factors for tellurium dimethanethiosulphonate.

l	F_{obs}	F_{calc}	l	F_{obs}	F_{calc}	l	F_{obs}	F_{calc}
	*h*0*l* zone			40*l*			80*l*	
	00*l*		0	40	+ 50	0	50	+ 54
			2	53	+ 27	2	< 15	− 12
2	117	− 135	4	112	− 94	4	23	− 48
4	118	− 118	6	137	+ 140	6	29	+ 37
6	34	+ 30	8	113	− 93	8	< 16	+ 10
8	69	− 73	10	16	+ 12	10	31	− 30
10	93	+ 89	12	55	+ 58	12	< 16	+ 8
12	31	+ 35	14	44	− 48	14	< 14	− 16
14	37	− 43	16	19	+ 20	16	< 10	+ 3
16	29	+ 24	18	< 13	+ 10			
18	16	− 14	20	< 7	− 10		90*l*	
20	< 10	− 7						
						1	55	− 55
	10*l*			50*l*		3	< 16	+ 4
						5	19	− 30
1	82	− 89	1	122	− 188	7	< 16	− 6
3	166	+ 184	3	102	+ 111	9	31	+ 50
5	146	− 156	5	50	− 64	11	< 15	− 9
7	147	+ 116	7	64	− 74	13	< 14	− 17
9	37	+ 8	9	104	+ 107	15	< 10	+ 12
11	79	− 79	11	69	− 68			
13	37	+ 29	13	< 16	+ 5		10,0,*l*	
15	29	+ 22	15	< 16	+ 2			
17	19	− 14	17	< 13	− 12	0	37	+ 46
19	16	+ 15	19	< 9	+ 16	2	25	+ 47
21	< 6	− 4				4	40	− 58
						6	31	+ 50
	20*l*			60*l*		8	19	− 19
						10	< 15	− 13
0	80	+ 80				12	16	+ 23
2	257	− 247	0	128	+ 131	14	< 9	− 4
4	57	− 81	2	< 11	− 13			
6	166	+ 167	4	47	− 45			
8	91	− 81	6	31	+ 48		11,0,*l*	
10	< 14	+ 5	8	16	− 30			
12	< 15	− 2	10	23	+ 15	1	55	− 68
14	< 16	− 26	12	23	+ 35	3	< 13	− 10
16	< 16	0	14	31	− 43	5	< 16	+ 16
18	< 14	+ 9	16	< 14	+ 15	7	< 15	− 14
20	< 9	− 10	18	< 10	− 1	9	< 14	+ 16
						11	11	− 21
	30*l*			70*l*		13	< 8	0
1	135	− 133					12,0,*l*	
3	152	+ 150	1	19	− 10			
5	19	− 20	3	91	+ 91			
7	59	− 46	5	44	− 44	0	< 15	+ 3
9	67	+ 66	7	25	− 28	2	< 15	+ 1
11	29	− 45	9	< 16	+ 35	4	< 15	− 22
13	29	+ 29	11	< 16	− 16	6	< 14	+ 22
15	< 16	+ 8	13	< 16	+ 2	8	< 13	− 7
17	23	− 24	15	16	+ 27	10	< 10	− 4
19	< 11	+ 10	17	< 10	− 13	12	< 6	+ 11

l	F_{obs}	F_{calc}
	13,0,l	
1	< 14	− 8
3	< 13	+ 10
5	< 11	+ 10
7	11	− 18
9	< 8	+ 10
	14,0,l	
0	< 10	− 4
2	< 10	+ 9
4	< 2	− 9
6	< 7	+ 1
	$\bar{1}0l$	
1	48	− 60
3	76	+ 84
5	266	− 244
7	80	+ 49
9	170	+ 137
11	108	− 92
13	< 16	+ 3
15	< 16	− 8
17	25	− 30
19	25	+ 28
21	< 6	− 7
	$\bar{2}0l$	
2	19	− 3
4	40	+ 40
6	61	+ 72
8	91	− 96
10	< 14	+ 2
12	29	+ 28
14	59	− 49
16	58	+ 56
18	< 14	− 5
20	< 10	− 11
	$\bar{3}0l$	
1	112	+ 100
3	117	+ 132
5	81	− 86
7	57	− 39
9	76	+ 72
11	34	− 34
13	47	+ 49
15	< 16	− 6
17	< 15	− 15
19	< 11	+ 7

l	F_{obs}	F_{calc}
	$\bar{4}0l$	
2	98	− 106
4	50	+ 30
6	40	+ 29
8	97	− 96
10	96	+ 84
12	< 16	− 16
14	44	− 42
16	< 15	+ 12
18	< 13	− 3
20	< 8	+ 1
	$\bar{5}0l$	
1	23	− 21
3	128	+ 116
5	123	− 128
7	50	+ 56
9	25	− 9
11	31	− 36
13	55	+ 48
15	31	− 28
17	14	+ 4
19	11	+ 14
	$\bar{6}0l$	
2	127	− 117
4	59	+ 59
6	68	+ 72
8	113	− 103
10	65	+ 69
12	< 16	− 3
14	< 12	− 8
16	< 14	+ 16
18	11	− 11
	$\bar{7}0l$	
1	41	− 28
3	25	+ 38
5	81	− 88
7	87	+ 69
9	< 16	0
11	37	− 36
13	31	+ 28
15	< 14	− 10
17	16	− 15
	$\bar{8}0l$	
2	101	− 96
4	25	+ 22
6	31	+ 18
8	< 16	− 10
10	< 16	+ 10
12	19	− 10

l	F_{obs}	F_{calc}
14	< 14	− 13
16	19	+ 18
18	< 5	− 6
	$\bar{9}0l$	
1	88	+ 98
3	< 12	+ 10
5	43	− 45
7	29	+ 26
9	< 16	− 3
11	< 16	− 9
13	16	+ 18
15	< 11	− 2
17	< 6	− 3
	$\bar{10}$,0,l	
2	29	− 33
4	< 16	+ 16
6	31	− 38
8	< 16	− 7
10	31	+ 37
12	< 14	− 10
14	< 10	− 8
16	< 2	+ 6
	$\bar{11}$,0,l	
1	< 16	+ 20
3	< 12	+ 8
5	19	− 27
7	25	+ 26
9	< 14	− 2
11	16	− 15
13	< 9	+ 12
	$\bar{12}$,0,l	
2	< 15	− 6
4	25	+ 27
6	< 14	+ 1
8	23	− 28
10	19	+ 21
12	< 8	− 8
	$\bar{13}$,0,l	
1	< 14	+ 5
3	< 13	+ 7
5	< 13	− 16
7	< 11	+ 8
9	< 9	− 4
	$\bar{14}$,0,l	
2	< 10	− 14
4	< 9	+ 13
6	< 8	− 3

l	F_{obs}	F_{calc}
	0*kl* zone	
	00*l*	
2	143	− 139
4	122	− 123
6	48	+ 40
8	79	− 86
10	94	+ 110
12	41	+ 44
14	46	− 61
16	37	+ 34
18	21	− 24
20	8	− 13
	01*l*	
1	8	− 10
2	98	− 98
3	49	+ 47
4	233	+ 230
5	57	+ 60
6	101	− 90
7	84	− 71
8	80	− 83
9	41	+ 36
10	75	+ 67
11	20	+ 8
12	86	− 86
13	21	+ 16
14	44	+ 50
15	16	− 14
16	17	+ 21
17	< 7	− 2
18	26	− 40
19	< 6	− 1
20	8	+ 15
21	< 3	+ 2
	02*l*	
0	134	− 122
1	86	+ 91
2	28	+ 29
3	42	+ 42
4	60	+ 50
5	< 6	− 24
6	69	− 58
7	38	− 48
8	109	+ 111
9	11	− 13
10	56	− 71
11	18	− 10
12	30	− 42
13	29	+ 30
14	59	+ 69
15	< 8	− 10
16	22	− 29
17	< 7	− 12
18	< 6	− 3
19	< 5	+ 8
20	< 3	+ 12
	03*l*	
1	8	+ 4
2	43	+ 26
3	35	− 41
4	80	− 77
5	49	+ 41
6	56	+ 58
7	32	− 28
8	< 7	+ 6
9	14	− 18
10	43	− 52
11	25	+ 29
12	20	+ 16
13	21	− 19
14	20	+ 13
15	15	− 17
16	26	− 30
17	20	+ 30
18	14	+ 29
19	< 2	− 8
	04*l*	
0	58	+ 26
1	35	− 34
2	50	− 48
3	11	+ 3
4	32	− 12
5	39	+ 36
6	51	+ 55
7	59	− 56
8	20	− 25
9	36	+ 53
10	11	− 16
11	12	− 19
12	< 7	+ 5
13	16	− 29
14	14	− 18
15	18	+ 30
16	12	+ 20
17	< 3	− 8
	05*l*	
1	56	− 38
2	42	− 42
3	65	+ 54
4	36	+ 24
5	35	− 44
6	10	− 4
7	40	+ 14
8	16	− 18
9	12	+ 20
10	32	+ 35
11	28	− 33
12	20	− 15
13	8	+ 12
14	< 3	+ 9
	06*l*	
0	30	− 19
1	49	+ 42
2	17	+ 24
3	26	− 28
4	< 6	− 12
5	20	− 21
6	< 6	− 16
7	26	+ 36
8	14	+ 17
9	6	− 4
10	< 3	− 8

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The Crystal Structure of Ammonium Telluropentathionate

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In a preceding article [1], the crystal structure of tellurium dimethanethiosulphonate was described. The present work is concerned with the crystal structure of another thio derivative of divalent tellurium, *viz.*, ammonium telluropentathionate. The compounds, $Te(S_2O_2CH_3)_2$ and $(NH_4)_2Te(S_2O_3)_2$, both contain an unbranched and non-planar S—S—Te—S—S chain, the dimensions and configuration of which have been found to be almost exactly the same in the two crystals.

Telluropentathionic acid, the ammonium salt of which was first prepared in 1950 [2], is derived from pentathionic acid by substitution of tellurium for the middle sulphur atom of the latter acid. The crystal structure of barium pentathionate dihydrate was published recently [3]. The telluropentathionate and pentathionate anions have analogous structures, except that they in these salts occur as rotational isomers of each other.

X-RAY DATA

The crystals of ammonium telluropentathionate, $(NH_4)_2Te(S_2O_3)_2$, are monoclinic prismatic, and occur as yellow prisms, elongated along the a axis and bounded by $\{011\}$.

From oscillation and Weissenberg photographs, taken with Cu$K\alpha$ radiation, $\lambda = 1.542$ Å, the following unit cell dimensions were derived: $a = 5.03$ Å, $b = 18.90$ Å, $c = 11.61$ Å, $\beta = 100°$. The axial lengths are believed to be accurate to $\pm$ 0.5 %. The values reported earlier [2] for the β angle and the c axis are in error.

There are four molecules per unit cell; density, calc. 2.35, found 2.34 g/cm^3. The systematic absences, $h0l$ when l is odd and $0k0$ when k is odd, determine the space group as C_{2h}^5—$P2_1/c$.

For intensity measurements, zero layer Weissenberg photographs were taken about the a and c axes, using a double film technique. The crystals employed had cross-sections of about 0.05 × 0.05 mm. 199 of the 282 $0kl$

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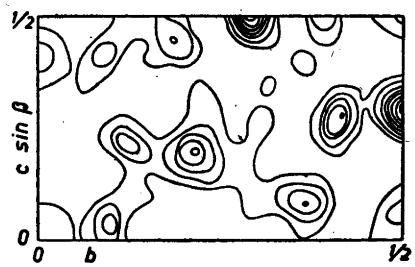

Fig. 1. Patterson projection along the a *axis. Contours at arbitrary but equal interval. The origin peak is not drawn.*



Fig. 2. Patterson projection along the c *axis. Contours as in Fig. 1.*

reflections and 103 of the 128 *hk*0 reflections attainable with Cu$K\alpha$ radiation were recorded on the films with measurable intensities. The intensities were estimated visually, and corrected for the Lorentz and polarization factors as described by Goldschmidt and Pitt [4]. No correction for absorption was applied. The structure factors were later placed on an approximately absolute scale by comparison with the calculated values.

The calculated structure factors were based on the Hartree atomic scattering curves for sulphur and oxygen, and the Thomas-Fermi curve for tellurium. The same curve was used for all sulphur atoms, and the oxygen curve was used for the nitrogen atom of the ammonium ions. A temperature factor exp ($-B \sin^2\Theta/\lambda^2$) with $B = 4.0$ Å^2 for the 0*kl* reflections and $B = 3.8$ Å^2 for the *hk*0 reflections was applied to the calculated structure factors. The values of B, and the scale factors, were determined from plots of log $|F_{obs}|/|F_{calc}|$ against $\sin^2\Theta$.

The Patterson and Fourier summations were made at 6° intervals along all three axes, using Beevers-Lipson strips. Peaks were located by Booth's method [5].

DETERMINATION OF THE STRUCTURE

As in the case of tellurium dimethanethiosulphonate [1], the structure was solved by means of the heavy atom technique. The tellurium atom, accounting for 28 % of the electrons in the asymmetric unit, was found to dominate the signs of the reflections to a sufficient degree.

The position of the tellurium atom was located from Patterson projections along the *a* and *c* axes. The asymmetric parts of these projections are reproduced in Figs. 1 and 2. The Fourier symmetries of the plane groups being *pgg* and *pgm*, respectively, equivalent vectors, of double weight, occur at $v = \frac{1}{2}$, $w = \frac{1}{2} + 2z$ and $v = \frac{1}{2} + 2y$, $w = \frac{1}{2}$ in Fig. 1 and at $u = 0$, $v = \frac{1}{2} + 2y$ and $u = 2x$, $v = \frac{1}{2}$ in Fig. 2. The Te—Te vectors are readily recognized as the highest peaks along the appropriate lines in Figs. 1 and 2, and gave the follow-

ing tellurium coordinates: $x = 0.132$, $y = 0.105$, $z = 0.102$. Only the x coordinate differs, by —0.002, from the final value.

Fourier projections along the a axis were subsequently carried out, starting with signs calculated from the tellurium contributions alone. The positions of the sulphur atoms were recognized in the first map, and peaks due to oxygen atoms and ammonium ions appeared during the refinement process. Essentially the same procedure was followed with regard to the projection along the c axis. Here, however, overlapping occurs to a large extent, and some of the atoms had to be placed by trial and error methods.

The positive direction of the a axis relative to that of the c axis was established by comparison of some observed and calculated $h0l$ and $\bar{h}0l$ structure factors.

The final electron density maps, $\varrho(yz)$ and $\varrho(xy)$, are reproduced in Figs. 3 and 5, and a diagram showing the outline of the ions as seen along the a axis is shown in Fig. 4.

The atomic coordinates are listed in Table 1. The y coordinates are those determined from $\varrho(yz)$, since the resolution is much better in this projection. For the same reasons, the x coordinates, particularly of S_2, O_3 and N_1, are probably less accurate than the y and z coordinates.

Errors in the y and z coordinates due to finite termination of Fourier series were estimated from a Fourier synthesis based on the calculated $0kl$ structure factors [6]. The tellurium coordinates and the y coordinates of the sulphur atoms remained unchanged, whereas average shifts of 0.025 Å occurred in the sulphur z coordinates, with a maximum shift of 0.04 Å in zS_1. The S_1 peak is subject to overlapping by O_2, and the significance of the shift may be difficult to judge. For the oxygen atoms and the ammonium ions, the average shifts in the y and z coordinates were 0.04 Å and 0.03 Å, respectively. The presence of the heavy tellurium atom introduces a further uncertainty in the coordinates of these lighter atoms.

Table 1. Atomic coordinates, in fractions of cell edges. Origin at centre of symmetry.

	x	y	z		x	y	z
S_1	0.434	−0.022	0.309	O_1	0.622	−0.077	0.320
S_2	0.419	0.005	0.132	O_2	0.151	−0.030	0.330
Te	0.134	0.105	0.102	O_3	0.565	0.034	0.375
S_4	0.432	0.199	0.161	O_4	0.746	0.288	0.052
S_5	0.539	0.236	0.003	O_5	0.317	0.270	−0.063
N_1	0.067	0.097	0.455	O_6	0.646	0.182	−0.063
N_2	0.033	0.334	0.270				

In Table 5, observed and calculated values of structure factors are compared for all $0kl$ and $hk0$ reflections within the range of Cu$K\alpha$ radiation. The reliability factor, $R = \Sigma||F_{obs}|-|F_{calc}||/\Sigma|F_{obs}|$, with unobserved reflections included when $|F_{calc}|$ is greater than the smallest observable value of $|F_{obs}|$, is 0.18 for the $0kl$ data and 0.15 for the $hk0$ data, with an overall value of 0.17 for the two zones.

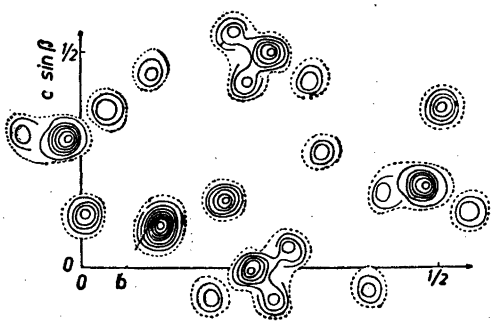

Fig. 3. Electron density projection of $(NH_4)_2Te(S_2O_3)_2$ along the a axis, ϱ(yz). The 4-electron line is dotted. Contour intervals: 8 e.Å^{-2} for the tellurium atom, 4 e.Å^{-2} for the sulphur atoms, and 2 e.Å^{-2} for the oxygen atoms and ammonium ions.

THE TELLUROPENTATHIONATE ION

Distances and angles, calculated on the basis of the coordinates of Table 1, are given below. The numbering of atoms is shown in Fig. 4. The average Te—S, S—S and S—O bond lengths are estimated as reliable to within ± 0.03 Å, ± 0.03 Å and ± 0.04 Å, respectively, whereas N—O distances may be in error by as much as ± 0.1 Å. Angles involving the heavier atoms, tellurium and sulphur, are probably correct to within ± 3°.

The dihedral angles, S_1S_2Te/S_2TeS_4 and S_2TeS_4/TeS_4S_5, are 86° and 95°, respectively.

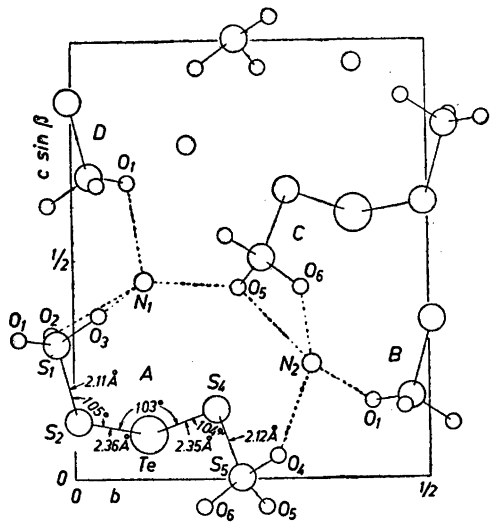

Fig. 4. Arrangement of the ions as seen along the a axis. Half a unit cell (two asymmetric units) is shown.

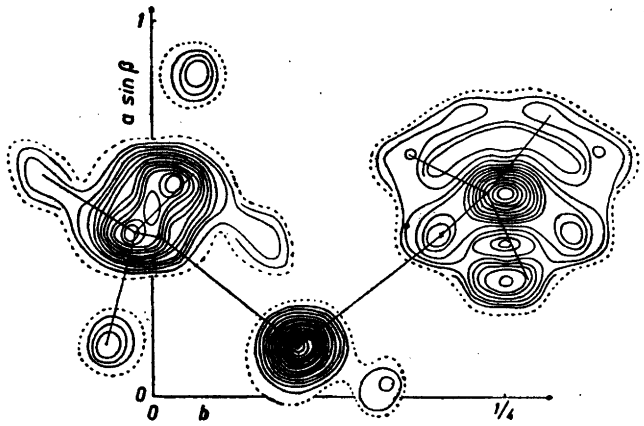

Fig. 5. Electron density projection of $(NH_4)_2Te(S_2O_3)_2$ along the c axis, $\varrho(xy)$. The 6-electron line is dotted. Contour intervals as in Fig. 3. Lines are drawn to show the outline of one telluropentathionate ion. One of the ammonium ions is resolved, whereas the other coincides with the tellurium peak.

Table 2. Sulphur-sulphur and tellurium-sulphur bond lengths and bond angles.

$S_1-S_2 = 2.11$ Å	$\angle S_1S_2Te = 105°$	$S_4-S_5 = 2.12$ Å
$S_2-Te = 2.36$	$\angle TeS_4S_5 = 104°$	$Te-S_4 = 2.35$
	$\angle S_2TeS_4 = 103°$	

Non-bonded distances

$S_1-Te = 3.57$ Å	$S_2-S_4 = 3.68$ Å	$Te-S_5 = 3.52$ Å
$S_1-S_4 = 4.52$	$S_1-S_5 = 6.11$	$S_2-S_5 = 4.69$

The S—S—Te—S—S chain of the telluropentathionate ion is unbranched and non-planar, and occurs in a *trans* form [7], as does the analogous chain of tellurium dimethanethiosulphonate [1]. In fact, the bond lengths and angles of Table 2 are, within the probable errors, the same as those found for tellurium dimethanethiosulphonate.

The only differences between the S—S—Te—S—S chains of the two compounds occur in the dihedral angles, these being susceptible to variations with rotations about the Te—S bonds. In tellurium dimethanethiosulphonate [1], the dihedral angles are both 81°. The larger values in the telluropentathionate ion, 86° and 95°, are in accordance with the larger $S_1—S_4$, $S_1—S_5$ and $S_2—S_5$ distances, 4.52 Å, 6.11 Å and 4.69 Å, as compared with 4.38 Å, 5.74 Å and 4.36 Å, respectively, in the methanethiosulphonate.

The terminal sulphur atoms are each surrounded by three oxygen atoms, at an average distance of 1.43 Å. Together with the sulphur atoms S_2 and S_4, respectively, each sulphonate group forms a thiosulphate group having the shape of a distorted tetrahedron. The dimensional data are listed in Table 3.

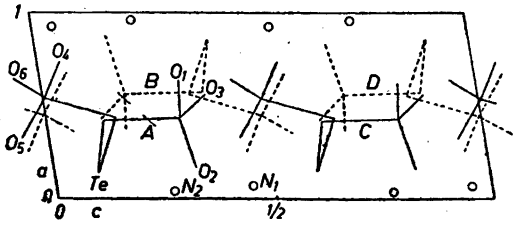

Fig. 6. Arrangement of the ions as viewed along the b *axis, showing the layer structure.*

Table 3. Sulphur-oxygen bond lengths and bond angles.

S_1-O_1 = 1.40 Å	$\angle S_2S_1O_1$ = 100°	$\angle O_1S_1O_2$ = 124°
S_1-O_2 = 1.48	$\angle S_2S_1O_2$ = 109°	$\angle O_1S_1O_3$ = 106°
S_1-O_3 = 1.40	$\angle S_2S_1O_3$ = 107°	$\angle O_2S_1O_3$ = 109°
S_5-O_4 = 1.47	$\angle S_4S_5O_4$ = 99°	$\angle O_4S_5O_5$ = 111°
S_5-O_5 = 1.40	$\angle S_4S_5O_5$ = 109°	$\angle O_4S_5O_6$ = 112°
S_5-O_6 = 1.43	$\angle S_4S_5O_6$ = 114°	$\angle O_5S_5O_6$ = 112°
Average: 1.43 Å	Average: 106°	Average: 112°

The average distance from the oxygen atoms to the nearest non-bonded sulphur atom is 2.86 Å, and the average non-bonded oxygen-oxygen distance within sulphonate groups is 2.37 Å, with individual values ranging from 2.73 to 3.00 Å and 2.23 to 2.54 Å, respectively. The average dimensions of the thiosulphate groups are the same, within the limits of error, as those found in barium pentathionate dihydrate [3] and barium tetrathionate dihydrate [8].

The telluropentathionate ion consists of two thiosulphate groups attached to divalent tellurium through covalent Te—S bonds. In displacement reactions with nucleophilic reagents [9,10] the tellurium atom acts as the electrophilic part of the ion. The telluropentathionate ion may thus be regarded as a thiosulphate complex of divalent tellurium.

THE IONIC ENVIRONMENT

The relative arrangement of oxygen atoms and ammonium ions is of interest in connection with the frequent occurrence of N—H···O hydrogen bonds in ammonium salts of oxy-acids and in organic crystals. The lengths of N—H···O bonds usually lie in the range [11,12] 2.8—3.0 Å.

In the present structure, each ammonium ion appears to form hydrogen bonds to four oxygen atoms, which in each case belong to four different telluropentathionate ions. The arrangement is a distorted tetrahedral one, with an average nitrogen-oxygen distance of 2.83 Å for N_1 and 2.88 Å for N_2. The data are collected in Table 4. The letters *A* to *D* refer to different telluropentathionate ions, as shown in Figs. 4 and 6. A prime denotes nearest corresponding atom in adjacent unit cell along the *a* axis.

Table 4. Distances, and angles between directions, from ammonium ions to oxygen atoms.

N_1-O_{1D}	= 2.83 Å	N_2-O_{1B}	= 2.75 Å
N_1-O_{2A}	= 2.87	$N_2-O_{4A'}$	= 2.82
$N_1-O_{3A'}$	= 2.80	N_2-O_{5C}	= 2.95
N_1-O_{5C}	= 2.83	$N_2-O_{6C'}$	= 2.99
$\angle O_{1D}-N_1-O_{2A}$	= 105°	$\angle O_{1B}-N_2-O_{4A'}$	= 96°
$\angle O_{1D}-N_1-O_{3A'}$	= 125°	$\angle O_{1B}-N_2-O_{5C}$	= 113°
$\angle O_{1D}-N_1-O_{5C}$	= 90°	$\angle O_{1B}-N_2-O_{6C'}$	= 147°
$\angle O_{2A}-N_1-O_{3A'}$	= 73°	$\angle O_{4A'}-N_2-O_{5C}$	= 120°
$\angle O_{2A}-N_1-O_{5C}$	= 127°	$\angle O_{4A'}-N_2-O_{6C'}$	= 123°
$\angle O_{3A'}-N_1-O_{5C}$	= 138°	$\angle O_{5C}-N_2-O_{6C'}$	= 79°

Other nitrogen-oxygen approaches shorter than 3.3 Å are $N_1—O_{4C'}$ = 3.04 Å, $N_1—O_{3A}$ = 3.06 Å, $N_1—O_{2D'}$ = 3.16 Å, and $N_2—O_{2B'}$ = 2.91 Å. The latter has not been included in Table 4 because the angles, $O_{2B'}—N_2—O_{1B}$ = 59° and $O_{2B'}—N_2—O_{5C}$ = 159°, appear to deviate too much from the tetrahedral value for hydrogen bonding to occur.

According to the above picture, the six oxygen atoms of a telluropentathionate ion participate in hydrogen bonding to eight different ammonium ions. Two oxygen atoms, one from each sulphonate group, form two hydrogen bonds, whereas each of the other four oxygen atoms form one bond only.

From Fig. 6, which shows the contents of the unit cell as viewed along the *b* axis, it appears that the telluropentathionate ions are arranged in layers normal to the *a* axis, with intermediate layers of ammonium ions. Twelve N—H···O bonds pass across each *a* face and hold the layers together.

SUMMARY

The crystal structure of ammonium telluropentathionate has been determined from X-ray data, by two-dimensional Patterson and Fourier methods.

The telluropentathionate ion contains an unbranched S—S—Te—S—S chain, which is non-planar and has a *trans* configuration. The dihedral angles are 86° and 95°.

The average Te—S and S—S bond lengths are 2.36 Å and 2.12 Å, respectively. The Te—S—S bond angles are 105° and 104°, and the S—Te—S bond angle is 103°.

Three oxygen atoms, in approximately tetrahedral positions, surround each of the terminal sulphur atoms, at an average distance of 1.43 Å.

Hydrogen bonding occurs between the oxygen atoms and the nitrogen atoms of the ammonium ions.

The authors wish to express their thanks to Dr. Sven Furberg for valuable assistance in the early part of the work.

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Table 5. Observed and calculated values of structure factors for ammonium telluropentathionate. The figures represent one fourth of the absolute values.

k	F_{obs}	F_{calc}	k	F_{obs}	F_{calc}	k	F_{obs}	F_{calc}
	0kl zone		9	11	+ 11	10	27	− 29
			10	15	+ 5	11	4	0
	0k0		11	34	− 31	12	9	+ 4
			12	6	+ 2	13	< 4	0
2	12	+ 13	13	17	− 14	14	7	+ 8
4	10	+ 6	14	7	− 6	15	7	+ 6
6	17	− 16	15	< 4	0	16	< 4	+ 2
8	27	+ 23	16	< 4	0	17	11	+ 11
10	40	+ 39	17	< 4	+ 5	18	7	− 5
12	5	+ 3	18	5	+ 6	19	< 3	− 1
14	21	− 19	19	< 4	+ 1	20	7	− 7
16	6	− 8	20	< 3	+ 3	21	< 3	− 3
18	5	+ 6	21	11	− 10	22	< 2	+ 2
20	5	+ 6	22	3	+ 3	23	< 2	− 2
22	3	− 3	23	< 2	− 4			
24	4	− 5	24	< 1	− 4		0k5	
	0k1			0k3		1	37	− 39
						2	11	+ 6
1	21	+ 22	1	6	− 2	3	8	− 4
2	21	− 20	2	43	− 46	4	17	− 19
3	24	− 25	3	28	+ 25	5	27	+ 29
4	2	− 1	4	26	− 27	6	7	+ 8
5	17	− 17	5	7	+ 8	7	10	− 8
6	18	+ 18	6	17	+ 21	8	< 3	+ 2
7	5	+ 3	7	< 2	+ 1	9	22	− 21
8	27	+ 30	8	19	+ 18	10	< 3	− 3
9	26	+ 24	9	16	− 15	11	20	− 19
10	10	− 6	10	17	− 14	12	8	+ 5
11	18	+ 17	11	14	− 16	13	13	+ 12
12	11	− 12	12	23	− 21	14	8	− 6
13	7	− 6	13	11	+ 6	15	8	+ 8
14	10	+ 6	14	11	− 10	16	< 4	+ 3
15	7	− 7	15	10	− 7	17	< 4	+ 1
16	9	+ 8	16	9	+ 11	18	< 4	− 1
17	10	+ 10	17	6	− 1	19	9	− 8
18	6	+ 6	18	4	+ 5	20	3	+ 2
19	6	+ 7	19	13	− 12	21	< 3	+ 1
20	< 3	− 2	20	5	− 5	22	< 2	+ 1
21	5	+ 6	21	< 3	− 3			
22	6	− 6	22	6	− 6		0k6	
23	4	− 4	23	< 2	− 1			
24	< 2	+ 1				0	2	− 2
				0k4		1	14	+ 15
	0k2					2	7	0
			0	30	− 39	3	12	+ 14
0	15	+ 17	1	8	+ 3	4	28	+ 32
1	30	− 34	2	26	− 32	5	15	− 10
2	30	− 28	3	8	− 10	6	10	+ 11
3	37	− 41	4	20	+ 17	7	21	− 18
4	6	− 6	5	14	+ 14	8	3	− 6
5	3	− 4	6	< 3	+ 3	9	8	− 10
6	32	− 30	7	25	+ 26	10	9	− 10
7	24	+ 23	8	12	− 10	11	8	+ 5
8	13	+ 13	9	8	+ 5	12	< 4	− 1

k	F_{obs}		F_{calc}
13	6	+	8
14	9	+	9
15	9	−	5
16	5	+	4
17	6	−	6
18	8	−	9
19	3	+	1
20	< 3	−	3
21	4	+	5
22	< 1		0

0*k*7

1	7	+	1
2	20	+	22
3	5	+	5
4	19	+	20
5	18	+	17
6	9	−	8
7	< 3	−	1
8	5	−	6
9	14	+	10
10	11	+	10
11	5	−	3
12	18	+	15
13	< 4	+	7
14	6	+	6
15	4	+	4
16	5	−	5
17	4	+	2
18	≤ 3	−	2
19	≤ 3	−	2
20	5	+	4
21	< 2	+	2

0*k*8

0	5	+	5
1	≤ 3	+	3
2	≤ 3	+	2
3	19	+	20
4	5	−	3
5	< 4	−	4
6	5	−	5
7	16	−	21
8	13	+	10
9	≤ 4	−	2
10	≤ 4	+	2
11	5	+	8
12	< 4	+	4
13	9	+	8
14	4	−	2
15	3	−	4
16	5	+	2
17	8	−	8
18	6	+	4
19	≤ 2		0
20	≤ 2	+	3

0*k*9

1	12	+	17
2	≤ 4	+	4
3	≤ 4	−	2
4	≤ 4		0
5	12	−	12
6	9	−	12
7	< 4	−	1
8	10	−	9
9	15	+	14
10	6	−	2
11	≤ 4	+	2
12	≤ 4		0
13	3	+	2
14	< 3	−	2
15	9	−	9
16	4	−	5
17	7	+	3
18	5	−	3
19	1	+	3

0,*k*,10

0	11	+	10
1	< 4	+	2
2	6	+	5
3	< 4	+	3
4	5	−	9
5	< 4	+	2
6	9	−	11
7	7	+	5
8	11	+	10
9	≤ 4		0
10	≤ 4	+	4
11	≤ 4	+	2
12	≤ 3	+	1
13	≤ 3		0
14	8	−	8
15	3	+	1
16	3	−	3
17	< 2	+	1

0,*k*,11

1	< 4	+	2
2	4	−	7
3	8	−	9
4	4	−	6
5	8	−	10
6	9	+	5
7	9	−	6
8	3	+	4
9	4	+	7
10	≤ 3	−	1
11	≤ 3	−	1
12	≤ 3	−	4
13	≤ 3	−	3
14	< 2	−	2
15	5	−	5
16	4	+	4

0,*k*,12

0	4	+	3
1	5	−	7
2	7	−	5
3	6	−	8
4	≤ 3	−	1
5	≤ 3		0
6	6	−	4
7	4	+	6
8	3	−	1
9	≤ 3		0
10	≤ 3		0
11	2	−	4
12	< 2	−	1
13	5	−	5
14	< 1	−	1

0,*k*,13

1	< 3	+	1
2	4	−	6
3	≤ 3		0
4	≤ 3		0
5	6	+	6
6	< 3	+	4
7	2	−	1
8	5	+	6
9	≤ 2	−	1
10	≤ 2		0
11	≤ 2	−	1

0,*k*,14

0	≤ 2	−	4
1	≤ 2	−	3
2	3	−	3
3	< 2	−	1
4	5	+	5
5	≤ 2		0
6	≤ 2	+	3
7	≤ 1	+	2

*hk*0 zone

0*k*0

2	12	+	13
4	9	+	7
6	14	−	16
8	26	+	22
10	38	+	40
12	4	+	3
14	20	−	20

k	F_{obs}		F_{calc}	k	F_{obs}		F_{calc}	k	F_{obs}		F_{calc}
16	6	−	8	11	13	−	17	6	5	+	7
18	4	+	6	12	6	+	5	7	< 3	+	1
20	5	+	7	13	15	−	18	8	7	−	6
22	3	−	3	14	< 3	−	1	9	12	−	10
24	4	−	5	15	7	+	8	10	13	−	13
				16	2	+	1	11	3	+	2
	1k0			17	9	+	12	12	< 3		0
				18	3	−	3	13	2	−	2
0	20	−	19	19	2	+	1	14	7	+	7
1	19	−	18	20	< 2	+	1	15	< 3	−	2
2	5	+	6	21	4	−	5	16	< 2	+	2
3	31	−	32	22	< 2	−	1	17	< 2		0
4	56	−	64	23	2	−	3	18	3	−	3
5	9	+	7					19	< 1		0
6	22	−	23		3k0						
7	37	+	38						5k0		
8	3	+	4	0	29	−	22				
9	13	+	15	1	10	−	11	0	< 3	−	1
10	8	+	10	2	9	−	9	1	8	+	7
11	12	−	11	3	14	−	13	2	3	+	4
12	5	−	2	4	8	+	8	3	6	+	7
13	6	−	9	5	9	+	7	4	5	+	6
14	13	−	15	6	13	+	13	5	2	+	3
15	5	+	5	7	13	+	15	6	7	+	7
16	7	−	9	8	13	−	13	7	10	−	9
17	7	+	8	9	15	+	18	8	< 3	−	2
18	8	+	9	10	12	−	11	9	3	+	2
19	< 3		0	11	8	−	10	10	< 2	−	1
20	3	+	5	12	3	+	4	11	4	+	3
21	5	−	7	13	< 3		0	12	< 2	+	1
22	< 2	−	1	14	10	+	11	13	5	+	5
23	1	−	3	15	< 3	+	2	14	2	+	3
24	< 1	−	2	16	2	+	4	15	2	−	1
				17	5	+	4				
	2k0			18	3	−	3		6k0		
				19	2	+	1				
0	27	+	22	20	3	−	4	0	< 2	−	2
1	21	−	19	21	3	−	5	1	2	+	3
2	7	+	5					2	< 2	−	2
3	30	−	33		4k0			3	6	+	4
4	13	+	13					4	5	−	4
5	7	+	4	0	15	−	18	5	3	−	4
6	8	+	8	1	< 3	+	1	6	3	−	3
7	25	+	29	2	11	−	11	7	4	−	4
8	< 2	+	4	3	5	+	1	8	< 2		0
9	< 2	+	1	4	14	+	13				
10	< 3		0	5	8	−	10				

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The First Step in the Swelling of Gelatin with Water

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In two earlier treatises I have examined the preparation, the properties and the theory of a chromated gelatin interference plate [1] and the treatment of this plate with water and water vapour [2] which, *i. a.*, resulted in a purely optical method of measuring the depression of the vapour pressure of diluted aqueous solutions. The examination comprised measurements of the rate of water absorption of the gelatin, either by immersion of the plate in water or by placing it over aqueous solutions of different water vapour pressure, and measurement of the absorbed water after equilibrium between the gelatin and water vapour of different pressures had been obtained.

In the following these two treatises will be referred to as I and II, respectively.

The said interference plate, which was invented by the late civil engineer J. H. Christensen, in transmitted light shows a spectrum which in part consists of a broad band, BB, in part of a number of single narrow bands with which we shall not occupy ourselves here. Through swelling with water or water vapour these bands shift towards larger wave lengths which in many instances lie in infrared. Bands photographed on an ordinary panchromatic plate must, therefore, be of the 2nd or 3rd order. In both cases it is possible by means of a previously given formula to calculate the absorbed amounts of water (*cf.* II, p. 4), which, of course, must be greatest if bands of the 3rd order are reckoned with. In the treatise I have, for a reason which will be explained below, reckoned with 3rd order, but later investigations have shown that this is not correct. These investigations were made in the following way.

The following measurements were made on small pieces of plate No. 48.

1) Photographic recording of the extinction spectrum
2) Weighing
3) Swelling in water for 5 days
4) Photographic recording of the extinction spectrum of the wet plate
5) Weighing of the wet plate in a weighing bottle
6) Fixation in 20 % sodium thiosulphate for 30 minutes, watering, drying
7) Weighing
8) Removal of the gelatin with hydrofluoric acid
9) Weighing of the glass.

From these measurements the following calculations are made:

From (1), (4), (7) and (9): the maximum amount of water v_v absorbed per 100 volumes of gelatin (*cf.* II, p. 4), corresponding to BB (*cf.* I, p. 27),

from (2) and (5): the average amount of water absorbed throughout the coating,

from (7) and (9): the amount of gelatin.

From the two last values v_M is obtained, which is the average amount of water per 100 volumes of gelatin.

Table 1 contains: 1) The plate number, 2) λ_t, the wave length of BB on the dry plate, 3) λ_v, the directly measured wave length on the wet plate, 4) and 6) this wave length converted to the 1st order under the assumption that it is of the 2nd respectively the 3rd order, 5) and 7) the corresponding percentages by volume of water, 8) the average amount of water v_M, 9) and 10) the quotients v_v:v_M, calculated on basis of the 2nd and 3rd order, respectively.

Table 1.

No.	λ_t	λ_v	2nd order		3rd order		v_M	$v_v : v_M$	
			conv.	v_v	conv.	v_v		2nd order	3rd order
4	582	588	1 170	170	1 737	342	101	1.68	3.39
8	579	615	1 228	187	1 840	364	118	1.59	3.09
10	583	529	1 050	134	1 575	286	77	1.74	3.71
12	583	599	1 195	176	1 790	348	96	1.83	3.63
13	588	596	1 186	173	1 782	345	92	1.88	3.75
14	586	615	1 228	185	1 840	362	91	2.03	3.98

In the exposed chromated gelatin plate the amount of unhardened gelatin decreases evenly from the air surface which during the exposure has been in optical contact with mercury, to the interface between the coating and the glass. The broad band, BB, is formed in the outermost part of the coating and the upper edge of BB, which always forms the basis of the calculations, accordingly corresponds to the layer which is situated closest to the air surface. Here, consequently, the amount of unhardened gelatin is larger than the average value for the whole coating. The amount of unhardened gelatin g is calculated from the formula

$$g' = 16.3—8.8 \cdot c''$$

in which 16.3 is the total amount of gelatin, c'' the amount of chromic oxide formed by the exposure, and 8.8 the proportionality factor between the amounts of hardened gelatin and chromic oxide (*cf.* I, p. 18).

For the three plates Nos. 27, 9 and 28 this calculation has been performed for the outermost layer of the coating, corresponding to BB (*cf.* I, Table 24), and for the entire coating (*cf.* I, Table 25). The results were as recorded in Table 2.

Provided the absorbed amounts of water are proportional to the amounts of unhardened gelatin, and this assumption seems reasonable, these quotients must correspond to the quotients v_v:v_M. A comparison with these values in Table 1 clearly shows that the measured spectra are of the 2nd order.

Table 2.

No.	g'		quotient
	BB	the coating	
27	5.0	3.31	1.51
9	6.0	3.82	1.57
28	7.0	4.05	1.73

This cognition of course entails alterations of some of the formulae which have been set up in II, both for the rate of the water absorption and for the relation between the absorbed amounts of water and the vapour pressures. However, it appears that the formulae are still applicable, only the constants take other values.

For the rate of swelling with water the formula will be:

$$v_v - 84.1 = (v_\infty - 84.1)\ (1 - 10^{-0.028 \cdot t^{0.5}})$$

and Table 3 will be:

Table 3.

t	λ_{BB}		v_v	
	obs.	calc.	obs.	calc.
0	558	—	—	—
1	868	873	89.3	90.9
2	881	881	93.1	93.0
4	889	891	95.4	95.9
8	906	905	100.5	99.9
15	923	921	105.2	104.6
30	950	945	113.0	111.4
60	975	974	120.1	119.9
120	1 010	1 010	130.3	130.3
240	1 047	1 050	141.0	141.8
1 920	1 158	1 157	173	172.7
4 320	1 182	—	180	—

The new formula has the great advantage over the previous one that the time t will have the same exponent as was previously found for the swelling with water vapour for which the formula reads:

$$v_v = v_\infty\ (1 - 10^{-k \cdot t^{0.5}})$$

Add to this the altogether unexpected information that 84.1 % by volume of water is absorbed instantaneously — at any rate in the surface of the coating —, whereafter the absorption of water continues after the same law as in the treatment with water vapour.

The original calculation had been performed on basis of spectra of the 3rd order just to avoid this instantaneous absorption of water which already at

Table 4.

t	λ_{BB}		v_v	
	obs.	calc.	obs.	calc.
5	618	626	17.8	19.7
10	656	652	28.6	27.2
20	692	687	39.0	37.2
45	740	741	53.1	52.8
60	765	764	60.0	59.5
90	798	800	69.7	69.8
120	834	829	79.6	78.0
180	868	871	89.6	90.1
∞	1 182	—	180	—

that time was found as the result of the calculation on basis of spectra of the 2nd order. However, the new experiments have shown the impossibility of avoiding this assumption.

Also the formula for the absorption of water from saturated water vapour must be altered. The constant will now be 0.0225, and the corresponding parts of Tables 3a and 3b will be as recorded in Table 4.

It will be seen that after the first film has been formed the absorption of water proceeds more slowly than the absorption of saturated water vapour. Presumably this must be due to the fact that this film of water restrains the diffusion of water into the interior gelatin coating.

In Table 5 are the values of k for a number of varying vapour pressures.

Table 5.

$\frac{p}{p_0}$	k	$v\infty$
1	0.0225	180
0.9964	0.0250	140
0.98	0.0521	58.6
0.95	0.1001	26.0
0.84	0.1376	14.8

For the equilibrium with the vapour pressure formula (4) will be:

$$v_v = v_1 \left(1 - 10^{-0.0126\left(\frac{p/p_0}{1-p/p_0}\right)^{0.7}}\right)$$

The corresponding values are found in Table 6.

The measurement of the depression of the vapour pressure is based on the fact that the curve whose abscissa is the degree of moisture p/p_0 and whose ordinate is the corresponding amounts of water in the gelatin (*cf.* II, Fig. 5) rises very sharply when p/p_0 approaches 1. In Table 7 are stated some (now

Table 6.

$\frac{p}{p_0}$	λ_{BB}		v_v	
	obs.	calc.	obs.	calc.
1	1 182	—	180	—
0.9964	1 044	1 047	140	141
0.98	753	783	58.6	65
0.95	672	687	26.0	37.1
0.88	624	627	19.1	20.0
0.84	609	614	14.9	16.0
0.795	599	604	12.1	13.2
0.726	592	593	9.7	10.1
0.66	591	586	9.5	8.1
0.58	582	580	7.1	6.4
0.45	580	574	6.3	4.5
0.323	572	569	4.3	3.1
0.20	562	565	1.3	1.9

corrected) values of the shift of v_v and of the wave length of BB as compared with the value for $p = p_0 = 1$ for various values of p/p_0 near 1.

Finally, Table 8 shows the result of a number of measurements of the depression of vapour pressure. Four pieces of a plate were first saturated with

Table 7.

$-\log\ (1-\frac{p}{p_0})$	Δv_V	$\Delta\lambda_{BB}$
1	155.2	538
2	85.2	295
3	4	14

water for several days and then placed over water and 0.01, 0.03, and 0.1 *M* solutions of potassium chloride, respectively, until equilibrium had been established. Table 8 contains the depression of vapour pressure in question and the corresponding directly observed wave lengths and the wave lengths converted to 1st order.

With a plate which is strongly sensitized to infrared a direct measurement of the bands of the 1st order should be possible. Incidentally, it may be men-

Table 8.

	$1-\frac{p}{p_0}$	λ_{BB}	
		obs.	conv.
water	0	525	1 040
0.01-KCl	0.00036	517	1 025
0.03-KCl	0.001026	501	990
0.1 -KCl	0.00328	443	870

tioned that attempts are being made to avoid the photographic recordings entirely and instead to measure the differences directly by visual, optical means.

SUMMARY

By treatment of gelatin with water the surface instantaneously absorbs a considerable amount of water whereafter the swelling proceeds slowly and after the same law but at a somewhat lower rate than in the case of absorption of saturated water vapour.

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The Complex Formation between Iron(III)Ion and Some Phenols. II. Salicylic Acid and *p*-Amino Salicylic Acid *

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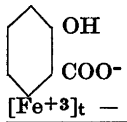
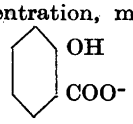
In a previous work [1] this author has studied the complex formation between iron(III)ion and sulfosalicylic acid. The complex formation between iron(III)ion and salicylic acid and *p*-aminosalicylic acid has now been investigated.

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

h = hydrogen ion concentration, mC.
[ ] = concentration of substance indicated, mC.
$[\ ]_t$ = stoichiometrical concentration, mC.
RH =  or NH_2 

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

ε = extinction, $cm^{-1}\ mC^{-1}$
E = extinction, cm^{-1}.
K_a = dissociation constant of acid.
$\varkappa$ = complexity constant.

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

$\varkappa_{nm}$ = complexity constants of the hydroxyl complexes $Fe_n(OH)_m$.

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

SALICYLIC ACID

Salicylic acid, like sulfosalicylic acid, forms complexes built up from one atom of iron(III) and one, two or three molecules of salicylic acid. This complex formation has been used in quantitative photometric determinations of iron [2,3] and salicylic acid and some derivatives of this acid [4].

The complexes have been studied by, among others, Babko [5] and Bertin-Batsch[6]. The former, by a photometric method, has determined the dissociation constants of the three complexes. The latter has, also photometrically, determined the first dissociation constant.

* Part I: *Acta Chem. Scand.* 8 (1954) 266.

Table 1. Survey of previous investigations.

Author	FeR			FeR_2			FeR_3			Method	Temp.	Ionic strength
	$\varkappa_1$	ε_1	λ	$\varkappa_2$	ε_2	λ	$\varkappa_3$	ε_3	λ			
Babko *	900	—	—	9.3	—	—	$1.7 \cdot 10^{-6}$	—	—	photom.	—	—
Bertin-Batsch *	1 000	1 630	—	—	—	—	—	—	—	photom.	15°	$3.6 \cdot 10^{-3}$
This work	500	1 350	530	18	3 600	480	$8 \cdot 10^{-4}$	4 300	430	photom.	25°	3.0
» »	470	—	—	15	—	—	$1.5 \cdot 10^{-4}$	—	—	potentiom.	25°	3.0

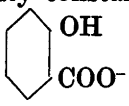
* $\varkappa_n$ is calculated using the dissociation constant of salicylic acid $\frac{[R] \cdot h}{[RH]} = 3.6 \cdot 10^{-14}$

The determinations in this work are made potentiometrically and photometrically. In spite of the determinations having been made under other conditions the results agree rather well with the results of Babko and Bertin-Batsch (Table 1). The complexity constants are defined by the following equations:

$$\varkappa_n = \frac{h^n [FeR_n]}{[RH]^n [Fe]} \quad (n = 1, 2 \text{ and } 3) \tag{1}$$

In a previous work [1] the following equation was shown to be valid:

$$\frac{(b - \varkappa') h}{[RH]} = \varkappa_1 + \varkappa_2 \frac{[RH]}{h} + \varkappa_3 \left(\frac{[RH]}{h}\right)^2 \tag{2}$$

By this equation the complexity constants could be calculated. The same equation is valid here if RH is  .

b was calculated from the redox potential of the solution. At small values of [RH]/h the last term could be omitted and a straight line was obtained from which $\varkappa_1$ and $\varkappa_2$ were calculated. At high values of [RH]/h, $\varkappa_3$ could then be calculated.

In the photometric determinations the following equations, derived earlier, were used:

$$\frac{c}{E} = \frac{1}{\varepsilon_1} + \left[\sqrt{\frac{h\,(h + K_a)}{E\,K_a}}\right] \sqrt{\frac{1}{\varepsilon_1 \varkappa_1}} \tag{3}$$

This equation is valid if Fe and FeR are the only forms of iron(III) present in measurable amounts. $c = [Fe]_t = [R]_t$.

$$\frac{c}{E} = \frac{1}{\varepsilon_n} + \left[\frac{h}{[RH]} \cdot \frac{E - c\,\varepsilon_{n-1}}{E}\right] \frac{\varkappa_{n-1}}{\varepsilon_n \varkappa_n} \tag{4}$$

This equation was used when FeR and FeR_2, or FeR_2 and FeR_3 are the only forms of iron(III) present in measurable amounts. $c = [Fe]_t$.

Experimental

Apparatus. The apparatus was the same as that in the previous work [1].

Chemicals. Iron(II) and iron(III) perchlorate were prepared in the same manner as described before [1]. From sodium salicylate (A.R.) a solution was prepared, the concentration of which was determined bromometrically as described in the Danish Pharmacopeia (1948). To all solutions $NaClO_4$ (A.R.) was added to make the concentration of ClO_4^- 3 000 mC. Under these conditions it is assumed that the activity factors are constant and hence the concentrations may be used instead of activities in the equilibria.

Determination of the dissociation constant of salicylic acid. In acidic solutions only the carboxylic group is dissociated. The phenolic group is dissociated in alkaline solutions only. The dissociation constant of the carboxylic group was determined by titrating a 20 mC solution of sodium salicylate with perchloric acid. The hydrogen ion concentration was determined with a glass electrode. The mean value of the dissociation constant was

$$K_a = \frac{h\,[RH]}{[RH_2]} = 0.718 \pm 0.005 \text{ mC} = 10^{-3.86 \pm 0.01} \text{ C}.$$

Potentiometric determination of the complexity constants. The experiments were made in the following manner. A solution with known concentration of iron(III) and iron(II) and perchloric acid was titrated with a sodium salicylate solution of known concentration. At the same time an iron solution was added from another burette, whereby the total oncentrations of iron(III) and iron(II) were kept constant.

Table 2. Salicylic acid. Potentiometric determination of $\varkappa_1$ and $\varkappa_2$. Compare Table 3, No. 1.

$[R]_t$	$[H]_t$	h	b	$\frac{[RH]}{h}$	$\frac{(b-\varkappa')\,h}{[RH]}$
2.186	0.647	1.177	137.03	0.332	410
3.060	0.402	0.7072	565.96	1.312	431
3.825	0.187	0.4737	1 436.3	3.219	446
4.500	−0.002	0.3537	2 773.6	5.900	470
5.100	−0.171	0.2856	4 655	9.054	514
5.637	−0.321	0.2473	6 951	12.137	573
6.120	−0.457	0.2108	10 060	16.295	617
6.557	−0.579	0.1943	13 160	19.310	682
6.955	−0.691	0.1735	17 620	23.666	745

Table 3. Salicylic acid. Survey of potentiometric determinations.

No.	Symbol	Concentrations		$\varkappa_1$	$\varkappa_2$	$\varkappa_3$
		Fe (III)	Fe (II)			
1	○	1.273	0.433	400	14	—
2	□	1.280	1.023	510	17	—
3	△	1.173	1.502	450	20	—
4	▽	1.201	0.312	300	18	—
5	×	1.192	3.004	470	23	—
6	◐	1.136	0.295	600	10	$1.7 \cdot 10^{-4}$
7	+	1.192	3.004	490	11	—
8	⅄	1.173	1.502	460	13	—
9	●	1.136	0.295	550	11	$1.2 \cdot 10^{-4}$
10	■	0.265	1.430	—	11	$1.4 \cdot 10^{-4}$
11	▲	0.256	0.604	500	12	$1.8 \cdot 10^{-4}$

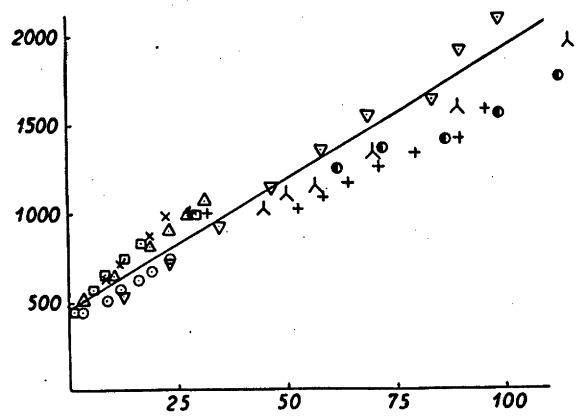


Fig. 1. Salicylic acid. Survey of potentiometric determinations of $\varkappa_1$ *and* $\varkappa_2$. *Compare Table 3.* $\frac{(b-\varkappa')\ h}{[RH]}$ *vs.* $\frac{[RH]}{h}$

As an example the results of one titration are given in detail in Table 2. From these values the complexity constants were calculated: $\varkappa_1 = 400$, $\varkappa_2 = 14$. Several such titrations were made, and the results are collected in Table 3 and Fig. 1. Some of the titrations were made at very low hydrogen ion concentrations. In these solutions the complex FeR_3 was formed. The results of these experiments are found in Fig. 2.

Photometric experiments. The values of the complexity constants, found potentiometrically, were verified by photometric determinations. These experiments also give the extinction values.

Using the values of $\varkappa_n$ found potentiometrically, conditions were chosen so that one or two complexes but not more were formed in appreciable amounts in the same solution.

The results of the photometric determinations are shown in Tables 4—6 and Figs. 3—5. Fig. 6 shows how iron(III) is distributed over the complexes when [RH] / h is varied.

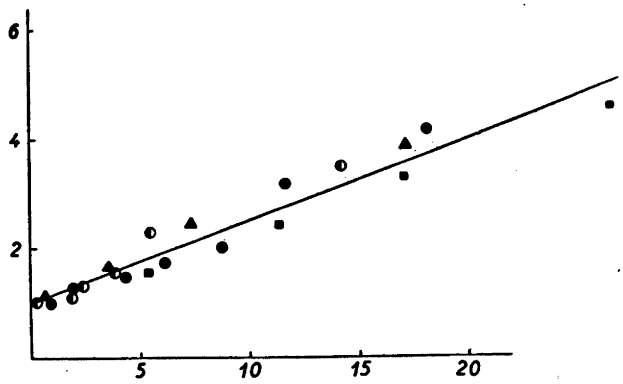


Fig. 2. Salicylic acid. Survey of potentiometric determinations of $\varkappa_2$ *and* $\varkappa_3$. *Compare Table 3.* $\left(\frac{(b-\varkappa')\ h}{[RH]} - \varkappa_1\right)\frac{h}{[RH]}$ *vs.* $\frac{[RH]}{h}$

Table 4. Salicylic acid. Photometric determination of $\varkappa_1 \cdot [Fe]_t = [R]_t = 1.514\,mC$. *In the left hand section are given the extinction values at 530 mμ. In the right hand section are given the extinction at different wave lengths when* $h = 31.55\ mC$.

h	E	λ	E
11.29	1.339	400	0.154
16.36	1.066	425	0.191
21.42	0.848	450	0.307
26.49	0.697	475	0.426
31.55	0.565	500	0.514
36.62	0.492	530	0.565
41.68	0.413	550	0.556
46.75	0.315	575	0.505
		600	0.439

Table 5. Salicylic acid. Photometric determination of $\varkappa_2$. $[Fe]_t = 0.3805\ mC$; $[R]_t = 10.10\ mC$.

h	Extinction at wave length								
	400	425	450	460	470	480	490	500	525
1.248	0.373	0.805	1.105	1.195	1.225	1.248	1.232	1.185	1.000
1.270	0.400	0.826	1.136	1.203	1.250	1.270	1.250	1.208	1.028
1.284	0.428	0.850	1.196	1.225	1.270	1.284	1.263	1.220	1.020
1.300	0.435	0.875	1.166	1.235	1.282	1.300	1.278	1.224	1.015

Table 6. Salicylic acid. Photometric determination of $\varkappa_3$. $[Fe]_t = 0.2154\ mC$; $[R]_t = 10.10\ mC$.

h	Extinction at wave length								
	400	430	450	475	500	525	550	575	600
5.951	0.500	0.715	0.775	0.730	0.609	0.468	0.320	0.195	0.108
4.121	0.535	0.755	0.780	0.715	0.580	0.440	0.295	0.175	0.100
3.229	0.585	0.775	0.720	0.701	0.575	0.420	0.275	0.163	0.095
2.228	0.605	0.800	0.805	0.712	0.550	0.400	0.265	0.156	0.090
1.425	0.655	0.840	0.835	0.725	0.550	0.395	0.255	0.155	0.090
1.104	0.660	0.845	0.835	0.685	0.500	0.345	0.210	0.120	0.068
0.835	0.700	0.880	0.840	0.690	0.500	0.325	0.205	0.115	0.070

p-AMINOSALICYLIC ACID (PAS)

The colour reaction between iron(III) and PAS has been used in a photometric determination of Fe by Rosdahl [11]. Like salicylic acid, PAS forms coloured complexes with iron(III) and the colour shifts as the pH is changed. In rather strong acidic solutions the colour is violet and when the hydrogen ion concentration is decreased the colour shifts through red to yellow. The violet complex is built up from one atom Fe and one molecule of PAS, which may be shown by a method introduced by Job [7] (Fig. 7).

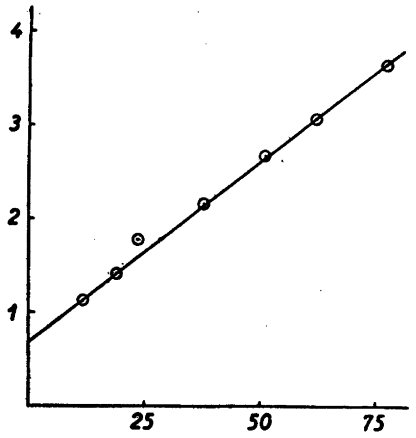


Fig. 3. Salicylic acid. Photometric determination of $\varkappa_1$.
$\frac{c}{E}$ *vs.* $\sqrt{\frac{h\ (h + K_a)}{E \cdot K_a}}$; $c = [Fe]_t = [R]_t = 1.514$ *mC.* $\lambda = 530$ *mμ.*

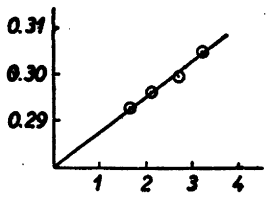


Fig. 4. Salicylic acid. Photometric determination of $\varkappa_2$.
$\frac{c}{E}$ *vs.* $\frac{h}{[RH]} \cdot \frac{E - c \cdot \varepsilon_1}{E}$; $c = [Fe]_t = 0.3805$ *mC.* $[R]_t = 10.10$ *mC.* $\lambda = 480$ *mμ.*

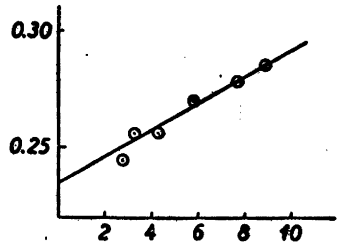


Fig. 5. Salicylic acid. Photometric determination of $\varkappa_3$.
$\frac{c}{E}$ *vs.* $\frac{h}{[RH]} \cdot \frac{E - c \cdot \varepsilon_2}{E}$; $c = [Fe]_t = 0.2154$ *mC.* $[R]_t = 10.10$ *mC.* $\lambda = 430$ *mμ*

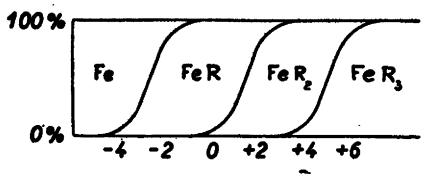


Fig. 6. Salicylic acid. Distribution of $Fe(III)$ *in per cent over* Fe^{+3}, FeR^{+}, FeR_2^{-} *and* FeR_3^{-3} *as a function of log* $\frac{[RH]}{h}$.

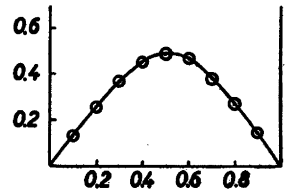
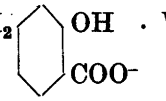


Fig. 7. PAS. Determination of composition of the violet complex.
E *vs.* $\frac{[Fe]_t}{[Fe]_t + [R]_t}$; $[Fe]_t + [R]_t = 0.75$ *mC. h = 5,0 mC.*

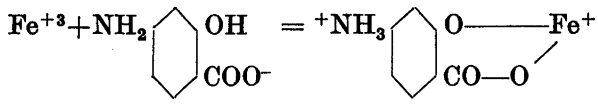
When the hydrogen ion concentration is decreased, complexes built up from two and three molecules of PAS per atom Fe are formed. In order to determine the complexity constants, defined by equation (1) the same method was used as in the investigation of sulfosalicylic acid [1] and salicylic acid.

Theoretical

Potentiometric determinations. It is easy to see that equation (2) should also be valid if RH is  . When [RH]/h is rather small, the last term in this equation may be omitted. Thus a straight line should be obtained if $\frac{(b-\varkappa')h}{[RH]}$ is plotted against [RH]/h.

During this investigation, however, it was observed that when [RH]/h was very low or h rather great, the line was bent upwards (*cf.* Fig. 8). This was not observed with the phenols hitherto investigated. This phenomenon may be explained in the following manner.

PAS has an amino group with weakly basic properties. In acid solutions one hydrogen ion can be added, and thus there is a possibility that another complex is formed:



$(Fe + RH = FeRH)$

the equilibrium constant being

$$\varkappa_{10} = \frac{[FeRH]}{[RH]\,[Fe]} \qquad (5)$$

In analogy to the complex FeRH one would expect the complexes FeR_2H, FeR_2H_2 and so on, but complexes with more than one R per Fe atom are formed only in very weakly acidic solutions and no such complexes have been found in fact.

A similar phenomenon was observed by Schwarzenbach and Willi [8] with pyrocatechol-3,5-disulfonic acid, which forms complexes of the type FeBz and FeHBz (Bz = $C_6H_2(O^-)_2(SO_3^-)_2$). Schwarzenbach and Willi believe that the hydrogen atom is bound to the sulfonic group but not to the phenolic groups.

It is easy to show, that here equation (2) has to be changed to

$$\frac{(b-\varkappa')\ h}{[RH]} = \varkappa_{10}h + \varkappa_1 + \varkappa_2 \frac{[RH]}{h} + \varkappa_3 \left(\frac{[RH]}{h}\right)^2 \qquad (6)$$

At low h values this equation, like equation (2), gives a straight line, from which $\varkappa_1$ and $\varkappa_2$ may be calculated. But if the hydrogen ion concentration is appreciably increased the first term of the right member increases, which results in a bending upwards of the curve. If [RH]/h is small and h increases the last two terms of equation (6) may be omitted:

$$\frac{(b-\varkappa')\ h}{[RH]} = \varkappa_{10}h + \varkappa_1 \qquad (7)$$

A straight line should be obtained if the left member is plotted against h. The slope of this line is $\varkappa_{10}$ and the intercept gives $\varkappa_1$.

When [RH]/h is appreciably increased the complex FeR_3 is formed. However, the complexity constant $\varkappa_3$ could not be determined potentiometrically because PAS is sparingly soluble. Thus [RH] may not be increased too much, and h may not be decreased too much because the hydrolysis of Fe(III) and Fe(II) will then be too great.

Photometric determinations. The hydrogen ion concentration was kept within a range where only one or two complexes but not more were formed in appreciable amounts. This could be calculated from Fig. 9, which shows the distribution of iron between the complex forms at different h values. These curves are calculated from $\varkappa_n$ measured potentiometrically. The total concentration of PAS is 10 mC and the concentration of iron so low that only a very small portion of PAS is bound to the iron.

The equations used before, (3) and (4), in the photometric determinations could not be used here due to the formation of the complex FeRH. Instead the following equations are valid.

$$[Fe]_t = [Fe] + [FeRH] \qquad (8)$$

$$E = \varepsilon_{10}\,[FeRH] \qquad (9)$$

(Only Fe and FeRH are present in appreciable amounts, and only the latter has a measurable extinction at the concentration and wave length used).

If these equations are combined with equation (5) the following equation is formed:

$$\frac{[Fe]_t}{E} = \frac{1}{\varepsilon_{10}} + \frac{1}{[RH]} \frac{1}{\varepsilon_{10}\varkappa_{10}} \qquad (10)$$

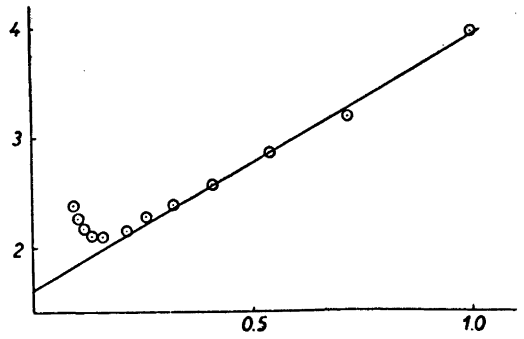


Fig. 8. PAS. Potentiometric determination of $\varkappa_1$ *and* $\varkappa_2$. *Expt. No. 3 of Table 7.* $\frac{(b-\varkappa')\,h}{[RH]}\,10^{-3}$ *vs.* $\frac{[RH]}{h}$.

If $[Fe]_t/E$ is plotted as a function of 1/[RH] a straight line should be formed, the intercept of which is $1/\varepsilon_{10}$. From the slope of the line $\varkappa_{10}$ could be calculated.

When FeRH and FeR are formed, the following equations are valid:

$$E = \varepsilon_{10}\,[FeRH] + \varepsilon_1\,[FeR] \tag{11}$$

$$[Fe]_t = [FeRH] + [FeR] \tag{12}$$

(No forms of iron other than FeRH and FeR are supposed to be present). From these equations and equations (1) and (5), the following expression may be derived:

$$\frac{[Fe]_t}{E} = \frac{1}{\varepsilon_1} + \left[\mathrm{h}.\frac{E-\varepsilon_{10}[Fe]_t}{E}\right]\frac{\varkappa_{10}}{\varepsilon_1\varkappa_1} \tag{13}$$

If $[Fe]_t/E$ is plotted against the expression in the square brackets a straight line should be obtained. From the slope and intercept, ε_1 and $\varkappa_1$ may be calculated.

From Fig. 6 it is seen that under the conditions used there, $\varkappa_{10}$ may be determined within a range of log h from +3 to +2, where Fe^{+3} and FeRH but no other forms of iron are present in measurable amounts. In the range from +1 to 0 the expression $\varkappa_{10}/\varkappa_1$ may be determined. The expression $\varkappa_1/\varkappa_2$ cannot be determined, however, in this manner since the complexes FeR and FeR_2 cannot be formed without an appreciable amount of FeRH being present.

At h values which are so low (log h = —3 to —6) that the yellow complex FeR_3 is formed, $\varkappa_3$ can also be estimated. From equation (4) where n = 3, ε_3 and $\varkappa_3$ may be determined if ε_2 and $\varkappa_2$ are known. However, $\varkappa_2$ could not be determined photometrically in the usual manner as shown above. Instead the following method was used.

$[Fe]_t/E$ plotted against the expression in the square brackets of equation (4) should give a straight line. This is only true if the right value of ε_2 is used.

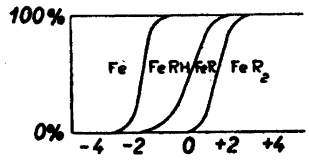


Fig. 9. PAS. Distribution of Fe(III) in per cent over Fe^{+3}, $FeRH^{+2}$, FeR^{+} and FeR_2^{-} as a function of -log h. $[R]_t$ = 10 mC.

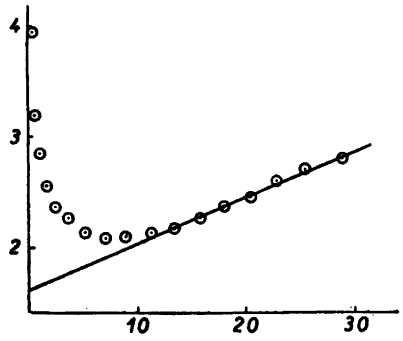


Fig. 10. PAS. Potentiometric determination of $\varkappa_{10}$ and $\varkappa_1$. $\frac{(b-\varkappa')\ h}{[RH]}\ 10^{-3}$ vs. h.

If a wrong value is used the curve will bend in one direction or another. When several values were tried only a certain value gave a straight line but small variations in ε_2 gave strong deviations from the straight line. In order to calculate $\varkappa_3$ the value of $\varkappa_2$ found potentiometrically was used.

Experimental

Chemicals. In addition to the chemicals used before, solutions of PAS were used. These were prepared immediately before each experiment from pure PAS, which was dissolved in an excess of NaOH 100 mC. Then a calculated amount of $HClO_4$ 100 mC was added.

Table 7. PAS. Survey of potentiometric experiments.

No.	Symbol	Concentrations		$\varkappa_{10}$	$\varkappa_1$	$\varkappa_2$
		Fe (III)	Fe (II)			
1		0.651	0.591	1 700	1 500	—
2		0.660	0.573	1 700	—	—
3		1.663	0.595	(2 300)	1 600	43
4	○	0.558	0.507	—	1 400	24
5	□	0.651	0.591	—	1 600	21
6	△	0.660	0.573	—	2 500	32
7	×	0.669	0.573	—	2 500	38
8	+	0.669	0.573	—	2 000	43
9	⅄	1.500	0.591	—	1 400	35
10	◊	1.663	0.573	—	1 600	43
11	●	2.981	0.551	—	1 600	66

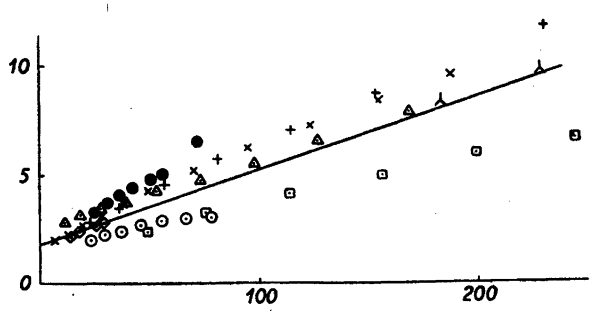


Fig. 11. PAS. Survey of potentiometric determinations of $\varkappa_1$ and $\varkappa_2$. Compare Table 7. $\frac{(b-\varkappa')\ h}{[RH]}$ *vs.* $\frac{[RH]}{h}$.

To the solutions of PAS, as to all other solutions, $NaClO_4$ was added up to a concentration of 3 000 mC ClO_4^-.

Acidic solutions of PAS are not quite stable [9,10] but at the hydrogen ion concentrations and the temperature used in this investigation the decomposition, as was experimentally proved, is insignificant.

Determination of dissociation constants. In acidic or neutral solutions the following constants are of interest:

$$K_{a1} = \frac{[RH]\,h}{[RH_2]} \qquad K_{a2} = \frac{[RH_2]\,h}{[RH_3]}$$

Table 8. PAS. Photometric determination of $\varkappa_{10}$.

h	Extinction at wave length								
	400	425	450	475	500	525	550	575	600
102.7	0.174	0.265	0.417	0.532	0.589	0.575	0.500	0.400	0.296
110.6	0.155	0.240	0.380	0.499	0.541	0.522	0.462	0.358	0.260
118.7	0.141	0.230	0.350	0.459	0.493	0.490	0.424	0.328	0.238
126.8	0.132	0.212	0.324	0.422	0.462	0.460	0.390	0.301	0.218
134.9	0.123	0.199	0.301	0.392	0.437	0.420	0.360	0.279	0.201
143.1	0.105	0.180	0.276	0.358	0.396	0.382	0.332	0.254	0.185

Table 9. PAS. Photometric determination of $\varkappa_1$.

h	Extinction at wave length								
	400	425	450	475	500	525	550	575	600
0.4199	0.277	0.405	0.500	0.516	0.479	0.390	0.285	0.186	0.120
1.004	0.208	0.310	0.417	0.450	0.427	0.360	0.277	0.190	0.127
1.564	0.151	0.250	0.345	0.400	0.398	0.350	0.279	0.200	0.136
2.595	0.130	0.220	0.313	0.370	0.378	0.348	0.280	0.212	0.148
3.556	0.114	0.200	0.291	0.333	0.360	0.333	0.288	0.217	0.151
4.838	0.100	0.170	0.269	0.330	0.355	0.336	0.287	0.213	0.152
6.277	0.096	0.164	0.262	0.327	0.351	0.330	0.280	0.211	0.150

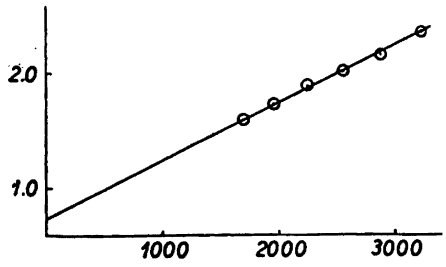


Fig. 12. PAS. Photometric determination of $\varkappa_{10}$. $\frac{[Fe]_t}{E}$ *vs.* $\frac{1}{[RH]}$; $[Fe]_t = 0.9318$ *mC,* $[R]_t = 9.774$ *mC.* $\lambda = 500$ *mμ.*

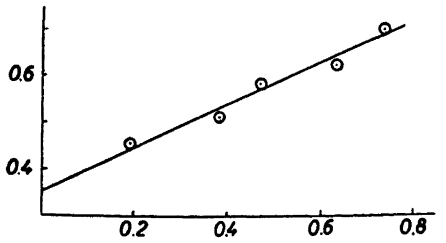


Fig. 13. PAS. Photometric determination of $\varkappa_1$. $\frac{c}{E}$ *vs. h.* $\frac{E - c \cdot \varepsilon_{10}}{E}$; $c = [Fe]_t = 0.2330$ *mC.* $[R]_t = 9.776$ *mC.* $\lambda = 475$ *mμ.*

They were determined by titrating a 25 mC solution of PAS-Na with $HClO_4$ 100 mC. From the titration curve obtained the constants could be calculated. Mean values are

$$K_{a1} = 8.3 \pm 0.1 \text{ mC} = 10^{-2.08 \pm 0.01} \text{ C}$$
$$K_{a2} = 0.0826 \pm 0.0005 \text{ mC} = 10^{-4.08 \pm 0.01} \text{ C}.$$

Potentiometric determinations of complexity constants. The experiments were made in complete analogy to those described under "salicylic acid". Fig. 8 shows how the formation of the complex FeRH influences the results. Here $\frac{b.h}{[RH]}$ is plotted as a function of [RH] / h. Disregarding the upwards bending of the left part of the curve one may calculate $\varkappa_1 = 1\,600$ and $\varkappa_2 = 43$.

If $\frac{b.h}{[RH]}$ is plotted against h instead, the curve in Fig. 10 is obtained. At high h values the complexes FeRH and FeR dominate. When h is small (< 0.2 mC) FeR_2 is being formed in an appreciable amount, which results in the bending upwards of the left part of this curve. Here the following values are calculated: $\varkappa_1 = 1\,600$ and $\varkappa_{10} = 2\,300$.

Several such experiments are collected in Table 7. Nos. 1 and 2 are made in strongly acidic solutions (h $\sim$ 50 mC). Fig. 11 shows those experiments from which $\varkappa_1$ and $\varkappa_2$ are calculated.

Table 10. PAS. Photometric determination of $\varkappa_3$.

h	Extinction at wave length						
	400	425	450	475	500	525	550
$1.412 \cdot 10^{-6}$	0.860	0.920	0.807	0.587	0.350	0.166	0.050
$2.646 \cdot 10^{-5}$	0.815	0.879	0.820	0.657	0.469	0.294	0.169
$1.202 \cdot 10^{-4}$	0.708	0.802	0.794	0.704	0.559	0.420	0.278
$5.488 \cdot 10^{-4}$	0.625	0.730	0.779	0.748	0.654	0.518	0.380
$1.035 \cdot 10^{-3}$	0.607	0.714	0.772	0.760	0.676	0.544	0.400

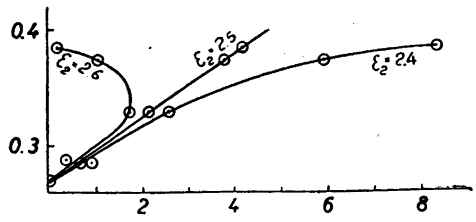


Fig. 14. PAS. Photometric determination of $\varkappa_3 \cdot \frac{c}{E}$ *vs.* $\frac{h}{[RH]} \cdot \frac{E - c \cdot \varepsilon_2}{E}$ 10^6; $c = [Fe]_t = 0.2330$ *mC.* $[R]_t = 9.758$ *mC.* $\lambda = 400$ *mμ.*

Photometric experiments. The results of the photometric experiments, from which $\varkappa_{10}$ and $\varkappa_1$ are determined are shown in Tables 8 and 9. From these values the curves shown in Figs. 12 and 13 are constructed.

In order to estimate $\varkappa_3$ a series of experiments was done, the results of which are shown in Table 10. Fig. 14 gives the results at 400 mμ. Three curves are drawn, which result if ε_2 is given the values 2.4, 2.5 and 2.6. Only the value 2.5 gives a straight line, and thus this value was taken as the right one. From this straight line ε_3 and $\varkappa_3$ were calculated. The value of $\varkappa_2 = 34$, which was determined potentiometrically, has been used in this calculation. In the same manner a straight line was constructed from the values at 425 mμ.

In the least acidic solution it is seen, that FeR_3 is almost the only complex present. Thus the extinction curve of this solution could be used to determine the extinction maximum.

Table 11 shows the collected results from the potentiometric and photometric determinations.

Table 11. PAS. Survey of the potentiometric and photometric determinations.

Complex	Potentiometric.	Photometric.		
	$\varkappa$	$\varkappa$	ε	λ_{max}
FeRH	1 700 ± 200	1 500 ± 200	1.33	510
FeR	1 800 ± 500	1 200 ± 200	2.8	460
FeR_2	34 ± 10	—	3.4	470
FeR_3	—	$3.4 \cdot 10^{-4}$ $\pm 1 \cdot 10^{-4}$	4.0	425

SUMMARY

The complex formation between iron(III)ion and salicylic acid or *p*-aminosalicylic acid (PAS) has been investigated at 25.0° ± 0.1° C in solutions with a concentration of ClO_4^- of 3 000 mC. Using potentiometric and photometric methods the complexity constants and the molar extinctions have been determined.

Salicylic acid forms the complexes FeR, FeR_2 and FeR_3: Table 1.

PAS forms the complexes FeRH, FeR, FeR_2 and FeR_3: Table 11.

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The Chemistry of the Natural Order Cupressales

XI. Heartwood Constituents of *Chamaecyparis nootkatensis* (Lamb.) Spach. Nootkatin

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The tropolone nootkatin, $C_{15}H_{20}O_2$, has been shown to have structure (I). Nootkatin yields a glycol (XI) which on oxidation with periodic acid gives acetone; in addition *iso*butyric acid is obtained on oxidation with chromic acid. Rearrangement of either of the two nootkatin methyl ethers (II) with sodium methoxide yields a mixture of two isomeric acids, 3-*iso*propyl-4-(3′-methylbut-2′-enyl)-benzoic acid (III), and 3-*iso*propyl-4-(3′-methylbut-1′-enyl)-benzoic acid (IV), which are reduced to the same dihydrocompound, 4-*iso*amyl-3-*iso*-propyl-benzoic acid (V). On nitric acid oxidation all three acids yield 3,3-dimethylphthalide-5-carboxylic acid (VI), which has been synthesised by oxidation of *iso*propyl-*p*-xylene (V), obtained by Friedel-Crafts' reaction between *p*-xylene and *iso*propyl chloride. Further oxidation of (VI) yields trimellitic acid (VIII). Decarboxylation of (V) with copper chromite and quinoline affords *o*-*iso*amyl-cumene (IX) which was not isolated, but was oxidised to phthalic acid (X). It follows from this series of reactions that nootkatin is β-*iso*propyl-γ--(3-methylbut-2-enyl)-tropolone (I). Preliminary accounts of the investigation have been published [8,10].

The isolation of the tropolone nootkatin (I), $C_{15}H_{20}O_2$, from the heartwood of *Chamaecyparis nootkatensis* has been described [1] and a preliminary note on its chemistry has appeared [8] together with a report of an X-ray examination of its copper complex, $C_{30}H_{38}O_4Cu$, kindly carried out by Campbell and Robertson [2]. Recently the isolation of nootkatin from the heartwood of *Cupressus macrocarpa* Hart. has been reported [3] *.

The tropolone nature of nootkatin is clearly shown by the U.V. [4] and I.R. [5] spectra, and the X-ray studies favour structure (I) for nootkatin.

The molecular formula, the tropolone nature of nootkatin and the fact that on complete hydrogenation it absorbs five moles of hydrogen, indicate that

* The genera *Chamaecyparis* and *Cupressus* are botanically very closely related and nootkatin may be expected to have a more widespread occurence in this group of the N. O. Cupressales.

nootkatin contains an exocyclic double bond, and hence is monocyclic. The U.V. spectrum shows that this double bond is not conjugated with the nucleus. It might be expected that it would be possible to hydrogenate this exocyclic double bond without at the same time reducing the ring system. Such however is not the case, and even when a mild palladised charcoal catalyst in ethanol is used, continuous absorption of hydrogen takes place, four moles being absorbed rapidly, and the fifth, probably reducing the carbonyl group, slightly less quickly. Even when the reaction is stopped after four moles have been absorbed it is not possible to obtain the ketonic octahydro-derivatives uncontaminated with the decahydronootkatins. That the tropolone ring is approximately equally reactive as the exocyclic double bond to catalytic hydrogenation is an interesting indication of the lability of this system, and demonstrates the relatively low reactivity of the double bond.

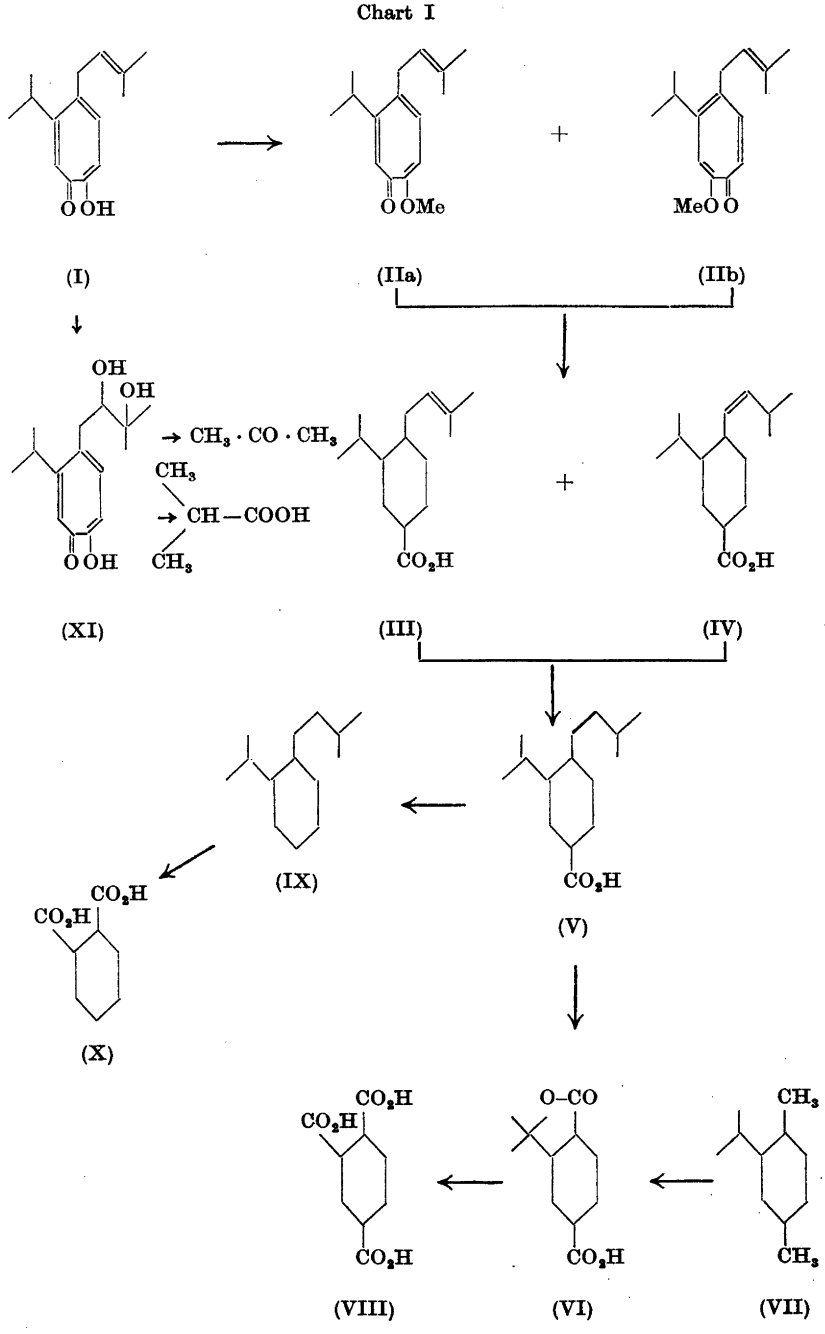
The presence of the double bond is also indicated by the ready formation of a glycol monoformate when nootkatin is treated with hydrogen peroxide in formic acid. The monoformate is readily hydrolysed to the parent glycol (XI), which is cleaved by periodic acid, affording acetone in good yield as the only identified oxidation product. Permanganate oxidation of the decahydronootkatin mixture and subsequent ring closure of the acid product yields ketonic material, possibly a mixture of diastereoisomeric *iso*propyl*iso*amyl-*cyclo*hexanones. Oxidation of the glycol with chromic acid yields *iso*butyric acid in addition to acetone.

The above evidence indicates that nootkatin is a tropolone containing one or two side-chain substituents, but does not indicate which of the possible formulae is the most likely. An *iso*propyl-3-methylbut-2-enyl tropolone structure seemed most satisfactory, and Campbell and Robertson suggested that formula (I) is probably the correct representation of nootkatin. This X-ray work was of considerable practical assistance in the chemical investigation, in indicating the best manner in which to employ the limited amounts of nootkatin available. A rigorous proof of this structure, by chemical methods, has now been obtained, and is summarized in Chart I. For the sake of brevity the argument will be developed on the basis of formula (I) for nootkatin.

The chemical approach used in the elucidation of the structure of the thujaplicins; oxidation of the fully saturated derivative, an α-glycol, to the corresponding pimelic acid, is unpromising in the case of a disubstituted tropolone, as it would involve the purification of complex mixtures of isomeric acids. Attention was therefore turned to the possibility of rearranging the tropolone to the corresponding benzoic acid, a type of reaction frequently observed in this field, and preliminary studies carried out with very small amounts of nootkatin, indicated that nootkatin methyl ether may be rearranged to a benzoic acid derivative containing two side chains, and also showed that nootkatin itself is unusually stable against alkaline rearrangement. This is in agreement with the observation that tropolone methyl ethers generally rearrange more readily than the parent compounds [6].

Unsymmetrically substituted tropolones will be expected to yield two isomeric methyl ethers, and this was found to be the case with nootkatin. The methylation product previously described as an oil has been separated into a crystalline and an oily methyl ether (II). The latter is probably not a pure

Chart I



compound, but in the case of nootkatin glycol both isomeric ethers were obtained pure, in the crystalline state.

Both the crystalline and oily nootkatin methyl ethers (II) could be rearranged by treatment with a methanolic solution of sodium methoxide, to give a mixture of two isomeric acids, $C_{15}H_{20}O_2$, acid A (III) and acid B (IV), in which the former greatly preponderated. The isomerism is obviously due to a migration of the side chain double bond since acid A has a maximum in the U.V. spectrum at 242 mμ whereas in acid B the maximum is at 270 mμ. This conclusion was confirmed by treating acid A with sodium methoxide, whereupon a good yield of acid B was obtained. Moreover both acids yield the same dihydro-compound (V) on catalytic hydrogenation.

That acid A, the less stable isomer, is the chief rearrangement product is significant as proof that the double bond in nootkatin occupies a similar position and is unconjugated with the ring system, in agreement with the spectral evidence. It is also of interest that rearrangement of the tropolone to the benzene ring takes place more readily than that of the unconjugated double bond to the conjugated position.

In the rearrangement reaction a quantity of oily material was obtained together with the pure acids. It could not be purified by crystallisation but yielded further quantities of acid B on treatment with alkali. Alternatively it could be oxidised to the oxidation product described below. It is obviously a mixture of the two acids.

Oxidation with dilute nitric acid of acid A (III), acid B (IV), the dihydro-acid (V), or the oily residues resulted in the production of only one identified product, an acid lactone, $C_{11}H_{10}O_4$, m.p. 265—266° (VI). Evidence of the structure of this oxidation product was obtained by synthesis from *p*-xylene which on Friedel-Crafts' reaction with *iso*propylchloride gave *iso*propyl-*p*-xylene (VII) oxidised by dilute nitric acid chiefly to the above acid lactone. The presence of the lactone ring in the oxidation product from the natural material was demonstrated by hydrolytic titration, and in view of its great stability the ring may be formulated as a γ-lactone system. It appears therefore that oxidation has been accompanied by hydroxylation, which is readily explained by the presence of an *iso*propyl substituent in nootkatin, as indicated also by the production of *iso*butyric acid on oxidation of nootkatin glycol. It is well-known that this group is easily hydroxylated, and the production of 3,3-dimethylphthalide by oxidation of *o-iso*propyl toluene affords a ready parallel with the above reaction [12].

Nitric acid oxidation of (VI) under more severe conditions yields trimellitic acid (VIII) as the sole product. The acid was characterised by the preparation of its anhydride and anhydride methyl ester, which were identical with authentic specimens obtained from β-naphthoic acid [7].

That this compound (VI) produced by the loss of four carbon atoms, is obtained from all three pure acids is proof of the occurrence of a five-carbon side chain in these acids, and hence in nootkatin. Moreover the presence of the double-bond unconjugated with the ring system in acid A, and the production of acetone on oxidation of nootkatin glycol, indicating branching of the chain, are conclusive proof that nootkatin contains a 3-methylbut-2-enyl substituent. The proof of structure of (VI) shows that the rearrangement acids,

and nootkatin also, must contain an *iso*propyl substituent. Moreover it proves that the five-carbon substituent in the rearrangement acids, and the dihydroacid, is *para* to the carboxyl group, and hence the 3-methylbut-2-enyl side chain in nootkatin is in the γ-position in the tropolone ring. But the *iso*propyl group can be in the α- or β-position as either of these compounds would, on rearrangement and oxidation, yield (VI) and trimellitic acid.

This point was settled by decarboxylation of dihydroacid A (V), whereby the carboxyl group obtained by rearrangement of the tropolone ring was removed. The resulting hydrocarbon (IX) was not isolated, but on oxidation yielded phthalic acid showing that the two alkyl substituents are adjacent to one another in the acid, and hence in nootkatin. With this additional evidence acid A may be formulated as 3-*iso*propyl-4-(3'-methylbut-2'-enyl)-benzoic acid (III), acid B as, 3-*iso*propyl-4-(3'-methylbut-1'-enyl)-benzoic acid (IV), and dihydroacid A as 3-*iso*propyl-4-*iso*amylbenzoic acid (V), and it follows, therefore, that nootkatin is β-*iso*propyl-γ-(3-methylbut-2-enyl)-tropolone (I).

EXPERIMENTAL

M. p.'s are uncorrected. "Petrol" refers to the fraction having b. p. 40—60°.

Nootkatin was isolated by essentially the same procedures as previously described [1]. It could also be obtained from the preliminary ethereal extract, by working up in the manner described for the acetone extract.

The *copper complex*, m. p. 234—235°. (Found: C 68.5; H 7.16; ash 15.1. $C_{30}H_{38}O_4Cu$ requires: C 68.5; H 7.28; ash (CuO) 15.1) has been submitted to X-ray crystallographic analysis by Prof. J. M. Robertson [2].

Nootkatin glycol. A solution of hydrogen peroxide (43.5 mg, 1 mole) in formic acid (85 %, 3 ml) made by dilution of a standardised solution was added to nootkatin (0.3 g). Heat was evolved on mixing and the solution darkened slightly in colour. The mixture was cooled until the reaction moderated, then heated at 40° for two hours, with intermittent shaking. Water (25 ml) was added followed by a few drops of dilute sulphuric acid, and the solution was shaken vigorously and set aside until precipitation was complete. The crystalline material was collected by filtration (250 mg, m. p. 160—165°) and after crystallisation from methanol formed colourless needles of *nootkatin glycol monoformate* m. p. 170—171°. (Found: C 65.4; H 7.43. $C_{16}H_{22}O_5$ requires: C 65.3; H 7.53). Light absorption: λ_{max} 241, 322 mμ; logε_{max} 4.52, 3.97. λ_{min}. 274 mμ; logε_{min} 3.14. This substance gave an intense ferric chloride reaction, did not respond to the periodate spray test [3], and reacted with sodium periodate solution only very slowly (0.17 mole/mole after 41 hrs., 0.275 mole/mole after 90 hrs).

The monoformate (1 g) was added to a solution of potassium hydroxide (20 ml, 20 %). The solid did not dissolve completely in the cold but did so on slight warming and the resulting solution was heated in a boiling water bath for 2 hours and then cooled. No precipitate formed, so the mixture was diluted with water (50 ml), acidified with hydrochloric acid (some oil separated) and extracted with ether (4 × half volume). The ethereal extracts were combined, washed twice with a little water, dried (Na_2SO_4) and evaporated, giving a solid product which was triturated with petrol several times to remove traces of formic acid: m. p. 114—117°: yield 750 mg. Crystallisation from acetonitrile gave large needles which, however, melted at approximately 82—84° the actual melting point depending on the rate of heating and never being very sharp, and clearly contained some solvent of crystallisation. (Found: C 63.9; H 7.87.) If this material is dried at 70° in a high vacuum it gives *nootkatin glycol* m. p. 118—119°. (Found: C 68.1; H 8.29. $C_{15}H_{22}O_4$ requires: C 67.6; H 8.33.) This substance gives an intense ferric chloride reaction, and a positive result in the periodate spray test. It is soluble in polar organic solvents, and sparingly soluble in hot water. It is soluble to some extent in sodium bicarbonate solution and can be extracted from ethereal solution by this reagent. The sodium salt is very sparingly soluble in dilute sodium hydroxide solution, and if the potassium

hydroxide used in the hydrolysis of the monoformate is replaced by sodium hydroxide, a precipitate forms during the reaction and it is more difficult to work up the product.

Nootkatin glycol methyl ether. Nootkatin glycol (200 mg) was dissolved in ether (30 ml), and an excess of diazomethane in ether (10 ml) added. After standing for 2 days in the refrigerator white crystalline material was found to have separated and was collected. Yield, 174 mg. (Found: OMe 11.8. $C_{16}H_{24}O_4$ req.: OMe 11.1.) Evaporation of the ethereal solution afforded an oil which solidified on rubbing with petrol, and was crystallised from ethyl acetate-ether. Yield, 21 mg. M. p. 113—126°.

Examination of the main fraction under the microscope showed that it consisted of two crystalline species, large prisms and smaller less well defined crystals. They were separated by hand, and the former were crystallised from ether in prisms, m. p. 129—130°. (Found: C 68.9; H 8.68. $C_{16}H_{24}O_4$ req.: C 68.6; H 8.57). The other material crystallised from ethyl acetate in prisms, m. p. 161—2°. (Found: C 68.7; H 8.63). When mixed the substances melted at 124—129°.

Oxidation of nootkatin glycol with chromic acid. Sodium dichromate (1.8 g) in water (4 ml) was added slowly with vigorous stirring and cooling to a solution of nootkatin glycol (250 mg) in 50% sulphuric acid (15 ml). A vigorous reaction took place with the evolution of a heat and a brisk evolution of gas. When the reaction moderated the mixture was set aside at room temperature for 2.5 hours after which time excess chromic acid was still present. The solution was diluted with water (40 ml) and distilled in steam in a steamed out apparatus until the distillate was no longer acid (total vol. *ca.* 250 ml). The residual solution was continuously extracted with ether for 16 hrs. but no acidic material was present in the ether extract. The above steam distillate was neutralised with standard sodium hydroxide (required 8.3 ml 0.1083 *N* NaOH, corresponding to 0.96 moles acid per mole of nootkatin glycol) and the solution was concentrated to half volume, the distillate being allowed to run into a solution of 2,4-dinitrophenylhydrazine hydrochloride in water. The orange precipitate that formed was collected by filtration and crystallised from alcohol forming long needles of *acetone 2,4-dinitrophenylhydrazone*, m. p. 124—125° undepressed in admixture with an authentic specimen. The yield of crude hydrazone (m. p. 121—122°) was 73 mg (0.32 mole/mole glycol). The neutral solution from which the acetone had been distilled was evaporated to dryness on a water bath. Paper chromatography using propanol-ammonia-water (80 : 4 : 16) as solvent and ninhydrin as spraying reagent revealed the presence of at least two acids with R_F-values corresponding to acetic (or formic) and butyric (or *iso*butyric) acids.

The mixed sodium salts were dissolved in the minimum amount of the same solvent mixture and transferred to the top of a column of powdered cellulose (15 × 1.5 cm) which had been packed in the dry state and then washed thoroughly with the solvent. The column was run with the same solvent and the effluent was collected in fractions of *ca.* 2 ml. These fractions were spotted on filter paper and treated with the ninhydrin reagent and those which gave a positive reaction were treated with 0.5 ml of 0.1 *N* NaOH and evaporated to dryness in a stream of air, and then samples were run on paper chromatograms in the usual way. The elution of the column was continued only until the faster moving component of the original mixture had been eluted and this appeared to be recovered in a pure state. The fractions containing this material were combined, dissolved in a little water, and passed through a column of Amberlite IR 120 ion exchange resin to remove the sodium ions. The eluate from this column was neutralised with standard alkali and evaporated to dryness on the water bath. Paper chromatography showed that it contained only one component and that this travelled at the same rate as *iso*butyric acid. The sodium salt was converted into the *p*-bromophenacyl ester using the calculated amount of *p*-bromophenacyl bromide and the ester isolated in the usual way and crystallised from aqueous alcohol giving the *p-bromophenacyl ester of isobutyric acid*, m. p. 76° undepressed in admixture with an authentic specimen.

Periodate oxidation of nootkatin glycol. Sodium periodate solution (5 ml, 0.1 *M*) was added to a solution of nootkatin glycol (100 mg) in pure methanol (5 ml) and the mixture was set aside at room temperature overnight, during which time some inorganic material separated. Water was added and the solution was distilled, the distillate being run into excess 2 : 4-dinitrophenylhydrazine hydrochloride solution. Distillation was continued until no further reaction occurred and the crystalline material was collected and crystallised from methanol giving yellow needles of acetone 2 : 4-dinitrophenyl hydrazone (53 mg, 60 %) m. p. 124—125°, undepressed on admixture with an authentic specimen.

The residue in the distilling flask was acidified with dilute sulphuric acid, and the gummy material collected by extraction with ether (4 × equal vol.). Evaporation of the dried ethereal solution gave a gum which solidified on trituration with petrol. This material reacted with Brady's reagent, and after crystallisation from acetonitrile melted at 128—129°.

Oxidation of decahydronootkatin. A solution of sodium periodate (60 ml 0.0967 *M*, 1 mole) was added to a solution of decahydronootkatin (1.38 g) in methanol (75 ml), shaken at room temperature for 4 hours, and then set aside for 2 days. Solid inorganic material which formed was removed and the solution concentrated under reduced pressure. The oil which separated was collected with the aid of ether and the ethereal extracts were combined, washed with water, dried (Na_2SO_4) and evaporated to a colourless oil which was suspended in water (50 ml). Sodium carbonate (1 g) and potassium permanganate (1.4 g) were added and the mixture was shaken overnight (unreacted permanganate was still present). The solution was treated with sulphur dioxide in the usual manner, a little dilute sulphuric acid was added and the mixture was extracted with ether (2 × equal volume). The ether extracts were extracted with sodium bicarbonate and the acidic material recovered by acidification and extraction with ether. Evaporation of the dried ethereal solution gave a colourless oil which was distilled in a high vacuum giving a very viscous gum (0.69 g) which did not crystallise even on long standing, and is presumably a mixture of isomers.

The barium salt of this acid mixture (2 g) was dry distilled in a stream of nitrogen at 300—320° and the pale yellow oily distillate was taken up in ether. The ethereal solution was washed once with a little sodium bicarbonate, then with water, dried (Na_2SO_4) and evaporated and the residue was distilled *in vacuo* giving a pale yellow liquid, n_D^{19} 1.4688 (223 mg).

A sample of the ketonic material was treated with an ethanolic solution of 2,4-dinitrophenylhydrazine hydrochloride in the usual way and the dinitrophenylhydrazone which precipitated was collected and crystallised from a large volume of alcohol forming yellow plates, m.p. 171—172° the analysis of which was in reasonable agreement with that expected for an *iso*propyl*iso*amyl*cyclo*hexanone dinitrophenylhydrazone. (Found: C 58.75; H 7.38; N 13.6. $C_{30}H_{30}O_4 . H_2O$ requires: C 58.8; H 7.90; N 13.7.)

Nootkatin methyl ether previously described as a gum was prepared on a larger scale, and the product distilled in high vacuum. The resultant pale yellow oil (Found: OMe 12.3. Calc. for $C_{16}H_{22}O_2$: OMe 12.6) was chromatographed on an alumina column using the method of fractional elution. Coloured end fractions were obtained but the compound came off the column over a wide range, and no crystalline material was obtained. The end fractions were rejected and the others were combined and distilled. The resultant oil was allowed to stand for some weeks whereupon long needle-shaped crystals appeared. They were collected and crystallised from petrol in needles, m.p. 72°. (Found: C 78.0; H 8.88; OMe 12.6. $C_{16}H_{22}O_2$ req.: C 78.0; H 8.94; OMe 12.6). The residual oily methyl ether could not be obtained crystalline.

Action of sodium methoxide on nootkatin methyl ether. Pure crystalline nootkatin methyl ether (600 mg) was dissolved in a solution of sodium methoxide (from sodium (1.2 g) in pure methanol (20 ml)), giving a dark red solution, and was heated in a sealed tube at 145° for 14 hours. The solvent was removed under reduced pressure and water added until the residue was completely in solution. The solution was extracted with ether, and the ethereal extract washed with water, dried ($NaSO_4$), and the solvent removed to give a dark oil (115 mg) from which it was not found possible to obtain an identifiable product.

The aqueous solution was acidified with sulphuric acid and extracted exhaustively with ether. The ethereal solution was extracted with sodium bicarbonate solution, and then with sodium carbonate solution.

The aqueous sodium bicarbonate extract was acidified, extracted with ether, the ethereal extract washed, dried (Na_2SO_4), and the solvent removed to yield a dark oil (61 mg) which was distilled to give a pale yellow gum. No pure material could be obtained from the gum, but it appeared to be a mixture of the acids described below.

The aqueous sodium carbonate extract was worked up in the same way to yield a dark oil (357 mg) which on distillation *in vacuo* gave an oil which crystallised on standing. The solid was crystallised from petrol to give *acid A* (III) (207 mg) in thick plates, m.p.

114—116°. Concentration of the petrol mother liquour gave a small quantity of impure *acid B* (IV) in needles, m.p. 84—92°. An oily acid residue from which pure material could not be obtained except by conversion to acid B, or by oxidation (see below) was obtained from the mother liquors.

Treatment of the oily nootkatin methyl ether in the manner described above afforded the same products:

Acid A, 3-*iso*propyl-4-(3′-methylbut-2′-enyl)-benzoic acid (III) m.p. 114—116°; λ_{max} 242 mμ; log ε_{max} 4.1; inflection 275 mμ; log ε 3.4. (Found: C 77.9; H 8.58. $C_{15}H_{20}O_2$ req.: C 77.55; H 8.68).

Acid B, 3-*iso*propyl-4-(3′-methylbut-1′-enyl)-benzoic acid (IV), was obtained from the mother liquors from the rearrangement of nootkatin methyl ether (10 g). It crystallised in needles, m.p. 108—109°; λ_{max} 270 mμ; log ε_{max} 4.0. (Found: C 77.8; H 8.50; Eq. wt. 238. $C_{15}H_{20}O_2$ req.: C 77.55; H 8.68.)

On admixture the acids melted at 96—106°, with softening at 80°.

Dihydro-acid A, 4-isoamyl-3-*iso*propyl-benzoic acid (V). On hydrogenation in ethyl acetate, using a palladium charcoal catalyst, both acid A and acid B absorbed one mole of hydrogen rapidly, after which no further absorption took place. Both acids gave the same product, *dihydro-acid A*, which crystallised from petrol in fine needles, m.p. 126—127.5°. (Found: C 77.35; H 9.40. $C_{15}H_{22}O_2$ req.: C 76.9; H 9.40.)

Conversion of acid A to acid B. Acid A (45 mg) was dissolved in a solution of sodium methoxide, (from sodium (100 mg) in methanol (2 ml)) and heated in a sealed tube at 145° for 15 hours. On cooling sodium salts separated but were dissolved by the addition of water, and the solution was acidified and extracted with ether. The ethereal extract was washed with water and dried ($CaCl_2$,) the solvent removed and the product crystallised from petrol to give acid A (3 mg) and acid B (28 mg), identified by melting point and mixed melting point with an authentic specimen.

Acid B could be obtained by treatment, as above, of the impure residues from the rearrangement of nootkatin methyl ether.

Oxidation of acid A, acid B, and dihydro-acid A. Acid B (100 mg) was added to a solution of conc. nitric acid (3.5 ml) in water (6.5 ml) and heated in a sealed tube at 146° for 16 hours. The resultant solution on cooling to 0° deposited crystalline material (32 mg) which was collected, and purified by sublimation at 196—205°/4 mm, yielding white clusters of plates, m.p. 265—266°; equivalent weight 209; equiv. wt. by hyrolytic titration 98; λ_{max} 240, 285 mμ; log ε_{max} 4.2, 3.43. (Found: C 63.9; H 5.3. $C_{11}H_{10}O_4$ req.: C 64.08; H 5.3. Equiv. wt. 206; Equiv. wt. by hydrolytic titration 103.) On concentrating the mother liquors a solid residue was obtained from which pure compounds could not be obtained.

Oxidation of acid A (110 mg) in the same manner gave the same acid lactone (40 mg) m.p. and mixed m.p. 262°: methyl ester, m.p. 141°.

Oxidation of dihydro-acid A (85 mg) gave the same acid lactone (30 mg), m.p. and mixed m.p. 263°, equiv. wt. 213; methyl ester, m.p. 143°.

The acid lactone could also be obtained by oxidation of the oily residues from the oxidation of nootkatin methyl ether, and the bicarbonate soluble fraction.

Methyl ester of the acid lactone. The acid lactone dissolved or suspended in ether was treated with excess of diazomethane in ether, the volatile compounds removed, and the residue sublimed at 120°/4 mm. The crystalline sublimate was crystallised from ether in needles, m.p. 143°. (Found: C 65.35; H 5.61; OMe 14.14. $C_{12}H_{12}O_4$ req.: C 65.5; H 5.48; OMe 14.09.)

The acid lactone was shown to be 3 : 3-dimethylphthalide-5-carboxylic acid, and will be referred to as such below.

Isopropyl-p-xylene: cf. Nightingale and Carton [12]. *Iso*propyl chloride (40 g) was added with stirring over 1 hr., to a mixture of *p*-xylene (106 g) and finely powdered aluminium chloride (30 g). Stirring was continued for a further 3 hrs and the product worked up in the usual manner, and roughly fractionated by distillation through a 30 cm column packed with glass cylinders. The fraction (33 g), b.p. 190—200° was distilled through a Vigreux column, yielding *iso*propyl-*p*-xylene (18.4 g) b.p. 207—214°, n_D^{22} ,1.4964. (Found: C 89.02; H 10.70. $C_{11}H_{16}$ requires: C 89.12; H 10.88.)

*3,3-dimethylphthalide-5-carboxylic acid. Iso*propyl-*p*-xylene (0.5 ml) was heated with dilute nitric acid (10 ml, 1 : 2) for 15 hrs, at 145°. The reaction mixture was cooled and the crystalline material (200 mg) collected, treated with charcoal, and crystallised from

water, yielding 3,3-dimethylphthalide-5-carboxylic acid, m.p. 263—265°, undepressed on admixture with the acid lactone obtained from nootkatin as described above. Treatment with diazomethane afforded the methyl ester, m.p. 140—142°, undepressed on admixture with a specimen obtained from nootkatin.

The mother liquors from the oxidation were concentrated to *ca.* 2 ml volume, cooled, and the crystalline material (136 mg) collected, and crystallised from water in needles, which did not melt but sublimed above 300°. Treatment with diazomethane afforded an ester, m.p. 107—108°. The identity of this compound was not established.

Oxidation of 3,3-dimethylphthalide-5-carboxylic acid. The above acid (73 mg) was heated with a solution of conc. nitric acid (4 ml) in water (8 ml) at 172° for 12 hours, in a sealed tube. On concentrating the solution to a small volume trimellitic acid (63 mg) crystallised and was collected. It had m.p. 210—216°, and equiv. wt. 80. (Required equiv. wt. 70.)

The acid was sublimed at 170—220°/4 mm yielding the anhydride m.p. 162° undepressed on admixture with an authentic specimen.

The acid anhydride was treated with diazomethane in the usual way, yielding the anhydride methyl ester, m.p. 104—105° after sublimation and crystallisation from ether. The melting point was undepressed on admixture with an authentic sample.

Trimellitic acid.(*cf.* Ekstrand[7]). β-Naphthoic acid (10 g) was dissolved in a solution of sodium hydroxide (2.5 g) in water (160 ml). The solution was heated on the waterbath and a warm saturated aqueous solution of potassium permanganate was added in portions, with shaking, until a permanent red colour was obtained. Excess reagent was destroyed by the addition of ethanol, and the manganese sludge was separated by filtration. The filtrate was made acid (H_2SO_4) and concentrated until salts began to separate. It was extracted continuously with ether, and the ethereal solution was evaporated to dryness. The residue was heated at 200°/2 mm until no more phthalic anhydride sublimed. The residue was dissolved in water, treated with charcoal, the charcoal separated, and the solution evaporated to dryness. The residue was sublimed at 2 mm pressure by heating gently with a free flame, giving trimellitic anhydride, which crystallised from ether-petrol in needles, m.p. 162—164°.

Treatment of the anhydride with diazomethane in the usual way gave trimellitic anhydride methyl ether, which crystallised from ether-petrol in needles, m.p. 100—103°.

Decarboxylation of dihydro-acid A: Dihydro-acid A (581 mg), copper chromite (200 mg) and quinoline were heated at 230°. Evolution of carbon dioxide commenced about 200°, and became vigorous at 230°. After 1 1/4 hrs evolution had slowed up appreciably, and after a further 1/2 hr appeared to have ceased. The reaction mixture was cooled, diluted with ether (100 ml), filtered, and the ethereal solution washed with 5 *N* hydrochloric acid (5 × 1/2 vol.), then with sodium bicarbonate solution (3 × 1/2 vol.) and finally with water. The solvent was removed yielding a dark oil with an aromatic hydrocarbon odour, which was not further purified, but was oxidised as described below.

Oxidation of decarboxylation product: The above oil was heated with dilute nitric acid (10 ml 1 : 2) at 145° for 15 hrs. The resulting solution was freed from traces of gummy material, concentrated to small volume (*ca.* 1 ml), and final traces of solvent removed under reduced pressure. The gummy solid residue was distilled in four portions at 1 mm pressure. An oil was obtained which crystallised on standing. The crystalline material was freed from oil (probably impure 3,3-dimethylphthalide) on a porous plate, and sublimed by heating on the waterbath, at water-pump pressure. Phthalic anhydride was obtained in long needles, m.p. and mixed m.p. 129—130°. The identity of the phthalic anhydride was checked by the preparation of fluorescein, and of phthalanil, m.p. 204—204.5°, mixed m.p. 204—205° with an authentic specimen of m.p. 205°.

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Short Communications

On the Preparation of bis-[Trimethylsilylmethyl]-acetic acid

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In a newly published paper [1] Sommer and co-workers reported that they were unable to prepare diethyl bis-[trimethylsilylmethyl]-malonate from diethyl trimethylsilylmethylmalonate and chloromethyltrimethylsilane. I consider that this was due to the unreactivity of the chlorine atom in chloromethyltrimethylsilane and that better results might be expected if the corresponding bromide was used instead.

In connection with other work [2] on aliphatic organosilicon acids the synthesis of diethyl bis-[trimethylsilylmethyl]-malonate from diethyl trimethylsilylmethylmalonate and bromomethyltrimethylsilane has been carried out successfully, the yield being 73 % (based on unrecovered material). The malonic ester was readily hydrolysed and decarboxylated to give bis-[trimethylsilylmethyl]-acetic acid in 93 % yield.

When treated with concentrated sulphuric acid at room temperature the acid liberated methane amounting to 80 % of the theoretically calculated volume (two moles of methane per mole of acid). Heating to 100° caused discolouration of the reaction mixture and carbon dioxide and sulphur dioxide were evolved.

Experimental. Diethyl bis-[trimethylsilylmethyl]-malonate. In a 250 ml flask equipped with a stirrer, a reflux condenser, a dropping funnel, and a thermometer reaching into the flask 4.6 g (0.200 g at.) of sodium was dissolved in 70 ml of absolute ethanol. 49.2 g (0.200 mole) of diethyl trimethylsilylmethylmalonate was added in one portion, followed by the addition of 33.4 g (0.200 mole) of bromomethyltrimethylsilane during two hours. The temperature of the reaction mixture was maintained at 70—80°. The reaction mixture was then heated under reflux for twenty-four hours. After cooling it was neutralized with glacial acetic acid, the alcohol was distilled off, and 150 ml of water was added. The oil was taken up in ether, the ether solution washed with water and finally dried with anhydrous calcium chloride. After distilling off the ether the residue was fractionated through a glass-helix-packed column of about ten theoretical plates. There was obtained 25.6 (0.077 mole) of diethyl bis-[trimethylsilylmethyl]-malonate, b. p. 108—109°/1.5 mm, n_D^{20} 1.4483, d_4^{20} 0.9508. 23.2 g of diethyl trimethylsilylmethylmalonate was recovered. The yield, based on unrecovered material was 73 %. (Found: C 54.0; H 9.6; Si 16.7; r_D 0.2823. Calc. for $C_{15}H_{32}O_4Si_2$: C 54.2; H 9.7; Si 16.9; r_D 0.2817).

Bis-[trimethylsilylmethyl]-malonic acid. Diethyl bis-[trimethylsilylmethyl]-malonate was hydrolysed by boiling with excess alcoholic potassium hydroxide for twenty-four hours to give a quantitative yield of the corresponding acid. A small amount was recrystallized from acetone, m. p. 165—166° (decomp.). (Found: C 47.9; H 8.8; Si 20.5. Calc. for $C_{11}H_{24}O_4Si_2$: C 47.8; H 8.8; Si 20.3.)

Bis-[trimethylsilylmethyl]-acetic acid. The crude bis-[trimethylsilylmethyl]-malonic acid was decarboxylated by heating to 170—180° for twenty minutes. After cooling, the oil was dissolved in ether, the ether solution was filtered, and the ether was distilled off. The remaining oil was distilled *in vacuo* and there was obtained a 93 % yield of bis-[trimethylsilylmethyl]-acetic acid, b. p. 123—124°/1 mm. The compound solidified after standing overnight, m. p. 41—42°. One recrystallization from petroleum ether did not raise the melting point. (Found: C 51.7; H 10.5; Si 24.1; Eq.wt. 232.3. Calc. for $C_{10}H_{24}O_2Si_2$: C 51.7; H 10.4; Si 24.2; Eq. wt. 232.5.)

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Über die Reaktionsprodukte von einigen *n*-Butoxy-chlor-silanen mit Ammoniak und Äthylamin

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Im folgenden wird in aller Kürze über einige vorläufige Versuche berichtet, die ausgeführt wurden, um Verbindungen der allgemeinen Formel $(n\text{-}C_4H_9O)_{4-x}Si(NH.R)_x$ darzustellen. R ist ein Wasserstoffatom oder ein Kohlenwasserstoffrest, x = 1, 2 oder 3.

Tri-*n*-butoxy-chlor-silan ergibt mit Ammoniak Tri-*n*-butoxy-amino-silan, ein unter vermindertem Druck destillierbares Öl. Bei längerem Erhitzen wird Ammoniak unter Bildung von Hexa-*n*-butoxy-disilazan abgespaltet. Mit Äthylamin ergibt Tri-*n*-butoxy-chlor-silan Tri-*n*-N-äthylamino-silan.

Di-*n*-butoxy-dichlor-silan ergibt mit Ammoniak kein Di-*n*-butoxy-diamino-silan. Man erhält statt dessen Hexa-*n*-butoxy-*cyclo*trisilazan. Mit Äthylamin erhält man in normaler Weise Tri-*n*-butoxy-N-äthylaminosilan, das keine Neigung zu Cyclisierung zeigt.

Aus den Reaktionsprodukten von *n*-Butoxy-trichlor-silan und Ammoniak wurden keine einheitlichen Verbindungen isoliert. Das erwartete *n*-Butoxy-triamino-silan hat wohl unter Ammoniakabspaltung und Cyclisierung unlösliche und undestillierbare Produkte ergeben. *n*-Butoxy-trichlor-silan ergibt dagegen mit Äthylamin *n*-Butoxy-tri-N-äthylamino-silan, das ohne Schwierigkeit in guter Ausbeute isoliert werden kann.

*Beschreibung der Versuche: Tri-*n*-butoxy-amino-silan.* 70,4 g Tri-*n*-butoxy-chlor-silan in 100 ml abs. Äther wurden unter Kühlung und Rühren zu 50 g flüssigem Ammoniak und 100 ml abs. Äther hinzugetropft. Das Ammoniumchlorid wurde abgesaugt und mit abs. Äther gewaschen. Die Ätherlösungen ergaben nach Abdestillation des Äthers und Destillation des Rückstandes unter vermindertem Druck 49,5 g Tri-*n*-butoxy-amino-silan vom Sdp. 129—131° (13 mm). Gef.: C 54,2; H 11,2; N 5,25; Si 10,63. Ber. für $C_{12}H_{29}O_3NSi$: C 54,7; H 11,1; N 5,32; Si 10,65.

*Hexa-*n*-butoxy-disilazan.* 10,0 g Tri-*n*-butoxy-silan wurden 10 Stunden auf 150° erwärmt. Die darauf folgende Destillation ergab ausser unverändertem Ausgangsmaterial noch 4,6 g Hexa-*n*-butoxy-disilazan vom Sdp. 210—211° (14 mm).: Gef.: C 56,4; H 10,8; N 2,77; Si 11,1. Ber. für $C_{24}H_{55}O_6NSi_2$: C 56,5; H 10,9; N 2,75; Si 11,0.

*Tri-*n*-butoxy-N-äthylamino-silan.* In eine Lösung von 56,6 g Tri-*n*-butoxy-chlor-silan in 100 ml abs. Äther wurden etwa 20 g getrocknetes gasförmiges Äthylamin eingeleitet. Am folgenden Tage wurde die Hydrochloridfällung abgesaugt und mit Äther gewaschen. Die Ätherlösungen ergaben 51,5 g Tri-*n*-butoxy-N-äthylamino-silan vom Sdp. 139—140° (15 mm). Gef.: C 57,5; H 11,5; N 4,8; Si 9,6. Ber. für $C_{14}H_{33}O_3NSi$: C 57,7; H 11,4; N 4,8; Si 9,6.

*Hexa-*n*-butoxy-cyclotrisilazan.* 61,3 g Di-*n*-butoxy-dichlor-silan in 50 ml abs. Äther und 42,5 g flüssiges Ammoniak in 50 ml Äther ergaben, wie bei dem Umsatz zwischen Tri-*n*-butoxy-chlor-silan und Ammoniak beschrieben wurde, 28,3 g Hexa-*n*-butoxy-cyclotrisilazan vom Sdp. 245—250° (12 mm). Gef.: C 50,7; H 10,2; N 7,3; Si 14,7; M(Rast) 573. Ber. für $C_{24}H_{57}O_6N_3Si_3$: C 50,8; H 10,1; N 7,4; Si 14,8; M 568.

*Di-*n*-butoxy-di-N-äthylamino-silan.* Aus 14,7 g Di-*n*-butoxydichlor-silan in 60 ml abs. Äther und etwa 12 g gasförmigem Äthylamin wurden, wie bei der Darstellung von Tri-*n*-butoxy-N-äthylamino-silan 11,6 g Di-*n*-butoxy-di-N-äthylamino-silan vom Sdp. 129—132° (15 mm) erhalten. Gef.: C 54,8; H 11,6; N 10,6; Si 10,6. Ber. für $C_{12}H_{30}O_2N_2Si$: C 54,9; H 11,5; N 10,7; Si 10,7.

n-Butoxy-trichlor-silan und Ammoniak. 20 g *n*-Butoxy-trichlorsilan in 100 ml abs. Äther und 50 g Ammoniak ergab in gewöhnlicher Weise ein Reaktionsgemisch, aus dem keine Siliziumverbindung mit Äther, Benzol oder Toluol extrahiert werden konnte.

n-Butoxy-tri-N-äthylamino-silan. Aus 20,8 g *n*-Butoxy-trichlor-silan in 100 ml abs. Äther und 28 g gasförmigem Äthylamin wurden wie bei der Darstellung von Tri-*n*-butoxy-N-äthylamino-silan 16,9 g *n*-Butoxy-tri-N-äthylamino-silan vom Sdp. 115—116° (16 mm) erhalten. Gef.: C 51,7; H 11,8; N 18,2; Si 12,0. Ber. für $C_{10}H_{27}ON_3Si$: C 51,5; H 11,7; N 18,0; Si 12,0.

Eingegangen am 8. Juni 1954.

A New α-Aminodicarboxylic Acid, α-Aminopimelic Acid, in Green Plants

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When investigating free amino acids in green plants we have found an amino acid in the 70 % alcohol extract of *Asplenium septentrionale*, which on two-dimensional chromatograms (butanol-acetic acid and phenol-NH_3) gives a spot X the location of which appears from Fig. 1.

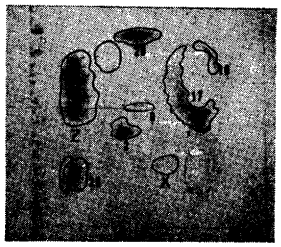

Fig. 1. Hydrolyzed alcohol extract from Asplenium septentrionale. Solvents: butanol-acetic acid and phenol-NH_3. 2 = Ala, 9 = Thr, 16 = Asp, 17 = Glu, 23 = Orn, 29 = γ-aminobutyric acid, X = new aminodicarboxylic acid, Z = unknown neutral amino acid formed through hydrolysis from an acidic aminoacid.

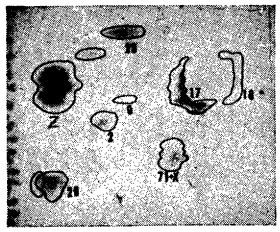

Fig. 2. As Fig. 1, but α-aminopimelic acid added to alcohol extract. 71 + X = synth. α-aminopimelic acid + the new aminodicarboxylic acid.



Fig. 3. One-dimensional chromatogram from a not hydrolyzed alcohol extract from Asplenium septentrionale. Solvent: butanol + acetic acid. 71 = synth. α-aminopimelic acid (control), X = the new aminodicarboxylic acid.

Paper electrophoresis shows that this amino acid is acidic. On the basis of the location of the spot on the chromatogram it can be suggested that the amino acid in question is α-aminopimelic acid. This amino acid has not earlier been found in any organisms as far as we know.

α-Aminopimelic acid, synthesized by Mr. M. Alfthan in this laboratory, travels exactly to the same place as our unknown amino acid on the chromatogram (Fig. 2).



Fig. 4. As Fig. 3. α-Aminopimelic acid added to alcohol extract (left), no addition (right). 71 + X = synth. α-aminopimelic acid + the new aminodicarboxylic acid, X = the new aminodicarboxylic acid. 17 = Glu + an unknown compound, which by hydrolysis gives an unknown neutral amino acid Z (cf. Figs. 1 and 2.)

When acidic amino acids are separated from the neutral and basic ones by an ion exchange treatment (Amberlite IR 4B) of the alcohol extract of *Asplenium*, and an one-dimensional chromatogram is made from the fractions obtained, using butanol-acetic acid as solvent, the unknown amino acid and α-aminopimelic acid cannot be separated from each other, even if the run is continued until the spots are at the lower end of a long paper strip (Figs. 3 and 4).

From chromatographic and paper electrophoretic runs we conclude that the new amino acid in *Asplenium septentrionale* is α-aminopimelic acid. The difficulty in obtaining enough of plant material has so far prevented us from isolating the acid in pure form. We think, however, that there is a good reason to publish these results while waiting for more plant material.

Later on one of us (V) will give an account of another new acidic amino acid found in different species of *Asplenium*.

Received June 3, 1954.

Reactions between Triethylsilane and Some Organic Bromides

HENRIK WESTERMARK

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It is well known that hydrogen bonded to silicon in alkyl-, and arylsilanes can be hydrolysed to silanols in concentrated alcoholic potassium hydroxide solution with the evolution of hydrogen [1], and it also reacts with chlorine, bromine [2], and iodine [3] to give the corresponding halogen silanes and hydrogen halides. Jenkins and Post [2] investigated the reactions of triethylsilane and tribenzylsilane with benzoyl chloride, benzoyl bromide and some derivatives of these compounds. Reductions to aldehydes were observed except in a few cases. Whitmore, Pietrusza, and Sommer [4] reported that in the presence of aluminium chloride hexyl chloride reacted with triethylsilane yielding triethylchlorosilane and hexane.

During work on organic silicon hydrides, I found that the bromine in allyl bromide and methyl α-bromopropionate could react with triethylsilane in the absence of aluminium chloride. The compounds were reduced to propene and methyl propionate according to the equations

$$(C_2H_5)_3SiH + BrCH_2 \cdot CH{:}CH_2 \rightarrow (C_2H_5)_3SiBr + CH_3 \cdot CH{:}CH_2 \quad (1)$$

$$(C_2H_5)_3SiH + BrCH_2 \cdot CH_2 \cdot COOCH_3 \rightarrow (C_2H_5)_3SiBr + CH_3 \cdot CH_2 \cdot COOCH_3 \quad (2)$$

The reactions were followed by iodine titration of the remaining triethylsilane. (See below). It does not seem likely that other reactions than those given by the formulae, *e. g.* splitting off hydrogen bromide, had occurred, because the reaction products were isolated in amounts which roughly corresponded to those calculated from the titrations. For *n*-propyl bromide the reaction was very slow.

It is not unlikely that reactions of the type described here may be extended to preparative methods.

Experimental. All syntheses were carried out on a steam bath in an atmosphere of dry nitrogen.

Allyl bromide and triethylsilane. 5.8 g (0.05 mole) of triethylsilane and 6.1 g (0.05 mole) of allyl bromide were refluxed. After 15 hours the silane was completely (97 %) transformed into triethylbromosilane as determined by iodine titration. The reaction mixture was distilled to yield 8.2 g of triethylbromosilane, b.p. 50—51° (11 mm). The equivalent weight was determined by alkali titration. Found 195.1; Calc. 195.2. According to eq. (1) a gas was evolved during the synthesis which decolorized bromine water.

Methyl β-bromopropionate and triethylsilane. 5.8 g (0.05 mole) of triethylsilane and 8.35 g (0.05 mole) of methyl β-bromopropionate were heated together. After 20 hours 40 %, and after 44 hours 50 % of the initial amount of the silane had reacted. Distillation yielded 1.5 g of methyl propionate, b. p. 80—81°.

n-Propylbromide and triethylsilane. 0.025 mole of each was refluxed on the steam bath. After 22 hours about 10 % of the triethylsilane had reacted.

Determination of triethylsilane by iodine titration can be performed in the following way: Into 50 ml of ethyl alcohol and 40 ml of 0.1 *N* iodine solution (containing potassium iodide) 50—70 mg of triethylsilane was introduced. After two hours 10 ml of 5 *N* hydrochloric acid

When acidic amino acids are separated from the neutral and basic ones by an ion exchange treatment (Amberlite IR 4B) of the alcohol extract of *Asplenium*, and an one-dimensional chromatogram is made from the fractions obtained, using butanol-acetic acid as solvent, the unknown amino acid and α-aminopimelic acid cannot be separated from each other, even if the run is continued until the spots are at the lower end of a long paper strip (Figs. 3 and 4).

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was added and the excess of iodine was determined with 0.1 *N* sodium thiosulphate. The sample had to be so small that less than half of the iodine was consumed. In this way the following values of the equivalent weight were obtained in different titrations: 118.3, 118.3, 118.9. Calculated for triethylsilane: 116.3.

When determining the amount of triethylsilane in the syntheses described above, a sample of about 0.2 ml was removed for titration. One test was made when the reaction components were mixed, and from this zero value the composition of the mixture could easily be determined at any time by titration.

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A Comment on the Ultraviolet Light Absorption Spectrum of Cytochrome c

K. G. PAUL and H. THEORELL

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The light absorption spectrum of ferrocytochrome c at neutral pH shows an absorption band at 316 mμ [1] in addition to the protein band at 280 mμ, the Soret band, and the bands in visible light. The 316 mμ-band has been interpreted as indicating the presence of a sulphoxide group [2]. This interpretation became unprobable when the six sulphur atoms in cytochrome c could be accounted for (two thioether, two methionine, and two cystine sulphur atoms) [3,4,5]. We have examined the spectra in the near ultraviolet range of the histidine and ammonia ferri and ferro meso-, proto-, and deuteroporphyrins. The results are given in Table 1 and Fig. 1.

Table 1. Wavelengths in mμ of absorption maxima.

Species	Histidine		Ammonia	
	Ox.	Red.	Ox.	Red.
Proto	Shoulder	327	Shoulder	327
Meso	347	322	350	317
Deutero	337	320	340	315

The solutions (20 ml) were 10 μ*M* in the haemin in question, 0.1 *M* in histidine (free base without chloride in phosphate, resulting pH 7.5) or 5 *M* in ammonia. After deaeration with nitrogen reduction was performed with Pt-H_2 under spectroscopic control. The solution was filtered anaerobically and transfered *via* a capillary tube directly to the cuvette (Beckman). This was sealed with a 4 mm rubber square, provided with a capillary outlet. A system of taps permitted the closed system, in-

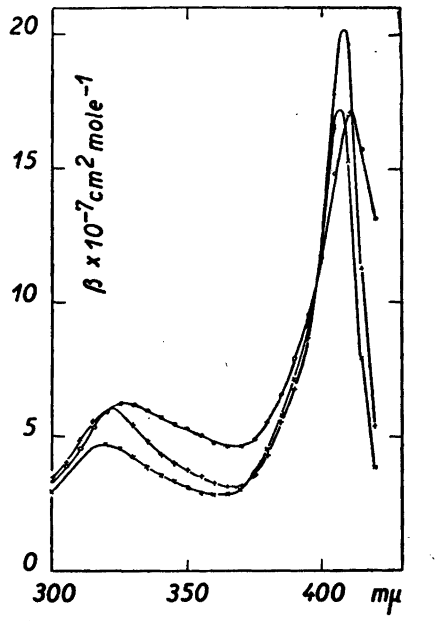

Fig. 1. Absorption spectra of ammonia proto- (○), meso- (+), and deuterohaemochromogens (×).

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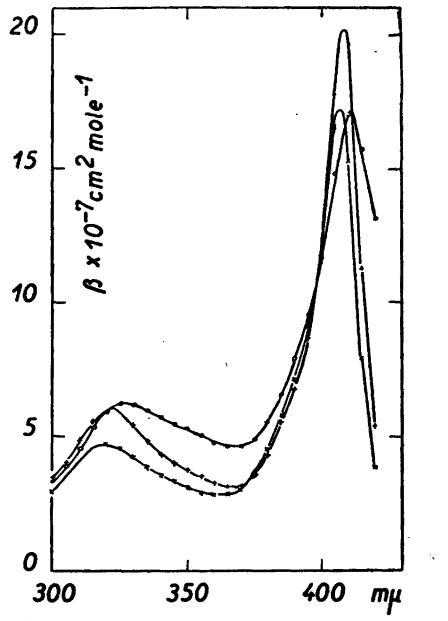

Fig. 1. Absorption spectra of ammonia proto- (○), meso- (+), and deuterohaemochromogens (×).

cluding the cuvette, to be washed free from air by means of nitrogen. The first 15 ml of the solution were used to rinse away remaining traces of oxygen. The solutions were stable in the cuvette for at least two hours. Fresh solutions were used for the determination of the base ferri porphyrin spectra, since reoxidation with air caused deterioration. We have found this arrangement – a number have been tried – to be most convenient for the handling of solutions of this kind.

The base ferro porphyrins (haemochromogens) gave one band within the range 315–330 mμ. The substitution of the hydrogen atoms at the β-positions 2 and 4 in deuterohaemin by two vinyl or two ethyl groups to give protohaemin and mesohaemin caused shifts in wavelength of the band analogous to what is found in visible light. We conclude that the 316 mμ-band in ferrocytochrome c is to be attributed to its nature of being a haemochromogen. Notably the spectra of oxy- and carboxyhaemoglobin [6] and of ferrocytochrome b_3 [7], which contain protohaemin, possess a band at 330–340 mμ.

The ferricytochrome c spectrum shows an absorption band at 364 mμ[1]. It is present also in base ferri porphyrin and free ferri porphyrin spectra, and its position varies as above with the substitution at the positions 2 and 4. Most, possibly all, haemproteids in the threevalent state show a peak at 350–370 mμ or a shoulder adjacent to the Soret band. The "sharpening" of the Soret band upon reduction is partly caused by its migration 10–15 mμ towards red and the vanishing of the band or shoulder at 350–370 mμ.

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Note on the Potassium Bromide Disk Technique for Measurements of Infrared Spectra

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Attempts to find a general method for quantitative measurements of infrared absorption of solids have led to the development of the potassium bromide disk technique (Stimson [1], Schiedt [2,3] and their associates, *cf.* also [4]). A few milligrams of the sample are mixed with some hundred milligrams of potassium bromide powder, the mixture pressed to a disk and the absorption of the disk measured. It is evident that the sample must be uniformly distributed throughout the disk in order to obtain quantitative results, and that the particles of the sample must be so small that further dispersion does not increase the absorption. The technique has been developed by Andersen and Woodall [5] for samples as small as 0.01 mg by using a beam-condensing system of silver chloride lenses to reduce the area of the sample beam.

We have used the potassium bromide technique in an investigation of the infrared absorption of furan compounds. Our disks were prepared essentially after the directions of Stimson [1] and of Schiedt [2,3], but the mixture of sample and potassium bromide was not evacuated during the pressing (*cf.*[5]). The pressing tools described by Schiedt were simplified. The potassium bromide powder was prepared by precipitation of an aqueous solution of the salt with acetone. This way of preparing powder, which gives transparent disks, is simpler than grinding. The technique was not only used for solids but also for viscous liquids.

At first we found it difficult to distribute the sample uniformly and in sufficient dispersion throughout the disk. Grinding the sample in a mortar [1,3] or in a small ball

cluding the cuvette, to be washed free from air by means of nitrogen. The first 15 ml of the solution were used to rinse away remaining traces of oxygen. The solutions were stable in the cuvette for at least two hours. Fresh solutions were used for the determination of the base ferri porphyrin spectra, since reoxidation with air caused deterioration. We have found this arrangement – a number have been tried – to be most convenient for the handling of solutions of this kind.

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Note on the Potassium Bromide Disk Technique for Measurements of Infrared Spectra

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Attempts to find a general method for quantitative measurements of infrared absorption of solids have led to the development of the potassium bromide disk technique (Stimson [1], Schiedt [2,3] and their associates, *cf.* also [4]). A few milligrams of the sample are mixed with some hundred milligrams of potassium bromide powder, the mixture pressed to a disk and the absorption of the disk measured. It is evident that the sample must be uniformly distributed throughout the disk in order to obtain quantitative results, and that the particles of the sample must be so small that further dispersion does not increase the absorption. The technique has been developed by Andersen and Woodall [5] for samples as small as 0.01 mg by using a beam-condensing system of silver chloride lenses to reduce the area of the sample beam.

We have used the potassium bromide technique in an investigation of the infrared absorption of furan compounds. Our disks were prepared essentially after the directions of Stimson [1] and of Schiedt [2,3], but the mixture of sample and potassium bromide was not evacuated during the pressing (*cf.*[5]). The pressing tools described by Schiedt were simplified. The potassium bromide powder was prepared by precipitation of an aqueous solution of the salt with acetone. This way of preparing powder, which gives transparent disks, is simpler than grinding. The technique was not only used for solids but also for viscous liquids.

At first we found it difficult to distribute the sample uniformly and in sufficient dispersion throughout the disk. Grinding the sample in a mortar [1,3] or in a small ball

mill [3] with potassium bromide usually did not give sufficient dispersion, in particular when the sample consisted of soft crystals or was liquid. Evaporation of a solution of the sample on the potassium bromide [2] gave good results with some compounds, but (1) could not be used for volatile compounds and (2) was not reliable, as solid samples occasionally crystallized in too large crystals during the evaporation. We have found that *grinding the sample with potassium bromide followed by pressing, regrinding of the disk, and pressing once more consistently gave reproduceable results.*

The experimental part only contains directions for the preparation of the potassium bromide and of the disks. No examples of the use of the method for quantitative measurements can be reported as a systematic investigation on a large number of compounds lies outside the scope of our laboratory program. However, judging from experiments carried out so far, we believe that the method is of general applicability.

Experimental. Preparation of the potassium bromide. Potassium bromide (analytical grade, 335 g) was dissolved in water (purified by heating under reflux with potassium permanganate and distilling, 500 ml) and the solution precipitated with acetone (analytical grade, heated under reflux with potassium permanganate and distilled, 500 ml). After about one minute the resulting precipitate was removed by filtration and dried at 110° C, with occasional grinding of the large lumps. The product was stored in the dark. The transmission of disks prepared from this product is 85—88 %.

The pressing tools, shown in Figure 1, were made of Sverker 3 (Uddeholm A/S, Copenhagen), hardened to 64° Rockwell C. The pressing surfaces were polished as recommended by Stimson [1]. The difference between the diameters of the plunger and the anvil and of the die shell is about 0.03 mm.

Preparation of the disks. The sample is mixed for 3—4 minutes in a small mortar of stainless steel with 500 mg of potassium bromide. The mixture is placed in the die and pressed for 30 seconds at 90 kg/mm². The disk is ground finely in the mortar and the resulting powder pressed again. The die shell holding the new disk is then placed in the spectrophotometer.

Liquid samples or samples of very soft crystals are weighed directly into the potassium bromide powder and the mixture pressed without prior grinding. The sample is then ground, pressed, ground and pressed again. Only rather viscous liquids, which are not squeezed out of the potassium bromide under the pressing may be measured in this way.

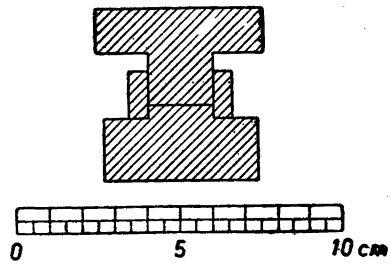

Fig. 1. Pressing tools.

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2. Schiedt, U. and Reinwein, H. *Z. Naturforsch.* **7b** (1952) 270.
3. Schiedt, U. *Z. Naturforsch.* **8b** (1953) 66.
4. Abstracts of five papers read at a recent conference, *Anal. Chem.* **26** (1954) 431.
5. Anderson, D. H. and Woodall, N. B. *Anal. Chem.* **25** (1953) 1906.

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γ-Glutamyl-Alanine in Pea Seedlings

ARTTURI I. VIRTANEN and ANN-MARIE BERG

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In an earlier paper [1] from this laboratory, it was reported that during germination of pea seeds a ninhydrin positive substance is formed, which on a two-dimensional chromatogram (solvents: butanol + acetic acid and phenol + NH_3), gives the spot "X" below serine. We have now investigated this substance more closely, and found it to be γ-glutamyl-alanine.

After 5 days of germination, pea seeds contain a comparatively great amount of the compound forming spot "X", and an extract was made from them with 70 % alcohol. Following a phenol run, a two-dimensional paper chromatogram was dried at 110° C, after which the spots formed were examined in UV-light. Spot "X" was cut off from many paper chromatograms, and the detached pieces of pa-

mill [3] with potassium bromide usually did not give sufficient dispersion, in particular when the sample consisted of soft crystals or was liquid. Evaporation of a solution of the sample on the potassium bromide [2] gave good results with some compounds, but (1) could not be used for volatile compounds and (2) was not reliable, as solid samples occasionally crystallized in too large crystals during the evaporation. We have found that *grinding the sample with potassium bromide followed by pressing, regrinding of the disk, and pressing once more consistently gave reproduceable results.*

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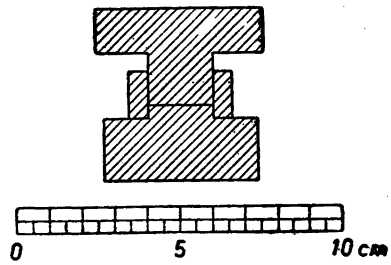

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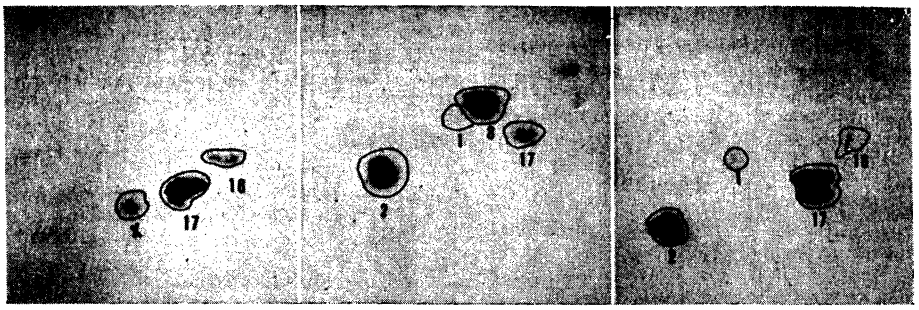

Fig. 1. Unhydrolyzed extract of spot "X". Aspartic acid = 16 and glutamic acid = 17 were added to the extract.

Fig. 2. With 1 N HCl hydrolyzed extract of spot "X". Alanine = 2 and serine = 8 were added to the extract. 1 = Gly.

Fig. 3. With 1 N HCl hydrolyzed extract of spot "X". Aspartic acid and glutamic acid were added to the extract.

per were throughly extracted with water. The extract was concentrated. The major part was hydrolyzed with 1 *N* hydrochloric acid at 108° C for 20 hours. The hydrochloric acid was carefully removed after hydrolysis. Chromatograms were prepared from the extracts by using the following additions:

1. unhydrolyzed extract ("X") + glutamic acid + aspartic acid
2. hydrolyzed extract + alanine + serine
3. hydrolyzed extract + glutamic acid + aspartic acid

1. gave a chromatogram showing, in addition to spot "X", the spots for glutamic acid and aspartic acid (Fig. 1).

2. gave a chromatogram showing clear and intense spots of glutamic acid, alanine, and serine (Fig. 2). The spot of alanine was much more intense than could have been expected on the basis of the amount of alanine added. Glutamic acid and alanine had thus been formed on the hydrolysis of spot "X". The origin of a very faint spot of glycine observed on the chromatogram is unknown. The possibility that spot "X", in addition to γ-glutamyl-alanine, should contain a small amount of γ-glutamyl-glycine cannot be overlooked. On the basis of our experimental material we cannot, however, say anything with certainty.

3. gave a chromatogram showing spots of alanine, glutamic acid and aspartic acid, and in addition a very faint spot of glycine (Fig. 3). The spot of glutamic acid was much more intense than in case 1, even if the same amount of glutamic acid had been added to the extract in both cases. This chromatogram too confirmed that only alanine and glutamic acid (and possibly a very small amount of glycine) are formed from the compound giving spot "X".

When the unhydrolyzed alcohol extract was run through Amberlite IR 4B column, we found that "X" travels together with acid amino acids. This is what could be expected from the acid nature of γ-glutamyl-alanine.

For the examination of the γ-glutamyl-alanine synthesis, we also made an experiment with an extract obtained by crushing peas which had germinated for 4 days. This extract did not give the spot "X" on paper chromatograms. When either 15 mg of alanine or 15 mg of glutathione were added to 1 ml of the extract a clear, even if faint, spot "X" appeared.

The results suggest that the same reaction which Hird and Springell [2] discovered with kidney homogenate between glutathione and alanine:

γ-glutamyl-cysteine-glycine + L-alanine

↓ γ-glutamyl transferase

γ-glutamyl-L-alanine + cysteinyl-glycine

also occurs in germinated peas.

1. Virtanen, A. I., Berg, A-M. and Kari, S. *Acta Chem. Scand.* **7** (1953) 1423.
2. Hird, F. J. R. and Springell, P. H. *Biochem. J. (London)* **56** (1954) 417.

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A New Type of Monoaminodicarboxylic Acid, γ-Hydroxy-α-Aminopimelic Acid and Its Lactone in Green Plants

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Hydroxyl derivatives of aminodicarboxylic acids have not so far been found in the plant or animal kingdom. Dakin [1] believed to have isolated hydroxyglutamic acid, but his preparation has, on reinvestigation, proved to be a mixture of commonly occurring amino acids, mainly of glutamic acid and aspartic acid [2].

Virtanen and Berg [3] have recently found α-aminopimelic acid in *Asplenium septentrionale*, and also noticed two unknown spots on the two-dimensional paper chromatogram (solvents: butanol – acetic acid and phenol-NH_3) prepared from the alcohol extract of this plant.

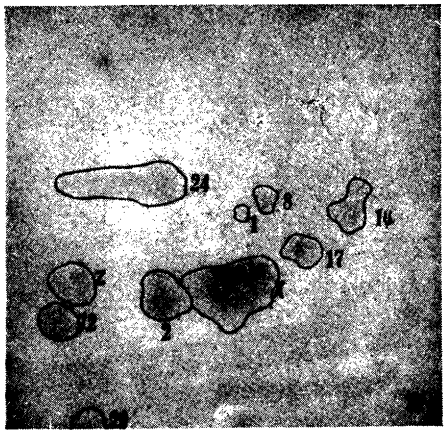


Fig. 1. Two-dimensional paper chromatogram of the unhydrolyzed 70 % alcohol extract of Asp. septentrionale. 1 = gly, 2 = ala, 16 = asp, 17 = glu, 24 = glu-NH_2 + some other amino acids, 72 = acetylornithine, X = unknown acidic amino acid which disappears on hydrolysis, Z = unknown neutral amino acid which becomes much more intense on hydrolysis.

We have closer investigated, and also succeeded to isolate, the amino acids which form these spots (X and Z in Fig. 1). Spot X moves somewhat faster with phenol than glutamic acid. The brownish colour of the spot distinguishes it from the spot of glutamic acid also in the case that the spots are not totally separated from each other. The colour of the other spot (Z) is in the beginning yellow but changes rapidly to reddish. It moves with phenol nearly with the same velocity as proline, but with butanol much slower.

When hydrolyzed with 1 *N* HCl at 108° C for 24 hours spot X disappears, and spot Z becomes much stronger. This observation already showed that there is a connection between the substances corresponding to spots X and Z on the paper chromatogram. It appeared very possible that the substance Z is the lactone of the amino acid X.

It could be shown by the paperelectrophoretic method that the amino acid X is acidic. It was separated in the same fraction as the other acidic amino acids (aspartic, glutamic, and α-aminopimelic acids) in the alcohol extract of *Asplenium septentrionale* when the extract was treated with Amberlite IR – 4B. In this way the unknown amino acid could be separated from neutral and basic amino acids.

The fraction of acidic amino acids was hydrolyzed with 1 *N* HCl at 108° C. The hydrolysate gave now the spots of aspartic, glutamic, and α-aminopimelic acids, and in addition a very strong spot Z. The spot was cut off before treatment with ninhydrin, and the piece of paper extracted with water. The extract contained practically only substance Z. Na_2CO_3 was added to the extract, and the 5 % Na_2CO_3-solution was boiled for 2 hours. It could be shown by the paper chromatographic method that spot Z to a great extent disappeared during this procedure, and that spot X reappeared. This result confirmed the opinion that the amino acid Z is the lactone of the hydroxy-α-aminodicarboxylic acid X. The properties of the lactone suggest that γ-lactone is in question.

The lactone was now isolated from the hydrolyzed fraction of acidic amino acids using again an Amberlite IR – 4B column. Lactone Z, which was formed through treatment with HCl, came from the column in the fractions 3 – 11. These were evaporated *in vacuo*. The paper chromatogram showed that the isolated substance contained practically only lactone Z and as im-

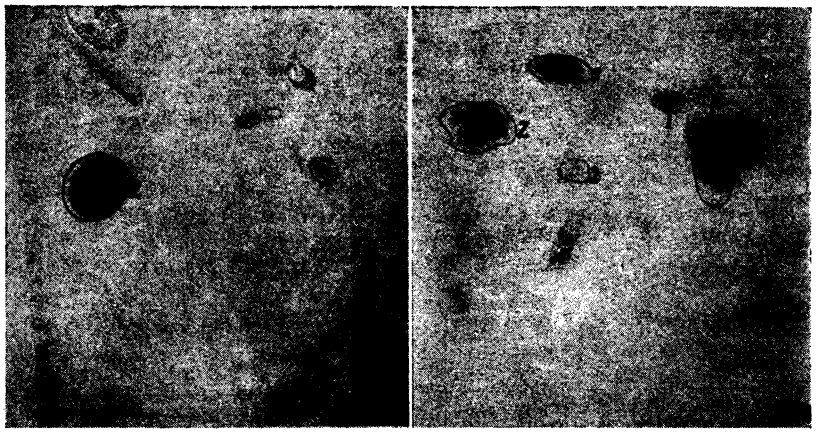


Fig. 2. A. Two-dimensional paper chromatogram from the isolated amino acid Z before treatment with Na_2CO_3. The preparation contained only traces of glycine, serine, and glutamic acid.

Fig. 2. B. Paper chromatogram of amino acid Z after boiling with 5 % Na_2CO_3. The amino acid X has been formed from Z in very large amounts. Traces of glycine (1) and serine (8) appear as impurities. A small amount of alanine and of an unknown substance (S) are formed during treatment with Na_2CO_3.

purities traces of glycine, serine, and glutamic acid (Fig. 2A). When boiled in a 5 % Na_2CO_3-solution for 2 hours large amounts of the amino acid X was formed, and small amounts of alanine and an unknown substance S (Fig. 2B).

The preparation of lactone Z was purified once more with Amberlite IR—4B.

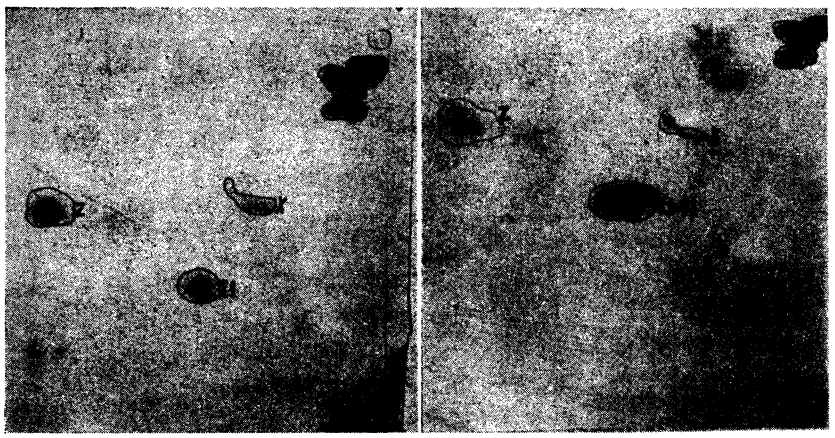


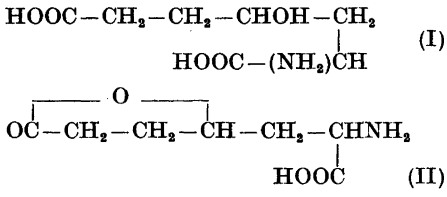
Fig. 3. A. Pure preparation of the amino acid Z after reduction with HJ and red P. α-Aminopimelic acid (71) is formed from Z through reduction. Part of Z has remained unchanged, and part is hydrolyzed to X.

Fig. 3. B. The same as A with the only difference that synt. α-aminopimelic acid was added to the solution (spot 71 + 71).

The solution was evaporated to a syrup. In the syrup appeared star-like clusters of needle-shaped crystals. The recrystallized substance which did not give other spots than Z on the paper chromatogram had a melting point 226–227° (decomposition). (Found: C 48.78; H 6.27; N 8.02. Calc. for $C_7H_{11}O_4N$: C 48.55; H 6.40; N 8.09.)

The substance was reduced with 66 % HJ (*d* 1.96) and red phosphorus at 136° C during 4 hours. α-Aminopimelic acid (Fig. 3) could be shown as the reduction product.

Our results show that *Asplenium septentrionale* contains γ-hydroxy-α-aminopimelic acid (I), and its lactone (II) as free amino acids. The γ-position of the OH-group was proved by oxidation of the deaminated lactone with permanganate in acid solution, whereby succinic acid was formed.



(I)

(II)

In the protein these amino acids could not be found. Also other *Asplenium* species seem to contain these amino acids. In addition to *Aspl. septentrionale* also *Aspl. nidus*, *Aspl. trichomonas*, and *Aspl. viviparum* have so far been investigated.

The amount of γ-hydroxy-α-aminopimelic acid in *Aspl. septentrionale* is relatively high (*cf.* Fig. 1). It is the most abundantly appearing free amino acid in this plant. The amount of its lactone is much smaller corresponding probably to the chemical equilibrium between the acid and the lactone.

We have not found in literature that any hydroxy-α-mono-aminopimelic acid should have been found earlier in living organisms Also synthetic products of this kind seem to be unknown.

Virtanen and Linko have found acetylornithine (Fig. 1) in *Asplenium* species, and in *Aspl. nidus* also free ornithin. They will communicate about the isolation of this acetyl compound.

We wish to thank Mr. R. E. Ruotsalo for his help in procuring *Asplenium septentrionale*, and Professor A. Kalela for *Asplenium* species obtained from the University Botanica Gardens.

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3. Virtanen, A. I. and Berg, A-M. *Acta Chem. Scand.* **8** (1954) 1085.

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Adsorptiochromism of 1,4-Naphthoquinones

JACK PETER GREEN * and HENRIK DAM

Department of Biochemistry and Nutrition, Polytechnic Institute, Copenhagen, Denmark

A number of organic compounds are adsorbed to inorganic salts with an accompanying color change [1-3]. We have observed that some 1,4-naphthoquinones (all of which are yellow) in petroleum ether solution were adsorbed to Al_2O_3 with a concomitant shift to deeper colors (Table 1). 1,4-Naphthoquinone and 1,2-naphtoquinone remained yellow.

Color intensity varied with the source of the aluminia. On Merck's *purissimo* colors were faint; they were more evident on Merck's Al_2O_3 *nach Brockmann*, and most intense on May and Baker's Al_2O_3

Table 1. Colors of some 1,4-naphthoquinones when adsorbed to aluminia.

Derivatives of 1,4-naphthoquinone	Color of compound on Al_2O_3
2-hydroxy-	orange
2-methyl-	deep violet
2-methyl-3-hydroxy-	orange
2,3,6-trimethyl-	pink
2,6,7-trimethyl-	pink
3,5,7-trimethyl-	pink
2-hydroxy-3-(2-methyl)octyl-	pink
2-methyl-3-phytyl-	violet
2,3-oxide of 2-methyl-3-phytyl	violet
2-methyl-3-difarnesyl-	violet

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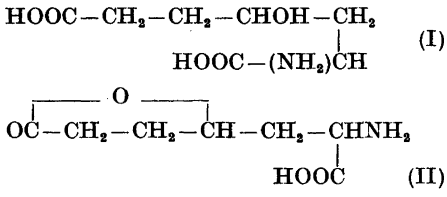

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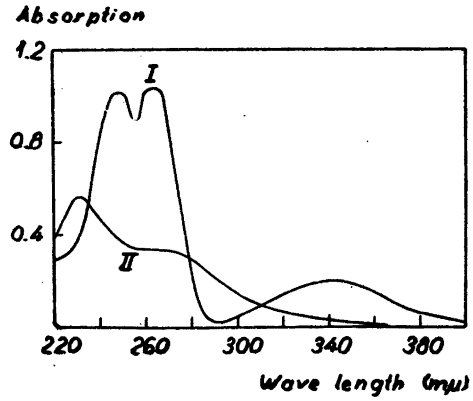

Fig. 1. Absorption spectra of pure menadione in water (curve I) and the water eluate after Al_2O_3 adsorption (curve II).

for chromatography. Pre-treatment of the adsorbent with water prevented color formation.

At least in one instance the colored compound can be eluted, as shown by the following procedure which was carried out in the dark. One hundred mg of 2-methyl-1,4-naphthoquinone (*i.e.*, menadione) were dissolved in 100 ml petroleum ether and poured through an 8 × 1.5 cm column of May and Baker's Al_2O_3. The upper violet zone which remained after elution with 200 ml benzene was eluted with 200 ml H_2O. The ultraviolet absorption spectrum of the violet water-eluate was measured in a Beckman DU Spectrophotometer; the blank was a water-"eluate" of a fresh column treated with the same solvents. The spectrum is shown in Figure 1, along with that of the pure untreated menadione in H_2O.

The nature of the change induced by adsorption to Al_2O_3 is unknown, but it is interesting that the spectrum of the resultant compound(s) is similar to that induced by light-activation of 1,4-naphthoquinones [4]. It should be emphasized, however, that the spectrum differs markedly from the spectrum of one of the products resulting from light-activation of menadione (*viz.*, the dimer of menadione) [5] which we have found absorbs maximally at 265 mμ and 272 mμ. That the violet compound can be eluted with water but not with benzene suggests highly polar groupings, such as hydroxy groups. Perhaps pertinent is the suggestion of Schoenberg *et al.*[3] that anthrones are changed to anthranols by Al_2O_3 adsorption.

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Ulf Lagerkvist and Peter Reichard

Department of Biochemistry, Karolinska Institutet, Stockholm, Sweden

In a previous communication we have proposed the following scheme for the synthesis of orotic acid in rat liver slices [1]. L-aspartic acid + CO_2 + NH_3 → L-ureidosuccinic acid → dihydro-orotic acid → orotic acid.

Reichard [2] has presented strong evidence that orotic acid is a normal intermediate in the biogenesis of polynucleotide pyrimidines and therefore the scheme given above probably represents the actual pathway of pyrimidine synthesis in rat liver. The pyrimidine biogenesis of the Ehrlich ascites tumor [3] has now been investigated for comparison.

Two different experimental approaches were used. In the first one tumor cells were incubated in Krebs-Henseleit medium with any of the following N^{15} labeled substances: Ammonia, aspartic acid, L-ureidosuccinic acid, orotic acid, uracil, dihydrouracil, ureidopropionic acid, β-alanine. The ribonucleic acid pyrimidines were isolated and their isotope content determined. In experiments with N^{15}-ammonia and aspartic acid the distribution of the isotope in the pyrimidine ring was determined [4].

In the second approach the tumor cells were incubated with an isotopically labeled precursor (known to give an incorporation of isotope into the polynucleotide pyrimidines) in the presence of a pool of the non labeled substance suspected to be an intermediate (ureidosuccinic acid, dihydroorotic acid or orotic acid). Both the substance under investigation and the polynucleotide pyrimidines were isolated at the end of the incubation and their isotope content was determined.

The results of these two experimental approaches show that the same precursors as in rat liver slices can be used by the ascites tumor for the synthesis of polynucleotide pyrimidines. The question of these substances being also normal intermediates tends to be obscured by permeability effects and will be discussed.

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The transformation of orotic acid into different uridine phosphates has been demonstrated in rat liver *in vivo* [1]: Since it was shown that one or several of these nucleotides are precursors of polynucleotide pyrimidines and since orotic acid has been demonstrated to be a normal intermediate in pyrimidine biosynthesis in rat liver [2] an investigation of the enzymatic conversion of C^{14}-orotic acid into uridine phosphates has been started.

The enzymes were obtained either from the dialyzed supernatant of homogenized liver (rat and pigeon) after high speed centrifugation or from extracts of pigeon liver acetone powder. In the presence of ribose-5-phosphate, Mg^{++} and ATP(or fructose-di-phosphate DPN, Mg^{++} and ATP) C^{14}-orotic acid was converted into several radioactive compounds, one of which was identified as uridine-5-phosphate (UMP). The other compounds gave rise to UMP upon acid hydrolysis and were probably pyrophosphates of uridine (*e. g.* uridine-diphosphate and uridinetriphosphate). In some cases enzymatic breakdown of the uridinephosphates to uridine and uracil took place. In a time study it was found that UMP was the first uridine compound formed from orotic acid. This result was confirmed by the finding that C^{14}-UMP was rapidly transformed into radioactive uridinepyro-

phosphates and that the presence of non labeled UMP diminished the incorporation of isotope from C^{14}-orotic acid into the pyrophosphates. Uridine was not an intermediate in the transformation of orotic acid into UMP.

Enzyme fractionation with $(NH_4)_2SO_4$ and methanol are being carried out to investigate the intermediate steps in these reactions.

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L. Ernster and O. Lindberg

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The question was left open whether the ATP-containing enzyme complex which catalyzes reaction 2 is responsible for the ultimate synthesis of ATP, or whether its function is merely restricted to the oxidative formation of the primary $\sim$ P bond. It has now been shown that the latter is the case.

2,4-dinitrophenol (DNP), which is known to inhibit ATP synthesis connected with hydrogen transfer between $DPNH_2$ and oxygen, stimulates respiration in mitochondrial systems (see Table 1). The latter action is, however, dependent on the concentration of ATP prevailing in the medium. Thus the stimulation can be turned into an inhibition when the concentration of ATP is kept low by adding hexokinase. Moreover, Mn^{++} ($0.5—1.0 \times 10^{-3}$ *M*) is able to prevent this inhibition. These facts indicate that $\sim$ P is formed in this system according to equation 2, while the action of DNP is confined to the blockage of the subsequent transfer of the formed $\sim$ P to the adenylic acid system.

Table 1. Effect of Mn^{++} on mitochondrial respiration inhibited by DNP. Each Warburg vessel contained: mitochondria (prepared in 0.25 M sucrose containing 0.01 M versene), 1/12 rat liver; glycylglycine, 200 µmoles; KCl, 150 µmoles; glucose, 180 µmoles; D,L-*glutamate, 60 µmoles; orthophosphate, 90 µmoles; AMP, 3 µmoles; Mg^{++}, 10 µmoles. Additions (where indicated): yeast hexokinase (prepared as described previously* [1]*), 0.1 ml; DNP, 0.4 µmoles; Mn^{++} 1.2 µmoles.—Final volume, 2.5 ml. pH, 7.8. Temp. 30° C. Gas phase, air. Time of incubation, 45 min.*

Additions	Respiration µatoms O	Phosphorylation µmoles P
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Hexokinase	36.5	85.5
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Free Amino Acids in Brewing Materials

L. Ljungdahl and E. Sandegren

AB Stockholms Bryggerier, Stockholm, Sweden

The free amino acids occurring in a sixrow barley (Stella) and in malt, wort and beer prepared from this barley have been quantitatively determined by means of chromatography on Dowex 50 [1].

The amino acids were determined in water extracts of barley and malt samples previously boiled in alcohol to inactivate the proteolytic enzymes. Barley contains very small amounts of free amino acids. Alanine, aspartic acid, asparagine and glutamic acid occur, however, in quite considerable quantities. Proline and γ-aminobutyric acid are also present in rather large amounts. During the malting there was a general increase of the free amino acids, but most remarkable was the increase of free proline.

The samples of wort, hopped wort, beer and beer refermented after addition of glucose, were treated with alcohol to precipitate proteins and carbohydrates before the determinations of the amino acids. During the mashing process there was a general increase of the free amino acids, which indicates that a considerable peptidase action occurred during the mashing. Arginine, leucine, lysine, phenylalanine and valine showed the greatest increase. When the wort was boiled with hops, there was an increase of some of the amino acids, particularly of aspartic acid, threonine and valine. There was a remarkable decrease, however, of arginine, histidine, lysine, glutamic acid and also of tyrosine during the boiling. A possible explanation for this decrease is that the boiling involves a reaction of amino acids and carbohydrates, with the formation of melanoidines. The fermentations were performed with bottom yeast *(Saccharomyces Carlsbergensis)*. During the first fermentation all of the amino acids decreased except γ-aminobutyric acid which increased. During the refermentation all amino acids decreased, but proline, γ-aminobutyric acid, glycine and alanine only to a rather small extent. In the chromatograms at least 10 unknown substances could be demonstrated, several of which are probably peptides. The results obtained in the fermentation experiments generally agree well with those of Barton-Wright [2] obtained with microbiological methods for the determination of the amino acids. The excretion of glutamic acid and the assimilation of glycine by the yeast found by Barton-Wright, however, could not be demonstrated in our experiments.

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Gunnar Ågren, Carl-Henric de Verdier and John Glomset

Institute of Medical Chemistry, University of Uppsala, Uppsala, Sweden

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The hyaluronidase activity was determined as described by Diczfalusy *et al.*[3] The crude fraction had an activity of 700 VRU per mg N. The resin used was Amberlite XE-64 as the ammonium salt.

In a typical experiment 200 ml of crude hyaluronidase with a potency of 3 500 VRU per ml was run through a 1 cm × 11 cm Amberlite XE-64 column (2 g) at room temperature. Rate of flow: 1 ml per min. A gold-coloured impurity passed into the effluent. About 95 % of the activity was adsorbed on the resin. The resin was rinsed with 200 ml of water, 150 ml 0.1 *M* ammonium acetate, and 25 ml 0.1 *M* ammonia, respectively. The hyaluronidase was then eluated with minimal volumes of a 0.1 *M* KCl-HCl-buffer, pH 1.5.

On a nitrogen basis the eluate had en activity about 100 times that of the original material. The purified hyaluronidase fractions, thus obtained, are homogeneous in electrophoretic analyses. The protein impurity not adsorbed has neither activating nor inhibitory effects on purified hyaluronidase.

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On the Action of the Intestinal Flora on Conjugated Bile Acids

Arne Norman

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Recently Bergström and Norman [1] have investigated the labelled products in bile and feces after injection of cholesterol-4-^{14}C in the rat. This compound is transformed and excreted into the bile to more than 90 % as taurocholic acid. However, only a minor part of the labelled product in feces consists of taurocholic acid. Most of the conjugates had been split and the cholic acid further modified. This is in accord with a number of older observations that the bile acids present in bile cannot be found in feces.

To obtain some information regarding the role played by the microorganisms in the biological splitting of the conjugated bile acids and the further modifications of the bile acid molecules, antibiotics were given to rats and the fecal bile acids were analyzed.

100 mg of terramycin and 250 mg of "sulftalyl"* was given by stomach-tube twice a day to rats. If the decrease in the total aerobic count was satisfactory, the rats were given 3—3.5 mg of cholic acid-24-^{14}C on the third day after administration of antibiotics. The feces were collected daily for five days. No increase in the amount of viable bacteria was found during this period. The labelled bile acid products were extracted and fractioned by reversed partition chromatography as described by Bergström and Norman [1].

Intraperitoneally administered cholic acid-24-^{14}C is excreted in the bile almost totally

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Torsten Ekstrand

Centrallaboratoriet, Astra, Södertälje, Sweden

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tions. Further details of procedures and figures will be published in the near future.

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The Use of Ion-exchange Resin in the Purification of Hyaluronidase

Bertil Högberg

Research Department, AB Leo, Hälsingborg, Sweden

It has been shown that high molecular weight polyanions produce a complete reversible inhibition of hyaluronidase even in small amounts [1-4]. This led to the idea that a cation exchanger of a weakly acid type could be used for the isolation and purification of hyaluronidase.

Decapsulated ground bull testes were extracted with equal volumes of 0.1 *M* acetic acid. Extracts fractionated with $(NH_4)_2SO_4$. Fraction obtained between 30 and 70 % saturation was retained. An aqueous solution of this fraction was dialysed salt free. In order to denature proteins of high molecular weight the solution was vigourously shaken at pH 6 at room temperature for 15 minutes with 1/2 parts of chloroform without loss of activity. After centrifugation the upper aqueous layer was separated and dialysed against distilled water.

The hyaluronidase activity was determined as described by Diczfalusy *et al.*[3] The crude fraction had an activity of 700 VRU per mg N. The resin used was Amberlite XE-64 as the ammonium salt.

In a typical experiment 200 ml of crude hyaluronidase with a potency of 3 500 VRU per ml was run through a 1 cm × 11 cm Amberlite XE-64 column (2 g) at room temperature. Rate of flow: 1 ml per min. A gold-coloured impurity passed into the effluent. About 95 % of the activity was adsorbed on the resin. The resin was rinsed with 200 ml of water, 150 ml 0.1 *M* ammonium acetate, and 25 ml 0.1 *M* ammonia, respectively. The hyaluronidase was then eluated with minimal volumes of a 0.1 *M* KCl-HCl-buffer, pH 1.5.

On a nitrogen basis the eluate had en activity about 100 times that of the original material. The purified hyaluronidase fractions, thus obtained, are homogeneous in electrophoretic analyses. The protein impurity not adsorbed has neither activating nor inhibitory effects on purified hyaluronidase.

The method described showed that bacterial hyaluronidase from different origin cannot be adsorbed. These phenomena make it possible to separate testes hyaluronidase from bacterial hyaluronidase.

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On the Action of the Intestinal Flora on Conjugated Bile Acids

Arne Norman

Department of Physiological Chemistry, University of Lund, Lund, Sweden

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The amount of isotope in the fecal lipids was determined and the amount of isotope absorbed was calculated. The lymph fat was subjected to chromatography on silicic acid according to Borgström [2]. A method was worked out for separating free cetyl alcohol from neutral fat.

From 63 to 96 % of the fed activity was absorbed. These results confirm earlier investigations that cetyl alcohol is well absorbed in the rat [1,3,4].

In four experiments from 31 to 64 % of the absorbed activity was recovered in the lymph lipids. The major portion of the activity in the lymph lipids was recovered in the neutral fat. After saponification and recrystallisation with inactive palmitic acid it was found that all the activity in the neutral fat fatty acids could be accounted for as palmitic acid. The oxidation of the cetyl alcohol must largely have taken place during its passage through the intestinal mucosa.

About 15 % of the absorbed cetyl alcohol passed unchanged through the intestinal mucosa and could be isolated as free cetyl alcohol in the lymph lipids. The remainder of the activity in the lymph lipids was present in the phospholipid fatty acids. The proportions of the activity between neutral fat and phospholipids were those characteristic for palmitic acid.

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Some Barbiturate Metabolites in Man, Studied by Paper Chromatography

Lars-Göran Allgén

Clinical Central Laboratory, Sabbatsberg Hospital, Stockholm Va., Sweden

In about 50 cases of humans suffering from acute or chronic barbiturate intoxication, concentrated chloroform extracts from 50 ml urine were studied by paper chromatography (Whatman No. 1 filter paper, descending in *iso*-amylalcohol — concentrated ammonia solution + 5 % ethylene glycol, and ammonia atmosphere [1,2]). The spots were identified by ultraviolet illumination after treatment with ammonia vapours [1]. Ultraviolet absorption curves were determined after elution of spots with a pH 10 borate buffer, and also in acid solution.

After ingestion of diethyl-BA (BA for barbituric acid), phenylethyl-BA, diallyl-BA or 5-ethyl-5(1-methyl-1-butenyl)-BA, only spots corresponding to unchanged barbiturates were obtained. With patients having taken ethyl*iso*amyl-BA, 5-ethyl-5(1-methylbutyl)-BA, ethyl*cyclo*hexenyl-BA, allyl*iso*propyl-BA and 5-methyl-5-*cyclo*hexenyl-N-methyl-BA, spots were obtained with R_F values lower than those for common barbiturates studied, but with characteristic barbiturate absorption curves. These spots would then correspond to barbiturates with oxidised side chains, with a resulting increase in hydrophilic properties.

A number of such oxidation products, mainly in dogs but in some cases in humans, have been reported recently [3,4]. Thus the spot obtained with ethyl*iso*amyl-BA would correspond to ethyl(3-hydroxy*iso*amyl)-BA [5], with ethyl(1-methylbutyl)-BA to the diastereoisomers of ethyl(3-hydroxy-1-methyl-butyl)-BA [6,7], and with 5-methyl-5-*cyclo*hexenyl-N-methyl-BA to 5-methyl-5(1-*cyclo*hexen-3?-on-1-yl)-BA [8]. In analogy with these findings the oxidation product obtained with allyl*iso*propyl-BA may be suggested to be 5-allyl-5(1-hydroxy*iso*propyl)-BA, as the pyrimidine nucleus is intact, the allyl group in diallyl-BA is not attacked and the *iso*propyl group is similar to the end group attacked in ethyl*iso*amyl-BA. Similarly the oxidation product obtained with ethyl-*cyclo*hexenyl-BA may be suggested to be a ketone, ethyl*cyclo*hexenonyl-BA in analogy with the case with methyl*cyclo*hexenyl-N-methyl-BA mentioned above. With ethyl-*cyclo*hexenyl-BA a spot corresponding to the unchanged barbiturate in low relative amount was also identified in some cases.

Thanks are due to miss Edith Andersson for valuable technical assistance.

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Untersuchungen über die Aktivität von Autolysaten aus Wanderlarven *(Hypoderma bovis)* auf Elemente des Bindegewebes

E. Lienert und W. Thorsell

Pharmakologisches Institut der Tierärztlichen Hochschule in Wien; Chemische Abteilung der Tierärztlichen Hochschule in Stockholm

Die Wanderlarven folgen, nachdem sie in die Haut eingedrungen sind, auf ihrem Weg zum Epiduralspalt des Wirbelkanals (der mit lockerem Binde- und Fettgewebe ausgefüllt ist) dem epineuralen Gewebe [1]. Nach Gebauer [2] sprengt die Wanderlarve das vor ihr liegende Gewebe mechanisch durch den in einzelne ihrer Körperpartien verlegten Druck. Wir nahmen an, dass am Vordringen der Larven auch enzymatische Vorgänge eine Rolle spielen, deren Hemmung eine Möglichkeit zur Bekämpfung der Dasselplage bieten könnte. Da das Bindegewebe neben zelligen Elementen (z.B. Fettzellen) aus Fasern (z.B. kollagene Fasern) und Grundsubstanz (z.B. Polysaccharide von Hyaluronsäure- und Chondroitinschwefelsäuretyp) besteht, wurde geprüft, ob Autolysate aus Wirbelkanallarven auf Fettgewebe, Kollagen, Hyaluronsäure und Chondroitinschwefelsäure eine Spaltwirkung ausüben.

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Some Barbiturate Metabolites in Man, Studied by Paper Chromatography

Lars-Göran Allgén

Clinical Central Laboratory, Sabbatsberg Hospital, Stockholm Va., Sweden

In about 50 cases of humans suffering from acute or chronic barbiturate intoxication, concentrated chloroform extracts from 50 ml urine were studied by paper chromatography (Whatman No. 1 filter paper, descending in *iso*-amylalcohol — concentrated ammonia solution + 5 % ethylene glycol, and ammonia atmosphere [1,2]). The spots were identified by ultraviolet illumination after treatment with ammonia vapours [1]. Ultraviolet absorption curves were determined after elution of spots with a pH 10 borate buffer, and also in acid solution.

After ingestion of diethyl-BA (BA for barbituric acid), phenylethyl-BA, diallyl-BA or 5-ethyl-5(1-methyl-1-butenyl)-BA, only spots corresponding to unchanged barbiturates were obtained. With patients having taken ethyl*iso*amyl-BA, 5-ethyl-5(1-methylbutyl)-BA, ethyl*cyclo*hexenyl-BA, allyl*iso*propyl-BA and 5-methyl-5-*cyclo*hexenyl-N-methyl-BA, spots were obtained with R_F values lower than those for common barbiturates studied, but with characteristic barbiturate absorption curves. These spots would then correspond to barbiturates with oxidised side chains, with a resulting increase in hydrophilic properties.

A number of such oxidation products, mainly in dogs but in some cases in humans, have been reported recently [3,4]. Thus the spot obtained with ethyl*iso*amyl-BA would correspond to ethyl(3-hydroxy*iso*amyl)-BA [5], with ethyl(1-methylbutyl)-BA to the diastereoisomers of ethyl(3-hydroxy-1-methyl-butyl)-BA [6,7], and with 5-methyl-5-*cyclo*hexenyl-N-methyl-BA to 5-methyl-5(1-*cyclo*hexen-3?-on-1-yl)-BA [8]. In analogy with these findings the oxidation product obtained with allyl*iso*propyl-BA may be suggested to be 5-allyl-5(1-hydroxy*iso*propyl)-BA, as the pyrimidine nucleus is intact, the allyl group in diallyl-BA is not attacked and the *iso*propyl group is similar to the end group attacked in ethyl*iso*amyl-BA. Similarly the oxidation product obtained with ethyl-*cyclo*hexenyl-BA may be suggested to be a ketone, ethyl*cyclo*hexenonyl-BA in analogy with the case with methyl*cyclo*hexenyl-N-methyl-BA mentioned above. With ethyl-*cyclo*hexenyl-BA a spot corresponding to the unchanged barbiturate in low relative amount was also identified in some cases.

Thanks are due to miss Edith Andersson for valuable technical assistance.

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Untersuchungen über die Aktivität von Autolysaten aus Wanderlarven *(Hypoderma bovis)* auf Elemente des Bindegewebes

E. Lienert und W. Thorsell

Pharmakologisches Institut der Tierärztlichen Hochschule in Wien; Chemische Abteilung der Tierärztlichen Hochschule in Stockholm

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reaction between R-1-P and GDP has been determined by following the formation of G-6-P. The optimum conditions for the preparation of RDP have been worked out. With the same enzyme system evidence has also been obtained for a reaction between GDP and deoxyribose-1-phosphate or galactose-1-phosphate. Preliminary experiments with pigeon liver acetone powder confirm the indications obtained by Saffran and Scarano [2] for RDP to be an intermediary in 5-adenylic acid formation from ^{14}C-adenine.

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2. Saffran, M. and Scarano, E. *Nature* **172** (1953) 1949.

Studies on the Inhibition of D-Amino Acid Oxidase

Otto Walaas and Eva Walaas

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

Studies on D-amino acid oxidase from pig kidney purified according to Negelein and Brömel [1] have been made. The flavineadenine dinucleotide (FAD) used was a pure sample obtained from baker's yeast by inophoresis on paper as the final step in the purification. The coupling between the apoenzyme and FAD caused no shift in the absorption band and no quenching of the fluorescense. Thus, D-amino acid oxidase is fluorescent and is similar to Straub's diaphorase in this respect.

Inhibition of enzyme activity by different anions and by partial structural analogs of the FAD molecule has been demonstrated. Anions were inhibitory in the following order:

$$J^- > Br^- > NO_3^- > Cl^- > PO_4^{--} > SO_4^{--} > CH_3COO^- \gg F^-$$

Thus, the anions of strong acids were most effective as inhibitors. At a concentration of 1.4×10^{-7} *M* FAD 50% inhibition was exerted by 8.5×10^{-2} *M* NaCl. The inhibition was reversible in the presence of high FAD concentration, suggesting competition of the inhibitor with the prosthetic group.

In similar experiments strong inhibition by adenylic acid, ATP and FMN was observed. 50 % inhibition was given by concentration of approximately 5×10^{-4} *M*. The inhibition by DPN was somewhat weaker, while adenosine, adenine and hypoxanthine exerted inhibition of the same order of magnitude at concentrations of 6×10^{-3} *M*. Cytosin and uracil were slightly inhibitory while riboflavin was without effect.

The experiments indicate that the phosphoric ester groups and the adenine part of the FAD molecule are of importance in the coupling to the apoenzyme. A possible physiological significance by some of these inhibitors in regulation of enzyme activity is suggested.

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Distribution in Rabbit Livers of Intravenously Injected Iron

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The Combination of Flavin Mononucleotide and Riboflavin with the Protein of "The Old Yellow Enzyme"

Hugo Theorell and Agnar P. Nygaard

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In a forthcoming paper *(Acta Chem. Scand.)* we have presented some kinetic data on the reversible dissociation of the old yellow enzyme (OYE) into FMN and apoenzyme obtained by the aid of fluorescence measurements. In water, the dissociation constant *K* of this

These results indicate that a complex between the uridyl group and the enzyme is formed, but a more obvious proof of the reaction would be to apply a C^{14}-labelled uridine compound to the same experiment with subsequent isolation of a C^{14}-labelled uridyl enzyme compound. For this purpose C^{14}-labelled uridine di- and triphosphate can be prepared from C^{14}-labelled uridylic acid by a new enzyme, found in extracts from *Saccharomyces fragilis* (grown on galactose). This enzyme brings about a phosphorylation of UMP to UDP and UTP in the presence of ATP. The separation of the uridine compounds from the adenine compounds is accomplished by paper ionophoresis in citrate buffer, pH 4.2. The uridine polyphosphate compound formed gives positive reaction in the hexokinase test for triphosphates [1].

This phosphorylation process is most likely a phosphorylation of UMP to UDP (with subsequent action of nucleoside diphosphokinase to form UTP from UDP) since equimolar amounts of UMP and ATP give UDP exclusively, whereas excess of ATP yields both UDP and UTP. Experiments with ATP, labelled in the terminal phosphate group, agree with this explanation.

1. Berg, P. and Joklik, W. K. *Nature* **172** (1953) 1008.

Metabolism of Uridinediphosphoglucose (UDPGlucose) in *Hemophilus Influenza* B.

H. M. Kalckar

Institute of Cytophysiology, University of Copenhagen, Denmark, and the Enzyme Section, National Institutes of Health, Bethesda, Md., U.S.A.

Hemophilus influenza (obtained through the courtesy of Drs. Alexander and Zamenhof, College of Physicians & Surgeons, New York) was grown on bloodagar and harvested (our thanks are due to Dr. M. Pittmann, Department of Pathology, National Institutes of Health, and Dr. N. Sloane, Lederle Laboratories, New York). The cells were ruptured in the sonic vibrator and lyophilized. Enzyme fractions could be isolated which brought about a rapid splitting of UDPglucose from the cell extracts. The split products were mainly 5-uridylate, uridine and α-glucose-1-phosphate. Unlike the usual nucleotide pyrophosphatases pyridine nucleotides did not undergo any splitting in the presence of the *Hemophilus* enzyme. UDP-N-acetyl glucosamine was not attacked to any detectable degree. Also, unlike correspondent preparations from yeast or *E. coli* (extract from sonic lysates) the *Hemophilus* enzyme operates independent of addition of inorganic pyrophosphate or 1-esters (galactose-1-phosphate, ribose-1-phosphate). Moreover, addition of pyrophosphatase does not interfere in any way with the enzymic splitting of UDPglucose. The formation of glucose-1-phosphate is preceded by an induction period which has attracted our special attention. The enzyme reaction may be either a simple specific hydrolysis or a combined hydrolysis and transfer reaction, or finally a two step reaction. The two latter possibilities are under investigation in an attempt to find a possible relation between the peculiar UDPglucose metabolism in this organism and the presence of polyglycophosphates in the capsules [1]. Dr. E. Maxwell and the author are investigating the presence of the UDPglucose splitting enzyme in various strains and mutants of the *Hemophilus* group with special reference to growth requirements and capsule formation.

1. Zamenhof, S., Leidy, G., Fitzgerald, P. L., Alexander, H. E. and Chargaff, E. *J. Biol. Chem.* **203** (1953) 695.

On the Interaction between Glucose-1,6-diphosphate and Ribose-1-phosphate

Hans Klenow, Rolf Emberland and Paul Plesner

Institute of Cytophysiology, University of Copenhagen, Denmark

Previous experiments[1] have shown that glucose-1,6-diphosphate (GDP) can serve as a coenzyme for the conversion of ribose-1-phosphate (R-1-P) to ribose-5-phosphate (R-5-P), and that this reaction can be catalysed by phosphoglucomutase from muscle. From incubation mixtures of GDP, R-1-P and phosphoglucomutase a ribose-1,5-diphosphate has been isolated. This led to the formulation of the reaction as follows: GDP + R-1-P $\rightleftharpoons$ RDP + G-6-P. This reaction has now been studied in details by following the formation of G-6-P spectrophotometrically in the presence of TPN and excess of Zwischenferment. The K_m value of R-1-P was found to be about 2×10^{-3} *M*. The optimum concentration of GDP was about 4×10^{-5} *M*, and higher concentrations inhibited the reaction in a competitive way. The K_m of GDP as inhibitor was about 2×10^{-3} *M*. The equilibrium of the

reaction between R-1-P and GDP has been determined by following the formation of G-6-P. The optimum conditions for the preparation of RDP have been worked out. With the same enzyme system evidence has also been obtained for a reaction between GDP and deoxyribose-1-phosphate or galactose-1-phosphate. Preliminary experiments with pigeon liver acetone powder confirm the indications obtained by Saffran and Scarano [2] for RDP to be an intermediary in 5-adenylic acid formation from ^{14}C-adenine.

1. Klenow, H. *Arch. Biochem. Biophys.* **46** (1953) 186.
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Studies on D-amino acid oxidase from pig kidney purified according to Negelein and Brömel [1] have been made. The flavineadenine dinucleotide (FAD) used was a pure sample obtained from baker's yeast by inophoresis on paper as the final step in the purification. The coupling between the apoenzyme and FAD caused no shift in the absorption band and no quenching of the fluorescense. Thus, D-amino acid oxidase is fluorescent and is similar to Straub's diaphorase in this respect.

Inhibition of enzyme activity by different anions and by partial structural analogs of the FAD molecule has been demonstrated. Anions were inhibitory in the following order:

$$J^- > Br^- > NO_3^- > Cl^- > PO_4^{--} > SO_4^{--} > CH_3COO^- \gg F^-$$

Thus, the anions of strong acids were most effective as inhibitors. At a concentration of 1.4×10^{-7} *M* FAD 50% inhibition was exerted by 8.5×10^{-2} *M* NaCl. The inhibition was reversible in the presence of high FAD concentration, suggesting competition of the inhibitor with the prosthetic group.

In similar experiments strong inhibition by adenylic acid, ATP and FMN was observed. 50 % inhibition was given by concentration of approximately 5×10^{-4} *M*. The inhibition by DPN was somewhat weaker, while adenosine, adenine and hypoxanthine exerted inhibition of the same order of magnitude at concentrations of 6×10^{-3} *M*. Cytosin and uracil were slightly inhibitory while riboflavin was without effect.

The experiments indicate that the phosphoric ester groups and the adenine part of the FAD molecule are of importance in the coupling to the apoenzyme. A possible physiological significance by some of these inhibitors in regulation of enzyme activity is suggested.

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The affinity of riboflavin for the protein is very weak as compared to the tight binding in the FMN-protein system. Thus, the dissociation constant in water is of the order of magnitude $5 \times 10^{-6} M$ as compared to $10^{-12} M$ or less for FMN-protein. This correlates with the low enzymatic activity of riboflavin as compared to FMN.

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Inosine triphosphate (ITP) — previously found to be inactive [2] without the addition of a magnesium salt [3] — gave a volume change of actomyosin gel comparable in magnitude to that given by the other nucleoside triphosphates, but somewhat slower. No addition of magnesium salt was required, although the concentration of magnesium in the reaction mixture was below 4×10^{-7} *M*.

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Deamination of Adenosine Diphosphate by Adenylate Deaminase Preparations

Adam Deutsch and Roy Nilsson

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The deamination of adenine nucleotides by muscle preparations is generally assumed to proceed via adenosine 5′-monophosphate (AMP) through the action of a specific adenylate deaminase associated with myosin. In recent reports, evidence was presented for an additional route of deamination: direct deamination of adenosine diphosphate (ADP) by actomyosin gel [1] and washed myofibrils [2]. Deamination of ADP can likewise be effected by preparations of adenylate deaminase obtained from water extracts of skeletal muscle by the method of Kalckar [3]. Whereas only AMP is deaminated under the usual conditions [3] of the adenylate deaminase test (1—5 μg protein/ml and a reaction time of up to 60 min.), higher protein concentrations and longer reaction times lead to the deamination of ADP as well. 80—90 % deamination was observed in reaction mixtures containing 5 mg protein and 7 μmoles ADP per ml in 0.1 *M* succinate buffer, pH 6.0 at 20° C and using a reaction time of 4 hours. Ion exchange [4] and two-dimensional paper chromatography [5] were used for analysis of the deproteinized reaction mixtures. Inosine diphosphate (IDP) was found to be the main product of the deamination of ADP and was isolated as the barium salt from pooled fractions of the effluent from ion exchange columns and identified by chemical analysis (hypoxanthine : ribose : acid labile P : total P). Beside IDP, minor amounts of inosine 5′-monophosphate (IMP) were detected and a further inosine nucleotide fraction, which precedes IDP in the ion exchange effluent and contains hypoxanthine, pentose, acid labile and acid stable phosphate in the proportions 2 : 2 : 1 : 2, corresponding thus in its composition to an equimolar mixture of IMP and IDP, or to a diinosine triphosphate.

Experiments on the pH-dependence of ADP deamination by adenylate deaminase preparations point to a pH optimum of near pH 6, in harmony with previous findings on actomyosin gel [1].

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2. Deutsch, A. and Nilsson, R. *Unpublished results.*
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On the Dissolving Power of the Bile for Cholesterol

Björn Isaksson

Department of Medical Chemistry, University of Gothenburg, Gothenburg, Sweden

The fractional extraction procedure on lyophilized bile described by Isaksson [1] gives the following separation of the main components: cholesterol is found in the ethyl ether extract, lecithin and part of the bile salts in the subsequent chloroform extract and the rest of the bile salts in the following ethanol extract. The constituents of the chloroform appear in rather constant proportions, 1 mole lecithin per 3 moles bile salts. This system is soluble in water and has the power to dissolve cholesterol. Thus, it seemed to be of value to interpret the importance of lecithin for the dispersion of the bile cholesterol. Since the works of Andrews *et al.*[2] the bile salts have been known as the only components of bile of interest in that respect.

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Experiments on artificial systems: Pure lecithin and bile salts were mixed in different proportions and tested for their power to dissolve added cholesterol with pH and temperature conditions as above. The best weight relation was found to be about 35/65 (*i.e.* 1 mole lecithin per 3 moles bile salts). The desoxycholic salts were slightly superior to the cholic salts, but no significant difference between tauro and glyco conjugates was found.

original nucleoside triphosphate. Identical results were obtained with synthetic UTP, obtained by courtesy of Professor A. R. Todd, Cambridge.

Inosine triphosphate (ITP) — previously found to be inactive [2] without the addition of a magnesium salt [3] — gave a volume change of actomyosin gel comparable in magnitude to that given by the other nucleoside triphosphates, but somewhat slower. No addition of magnesium salt was required, although the concentration of magnesium in the reaction mixture was below 4×10^{-7} *M*.

This work has been supported by grants from the *Swedish Natural Science Research Council* and the *Lilly Foundation*.

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Deamination of Adenosine Diphosphate by Adenylate Deaminase Preparations

Adam Deutsch and Roy Nilsson

Institute of Chemistry, University of Lund, Sweden

The deamination of adenine nucleotides by muscle preparations is generally assumed to proceed via adenosine 5′-monophosphate (AMP) through the action of a specific adenylate deaminase associated with myosin. In recent reports, evidence was presented for an additional route of deamination: direct deamination of adenosine diphosphate (ADP) by actomyosin gel [1] and washed myofibrils [2]. Deamination of ADP can likewise be effected by preparations of adenylate deaminase obtained from water extracts of skeletal muscle by the method of Kalckar [3]. Whereas only AMP is deaminated under the usual conditions [3] of the adenylate deaminase test (1—5 μg protein/ml and a reaction time of up to 60 min.), higher protein concentrations and longer reaction times lead to the deamination of ADP as well. 80—90 % deamination was observed in reaction mixtures containing 5 mg protein and 7 μmoles ADP per ml in 0.1 *M* succinate buffer, pH 6.0 at 20° C and using a reaction time of 4 hours. Ion exchange [4] and two-dimensional paper chromatography [5] were used for analysis of the deproteinized reaction mixtures. Inosine diphosphate (IDP) was found to be the main product of the deamination of ADP and was isolated as the barium salt from pooled fractions of the effluent from ion exchange columns and identified by chemical analysis (hypoxanthine : ribose : acid labile P : total P). Beside IDP, minor amounts of inosine 5′-monophosphate (IMP) were detected and a further inosine nucleotide fraction, which precedes IDP in the ion exchange effluent and contains hypoxanthine, pentose, acid labile and acid stable phosphate in the proportions 2 : 2 : 1 : 2, corresponding thus in its composition to an equimolar mixture of IMP and IDP, or to a diinosine triphosphate.

Experiments on the pH-dependence of ADP deamination by adenylate deaminase preparations point to a pH optimum of near pH 6, in harmony with previous findings on actomyosin gel [1].

This work has been supported by grants from the *Swedish Natural Science Research Council* and the *Lilly Foundation*.

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On the Deaminase Component of Actomyosin

Adam Deutsch and Roy Nilsson

Institute of Chemistry, University of Lund, Sweden

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Partition Chromatography of Brain Gangliosides on Cellulose

Lars Svennerholm

Department of Medical Chemistry, University of Gothenburg, Gothenburg, Sweden

The separation of the lipopolysaccharide ganglioside from a mixture of the naturally occurring brain lipids involves many difficulties and gives a low yield. Klenk [1] based his methods on solubility relations, including a separation of ganglioside from the last traces of sphingomyelin on aluminium oxide columns. Folch *et al.*[2] using partition dialysis were not able to obtain more than rather crude ganglioside. Trying to find a convenient method the author has explored the possibility of chromatographic separation on cellulose columns.

Gray matter from human brains was first extracted with boiling acetone and then in a Soxhlet apparatus with boiling chloroform: methanol 1 : 2 v/v. After removal of the solvent and re-solution in a small volume of watersaturated chloroform : methanol 8 : 1, the lipid extract was placed on the top of a column of cellulose packed as described by Hough *et al.*[3] The column was run with the solvent stated above. The solvent collected in 10 ml portions was removed and when no residue was obtained the solvent was changed to chloroform : methanol : water 3 : 9 : 1. Ganglioside determinations by the method of Klenk and Langerbeins [4] showed that ganglioside appeared as two bands; the first close behind the solvent front together with the bulk of the other lipids, the latter after the solvent had been changed. The hexosamine content was higher in the first ganglioside fraction. That is in good agreement with the fact that the hexosamine content of ganglioside is reduced by reprecipitations with chloroform: methanol.

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An Attempt to Crystallise β-Lactoglobulin from Human Milk Whey

Bengt Johansson

Department of Medical Chemistry, University of Gothenburg, Gothenburg, Sweden

In contrast to the fairly well known proteins of bovine milk whey the whey proteins in human milk have been subject to very few investigations. Wegelin [1] recently reported about the occurrence in human whey of a component with approx. the same electrophoretic mobility as bovine β-lactoglobulin at pH 6.8. This component amounted to about 10 % of the total whey protein and does not seem to have been isolated so far.

An attempt to isolate the possible β-lactoglobulin in human milk whey has recently been made in this laboratory as part of an investigation of the non-casein proteins of human milk.

Pooled human milk was freed from fat by centrifugation and dialysed against frequently changed distilled water at a temperature of + 3—4° C for 48 hours. The casein was precipitated at 30° C and pH 4.6 without dilution of the milk. The completely clear whey was treated with anhydr. sodium sulphate according to Palmer [2] or ammonium sulphate according to Sörensen [3]. In both cases the resulting precipitate of "lactalbumine" appeared to be faintly salmon coloured. This colour has been observed in all preparations. After redissolving in water this fraction was dialysed against distilled water for a long time and the solution adjusted to pH 5.2. In this step crystals of β-lactoglobulin are obtained when the initial material is bovine whey. Despite repeated preparations it has not been possible to obtain any crystals in the corresponding human fraction. Paper electrophoresis of the red coloured dialysed protein solution reveals three components of which the one with the lowest mobility appeared as a faintly red coloured spot on the paper strips. None of the components seems to be identical with bovine β-lactoglobulin. The properties of this red coloured protein fraction are under investigation.

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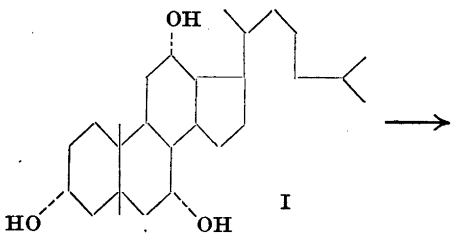
Synthesis and Metabolism of 3α,7α,12α [4—^{14}C] coprostane

S. Bergström, K. Pääbo and J. A. Rumpf

Department of Physiological Chemistry, University of Lund, Lund, Sweden

During the transformation of cholesterol into bile acids, the cholesterol side chain is presumably degraded through a ω- or methyl-oxidation followed by β-oxidation of an intermediary α-methyl-C_{27} acid. It is not known when these reactions take place in relation to the various changes on the ring system.

We have now prepared 3α-, 7α, 12α-coprostane (I) *i.e.* a compound with the cholesterol side chain and the same configuration in the cyclic part as cholic acid.



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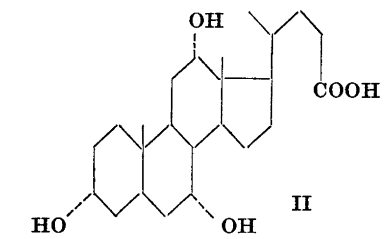

II

It has been prepared by electrolysis of a mixture of cholic acid (II) and 2-methylbutyric acid; m.p. 186°. (Found: C 76.7; H 11.4. Calc. for $C_{27}H_{46}O_3$: C 77.1; H 11.5; $[\alpha]_D^{20} = 15.1° \pm 2°$ [0.9 ethanol]).

Starting from [4—^{14}C] cholic acid and a large excess of 3-methylbutyric acid the corresponding ring labelled compound was obtained.

This compound has been administered intraperitoneally to rats with a bile fistula. Practically all activity was recovered in the bile during the following 10 hours. The isotope was to more than 90 per cent contained in taurocholic acid. A small amount of a labelled acidic compound was found in the chromatograms in the region where free trihydroxy acids appear but has not yet been identified.

Thus, when the ring system has the configuration of cholic acid, the sterol side chain is rapidly and completely degraded to that of a bile acid (I → II).

In contradistiction, when ring labelled cholesterol is administered intraperitoneally in the same manner, it is distributed in the body and the isotope only slowly appears in the bile mainly as bile acids during the following weeks.

Metabolism of Bile Acids in Liver Slices and Homogenates

Sune Bergström and Urs Gloor

Department of Physiological Chemistry, University of Lund, Lund, Sweden

The conjugation and 7 α-hydroxylation of desoxycholic acid to taurocholic acid found to take place in the rat *in vivo*, has also been demonstrated in rat liver slices [1,2].

In continuing this work, these reactions have now been further studied in human and rat liver slices and homogenates. Carboxyl labeled bile acids have been used as substrate and the reaction products have mainly been characterized by paper chromatographic methods [3]. Quantitative results have been obtained by measuring the radioactivity of the paper strips by means of an automatic scanner. The homogenizing medium contained 10.1 g sodium phosphate, 1.4 g potassium phosphate and 43 g sucrose per liter. The pH was 7.4. A short homogenizing time and a loose fitting pestle are essential. The conjugation with taurine in rat liver homogenates was inhibited at concentrations of bile acids in the range of 10—30 mg % (w/v, 30 % homogenate). However, the hydroxylation of taurodesoxycholic acid proceeds at much higher concentrations.

With the aid of the same technique we have investigated the metabolism of cholic, desoxycholic, chenodesoxycholic and lithocholic acid in human liver homogenates. The capacities to conjugate bile acids in these preparations were in the same range as for the rat liver homogenates. The average value for the conjugation of cholic acid without addition of any amino acids was 60 % taurocholic and 40 % glycocholic acid. The addition of 3 moles taurine changed the composition to 90 % taurocholic and 10 % glycocholic acid. Glycine (3—30 moles per mole bile acid) added to the homogenates of human liver caused smaller changes. Other substances such as cysteine

or cysteine-sulfonic acid had minor but distinct effect on this ratio.

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Synthesis and Metabolism of 2,2,17,17-Tetramethyloctadecanoic Acid

N. Tryding and G. Westöö

Department of Physiological Chemistry, University of Lund, Lund, Sweden

Recently 2,2-dimethyl [1-^{14}C] octadecanoic acid [1] and 2-methyl [1-^{14}C] octadecanoic acid [2] have been prepared and administered to adult white rats. According to the activity recovered in the expired carbon dioxide, 2-methyloctadecanoic acid is metabolized in the same way as stearic acid. 2,2-Dimethyl [1-^{14}C] octadecanoic acid was well absorbed, but the isotope was mainly excreted in the urine as 2,2-dimethyl [1-^{14}C] adipic acid [1].

We have now prepared 2,2,17,17-tetramethyl [1-^{14}C] octadecanoic acid from 15,15-dimethylhexadecanoic acid and methyl hydrogen 2,2-dimethyl [1-^{14}C] succinate (3-[3^{1}-^{14}C] carbomethoxy-3-methylbutyric acid) by anodic synthesis. M.p. 46.6°—46.8°. (Found: C 77.5; H 12.9; equiv. wt. 341, Calc. for $C_{23}H_{44}O_2$ (340.6): C 77.6; H 13.0; equiv. wt. 341.)

When tetramethyl [1-^{14}C] octadecanoic acid was fed to white rats no activity could be found in the urine.

The different metabolism of these branched fatty acids will be discussed.

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Naturally Occurring *iso*Thiocyanates and their Possible Relationship with α-Amino Acids

Anders Kjær

Chemical Laboratory of the University of Copenhagen, Denmark

Systematic paperchromatographic investigations [1] of seed and fresh parts of more than one hundred plant species, mainly belonging to the family *Cruciferae*, have indicated the presence of about 10 different, glucosidically bound, volatile *iso*thiocyanates. In addition to the previously known allyl, *sec*-butyl, benzyl and β-phenylethyl *iso*thiocyanates, the occurrence of 3-butenyl [2], *iso*propyl [3] and ethyl [4] *iso*thiocyanate in certain species has been definitely established in this laboratory. Three or four spots have been observed attributable to *iso*thiocyanates of so far unrevealed structure.

Chemical similarities between naturally occurring *iso*thiocyanates and the common α-amino acids suggest a possible biochemical relationship which will be briefly discussed.

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4. Kjær, A. and Larsen, I. *Acta Chem. Scand* **8** (1954) 699.

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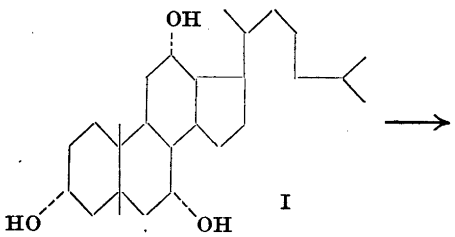
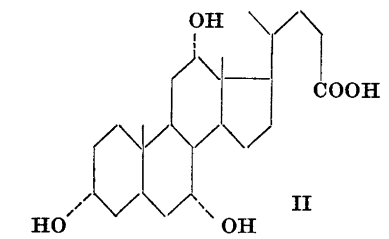


I



II

It has been prepared by electrolysis of a mixture of cholic acid (II) and 2-methylbutyric acid; m.p. 186°. (Found: C 76.7; H 11.4. Calc. for $C_{27}H_{46}O_3$: C 77.1; H 11.5; $[\alpha]_D^{20} = 15.1° \pm 2°$ [0.9 ethanol]).

Starting from [4—^{14}C] cholic acid and a large excess of 3-methylbutyric acid the corresponding ring labelled compound was obtained.

This compound has been administered intraperitoneally to rats with a bile fistula. Practically all activity was recovered in the bile during the following 10 hours. The isotope was to more than 90 per cent contained in taurocholic acid. A small amount of a labelled acidic compound was found in the chromatograms in the region where free trihydroxy acids appear but has not yet been identified.

Thus, when the ring system has the configuration of cholic acid, the sterol side chain is rapidly and completely degraded to that of a bile acid (I → II).

In contradistiction, when ring labelled cholesterol is administered intraperitoneally in the same manner, it is distributed in the body and the isotope only slowly appears in the bile mainly as bile acids during the following weeks.

Metabolism of Bile Acids in Liver Slices and Homogenates

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The conjugation and 7 α-hydroxylation of desoxycholic acid to taurocholic acid found to take place in the rat *in vivo*, has also been demonstrated in rat liver slices [1,2].

In continuing this work, these reactions have now been further studied in human and rat liver slices and homogenates. Carboxyl labeled bile acids have been used as substrate and the reaction products have mainly been characterized by paper chromatographic methods [3]. Quantitative results have been obtained by measuring the radioactivity of the paper strips by means of an automatic scanner. The homogenizing medium contained 10.1 g sodium phosphate, 1.4 g potassium phosphate and 43 g sucrose per liter. The pH was 7.4. A short homogenizing time and a loose fitting pestle are essential. The conjugation with taurine in rat liver homogenates was inhibited at concentrations of bile acids in the range of 10—30 mg % (w/v, 30 % homogenate). However, the hydroxylation of taurodesoxycholic acid proceeds at much higher concentrations.

With the aid of the same technique we have investigated the metabolism of cholic, desoxycholic, chenodesoxycholic and lithocholic acid in human liver homogenates. The capacities to conjugate bile acids in these preparations were in the same range as for the rat liver homogenates. The average value for the conjugation of cholic acid without addition of any amino acids was 60 % taurocholic and 40 % glycocholic acid. The addition of 3 moles taurine changed the composition to 90 % taurocholic and 10 % glycocholic acid. Glycine (3—30 moles per mole bile acid) added to the homogenates of human liver caused smaller changes. Other substances such as cysteine

or cysteine-sulfonic acid had minor but distinct effect on this ratio.

1. Bergström, S., Rottenberg, M. and Voltz, J. *Hoppe-Seyler's Z. Physiol. Chem.* **295** (1953) 278.
2. Bergström, S., Dahlqvist, A. and Ljungqvist, U. *Kgl. Fysiograf. Sällskap. Lund. Förh.* **23** (1953) No. 12.
3. Sjövall, J. *Acta Chem. Scand.* **8** (1954) 329.

Synthesis and Metabolism of 2,2,17,17-Tetramethyloctadecanoic Acid

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Recently 2,2-dimethyl [1-^{14}C] octadecanoic acid [1] and 2-methyl [1-^{14}C] octadecanoic acid [2] have been prepared and administered to adult white rats. According to the activity recovered in the expired carbon dioxide, 2-methyloctadecanoic acid is metabolized in the same way as stearic acid. 2,2-Dimethyl [1-^{14}C] octadecanoic acid was well absorbed, but the isotope was mainly excreted in the urine as 2,2-dimethyl [1-^{14}C] adipic acid [1].

We have now prepared 2,2,17,17-tetramethyl [1-^{14}C] octadecanoic acid from 15,15-dimethylhexadecanoic acid and methyl hydrogen 2,2-dimethyl [1-^{14}C] succinate (3-[3^{1}-^{14}C] carbomethoxy-3-methylbutyric acid) by anodic synthesis. M.p. 46.6°—46.8°. (Found: C 77.5; H 12.9; equiv. wt. 341, Calc. for $C_{23}H_{44}O_2$ (340.6): C 77.6; H 13.0; equiv. wt. 341.)

When tetramethyl [1-^{14}C] octadecanoic acid was fed to white rats no activity could be found in the urine.

The different metabolism of these branched fatty acids will be discussed.

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Naturally Occurring *iso*Thiocyanates and their Possible Relationship with α-Amino Acids

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Systematic paperchromatographic investigations [1] of seed and fresh parts of more than one hundred plant species, mainly belonging to the family *Cruciferae*, have indicated the presence of about 10 different, glucosidically bound, volatile *iso*thiocyanates. In addition to the previously known allyl, *sec*-butyl, benzyl and β-phenylethyl *iso*thiocyanates, the occurrence of 3-butenyl [2], *iso*propyl [3] and ethyl [4] *iso*thiocyanate in certain species has been definitely established in this laboratory. Three or four spots have been observed attributable to *iso*thiocyanates of so far unrevealed structure.

Chemical similarities between naturally occurring *iso*thiocyanates and the common α-amino acids suggest a possible biochemical relationship which will be briefly discussed.

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ACTA CHEMICA SCANDINAVICA 8 (1954) 1111—1119

Addition of Thioglycolic Acid to α-Alkenes

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In a recent study [1] one of us made use of the addition of thioglycolic acid to the ethylenic linkage in order to characterize the unsaturated hydrocarbons in a peat-tar fraction. It was found that alkenes, among other compounds, were present. Their structure was not definitely established, but the occurrence of normal α-alkenes was indicated.

To make a closer examination of this structural problem possible, it was decided to study in some detail the addition of thioglycolic acid to some α-alkenes in the range from octene to undecene. Holmberg [2] has studied the reaction between styrene and thioglycolic acid and Kharasch *et al.*[3] the reaction between styrene or *iso*butylene and thioglycolic acid. Cunneen [4] added thioglycolic acid to *cyclo*hexene and 1-methyl*cyclo*hexene whilst Hellström and Sandström [5] added the same acid to some methyl*cyclo*hexenes. Apart from an attempt by Hoog and Eichwald [6] to isolate some alkenes from mixtures containing saturated hydrocarbons by means of thioglycolic acid, no investigation of the reaction between this reagent and long chain alkenes has been reported, according to our knowledge.

THE ADDITION REACTION

Thioglycolic acid is not miscible at room temperature with the alkenes from C_8 to C_{11}, and no reaction is observed when thioglycolic acid is dropped into an equimolecular amount * of the alkene. Vigorous shaking, however, rapidly brings about a violent reaction, the temperature rising to 50—60°. A typical yield-time curve is shown in Fig. 1. After about 2 minutes the yield of octylthioglycolic acid is 80 % and after half an hour 90 %. A final value of 95—97 % is reached in about 20 hours. The yield-time curve for the reaction between equimolecular amounts of thioglycolic acid and styrene ** is also shown in Fig. 1. It is not possible to draw, from the shape of these curves, any definite conclusions concerning the relative reactivities of the above mentioned alkenes and styrene towards thioglycolic acid, as several factors influence the reaction (see below).

* Equimolecular amounts of alkene and thioglycolic acid were used throughout this work.
** Constructed from the values given by Holmberg [2].

Effect of peroxides and oxygen. It seems to be well established that the "abnormal" addition (see p. 1114) of thiols to unsaturated compounds is a chain reaction, proceeding through a radical mechanism [3,7]. Consequently peroxides, present either as olefine peroxides or specially added, and also oxygen are of importance in the reaction. Cunneen [4], for example, reported that *cyclo*hexene and 1-methyl*cyclo*hexene, both freshly distilled in an atmosphere of nitrogen, did not react with thioglycolic acid at room temperature. Addition of a little ascaridole, however, caused an immediate reaction. Several attempts in this work to prevent the reaction between alkenes and thioglycolic acid by exclusion of peroxides and oxygen failed. In one case octene-1 was shaken with acid ferrous sulfate solution and then distilled over sodium in an atmosphere of oxygen-free nitrogen. The thioglycolic acid used was also distilled in an atmosphere of oxygen-free nitrogen and the mixing of the reagents carried out with the exclusion of oxygen. Nevertheless an immediate reaction ensued and the yield of octylthioglycolic acid was over 90 %. Hence only minute traces of oxygen seem to be necessary to catalyze the reaction.

Effect of antioxidants. In view of these results it was of interest to study the influence of antioxidants on the reaction between alkenes and thioglycolic acid. Addition of as little as 0.1 mol.-% of hydroquinone had a distinct effect. Only after about 20 minutes shaking did the reaction mixture become homogeneous, whilst in the absence of hydroquinone about 10 seconds were sufficient. The yield after 30 minutes was about 40 %, after 20 hours about 80 %. The temperature rose only a few degrees, compared to 30—40° in the absence of hydroquinone. When 0.5 mol.-% of hydroquinone was used, the reaction mixture was nonhomogeneous even after shaking for 48 hours and the yield was 8 %. These experiments demonstrate the retarding effect of antioxidants on the reaction between alkenes and thioglycolic acid.

Effect of ultraviolet light. The majority of the experiments in this work was performed in diffuse daylight. However, the reaction between alkenes and thioglycolic acid proceeded as readily in total darkness. Thus no irradiation with ultraviolet light is necessary under ordinary conditions.

Effect of impurities in the thioglycolic acid. Thioglycolic acid is generally prepared from chloroacetic acid and sodium or potassium hydrosulfide. The boiling points of thioglycolic acid and chloroacetic acid are close and repeated fractionation is necessary to obtain a pure product which nevertheless may still contain small amounts of chloroacetic acid. The possibility of this impurity on storage of the product, giving rise to some thiodiglycolic acid, $S(CH_2COOH)_2$, must be taken in account.

However, the principal change resulting from the storage of thioglycolic acid in contact with air is the formation of dithiodiglycolic acid, $HOOCCH_2SSCH_2COOH$. It has been stated [6] that thioglycolic acid loses its activity on storage in contact with air, so some experiments were carried out to study the influence of dithiodiglycolic acid on the reactivity of thioglycolic acid towards alkenes.

Table 1 lists some semi-quantitative experiments with various specimens of thioglycolic acid and alkenes. For each experiment, this table shows the time elapsed from the mixing of the reagents (equimolecular amounts) and commencement of the shaking until the reaction mixture is homogeneous. Several experiments were made with each pair and the shortest time taken.

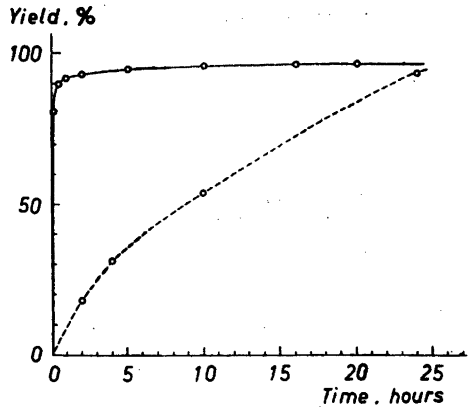


Figure 1. Yield-time curve for octene-1 and thioglycolic acid (———) and styrene and thioglycolic acid (– – –).

The decreasing reactivity of thioglycolic acid towards alkenes on storage is clearly demonstrated (Nos. 1—4). While the reaction time in the case of octene and nonene is only slightly increased, there is a marked difference in reactivity of freshly distilled and stored thioglycolic acid towards decene and 4-methyldecene and especially towards undecene. By addition of benzoyl peroxide to the pair, undecene and thioglycolic acid stored for 8 months, the reaction time was decreased from more than 60 minutes to 8 minutes.

In the experiments Nos. 5—8, various amounts of dithiodiglycolic acid were added to samples of freshly distilled thioglycolic acid and the effect on the reactivity of the latter noted. Comparison of the reaction times observed in these experiments with those in Nos. 1—4 indicates that the formation of dithiodiglycolic acid is not alone responsible for the decrease in reactivity of thioglycolic acid on storage as was assumed by Hoog and Eichwald [6].

From experiments Nos. 9 and 10 with thioglycolic acid containing 1 % of chloroacetic acid and 1 % of thiodiglycolic acid, respectively, it appears that the small amounts of these compounds which may be present in "pure" thioglycolic acid do not affect its reactivity greatly.

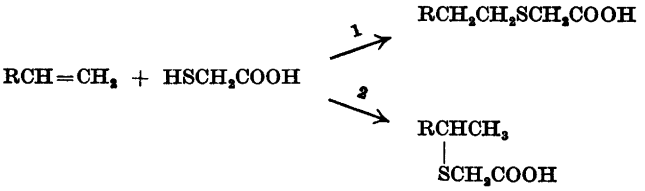
It is evident from the foregoing that thioglycolic acid, when stored in tightly stoppered bottles in contact with air (as was the case here) does not rapidly lose its reactivity towards alkenes. Even after 2 months it was as reactive as a freshly distilled sample.

Effect of impurities in the alkenes. The alkenes used were prepared by two methods, either from the appropriate alkyl magnesium bromides and allyl bromide or by pyrolysis of alkyl acetates. When the alkenes formed were carefully purified by prolonged boiling with metallic sodium and fractionation on an efficient column, there did not exist any difference in reactivity between specimens obtained by the two methods. However, in some cases, when using samples obtained by the allyl bromide method, the reaction did not start at all or gave low yields. It is possible that the presence of small amounts of impurities were responsible for this behaviour.

Table 1. Reactivity of thioglycolic acid towards alkenes as a function of time of storage and impurities.

Experiment No.	Specimen of thioglycolic acid	Octene-1	Nonene-1	Decene-1	4-Methyldecene-1	Undecene-1
1	Freshly distilled (99.7 % purity)	11 sec.	10 sec.	8 sec.	20 sec.	11 sec.
2	Stored for 2 months (99.4 % purity)	7 sec.	7 sec.	9 sec.	9 sec.	18 sec.
3	Stored for 5 months (95.0 % purity)	20 sec.	26 sec.	22 sec.	27 sec.	1 min. 43 sec.
4	Stored for 8 months (92.0 % purity)	21 sec.	25 sec.	1 min. 12 sec.	2 min.	>60 min.
5	With 1 % of dithiodiglycolic acid	11 sec.	15 sec.	16 sec.	15 sec.	32 sec.
6	With 2 % of dithiodiglycolic acid	13 sec.	14 sec.	18 sec.	22 sec.	33 sec.
7	With 5 % of dithiodiglycolic acid	12 sec.	15 sec.	16 sec.	20 sec.	50 sec.
8	With 10 % of dithiodiglycolic acid	12 sec.	19 sec.	26 sec.	30 sec.	2 min. 30 sec.
9	With 1 % of chloroacetic acid	11 sec.	12 sec.	15 sec.	17 sec.	30 sec.
10	With 1 % of thiodiglycolic acid	12 sec.	14 sec.	16 sec.	25 sec.	1 min. 9 sec

Structure of the addition products. The addition of thioglycolic acid to α-alkenes may result in the formation of two different products.



The reaction may proceed either contrary to Markownikoff's rule ("abnormal addition"), or according to this rule ("normal addition"). All alkenes investigated in this work reacted exclusively according to the former type of addition. In no instances could products formed by the reaction path 2 be traced. The structures of the alkylthioglycolic acids formed were established by comparison with the properties of alkylthioglycolic acids prepared from the appropriate alkyl bromides and sodium thioglycolate or with literature values (see Table 2).

Although the reaction between thiols and olefines is generally "abnormal", it has been possible to alter the addition in some instances by using catalysts such as sulfur or sulfuric acid [9,10]. However, sulfur did not change the "abnormal" reaction between octene-1 and thioglycolic acid, *n*-octylthioglycolic acid being formed exclusively. When sulfuric acid is used as a catalyst the

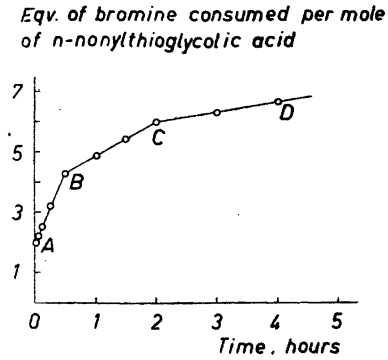


Figure 2. Diagram illustrating the reaction between n-nonylthioglycolic acid and bromine.

reaction is not to be considered as an addition of thiol to alkene. In this case sulfuric acid first adds to the double bond and the resulting ester subsequently reacts with the thiol [10]. As the addition of sulfuric acid to olefines proceeds according to Markownikoff's rule the reaction product between ester and thiol is the "normal" one. No experiments using sulfuric acid as a catalyst were made in this work.

Jones and Reid [9] found that ethane thiol reacted with propylene giving ethyl-*n*-propyl sulfide in the presence of peroxides and ethyl-*i*-propyl sulfide in their absence. In the previously described experiment (p. 1112) where octene-1 was allowed to react with thioglycolic acid in the absence of peroxides and oxygen, only *n*-octylthioglycolic acid was formed. This was also the case in the presence of hydroquinone.

Alkylsulfinylacetic and alkylsulfonylacetic acids

In order to compare the properties of some pure alkylsulfonylacetic acids with those of the sulfonylacetic acids obtained in the above-mentioned investigation of the alkenes contained in a peat-tar fraction, the alkylthioglycolic acids were oxidized by potassium permanganate to the corresponding alkylsulfonylacetic acids.

The reaction of the alkylthioglycolic acids with bromine presented some interesting points. Fig. 2 demonstrates the course of the reaction between *n*-nonylthioglycolic acid and bromine in excess. Two equivalents of bromine are used up instantly with the formation of the sulfinylacetic acid and subsequently (stage A—B) the oxidation of the sulfinylacetic acid to sulfonylacetic acid takes place. Stage B—C obviously represents substitution of hydrogen by bromine, probably in the methylene group situated between the carboxyl and sulfonyl groups. Further substitution meets with greater resistance (stage C—D).

As a result of the rapid oxidation of the alkylthioglycolic acids in question to the corresponding sulfinylacetic acids, the method of Siggia and Edsberg [11] for the quantitative determination of alkyl sulfides gave good results (see

Table 2. Alkylthioglycolic acids $RSCH_2COOH$.

R	B.p. °C (mm)	M.p. °C	% S		Equiv.wt. [a]		Equiv.wt. [b]		Method of preparation [c]
			Found	Calcd.	Found	Calcd.	Found	Calcd.	
n-C_8H_{17}	138 (0.35)	36.9 [d]	15.9	15.69	204.2	204.3	102.1	102.2	A
s-C_8H_{17} [e]	137 (0.70)	<0 [f]	15.6	15.69	204.7	204.3	102.6	102.2	B
n-C_9H_{19} [g]	157 (0.67)	53.4 [h]	14.6	14.68	218.6	218.4	109.1	109.2	A
n-$C_{10}H_{21}$	—	51.8 [i]	13.7	13.80	231.9	232.2	116.0	116.2	A
n-$C_{11}H_{23}$ [g]	—	63.0 [j]	13.1	13.01	247.5	246.4	123.8	123.2	A
4-CH_3-n-$C_{10}H_{21}$ [g,k]	126—127 (0.02)	<0 [l]	13.2	13.01	244.0	246.4	123.0	123.2	A

a. Titration with 0.1 N sodium hydroxide against phenolphthalein.
b. » » 0.05 N bromide-bromate solution.
c. Method A means addition of thioglycolic acid to the corresponding α-alkene.
» B » reaction of an alkyl bromide with sodium thioglycolate.
d. Literature value 36—37° [8].
e. $d_4^{20} = 0.9940$, $n_D^{20} = 1.4751$, $MR_D = 57.9$ (found) 57.87 (calcd.).
f. M. p. of benzyl thiuronium salt 137°.
g. New compound.
h. No depression of the m. p. on admixture with n-nonylthioglycolic acid prepared by method B, m. p. 53°.
i. Literature value 52—53° [8].
j. No depression of the m. p. on admixture with n-undecylthioglycolic acid prepared by method B, m. p. 63°.
k. $d_4^{20} = 0.9703$, $n_D^{20} = 1.4753$, $MR_D = 71.5$ (found) 71.57 (calcd.).
l. M. p. of benzyl thiuronium salt 147°. M. p. of the thiuronium salt of 4-methyldecylthioglycolic acid prepared by method B, 146°. Mixed m. p. 146°.

Table 2). This method involves direct titration with 0.05 N bromide-bromate solution in an acidic medium.

The method was also found suitable for the preparation of alkylsulfinylacetic acids from alkylthioglycolic acids. For this purpose, however, a 0.5 N bromide-bromate solution was used. The physical constants and analyses for the sulfinylacetic and sulfonylacetic acids are given in Tables 3 and 4.

*Table 3. Alkylsulfinylacetic acids $RSOCH_2COOH$ *.*

R	M.p. °C	% S		Equiv.wt. **	
		Found	Calcd.	Found	Calcd.
n-C_8H_{17}	60.4	14.5	14.55	221.0	220.3
n-C_9H_{19}	70.3	13.6	13.68	234.7	234.4
n-$C_{10}H_{21}$	74.9	12.7	12.91	249.0	248.4
n-$C_{11}H_{23}$	81.1—81.5	12.3	12.22	261.0	262.4

* All compounds in this table are new.
** Titration with 0.1 N sodium hydroxide against phenolphthalein.

Table 4. Alkylsulfonylacetic acids RSO_2CH_2COOH.

R	M.p. °C	% S		Equiv.wt. [a]	
		Found	Calcd.	Found	Calcd.
n-C_8H_{17} [b]	96.5	13.5	13.57	236.9	236.3
s-C_8H_{17} [c]	16—17.5	13.2	13.57	233.3	236.3
n-C_9H_{19} [c]	105.1	12.7	12.81	249.8	250.4
n-$C_{10}H_{21}$ [d]	105.7	12.0	12.13	264.7	264.4
n-$C_{11}H_{23}$ [c]	111.0—111.6	11.5	11.52	278.0	278.4

a. Titration with 0.1 *N* sodium hydroxide against phenolphthalein.
b. Prepared previously by Newman *et al.* [12], no m.p. reported.
c. New compound.
d. Literature value 104—105° [8].

Melting points of the acids

In the utilization of thioglycolic acid for the identification of alkenes, the melting points of the alkylthioglycolic, alkylsulfinylacetic, and alkylsulfonylacetic acids are of special interest. In Table 5 the melting points of some acids of these types, prepared in this work or taken from the literature, are compiled together with the melting points of the corresponding fatty acids, derived from the alkylthioglycolic acids by replacing the sulfur atom by a methylene group.

The melting points of the normal alkylthioglycolic acids increase as the series is ascended, but not regularly. The acids with an uneven number of carbon atoms in the alkyl group melt at higher temperatures than those which immediately follow them, and contain one carbon atom more. The normal alkylthioglycolic acids thus fall into two series as regards melting point, just as is the case with the normal fatty acids. In the series of normal alkylsulfinylacetic acids and normal alkylsulfonylacetic acids, however, irregularities exist. Replacement of a β-methylene group in a normal fatty acid by a sulfur atom raises the melting point by 9—11°.

Table 5. Melting points (°C) of some sulfur containing acids and the corresponding fatty acids.

R	$RSCH_2COOH$	$RSOCH_2COOH$	RSO_2CH_2COOH	RCH_2CH_2COOH [a]
n-C_7H_{15}	—	—	95.5—96 [b]	31.4
n-C_8H_{17}	36.9	60.4	96.5	28.0
n-C_9H_{19}	53.4	70.3	105.1	43.6
n-$C_{10}H_{21}$	51.8	74.9	105.7	40.5
n-$C_{11}H_{23}$	63.0	81.1—81.5	111.0—111.6	54.0
n-$C_{12}H_{25}$	61—62 [c]	—	108—109 [d]	52.1
s-C_8H_{17}	<0	—	16—17.5	liquid [e]
4-CH_3-n-$C_{10}H_{21}$	<0	—	—	—

a. M. p. from ref. [13]. b. Ref. [14]. c Ref. [8]. d. Ref. [15]. e. Ref. [16].

Branching of the chain causes the melting point to drop appreciably as demonstrated by the *s*-octylthioglycolic, *s*-octylsulfonylacetic, and 4-methyldecylthioglycolic acids. This fact is in accordance with the general rule that the melting point is raised by increasing the symmetry of the molecule. It is of value for the distinguishing of normal α-alkenes on one hand and α-alkenes with branched chain as well as alkenes with the double bond elsewhere than in the α-position on the other hand by means of thioglycolic acid.

For the identification of liquid alkylthioglycolic acids the benzyl thiuronium salts have proved very suitable.

EXPERIMENTAL

Alkenes

For the preparation of the alkenes two methods were utilized.

Method 1 involved the reaction of an alkyl magnesium bromide with allyl bromide in ether solution. After refluxing for one hour the reaction mixture was treated with water and then steam distilled. The organic layer was separated, dried and fractionated. The crude alkene was refluxed over sodium as long as any precipitate was formed and finally distilled using an efficient column. Using this method octene-1, nonene-1, and decene-1 were obtained as well as 4-methyldecene-1.

Method 2 involved the pyrolysis of alkyl esters of acetic acid at 500° C according to the procedure described by Geldof and Wibaut [17]. The crude alkenes were treated as above. Using this method octene-1, nonene-1, decene-1, and undecene-1 were obtained. The physical constants of the alkenes were in very good agreement with reliable literature values.

Alkylthioglycolic acids

Addition reactions. Equivalent amounts of alkene and thioglycolic acid were mixed and shaken. The reaction generally started immediately, the mixture becoming homogeneous and the temperature rising to 50–60°. After about half an hour the temperature had decreased to room temperature. The yield at this stage was about 90 %. Higher yields (about 95 %) could be obtained by warming the reaction mixture for another 15–20 hours to 50–60°. It was also possible to use glacial acetic acid or benzene as solvent, preferably the same volume as the volume of alkene. Too much solvent must be avoided. In this case the reaction mixture was homogeneous from the beginning. The reaction proceeded in much the same way as in the absence of solvent. After half an hour the reaction mixture had reached room temperature and the yield was about 85 %. After a further period of 15–20 hours at room temperature the yield increased to about 93 %.

In the addition reactions with octene-1, nonene-1, and 4-methyldecene-1, the solvent (if used) together with the alkene and thioglycolic acid not consumed were distilled from the reaction mixture and the remaining addition product then fractionated at low pressure. The alkylthioglycolic acid obtained was, if solid, recrystallized from ligroin to constant melting point.

In the addition reactions with decene-1 and undecene-1 the residue after removal of the low-boiling components was not distilled but directly recrystallized from ligroin.

Alkylthioglycolic acids from alkyl bromides and sodium thioglycolate were obtained according to Larsson [18] and purified as described above.

Alkylsulfinylacetic and alkylsulfonylacetic acids

Alkylsulfinylacetic acids. The alkylthioglycolic acid (0.01 mole) was dissolved in 50 ml of glacial acetic acid, containing 3 ml of conc. hydrochloric acid, and 40.0 ml of a 0.5 *N* bromide-bromate solution was added dropwise. Pouring of the mixture into ice water produced crystals of the alkylsulfinylacetic acid. They were separated by filtration. Extraction of the filtrate with warm chloroform (35°) furnished some more of the sulfinyl-

acetic acid. Yield 85—95 %. The crude product was washed with warm petroleum ether only since the alkylsulfinylacetic acids were liable to decompose when dissolved in boiling solvents (*e. g.* benzene, ethyl acetate). The washing procedure, by which remaining alkylthioglycolic acids were removed, was repeated until a constant melting point was obtained. The alkylsulfinylacetic acids slowly decomposed on storage.

Alkylsulfonylacetic acids. The alkylthioglycolic acids were oxidized with potassium permanganate essentially as described by Holmberg [2]. The yield of alkylsulfonylacetic acids was 90—100 %. The crude products were recrystallized from water to constant melting points.

SUMMARY

The addition of thioglycolic acid to some α-alkenes was studied under various conditions. The addition took place exclusively against Markownikoff's rule, with excellent yields of alkylthioglycolic acids.

Alkylsulfinylacetic and alkylsulfonylacetic acids were prepared from the alkylthioglycolic acids by oxidation with bromine and potassium permanganate, respectively.

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The Formal Theory of Diffusion, and its Relation to Self-diffusion, Sedimentation Equilibrium, and Viscosity

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Consequences of the generalization of the equation $D = RT/\Phi$ for the problem of self-diffusion in mixtures are discussed. Inequality relations are reported, showing that self-diffusion is slower than the "corrected" regular diffusion at the same composition of the solution.

It is shown that the concept of an over-all mass-flow according to the picture of Hartley and Crank, does not bring about any modification in the generalized theory. It has been tried to apply the simplest possible description compatible with the physical facts.

The use of Fick's (1st) law is discussed. The formulation of the general i-component case of the diffusion theory is discussed. It has been avoided to use Fick's laws, the deduction of which is instead contained in the theory.

The complete support given by the thermodynamic sedimentation-diffusion equilibrium, for the diffusion theory, is emphasized. The interesting connection to the molecular concept and to the osmotic theory is discussed.

Attention is directed towards the limitations, which seem necessarily connected with efforts to find a general relation between the product $D\eta$ and the concentration for a binary mixture, if the magnitudes expressing the friction within each component, contained in the formulae for self-diffusion in a mixture, are taken into account.

1. DIFFUSION AND SELF-DIFFUSION

In a previous paper [1], the self-diffusion of a fluid system was treated using the theory of the diffusion of a three component system. For the two identical (labelled) components, the indices 1 and 2 are used. Seen as a whole (1 + 2), this substance is denoted by the index 0. The self-diffusion proceeds in the presence of a c o n s t a n t concentration of an additional component 3. For the self-diffusion coefficient D_0, the following expression is obtained, which is a mathematically exact consequence of the system of equations (11) —(13) below.

$$D_0 = \frac{RT}{\Phi_{1(2)} + \Phi_{2(1)} + \Phi_{0(3)}} \equiv \frac{RT}{\Phi_{12} + \Phi_{0(3)}} \qquad (1)$$

$\Phi_{i(j)}$, the molar friction, is the friction between the components i and j in a volume containing one mole of component i. For the friction/cm³ we get $\varphi_{ij} = n_i\Phi_{i(j)} = n_j\Phi_{j(i)}$, n being concentration in moles/cm³. As the two molar frictions are not independent, we denote $\Phi_{1(2)} + \Phi_{2(1)}$ by Φ_{12}, omitting the Σ-sign used in the previous paper.

$\Phi_{0(3)}$ is the friction determining the (differential) diffusion of components 0 and 3 at the same concentrations n_0 and n_3 for which eq. (1) holds, accordingto

$$D_{03} = \frac{RTB_{03}N_3}{\Phi_{0(3)}} = \frac{RTB_{03}}{\Phi_{03}} = \frac{RTB_{03}N_0}{\Phi_{3(0)}} \tag{2}$$

N is the mole fraction. B_{03} is the symmetrical activity factor $\partial \ln a/\partial \ln N$.

Equation (1) refers to a system 0,3, in which component 0 is made up of two identical components 1 and 2. With the same right we may, instead, divide component 3 in two identical parts, and write the analogous equation for D_3. Indices 4 and 5 being the "labels" put on the two parts into which component 3 is divided, gives for the self-diffusion of component 3

$$D_3 = \frac{RT}{\Phi_{45} + \Phi_{3(0)}} \tag{3}$$

It seems natural to consider the connection between diffusion in a concentration gradient of the chemically different substances 0 and 3 (sometimes called chemical diffusion) and the two self-diffusion processes, which proceed simultaneously with the former process.

As the frictions are positive quantities, the equations show that $D_{03}/B_{03}N_3 > D_0$, and $D_{03}/B_{03}N_0 > D_3$. An example of this does the measurement of Wang [2] show, the corrected differential diffusion coefficient of glycine ($N_0 \simeq 1$) being greater than the selfdiffusion coefficient of glycine at the same concentration. $-\Phi_{45}$ of equation (3) rapidly approaches zero when $N_0 \to 1$ (if the molecules of component 3 seldom meet, the friction within the component will become very small). Thus in infinitely dilute solution we must have $D_3 = D_{03}$, as also shown by Wang's measurements. The fact that the self-diffusion at finite concentrations is slower than the corresponding, corrected ordinary diffusion, seems most natural to discuss in terms of the additional friction Φ_{45} in the former case, which may reach significant values already at moderate concentrations for a substance of pronounced dipole character like glycine. — Unpublished measurements in this Institute of C. L. Undeman on the self-diffusion of water in electrolytic solutions fulfil the same inequality relation.

Another inequality condition is $D_{03}/B_{03} > D_0D_3/(D_0 + D_3)$. If one of the components (3) is highly viscous and sufficiently concentrated, we expect $D_0 \gg D_3$. In such a case we must have $D_{03}/B_{03} > D_3$, that is the (differential) chemical diffusion, corrected thermodynamically, must be more rapid than the selfdiffusion of the viscous component at the same composition of the mixture.

It is thus seen that a knowledge of the self-diffusions D_0 (eq. 1) and D_3 (eq. 3) does not give the "chemical" coefficient D_{03} (eq. 2). As a matter of fact, the coefficients of self-diffusion are more complicated with respect to friction since they depend upon the friction w i t h i n the component 0 (or

3) as well as the friction b e t w e e n these components. This greater complication in the latter case is (independent of other conventions involved in our formulae) a consequence of the self-diffusion being described as three-component diffusion, whereas the chemical diffusion in question relates to a two-component case.

An equation of the type (1) shows that self-diffusion may be "slow" either owing to a high friction between the identical molecules of component 0 or owing to a high friction between this component and 3, or both.

At the same time, eq. (2) shows that ordinary diffusion of a two-component system may be rapid, even if the corresponding self-diffusion of the components is comparatively slow.

Even if the expressions "slow" and "rapid" of this qualitative description are, from reasons of molecular physics, restricted within certain very narrow limits, our formulae show that the two self-diffusion processes are not so quantitatively related to the ordinary diffusion as one is inclined to believe. Quite apart from the influence of the thermodynamic factor B_{03}, a quantitative description of the latter phenomenon in terms merely of the self-diffusion coefficients cannot be expected to be possible on an e x a c t basis in a formal sence, even though more or less empirical relations might serve practical requirements.

Developing the molecular theory is, of course, very important. The formal theory is based upon the conception that the free energy of the activity gradient is consumed through friction b e t w e e n components but not w i t h i n these. This has to be carefully considered when confronting the formal and the molecular aspects.

Regarding an experimental test of the equations, it has been shown (Ref.[1], eq. (35)), that

$$\frac{1}{D_0} - \frac{n_0 v_0^0}{D_0^0} \simeq \frac{B_{03} N_3}{D_{03}} \tag{4}$$

where v_0^0 and D_0^0 are the molar volume and self-diffusion of the p u r e component 0. The formula supposes that the addition of component 3 does not alter the specific frictional properties of component 0. Experiments are going on to test this connection between the self-diffusion in the mixture (D_0), in the pure component (D_0^0), and the differential diffusion coefficient (D_{03}) for an ideal mixture and, if this is successful, to study the interaction between the components in cases of disagreement.

From reasons of symmetry we would also have

$$\frac{1}{D_0} - \frac{n_3 v_3^0}{D_3^0} \simeq \frac{B_{03} N_0}{D_{03}} \tag{5}$$

so that B_{03}/D_{03} may be eliminated using eq. (5) and (4).

2. INTRINSIC DIFFUSION AND VOLUME-COMPENSATING FLOW

In the theory of Hartley and Crank [3] a distinction is made between "pure diffusion" and "real flow" as an expression for the natural tendency to look upon the interdiffusion of two substances as an overlap of two statistical

spreading effects and a necessary volume correcting convection, which must exist also if the mixing proceeds without specific contraction or expansion. The volume transports, resulting from the random motion (and migration) of the components, generally do not balance each other. The result is a volume-correcting flow, which is entangled in and unceasingly accompanies the "intrinsic" diffusion. An analogous picture is known in the kinetic theory of gaseous diffusion.

For the present we are, in connection with this treatment of the diffusion problem, mainly interested in the exact expressions of the authors mentioned, before they introduce the "viscous resistances" in a way which is of approximate character. In the treatment of the mutual diffusion coefficient D_{03}, Hartley and Crank use Fick's lst law in accordance with the theory of the present writer, in that it represents the flow relative to a volume-fixed reference frame. In addition to the volume-fixed frame, they use a coordinate moving with the above-mentioned "real flow", so that the transports through a level, fixed in this coordinate, result from "pure diffusion" of the components. These transports are expressed by Fick's law as $-\bar{v}_0\mathscr{D}_0\partial n_0/\partial x$ and $-\bar{v}_3\mathscr{D}_3\partial n_3/\partial x$ for the two components 0 and 3. $\mathscr{D}$ is called the intrinsic diffusion coefficient. We will proceed to show that this picture, however interesting in connection with a physical molecular interpretation, gives the same g e n e r a l result as our treatment, and thus is of no importance for the purpose, set by the present author.

We refer to a previous article [4] p. 1065. The linear velocities C of the components are obtained from the volume transport just mentioned, dividing the latter by the volume fractions $n_0\bar{v}_0$ and $n_3\bar{v}_3$ respectively. Thus

$$C_0 - C_3 = -\mathscr{D}_0\frac{\partial \ln n_0}{\partial x} + \mathscr{D}_3\frac{\partial \ln n_3}{\partial x} \tag{6}$$

and the condition for stationary movement becomes

$$RTB_{03}n_0\frac{\partial \ln N_0}{\partial x} = \left(\mathscr{D}_0\frac{\partial \ln n_0}{\partial x} - \mathscr{D}_3\frac{\partial \ln n_3}{\partial x}\right)\varphi_{03} \tag{7}$$

The "real flow" is supposed to proceed practically without friction. Equation (44) of Hartley and Crank reads in symmetrical notation

$$D_{03} = n_0\bar{v}_0\mathscr{D}_0 + n_3\bar{v}_3\mathscr{D}_3 \tag{8}$$

Applying simple exchange of variables, the last two equations give

$$D_{03} = RTB_{03}\frac{n_0n_3}{\varphi_{03}(n_0 + n_3)} \tag{9}$$

which is identical with equation (2) of the present paper. As the article of Hartley and Crank is easily misunderstood in this respect, it is of interest to state clearly that the special picture of intrinsic diffusion coefficients $\mathscr{D}$ does not introduce anything new regarding the exact generalization of the equation $D = RT/\Phi$ to finite concentrations and non-ideal solutions.

3. THE LAWS OF FICK AND RELATED QUESTIONS

The generalization of Fick's laws to more than two components is obviously of interest, although no special difficulties are met with, as shown by the example for three components [5]. The treatment given by Onsager and Fuoss in a famous paper [6] is concerned with dilute solutions, as stated on p. 2759 of that paper. Onsager, in another paper [7] treats the generalizations of Fick's laws in a way which may be subjected to some detailed remarks. The introductory statement for two components, where "the relative motion of the two components" is described as the flow $J_i = D\nabla n_i$ (i = 1,2), may need a comment. D_{12} being the symmetrical coefficient, we may define unsymmetrical coefficients according to

$$D_{12} \equiv D_1 Y_2 \equiv D_2 Y_1 \tag{10}$$

where $Y = n\bar{v}$ is the volume fraction, and write $J_i = -D_i\nabla n_i$, now describing the relative motion of the components. In order to show that this is true, consider the linear velocities $C_i = -D_i\nabla \ln n_i$. We expect $C_1 = -C_2$, which easily follows from eq. (10) and $\bar{v}_1 dn_1 + \bar{v}_2 dn_2 = 0$. — Using the symmetrical coefficient D_{12}, the equation $J_i = -D_{12}\nabla n_i$ expresses the flow relative to the local frame characterized by $\Sigma\, \bar{v}_i J_i = 0$, originally proposed in the same paper of Onsager and Fuoss, but not used there consistently owing to the unsymmetrical character of the system, water and dilute electrolyte. Also their expression for t e mobility of a component, although written under the heading of "the general equations for diffusion", is restricted to a dilute solution (*cf.* Ref.[8]). A more general expression is, in our notation, $1/\Phi_{0(3)}$ of equation (2).

As already discussed by Hartley and Crank [3], not all experimental methods give the symmetrical coefficient D (D_{03} of equations (2) and (9)). The latter is, however, most commonly measured (especially when the theory of the experimental method is based upon the Fick-Wiener expression $\partial n/\partial t = \partial(D\partial n/\partial x)/\partial x$), and should naturally be accepted as a standard value for tabulation purposes. Similar considerations hold also for m o b i l i t y as calculated from diffusion data.

In order to arrive at a simple formulation of the diffusion theory for the case of i components arbitrarily mixed with one another, let K_i be the force, originating from the chemical potential field, which acts on the amount of component i which is contained in one cm³ of the solution at a certain level of the diffusion coordinate x. The movements resulting from these forces are the linear component velocities C_i cm/sec. The movements are stationary owing to the high mutual frictional resistances, denoted by $\varphi_{ij} = \varphi_{ji}$. The latter are, as the forces, expressed per cm³ of the mixture. We obtain for the forces *

$$K_i = -n_i \frac{\partial \mu_i}{\partial x};\ \Sigma K_i = 0 \tag{11}$$

* If electrolytes are concerned (Onsager and Fuoss p. 2691), the chemical potential μ includes the electrochemical potential, and the electrical potential comes in as a new variable, whereas the (analytical) electroneutrality condition gives an additional relation.

The latter equation is equivalent to the well-known Gibbs-Duhem's relation. The condition for stationary movement provides

$$K_i = \sum_j (C_i - C_j)\varphi_{ij} \tag{12}$$

A conventional reference frame for the velocities is defined by

$$\Sigma C_i n_i \bar{v}_i = x' = 0 \tag{13}$$

At constant partial volumes, x' is constant and, for convenience, put equal to zero. This case is believed to be of quite dominating interest from the point of view of principles, so that we do not here give the integral equation which is necessary in order to settle the value of x' in terms of a bulk volume change *. Eliminating K_i, we have altogether the i independent equations which are necessary to determine the velocities C_i or the flows $C_i n_i$. Each of the resulting expressions for the flows represents a generalized Fick's first law. The corresponding "second law" (Fick-Wiener) is obtained by inserting the value of C_i into the equation for parallel flow

$$\frac{\partial n_i}{\partial t} = -\frac{\partial}{\partial x}(C_i n_i) \tag{14}$$

t being the diffusion time. — In this presentation, the mathematical solution contains concentrations, chemical potentials (or activities), frictions/cm^3, x and t. The introduction of "diffusion coefficients" is a matter of convention, and seems to be practical only in the two-component case. This is evident for three components [5], which is described by the frictions φ_{12}, φ_{23}, φ_{31}, and two independent thermodynamic factors. To describe a system of more than two components in terms of diffusion coefficients as proposed by Onsager [6] is certainly an unnecessary complication. A detailed discussion of Onsager's equations is outside the scope and competence of this article. However, in view of earlier calculations [9], it is concluded that a mixture of identical components may always be characterized by a diffusion coefficient, which for the special case of three components can be written

$$D_{123} = RTn_1n_2v/\varphi_{12} \equiv RTn_2n_3v/\varphi_{23} \equiv RTn_3n_1v/\varphi_{31} = \text{const.} \tag{15}$$

or in molar frictions

$$D_{123} = RT(n_1 + n_2)v/\Phi_{12} \equiv \text{etc.}; \quad \Phi_{12} = \Phi_{1(2)} + \Phi_{2(1)} \tag{16}$$

v is the molar volume of the substance.

Regarding the several component diffusion it is desirable to take standpoint here to the formal side of the approach made by Onsager, Ref. [7], p. 242, from which we quote

$$J_i = \sum_{k=1}^{s} D_{ik} \text{ grad } n_k \tag{17}$$

and

$$-\text{grad}\mu_i = \sum_{k=1}^{s} R_{ik} J_k \tag{18}$$

* This procedure is essentially different for liquids (constant pressure) and for gases (constant volume).

The work of Onsager and Fuoss contains the analogous equation

$$J_i = -\sum_{k=1}^{s} \Omega_{ik} \text{ grad } \mu_k \qquad (19)$$

The equations (17) and (19) express the flows J as linear functions of the gradients of concentration n or of the chemical potentials μ. Ω, R, and D are coefficients, of which Ω/n has the dimension of mobility, Rn of molar friction, and D of diffusion coefficient. In the last equation i is the number of solute species, but in equations (17) and (18) i is the total number of components as in our equations (11)—(14) above. The analogy between (17) and (19) (to be observed also between (18) and (12)), suggests however to look also upon equation (19) as a general expression for i components, although the coefficients Ω are undertermined without an additional condition for the flows. We will discuss the symmetry relation $R_{ik} = R_{ki}$. Calculations were made with i = 3, but there is no reason to question a general validity of the conclusions. The calculations, using equations (11) and (12) without assuming $\varphi_{ij} = \varphi_{ji}$, have shown that $R_{ik} = R_{ki}$ is equivalent to the assumption $\varphi_{ij} = \varphi_{ji}$. * The latter condition is, however, self-evident from physical reasons. — Further the calculations show that the flows J are obtained from (12) in the indefinite form 0/0, when considering that the sum of the forces/cm^3 is zero, (11), and that $\varphi_{ij} = \varphi_{ji}$. — The coefficients D_{ik} are easy to calculate and to express in terms of φ, concentrations, and thermodynamic factors. D_{ik} proves to be of no special simplicity. — Finally it is seen that the condition (11) regarding the sum of the forces/cm^3 makes it possible to express the flows, equation (19), by equations in which one of the forces has been eliminated. From symmetry reasons the choice $\Omega_{ii} = 0$ is obvious.

The equations (11)—(14) are simple to use and to survey, and seem to contain what can be expected of a generalization of the formal theory of ordinary diffusion, from the classical two component and dilute case, to a general (mechanically normal) fluid mixture. The supposed difficulty [7] of obtaining a general scheme of description, which covers also the simple 2-component case, appears irrelevant. It seems desirable to follow up the statistical-thermodynamic treatment made by Onsager, upon this simpler formal basis.

4. DIFFUSION THEORY AND SEDIMENTATION EQUILIBRIUM

The diffusion equation (9) may be written

$$D_{12} = \frac{RTB_{12}}{\left(\frac{1}{n_1} + \frac{1}{n_2}\right)\varphi_{12}} = \frac{L_{12}}{\varphi_{12}} \qquad (20)$$

* The equations are reproduced here for the sake of clearness

$$-\text{ grad } \mu_1 = \frac{\varphi_{12} + \varphi_{13}}{n_1^2} J_1 - \frac{\varphi_{12}}{n_1 n_2} J_2 - \frac{\varphi_{13}}{n_1 n_3} J_3$$

$$-\text{ grad } \mu_2 = -\frac{\varphi_{21}}{n_1 n_2} J_1 + \frac{\varphi_{21} + \varphi_{23}}{n_2^2} J_2 - \frac{\varphi_{23}}{n_2 n_3} J_3$$

$$-\text{ grad } \mu_3 = -\frac{\varphi_{31}}{n_1 n_3} J_1 - \frac{\varphi_{32}}{n_2 n_3} J_2 + \frac{\varphi_{31} + \varphi_{32}}{n_3^2} J_3$$

$$n_1 \text{ grad } \mu_1 + n_2 \text{ grad } \mu_2 + n_3 \text{ grad } \mu_3 = 0$$

Whereas B_{12}, n_1, and n_2 contain the molecular weights, this is not the case with L_{12}. The sedimentation diffusion equilibrium of a two-component incompressible solution has been shown to be given by (*cf.* Ref.[10])

$$L_{12}\frac{\partial \ln m_2}{\partial x} = (V_1 - V_2)V_1 m_1^2 m_2 \omega^2 x \tag{21}$$

where $\omega^2 x$ is the centrifugal acceleration and m the concentration in g / cm^3. As expected, this thermodynamical expression does not contain molecular weights. The analogy between equations (20) and (21) is believed to be an additional support for the appropriateness of the conventions underlying the diffusion theory, eq. (21) being thermodynamically exact. The factor L_{12} has no obvious physical meaning, but it is closely connected to the general differential osmotic pressure factor Q

$$-Q_{12} \equiv n_1 \frac{\partial P_2}{\partial n_1} \equiv n_2 \frac{\partial P_1}{\partial n_2} \tag{22}$$

by the relation

$$L_{12} = Q_{12} V_1 V_2 m_1 m_2 \tag{23}$$

where V is the partial specific volume.

The theory of sedimentation velocity falls naturally into the same scheme.

As is well known, molecular weight does not enter thermodynamical formulae and cannot be determined without an equation of state. The simplest assumption regarding the equation of state is in this connection to assume $B_{12} = 1$ (ideal solution). This means, according to equations (20) and (21), that the following functions of the molecular weights *

$$\frac{1}{n_1} + \frac{1}{n_2} \equiv \frac{M_1}{m_1} + \frac{M_2}{m_2} \tag{24}$$

is directly measured by the sedimentation equilibrium of an ideal mixture, (that is, without integration). The occurance of the function (24) in a thermodynamical equilibrium equation is of special interest, as it also plays an important rôle in the diffusion theory of two components. Thus, for instance, $1/n_1 + 1/n_2$ is the volume in which, in the case of self-diffusion, the friction between the components is independent of the labelling concentrations. This is true also in the presence of an additional, chemically different component. This is the case treated in section 1 of this paper, but is seen only in the fuller treatment [1].

If the solution is r e g u l a r (van Laar, Hildebrand), a more complicated function of the molecular weights is directly measured by the sedimentation equilibrium.

In the sedimentation equilibrium the self-diffusion proceeds without an equilization of concentrations. To study this theoretically, one way is to deduce the sedimentation diffusion equation for a three-component mixture, which ought not give rise to any considerable difficulties.

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* The molecular weights of this expression do not necessarily coincide with "kinetic" molecular weights (*cf.* Ref.[1]).

5. NOTE ON DIFFUSION AND VISCOSITY

The problem of diffusion in relation to viscosity η for ordinary liquid systems was treated by H. Eyring *et al.* in fundamental papers, and summarized by Kincaid, Eyring and Stearn [11]. One interesting point is the difficult question of how much simpler the product $D\eta$ might be than each of these magnitudes taken separately. For two-component systems, the results of Eyring *et al.* propose that, for normal liquids, $D\eta/B$ approximates to a linear function of the mole fraction of the mixture. Thus Onsager [7] writes this result in the form

$$D\eta/B = \beta_{21}N_1 + \beta_{12}N_2 \qquad (25)$$

Cases of non-ideal mixtures are well known to fall outside the range of validity of this formula. Later, Hartley and Crank [3] arrived at a similar expression.

A theory of $D\eta$ implies, as $D = RTB / \Phi$ (equation (2)), that we expect to be able to express the viscosity of a mixture as a function of the friction between the components; $\eta = f(\Phi)$. From the standpoint of the present paper, it would seem that this is too much to expect from the relation b e t w e e n the components and that too little consideration has been taken of the friction between equal parts within the mixture. A relation $\eta_{03} = f\ (\Phi_{03},\Phi_{12},\Phi_{45})$ would appear to be an increasingly necessary extension, the further the actual conditions of the mixture are from the hydrodynamic picture in the sense of Stokes' law.

Complete reference to the work of others is not repeated here, but is to be found in earlier publications, as far as they are known to the author.

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Studies on Carbamates

X. The Carbamates of Di-*n*-Propylamine and Di-*iso*-Propylamine

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1. The equilibrium conditions and reaction mechanism of the formation and decomposition in aqueous medium of the carbamate formed by di-*n*-propylamine have been studied. Analogous experiments have been carried out with di-*iso*-propylamine. The experimental and theoretical conditions are analogous to those of the carbamates previously investigated; we refer to previous investigations [1] for the detailed information concerning method, theory, significance of constants *etc.* It should be noted, though, that "Am" means dipropylamine and "AmH^+" dipropylammonium ion.

2. Di-*n*-propylamine and di-*iso*-propylamine, both from The British Drug Houses Ltd., were purified by distillation through a wiregauze column with 44 sets of platinum plates. The di-*n*-propylamine thus obtained boiled at 109.4—109.6° C (759 mm Hg) and by acidimetric titration we obtained a result corresponding to a content of 100.0 % $(C_3H_7)_2NH$. For the experiments with di-*iso*-propylamine we used a fraction with a boiling point 83.1—83.7° C, (750 mm Hg), $n_D^{20.0°} = 1.3922$ and by acidimetric titration we obtained a result corresponding to a content of 100.0 % $(C_3H_7)_2NH$.

3. The carbamate of di-*n*-propylamine was prepared in solution only by leading a deficit of carbon dioxide to aqueous solutions of the amine, practically all of the carbon dioxide thus being converted to carbamate.

Contrary to this no carbamate is formed by a similar treatment of an aqueous solution of di-*iso*-propylamine.

An attempt was then made to obtain the carbamate of di-*iso*-propylamine as substance. Di-*iso*-propylamine when treated with dry carbon dioxide yields a white microcrystalline compound which is very soluble in water and ether. A content of approx. 85 % of amine was found by titration with 0.1 *N* HCl but since the substance was very deliquescent a constant composition was not found. The theoretical content of amine in $(C_3H_7)_2NCOONH_2(C_3H_7)_2$ is 82 %. After dissolving the substance in 0.1 *N* NaOH and immediately adding barium chloride a precipitate was formed. The precipitate and the liquid were separated as quickly as possible by filtration and the filtrate heated. No precipitate

was formed showing that no carbamate was present. If on the other hand the substance is dissolved in a *M* solution of dimethylamine no precipitate appears after the addition of sodium hydroxide and barium chloride thus showing that no carbonate is present in the substance; dimethylamine reacts as previously shown very fast with carbon dioxide to form a carbamate but not with carbonate [2]. These facts indicate that the reaction product between di-*iso*-propylamine and carbon dioxide contains the carbon dioxide in a very labile form.

For both amines the solutions of carbonate were prepared by mixing equivalent amounts of solutions of dipropylammonium chloride and sodium carbonate.

4. The method of analysis was as in previous investigations precipitation with barium chloride, causing the precipitation of carbonate, but not of carbamate. All of the data presented in the later tables are corrected for blank experiments, *viz.* about 3 units of the percentage.

5. All of the experiments were done at 18° C, and the velocity constants were calculated by means of Briggs' logarithms, the unit of time being the minute. As in previous investigations, the activity coefficient f for a monovalent ion was calculated from the expression of Bjerrum, $-\log f = 0.3 \sqrt[3]{c_{ion}}$.

6. Bredigs [3] corrected value of the basic dissociation constant for di-*n*-propylamine is $10^{-3.09}$ at 25° C. Hall and Sprinkle [4] have found the basic dissociation constant for di-*iso*-propylamine to be $10^{-3.17}$ at 25° C. The heat effect of the reaction being very slight, these values may be used for 18° C as well.

ON THE REACTION "AMINE + CARBON DIOXIDE ⇌ CARBAMIC ACID"

The velocity constant for the reaction "amine + carbon dioxide" we usually determine by treating an aqueous solution of the amine and sodium hydroxide with a deficit of gaseous carbon dioxide mixed with atmospheric air. This method is also employed in this case. In the course of 5 minutes atmospheric air containing about 10 % of carbon dioxide was led into solutions containing both amine and sodium hydroxide. The mixture was immediately analysed. The analytical data obtained in the experiments with di-*n*-propylamine are listed in Table 1, where "% carbamate" indicates how many per cent of the carbon dioxide absorbed have been converted to carbamate. Fur-

Table 1. Carbon dioxide in di-n-propylamine + NaOH. 18°.

Initial solution		Absorb. CO_2 mole/litre	% carbamate	Final solution		Mean		$k_{CO_2 \cdot Am}$	
c_{NaOH}	c_{Am}			c_{NaOH}	c_{Am}	c_{NaOH}	c_{Am}		Mean
0.20	0.10	0.0209	58 [1]	0.17	0.09	0.19	0.09	$10^{5.46}$	
0.20	0.06	0.0211	47 [2]	0.17	0.05	0.18	0.06	$10^{5.48}$	$10^{5.47}$
0.10	0.06	0.0145	63 [3]	0.08	0.05	0.09	0.06	$10^{5.47}$	

[1], [2], [3] found by the analysis: 55.2, 43.7 and 55.6 %, respectively. The listed values are corrected to zero time.

thermore, the velocity constant $k_{CO_2 \cdot Am}$ for the reaction "amine + carbon dioxide → carbamic acid" was calculated. In analogous experiments with di-*iso*-propylamine no carbamate was found, nor was it found in an experiment in a far less basic solution containing 0.11 *M* Am and 0.09 *M* AmH^+ corresponding to a c_{OH^-} of about 10^{-3}.

In addition to the above, some experiments were carried out to illustrate that the percentage of carbamate formed in an aqueous solution of a definite amine (*in casu* ammonia) and sodium hydroxide when treated with carbon dioxide not only depends on the concentrations of amine and hydroxyl ion, but also on the manner of the treatment.

It has been previously observed that the calculated value of $k_{CO_2 \cdot Am}$ in the case of ammonia [2] depends on the percentage of carbon dioxide in the employed air in the way that a higher percentage yields a higher value for $k_{CO_2 \cdot Am}$.

Repeated experiments with ammonia have confirmed this phenomenon and analogous experiments with non-volatile amines as α-alanine and β-alanine gave a similar result, showing that the deviations observed in the case of ammonia are not entirely due to a side-reaction between gaseous ammonia and gaseous carbon dioxide.

The experiments were carried out in this way. Gaseous carbon dioxide or atmospheric air with an admixture of carbon dioxide was led into the solution in the form of fine bubbles.

In Table 2 are listed some of the experiments. It is seen that atmospheric air containing 1—10 % of carbon dioxide yields nearly the same value of

Table 2. Gaseous carbon dioxide in amine + NaOH. 18°.

Amine	% CO_2 in the gas mixture	Initial solution		Absorbed CO_2 $\frac{\text{mole}}{\text{litre}}$	% carbamate	Final solution		Mean		$k_{CO_2 \cdot Am}$
		c_{NaOH}	c_{amine}			c_{NaOH}	c_{amine}	c_{NaOH}	c_{amine}	
NH_3	100	0.10	1.0	0.0133	58	0.08	1.0	0.09	1.0	$10^{4.12}$
	80	—	—	0.0240	54	0.06	—	0.08	—	$10^{4.01}$
	35	—	—	0.0129	40	0.08	—	0.09	—	$10^{3.80}$
	11	—	—	0.0215	38	0.06	—	0.08	—	$10^{3.73}$
	7	—	—	0.0196	36	0.06	—	0.08	—	$10^{3.69}$
	5	—	—	0.0244	37	0.06	—	0.08	—	$10^{3.69}$
	3	—	—	0.0185	38	0.08	—	0.09	—	$10^{3.73}$
	2	—	—	0.0248	35	0.06	—	0.08	—	$10^{3.66}$
	1	—	—	0.0151	34	0.08	—	0.09	—	$10^{3.66}$
CH_3 | $CHNH_2$ | $COOH$	100	0.10	0.15	0.0112	60	0.09	0.14	0.09	0.15	$10^{5.00}$
	10	—	—	0.0134	51	0.08	—	—	—	$10^{4.82}$
	1	—	—	0.0113	48	0.08	—	—	—	$10^{4.78}$
CH_2NH_2 | CH_2 | $COOH$	100	0.10	0.10	0.0198	59	0.07	0.09	0.09	0.10	$10^{5.15}$
	10	—	—	0.0245	54	0.06	—	0.08	0.09	$10^{5.03}$
	1	—	—	0.0235	52	0.07	—	0.08	0.09	$10^{5.00}$

$k_{CO_2 \cdot Am}$, namely $10^{3.66}$ for ammonia, $10^{4.82}$ for α-alanine and $10^{5.04}$ for β-alanine which are the values published in earlier papers [2,1], while air mixtures being richer in carbon dioxide yield higher values of $k_{CO_2 \cdot Am}$, most typical in the case of ammonia.

In order to eliminate the gas phase some experiments were carried out in which a saturated aqueous solution of carbon dioxide was added to the amine-sodium hydroxide solution. In carbon dioxide water being about 0.03 *M* as to total carbon dioxide, 99.5 % of the total carbon dioxide is present as CO_2[2] and that fraction of the carbon dioxide existing *a priori* as carbonate (*viz.* H_2CO_3 and HCO_3^-) can consequently be ignored in the present case.

The experiments were carried out in this way, that 100 ml of the amine-sodium hydroxide solution was placed in a beaker. The liquid was agitated by means of two mechanical stirrers each consisting of a hollow shaft and two blades. The blades of one were also hollow and perforated with fine holes, and the shaft of this one was placed in that of the other. The two stirrers circulated in opposite directions, and through the stirrer with the hollow blades the carbon dioxide water was allowed to flow out in the solution for about 2 minutes.

From the experimental data listed in Table 3 it is seen that the percentage of carbamate depends on the rotation rate of the stirrers, and it is remarkable that the value of $k_{CO_2 \cdot Am}$ in the experiments with ammonia decreases, while in the experiments with the alanines it increases with the rising rate of rotation. The value of $k_{CO_2 \cdot Am}$ for the alanines becomes, even at moderate rates of rotation, identical with the one obtained in the experiments with diluted gaseous carbon dioxide. It is remarkable that this is not the case with the value of $k_{CO_2 \cdot Am}$ for ammonia even at the most rapid rotation obtainable with the applied apparatus.

The deviations observed in the experiments with gaseous carbon dioxide mixed with small volumes of atmospheric air or in the experiments where slow agitation was employed must be due to incalculable changes in the relation between the concentrations of amine and sodium hydroxide during the addition of the carbon dioxide. Employing diluted gaseous carbon dioxide or rapid agitation during the admission of the carbon dioxide water this error must decrease.

In order to eliminate the above mentioned source of error, a third method for the admission of the carbon dioxide was examined. The carbon dioxide was admitted in the form of sodium methyl carbonate which after being dissolved in an aqueous solution containing ammonia and sodium hydroxide comparatively rapidly decomposed into methyl alcohol and carbon dioxide.

$$CH_3CO_3^- \rightleftharpoons CH_3O^- + CO_2$$

followed by

$$NH_3 + CO_2 \rightleftharpoons NH_2COOH$$
$$OH^- + CO_2 \rightarrow HCO_3^-$$

The velocity constant for the first reaction from left to right is at 18° C 0.024[5], the velocity from right to left can be ignored, the concentration of methyl alcohol being very weak in comparison with the concentrations of

Table 3. Carbon dioxide water in amine + NaOH. 18°.

Amine	Number of revolutions per minute	Initial solution		c_{total} carbon dioxide	% carbamate	Final solution		Mean		$k_{CO_2 \cdot Am}$
		c_{NaOH}	c_{amine}			c_{NaOH}	c_{amine}	c_{NaOH}	c_{amine}	
NH_3	0	0.2	2.0	0.0148	56	0.08	1.0	0.14	1.5	$10^{4.09}$[1]
	20	—	—	0.0146	53	—	—	—	—	$10^{4.04}$
	40	—	—	0.0158	48	—	—	—	—	$10^{3.95}$
	60	—	—	0.0136	45	—	—	—	—	$10^{3.90}$
	65	—	—	0.0160	44	—	—	—	—	$10^{3.88}$
	160	—	—	0.0148	42	—	—	—	—	$10^{3.85}$
	170	—	—	0.0152	41	—	—	—	—	$10^{3.82}$
	210	—	—	0.0156	40	—	—	—	—	$10^{3.82}$
	220	—	—	0.0139	41	—	—	—	—	$10^{3.83}$
	260	—	—	0.0147	39	—	—	—	—	$10^{3.80}$
	275	—	—	0.0140	40	—	—	—	—	$10^{3.81}$
	700	—	—	0.0152	39	—	—	—	—	$10^{3.78}$
	850	—	—	0.0139	40	—	—	—	—	$10^{3.82}$
CH_3 | $CHNH_2$ | $COOH$	0	0.20	0.30	0.0163	45	0.08	0.14	0.14	0.22	$10^{4.73}$
	20	—	—	0.0156	46	—	—	—	—	$10^{4.74}$
	55	—	—	0.0149	46	—	—	—	—	$10^{4.75}$
	115	—	—	0.0157	46	—	—	—	—	$10^{4.74}$
	200	—	—	0.0163	48	—	—	—	—	$10^{4.78}$
	800	—	—	0.0134	48	—	—	—	—	$10^{4.79}$
CH_2NH_2 | CH_2 | $COOH$	0	0.20	0.20	0.0141	41	0.08	0.09	0.14	0.15	$10^{4.84}$[2]
	15	—	—	0.0143	42	—	—	—	—	$10^{4.86}$
	25	—	—	0.0151	49	—	—	—	—	$10^{4.97}$
	45	—	—	0.0148	51	—	—	—	—	$10^{5.01}$
	115	—	—	0.0158	50	—	—	—	—	$10^{5.00}$
	700	—	—	0.0137	49	—	—	—	—	$10^{4.99}$
	700	—	—	0.0132	49	—	—	—	—	$10^{4.99}$

[1] Not reproducible. Other experiments gave values from $10^{3.90}$ to $10^{4.51}$ for $k_{CO_2 \cdot Am}$
[2] In an other experiments we found $10^{4.93}$.

ammonia and hydroxylion. After about 150 minutes practically all of the monomethyl carbonate has disappeared and after this space of time the mixture was analysed, *cf.* Table 4. In calculation the listed values of $k_{CO_2 \cdot Am}$, the decomposition of the carbamate taking place during the 150 minutes, is taken into consideration.

Table 4. Sodium methyl carbonate in ammonia + NaOH. 18°.

Initial solution		c_{total} carbon dioxide	% carbamate	Final solution		Mean		$k_{CO_2 \cdot Am}$
c_{NaOH}	c_{NH_3}			c_{NaOH}	c_{NH_3}	c_{NaOH}	c_{NH_3}	
0.10	2.0	0.0186	45	0.08	2.0	0.09	2.0	$10^{3.61}$
0.10	1.0	0.0100	31	0.09	1.0	0.10	1.0	$10^{3.67}$
0.05	0.50	0.0195	33	0.03	0.50	0.04	0.50	$10^{3.66}$

Table 5. The solution carbonate-carbamate in equilibrium. 18°.

	Initial solution				% carbamate	Equilibrium				K_{Eq}	
	c_{Am}	c_{AmH^+}	$c_{(AmH)_2CO_3}$	$c_{carbamate}$		c_{Am}	c_{AmH^+}	$c_{carbamate}$	$c_{HCO_3^-}$		Mean
di-*n*-	0.19	0.10	0.02		5.4	0.19	0.14	0.0011	0.00084	$10^{-0.82}$	$10^{-0.81}$
	0.22	0.11		0.02	5.6	0.22	0.12	0.0011	0.00072	$10^{-0.80}$	
di-*iso*-	0.19	0.10	0.02		0	0.19	0.14	0	0.0013		

It is seen from Table 4 that the value of $k_{CO_2 \cdot Am}$ determined by this method is identical with the one determined in the experiments where gaseous carbon dioxide was admitted mixed with abundant atmospheric air (Table 2).

THE EQUILIBRIUM "CARBAMATE ⇌ CARBONATE"

Experiments with di-*n*-propylamine have been done from the carbonate side as well as from the carbamate side. Experiments with di-*iso*-propylamine showed that no carbamate is formed. In Table 5 are listed the compositions of the solutions and the equilibrium constant K_{Eq} for the reaction '$(CH_3 \cdot CH_2 \cdot CH_2)_2NCOO^- + H_2O \rightleftharpoons HCO_3^- + (CH_3 \cdot CH_2 \cdot CH_2)_2NH$'.

THE VELOCITY OF THE CONVERSION "CARBAMATE ⇌ CARBONATE"

In Table 6 are presented the experiments on velocity, which have been carried out in a di-*n*-propylammoniumion-di-*n*-propylamine buffer, where an easily measurable equilibrium is established between carbamate and carbonate. In Table 7 are presented those experiments which have been carried out in a medium containing sodium hydroxide, where carbamate is converted almost completely to carbonate.

No experiments could be done with di-*iso*-propylamine since no carbamate is present in aqueous medium.

The velocity constants calculated from the experiments are listed in Tables 6 and 7. These velocity constants may, provided the decomposition takes place through the reactions

$$\text{carbamate} \rightleftharpoons \text{amine} + CO_2$$
$$CO_2 \rightleftharpoons \text{carbonate}$$

be calculated in advance.

In Table 8 is given a survey of the experimental and calculated values of the velocity constants.

Table 6. Velocity constants for the process "carbamate ⇌ carbonate" pa_H = approx. 11.6. 18°.

Initial solution			Min.	% carbamate	$k_{amate} + k_{onate}$	
$c_{carbamate}$	c_{Am}	c_{AmH^+}				
0.019	0.22	0.11	99	70.4		0.00165
			237	45.3		0.00159
			352	32.6		0.00155
			468	23.5		0.00155
			∞	5.6	Mean:	0.00159
					k_{amate}:	0.0015
					k_{onate}:	0.00009

Table 7. Velocity constants for the process "carbamate → carbonate". pa_H = approx. 13. 18°.

Initial solution			Min.	%carbamate	k_{amate}	
$c_{carbamate}$	c_{NaOH}	c_{Am}				
0.012	0.17	0.09	45	81.0		0.00206
			123	58.5		0.00190
			217	38.3		0.00192
			310	26.9		0.00184
					Mean:	0.0019
0.010	0.17	0.05	25	87.2		0.00240
			48	75.6		0.00254
			75	64.6		0.00254
			97	58.4		0.00241
			125	48.7		0.00250
			176	36.7		0.00248
					Mean:	0.0025
0.009	0.08	0.05	26	80.2		0.00370
			46	65.8		0.00396
			70	54.7		0.00374
			134	31.5		0.00374
					Mean:	0.0038

Table 8. Velocity constants, experimental and calculated.

Initial solution				k_{amate}		k_{onate}	
c_{Am}	c_{AmH^+}	c_{NaOH}	$c_{carbamate}$	exp.	calc.	exp.	calc.
0.22	0.11		0.019	0.0015	0.0015	0.00009	0.00007
0.09		0.17	0.012	0.0019	0.0022		
0.05		0.17	0.010	0.0025	0.0029		
0.05		0.08	0.009	0.0038	0.0040		

SUMMARY

The velocity constant of the reaction $(CH_3 \cdot CH_2 \cdot CH_2)_2 \cdot NH + CO_2 = (CH_3 \cdot CH_2 \cdot CH_2)_2NCOOH$ and the equilibrium constant for the reaction $(CH_3 CH_2 CH_2)_2NCOO^- + H_2O \rightleftharpoons HCO_3^- + (CH_3 \cdot CH_2 CH_2)_2NH$ have been determined. The velocity of the decomposition of $(CH_3 \cdot CH_2 \cdot CH_2)_2NCOO^-$ in basic medium was investigated and may be explained by assuming that the decomposition is a two stage reaction, *viz.*

carbamate = amine + carbon dioxide
carbon dioxide = carbonate

Analogous experiments with di-*iso*-propylamine showed that this amine contrary to all the other secondary alifatic amines examined hitherto forms no carbamate in aqueous medium. Some experiments have proved that the percentages of carbamate and carbonate formed in an aqueous solution of an amine and sodium hydroxide when treated with carbon dioxide to some extent depend on the method of adding carbon dioxide.

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Reactions between Carbon Dioxide and Amino Alcohols

I. Monoethanolamine and Diethanolamine

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1. The present investigation deals with the equilibrium conditions and the reaction mechanism for the formation and decomposition of the carbamates of mono- and diethanolamine in aqueous medium. The theoretical conditions are not exactly analogous to those of previously investigated amines [1] because the alcohol groups of the amines may form monoalkyl carbonate simultaneously. The amount is, however, presumed to be negligible and at least cannot be detected in the results obtained. Allowing for errors in the experiments these may be explained by the assumption of a predominant formation of carbamate. This point will be the subject of further investigations.

2. The amino alcohols were purified by distillation, monoethanolamine through a fractionating column at atmospheric pressure, and diethanolamine *in vacuo* without fractionating devices. The fractions used were taken at 172.4 —172.6° C at 766 mm Hg and 160° C at 10 mm Hg, respectively. By acidimetric titration, using bromocresol green as an indicator, we obtained results corresponding to a content of 100.0 % and 99.9 %, respectively.

3. No solid carbamate was prepared. Solutions were made by adding carbon dioxide to suitable solutions of the amino alcohols.

4. The method of analysis was as in previous investigations precipitation with barium chloride, causing the precipitation of carbonate, but not of carbamate and contingent monoalkyl carbonate. All of the data presented in the tables are corrected for the blank values unless otherwise stated. "Corr." values are corrected not only with regard to blank values but also with regard to the decomposition mentioned in the next section. The blank values may amount to about 3 units of the percentage.

5. All of the experiments were done at 18° C, and the velocity constants were calculated by means of Briggs' logarithms, the unit of time being the minute. As in previous investigations the activity coefficient for a monovalent ion was calculated from the expression of Bjerrum: $-\log f = 0.3\sqrt[3]{c_{ion}}$.

6. Hall and Sprinkle [2] have found the pK's for monoethanolamine and diethanolamine at 18° C to be 9.58 and 9.02, respectively.

ON THE REACTION "AMINE + CARBON DIOXIDE → CARBAMIC ACID"

For a period of 5 minutes atmospheric air containing about 10 % of carbon dioxide was led into solutions containing both amine and sodium hydroxide.

In Table 1 we have listed the experimental results for the addition of carbon dioxide to the amines together with the calculated velocity constant. It has been necessary to correct the found percentages for the decomposition which has taken place before the separation of supernatant and precipitate in the case of diethanolamine.

Table 1. Carbon dioxide in amine + NaOH. 18°.

Amine	Initial solution		Absorbed CO_2/liter	%carbamate	%carbamate "corr"	$k_{CO_2 \cdot Am}$	
	c_{NaOH}	c_{Am}					Mean
mono-	0.1	0.1	0.0209	47.6		$10^{4.92}$	$10^{4.92}$
	0.2	0.1	0.0194	30.8		$10^{4.92}$	
	0.1	0.2	0.0199	63.8		$10^{4.92}$	
di-	0.1	0.1	0.0168	50.0	52	$10^{5.01}$	$10^{5.02}$
	0.2	0.1	0.0232	35.4	36	$10^{5.05}$	
	0.1	0.2	0.0169	65.7	67	$10^{4.92}$	

THE EQUILIBRIUM "CARBAMATE ⇌ CARBONATE"

Experiments with monoethanolamine have been done from the carbonate side only, while with diethanolamine experiments have been done from the carbamate side also. The results are listed in Table 2, K_{Eq} being the equilibrium constant.

THE VELOCITY OF THE CONVERSION "CARBAMATE ⇌ CARBONATE"

Experiments on the velocity have been carried out in buffer solutions of the corresponding amine, see Table 3, and in strongly basic solutions, Table 4.

In Table 5 is given a survey of the experimental and calculated values of the velocity constants.

SUMMARY

The velocity of the addition of carbon dioxide to mono- and diethanolamine has been investigated and may be explained by the assumption of a predominant formation of carbamate.

The decomposition into carbonate of the product formed has been investigated with regard to equilibrium and velocity.

Table 2. The solution of carbonate-carbamate in equilibrium. 18°.

Amine	Initial solution				% carb-amate	Equilibrium				K_{Eq}	
	c_{Am}	c_{AmH+}	$c_{(AmH)_2CO_3}$	$c_{carb-amate}$		c_{AmH+}	c_{Am}	$c_{carb-amate}$	$c_{HCO_3^-}$		Mean
mono-	0.10	0.10	0.02		80	0.12	0.10	0.016	0.003	$10^{-1.71}$	
	0.10	0.10	0.02		78	0.12	0.10	0.016	0.003	$10^{-1.68}$	$10^{-1.71}$
	0.01		0.02		45	0.02	0.02	0.009	0.009	$10^{-1.73}$	
di-	0.17	0.04	0.02		47	0.07	0.18	0.009	0.008	$10^{-0.81}$	$10^{-0.82}$
	0.16	0.08		0.013	48	0.08	0.17	0.006	0.005	$10^{-0.82}$	

Table 3. Velocity constants for the process "carbamate ⇌ carbonate." pa_H approx. 9.5. 18°.

Amine	Initial solution				Min.	% carb-amate	$k_{amate} + k_{onate}$
	c_{Am}	c_{AmH+}	$c_{(AmH)_2CO_3}$	$c_{carbamate}$			
mono-	0.10	0.10	0.020		44	13	0.00173
					93	25	0.00178
					140	35	0.00179
					190	43	0.00175
					250	50	0.00174
					350	61	0.00178
					∞	80	Mean: 0.00176
							k_{amate}: 0.00036
							k_{onate}: 0.0014
di-	0.17	0.04	0.020		40	17	0.00484
					63	24	0.00478
					94	31	0.00479
					119	36	0.00514
					164	41	0.00526
					198	42	0.00458
					∞	47	Mean: 0.0049
							k_{amate}: 0.0026
							k_{onate}: 0.0023
	0.16	0.08		0.013	23	89	0.00446
					47	80	0.00436
					75	73	0.00424
					106	66	0.00432
					142	60	0.00449
					178	58	0.00413
					216	55	0.00411
					∞	48	Mean: 0.0043
							k_{amate}: 0.0022
							k_{onate}: 0.0021

Table 4. Velocity constants for the process "carbamate → carbonate". p_{aH} approx. 13. 18°.

Amine	Initial solution			Min.	% carbamate	k_{amate}
	c_{Am}	c_{NaOH}	$c_{carbamate}$			
mono-	0.09	0.17	0.007	441	91	0.000100
				1 021	78	0.000104
				1 664	67	0.000104
				2 594	54	0.000106
				4 105	39	0.000100
				5 602	26	0.000105
						Mean: 0.000103
di-	0.09	0.08	0.009	40	86	0.00166
				82	74	0.00158
				121	65	0.00157
				164	56	0.00153
				207	48	0.00156
				247	43	0.00150
						Mean: 0.0016
	0.19	0.08	0.011	50	87	0.00126
				89	79	0.00118
				150	69	0.00109
				211	60	0.00104
				274	52	0.00109
				462	34	0.00102
						Mean: 0.0011
	0.09	0.16	0.008	50	86	0.00132
				80	79	0.00134
				121	72	0.00117
				167	66	0.00110
				223	55	0.00117
						Mean: 0.0012

Table 5. Velocity constants, experimental and calculated.

Amine	Initial solution					k_{amate}		k_{onate}	
	c_{Am}	c_{AmH+}	$c_{(AmH)_2CO_3}$	$c_{carbamate}$	c_{NaOH}	exp.	calc.	exp.	calc.
mono-	0.10	0.10	0.020			0.00036	0.00032	0.0014	0.0016
	0.09			0.007	0.17	0.00010	0.00013		
di-	0.17	0.04	0.020			0.0026	0.0018	0.0023	0.0021
	0.16	0.08		0.013		0.0022	0.0019	0.0021	0.0022
	0.09			0.009	0.08	0.0016	0.0019		
	0.19			0.011	0.08	0.0011	0.0012		
	0.09			0.008	0.16	0.0012	0.0013		

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Reactions between Carbon Dioxide and Amino Alcohols

II. Triethanolamine

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1. The present investigation deals with the equilibrium conditions and the reaction mechanism for the formation and decomposition of the monoalkyl carbonate of triethanolamine in aqueous medium. It should be mentioned that tertiary amines do not react with carbon dioxide to form carbamates. The conditions are thus analogous to those of previously investigated alcohols [1].

2. The triethanolamine used was purified by converting it into the chloride according to Germann and Knight [2]. Melting point was found to be 177—79° C, when heated 3—4°/min. By argentometric titration we obtained results corresponding to a content of 100.0 % triethanolammonium chloride. In the experiments the solutions of triethanolamine were prepared not of the free amine but of the chloride to which was added an equivalent amount of sodium hydroxide.

3. No solid monoalkyl carbonate was prepared. Solutions were made by dissolving carbon dioxide in basic solutions of the triethanolamine.

4. As to the method of analysis we refer to previous investigations [1]. The data presented are corrected with regard to blank values unless otherwise stated. "Uncorr." values are not corrected at all and "corr." values are corrected not only with regard to blank values but also with regard to the decomposition mentioned in the next section. The blank values may amount to about 3 units of the percentage of monoalkyl carbonate in some of the experiments.

5. The experiments were carried out at 0° C. Velocity constants are expressed on the basis of the Briggs' logarithms and the minute.

6. Hall and Sprinkle [3] have found pK for triethanolamine at 0° C to be 8.22. As pH in all the solutions used for experiments is above 10 it means that we can ignore the triethanolammonium ions in this connection.

ON THE FORMATION OF THE MONOALKYL CARBONATE FROM CARBON DIOXIDE AND TRIETHANOLAMINE IN BASIC SOLUTION AND ON THE REACTION:

$$N(CH_2{\cdot}CH_2OH)_2{\cdot}CH_2{\cdot}CH_2O^- + CO_2 = N(CH_2{\cdot}CH_2OH)_2{\cdot}CH_2{\cdot}CH_2{\cdot}CO_3^-$$

The experiments were carried out in a flask by vigorously shaking the solution of triethanolamine and sodium hydroxide with atmospheric air of

which about 30 % was substituted by carbon dioxide. The solution was immediately analysed to determine the percentage of monoalkyl carbonate. Due to the rapid decomposition of the monoalkyl carbonate, we had to correct the results by means of the velocity constants which we also have determined, see later.

By introduction of the adjusted results in the expression:

$$k' = \frac{\%\ \text{monoalkyl carbonate}}{\%\ \text{carbonate}} \cdot \frac{k_{CO_2 \cdot OH^-}}{c_{\text{alcohol}}}$$

the k' values presented in Table 1 are calculated. k' is the velocity constant of the overall reaction:

$$CO_2 + N(CH_2 \cdot CH_2OH)_3 + OH^- = N(CH_2 \cdot CH_2OH)_2 \cdot CH_2 \cdot CH_2 \cdot CO_3^- + H_2O$$

Table 1. Carbon dioxide in triethanolamine + NaOH. 0°.

Initial solution		Absorbed CO_2 mole/liter	% monoalkyl carbonate	% monoalkyl carbonate (corr.)	k'	Mean of k'
c_{NaOH}	$c_{N(CH_2 \cdot CH_2OH)_3}$					
0.10	0.10	0.0206	31.6	34.1	$10^{5.13}$	
0.10	0.20	0.0200	50.2	53.1	$10^{5.15}$	$10^{5.14}$
0.10	0.50	0.0199	70.6	72.7	$10^{5.13}$	

For the value of $k_{\text{alkyl} \cdot CO_3^-}$ we get 0.0073 when the value of K_{Eq} found in Table 2 is employed.

THE EQUILIBRIUM OF THE REACTIONS:

$$N(CH_2 \cdot CH_2OH)_2 \cdot CH_2 \cdot CH_2 \cdot CO_3^- + H_2O \rightleftharpoons HCO_3^- + N(CH_2 \cdot CH_2OH)_3$$

The above equilibrium was established in aqueous solutions of triethanolamine, potassium bicarbonate and sodium carbonate.

In Table 2 are presented the contents of monoalkyl carbonate calculated as a percentage of HCO_3^- initially present. In order to illustrate the accuracy that may be attached to the values, the uncorrected values have also been listed. From the experimental results the equilibrium constant of the reaction

$$K_{Eq} = \frac{c_{N(CH_2 \cdot CH_2OH)_3} \cdot c_{HCO_3^-} \cdot f}{c_{N(CH_2 \cdot CH_2OH)_2 \cdot CH_2 \cdot CH_2 \cdot CO_3^-} \cdot f}$$

may be calculated, the activity constants neutralizing each other.

Table 2. The solution of carbonate—monoalkyl carbonate in equilibrium. 0°.

Initial solution			% monoalkyl carbonate		K_{Eq}	Mean of K_{Eq}
$c_{N(CH_2 \cdot CH_2OH)_3}$	c_{KHCO_3}	$c_{Na_2CO_3}$	uncorr.	corr.		
0.50	0.10	0.10	5.56	4.94[1]	$10^{0.98}$	$10^{1.00}$
0.20	0.20	0.10	2.09	1.87[2]	$10^{1.02}$	

[1] Mean of 4 determinations: 4.85, 4.89, 5.02, 5.00.
[2] Mean of 4 determinations: 1.88, 1.84, 1.86, 1.91.

ON THE VELOCITY OF THE REACTIONS:

$$N(CH_2 \cdot CH_2OH)_2 \cdot CH_2 \cdot CH_2 \cdot CO_3^- + H_2O = HCO_3^- + N(CH_2 \cdot CH_2OH)_3$$

Determinations have been made from the monoalkyl carbonate side only and in strongly basic medium, the monoalkyl carbonate thus being almost completely converted into carbonate.

The solutions of monoalkyl carbonate have been made by shaking a solution of NaOH + triethanolamine with CO_2 and the solution, therefore, also contains ordinary carbonate, which, however, is of no importance to the present investigation.

In Table 3 are listed the experimental results of the decomposition of the monoalkyl carbonate. k_{Mono} is the value of $\frac{1}{t} \cdot \log \frac{a}{a-x}$.

Table 3. Monoalkyl carbonate in NaOH + alcohol. 0°.

Initial solution			Min.	%monoalkyl carbonate left	k_{Mono}
$c_{monoalkyl\ carbonate}$	c_{NaOH}	$c_{alcohol}$			
0.007	0.10	0.10	0	100	—
			5	93.0	0.00630
			11	87.1	0.00545
			19	76.4	0.00615
			33	65.1	0.00565
			62	46.4	0.00538
			102	29.9	0.00514
			164	14.7	0.00508
				Mean:	0.0056
0.011	0.10	0.20	0	100	—
			9	91.1	0.00450
			21	79.8	0.00467
			38	69.6	0.00414
			70	52.4	0.00401
			108	37.6	0.00393
			178	20.7	0.00384
			279	10.2	0.00355
				Mean:	0.0041
0.014	0.10	0.50	0	100	—
			13	93.8	0.00214
			30	86.8	0.00205
			50	78.8	0.00207
			88	65.0	0.00213
			137	52.1	0.00207
			219	36.2	0.00202
			346	18.4	0.00212
				Mean:	0.0021

The experiments are interpreted in a way similar to the one applied to the monoalkyl carbonates previously investigated. The decomposition takes place through the reactions:

$$1.\ \text{alkyl} \cdot CO_3^- = \text{alkylate}^- + CO_2$$

$$2.\ CO_2 + OH^- = HCO_3^-$$

k_{Mono} may be calculated at $pa_H > 10$ by means of the following expression:

$$k_{\text{Mono}} = \frac{k' \cdot K_{Eq} \cdot \frac{K_{H_2O}}{K_{CO_2}}}{1 + k' \cdot \frac{c_{\text{alcohol}}}{k_{CO_2} \cdot OH^-}}$$

the values of K_{H_2O} and K_{CO_2} being $10^{-14.93}$ and $10^{-6.85}$, respectively.

Upon introduction of the constants the equation for triethanolamine is:

$$k_{\text{Mono}} = \frac{0.0073}{1 + 5.6 \cdot c_{\text{alcohol}}}$$

k_{Mono} for the monoalkyl carbonate of triethanolamine in 0.5 *M*, 0.2 *M* and 0.1 *M* solutions of triethanolamine, pH > 10, will then be calculated to 0.0019, 0.0034 and 0.0047, respectively. The experimental and calculated values agree well.

SUMMARY

The velocity constants of the reaction "$N(CH_2 \cdot CH_2OH)_3 + OH^- + CO_2 = N(CH_2 \cdot CH_2OH)_2 \cdot CH_2 \cdot CH_2 \cdot CO_3^- + H_2O$" and the equilibrium constant for the reaction "$N(CH_2 \cdot CH_2OH)_2 \cdot CH_2 \cdot CH_2 \cdot CO_3^- + H_2O = N(CH_2 \cdot CH_2OH)_3 + HCO_3^-$" have been determined. The velocity of the decomposition of $N(CH_2 \cdot CH_2OH)_2 \cdot CH_2 \cdot CH_2 \cdot CO_3^-$ in strongly basic medium was investigated and may be explained by assuming that the decomposition is a two-stage reaction, *viz.*

monoalkyl carbonate = alcohol + carbon dioxide
carbon dioxide = carbonate.

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The Crystal Structure of Bi-1,3-Dithia*cyclo*pentyl(2)

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The crystal structure of the substance of mp. 135.5° C having the formula $C_6H_{10}S_4$ (a product obtainable from 2,3-dichloro-1,4-dioxan and 1,2-ethanedithiol) has been determined. The substance turned out to be bi-1,3-dithia*cyclo*pentyl(2) and therefore corresponds to the lower melting isomer (111° C) obtained in the reaction between 2,3-dichloro-1,4-dioxan and ethylene glycol.

If ethylene glycol reacts either with 2,3-dichlorodioxan or with glyoxal, two isomeric compounds are obtained. The structure of the lower melting isomer (m.p. 111° C) has been determined[1] and it has been found to be bi-1,3-dioxa*cyclo*pentyl(2).

By heating 2,3-dichloro-1,4-dioxan with 1,2-ethanedithiol in anhydrous benzene, Prošťenik and Balenović[2] obtained a compound with the empirical formula $C_6H_{10}S_4$ and m.p. 135.5° C. This compound is identical with glyoxaldiethylenmercaptal already obtained by Fasbender[3]. They also tried to obtain an isomer, but with negative results. As it could not be determined by the chemical reaction whether $C_6H_{10}S_4$ is bi-1,3-dithia*cyclo*pentyl or *cis*-naphthodithian, the structure has been determined by X-ray diffraction.

EXPERIMENTAL

Bi-1,3-dithia*cyclo*pentyl crystallizes monoclinic, and occurs in different habits. Twinning occurs constantly, especially about (001). Crystals grown from a hot benzene solution are needle-shaped, elongated along the *a*-axis, while crystals grown from a hot saturated solution in aceton are plates with well developed clinopinacoid planes.

A needle-shaped crystal with cross-section 0.24 × 0.56 mm² was used for a series of Weissenberg photographs of the $\{0kl\}$ zone. A tabular crystal with cross-section 0.35 × 0.25 mm² was used for the $(h0l)$ reflections.

Rotation, oscillation and Weissenberg photographs using Cu$K\alpha$ radiation ($\lambda = 1.54$ Å) led to the following data for the unit cell:

$a = 4.92$ Å; $b = 13.23$ Å; $c = 6.94$ Å (all ± 1 %); $\beta = 96°$

$C_6H_{10}S_4$: Formula weight = 210.3

$V = 454$ Å³; $D = 1.49$ g/cm³; $Z = 2$ (Z calc. = 1.95); $F_{000} = 200$

The density D was determined by flotation of some crystals in a $BaBr_2$ solution in water.

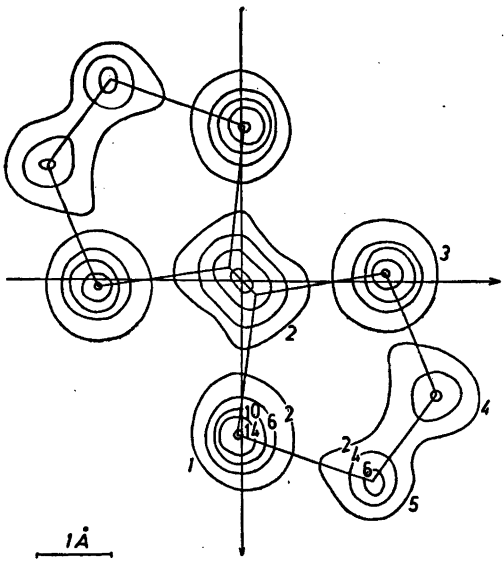

Fig. 1. The electron density map of the y—z projection.

Systematic absence of the *h0l* reflections for $h + l$ odd, and of the 0*k*0 reflections for k odd, occurs. Therefore the space group is $P\ 2_1/n$ and the general positions are

$x, y, z;\ \bar{x}, \bar{y}, \bar{z};\ 1/2-x,\ 1/2+y,\ 1/2-z;\ 1/2+x,\ 1/2-y,\ 1/2+z$

Intensity measurements were carried out visually on Weissenberg photographs, using the multiple film technique. Corrections for the Lorentz polarisation factor were made, whereas corrections for absorption were not thought quite necessary.

A total number of 78 0*kl* reflections were recorded, about 80 % of those theoretically accessible, and a total number of 24 *h0l* reflections were recorded, about 69 % of the theoretically accessible.

THE DETERMINATION OF THE STRUCTURE

The a-axis projection. A Patterson F^2 projection along the *a*-axis gave the coordinates of the sulphur atoms with moderate approximation. However, the phases of numerous observed structure factors were determined, and after successive refinements, the electron-density map shown in Fig. 1 was obtained. The contour line scale corresponds to 2 $e \cdot Å^{-2}$ for the carbon atoms and the first contour line of the sulphur atoms, whereas the inner contour lines of the sulphur atoms correspond to 4 $e \cdot Å^{-2}$.

All atoms are well resolved except the carbon C_2 atoms. The sulphur atoms are easily distinguished by the greater height of the peake. The maximum electronic density reached by the resolved carbon atoms is 6.68 $e \cdot Å^{-2}$, and for the sulphur atoms 18.56 $e \cdot Å^{-2}$, which is in good agreement with the theoretical values, (weight of carbon 6, of sulphur 16). No consideration has been given to the hydrogen atoms as they are not expected to show up clearly.

Hartree's model of the atomic scattering function f was used for the calculation of the structure amplitudes F. The theoretical f-curves were multiplied by the temperature factor $e^{-B\sin^2\Theta/\lambda^2}$ where the constant B was determined by comparison between the calculated and the observed structure factors F. B was found to equal 3.9 Å^2.

The agreement between calculated and observed amplitudes is given by $R = \frac{F_{calc} - F_{obs}}{F_{obs}}$ where the F_{calc} corresponding to observed values too weak to be detected, were taken in the summation with half of their value. The result obtained was $R = 0.21$. The final y and z coordinates are determined from the map, and given with three significant figures (Table 1). A detailed consideration of the error has not been attempted, but the coordinates are probably reliable to $\pm$ 0.002 Å, with exception of C_2 which may be less certain. (The numbering of atoms is given in Fig 1.) In Table 3 the structure factors calculated from the y and z coordinates are compared with the observed structure amplitudes.

Table 1.

	x/a	y/b	z/c	x	y	z
S_1	0.23	0.996	0.292	1.13 Å	−0.053 Å	2.026 Å
S_3	0.22	0.147	0.988	1.08 »	1.945 »	−0.014 »
C_2	0.15	0.013	0.026	0.74 »	0.172 »	0.180 »
C_4	0.42	0.194	0.218	2.07 »	2.567 »	1.513 »
C_5	0.27	0.131	0.379	1.33 »	1.733 »	2.630 »

The b- axis projection. The knowledge of the z-coordinates and the generally accepted bond lengths made it possible to work out this projection. The positions of the sulphur atoms were found from a Patterson projection. Once the positions of these atoms were established, a change in the x-coordinates of the carbon atoms to any acceptable position did not change the sign of calculated structure factors corresponding to any measurable amplitude, and the electron density map shown in Fig. 2 was obtained after only two refinements. As the peaks overlap extensively, it is not possible to derive accurate coordinates from this map. Table 1 gives the x-coordinates found by means of numerous successive calculations of the correlation factor. These calculations were made following the same procedure as before, but a better agreement between the observed and the calculated amplitudes was obtained in this case by choosing a B value of 2.5 in the temperature factor $e^{-B\sin^2\Theta/\lambda^2}$. In the a-axis projection B had a considerably higher value 3.9. This may indicate an oriented thermal vibration of the molecules.

A further study of the variation of B-values has not been attempted, but while bi-1,3-dioxa*cyclo*pentyl seems to vibrate chiefly in a direction normal to (100)[1] in our case the thermal vibration seems rather to take place in the (100) plane. Keeping in mind that the a-axis chosen in this projection is a diagonal axis, the disagreement may be apparent.

It is also of interest to point out that the c-axis of bi-1,3-dioxa*cyclo*pentyl(2) is the longest one, the cell dimensions are $a = 4.46$ Å, $b = 7.76$ Å, $c = 11.64$ Å, $\beta = 121.5$ °,while the cell dimensions of bi-1,3-dithia*cyclo*pentyl show that

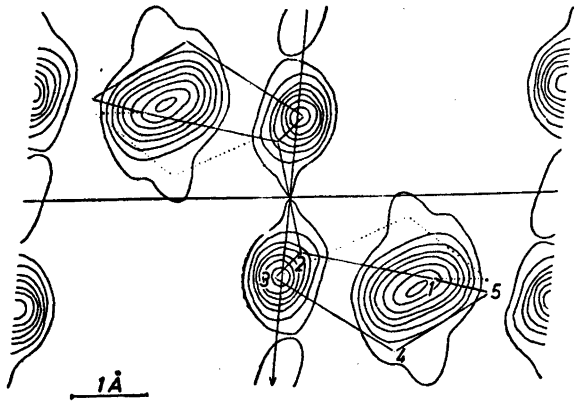

Fig. 2. The electron density map of the x—z projection. Full lines indicate structure I, dotted lines structure II.

the *b*-axis is the longest one. The different orientation of termal vibrations which the B value in the temperature factor seems to indicate, may be related to this difference in the cell dimensions. The *x* coordinates in Table 1 are given with only three significant figures because no greater accuracy can be expected with the unsatisfactory procedure in their determination.

The structure factors calculated from the *xz*-coordinates in Table 1 are compared with the observed amplitudes in Table 4. The correlation factor obtained is $R = 0.23$.

MOLECULAR DIMENSIONS AND DISCUSSION

The electron density map represented in Fig. 1 gives a good determination of the structure as far as the *y* and *z* coordinates are concerned, and there can be no doubt concerning the question whether the compound studied is bi-1,3-dithia*cyclo*pentyl or *cis*naphthodithian.

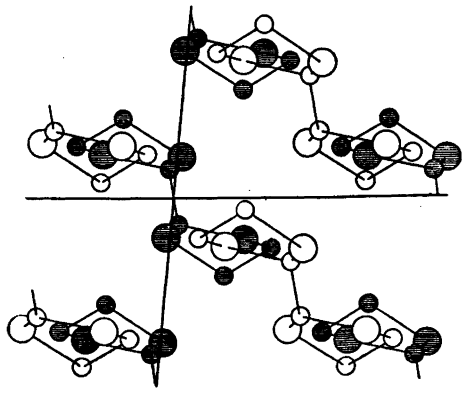

Fig. 3. The x—z projection of structure I.

Table 2.

S_1-S_3	2.86 Å	S_3-C_4	1.84 Å
S_1-C_2	1.86	C_4-C_5	1.52
S_1-C_5	1.89	C_2-C_2'	1.52
S_3-C_2	1.82		

Angles

$S_1C_2S_3 = 104°$	
$C_2S_1C_5 = 102°$	$C_5C_4S_3 = 100°$
$C_2S_3C_4 = 109°$	$C_2'C_2S_3 = 112°$
$S_1C_5C_4 = 109°$	$C_2'C_2S_1 = 107°$

The electron density map represented in Fig. 2 does not give so unquestionable results; a second structure (dotted on Fig. 2) is considered possible. In this case the x coordinates of the C_4 and C_5 are $x_4 = 0.30$ Å and $x_5 = 1.13$ Å. However, the refinement could not be improved beyond $R = 0.28$ in structure II whereas a value $R = 0.23$ was reached if the calculations were based upon structure I. Fig. 3 represents the xz projection of structure I. The bond lengths and angles calculated from the coordinates in Table 1 corresponding to structure I are give in Table 2. The four independent determinations of the C—S bond, agree whithin the limits of error with the generally accepted value[4], 1.81 Å. By using electron diffraction the single bond length C—S is found [5] within the range 1.78 to 1.90 Å. The C—C bond lengths are in close agreement with the accepted value of 1.54 Å. A detailed consideration of the errors has not been attempted, but on account of the very unsatisfactory determination of the x coordinates no greater accuracy than $\pm$ 4 % can be expected. The deviation of the bond angles from 109° are also whithin the limits of error.

A plane containing the three atoms S_1 C_2 S_3 will pass between the two carbon atoms C_4 and C_5 and the distance from these atoms to the plane is 0.47 Å for C_4 and for C_5 —0.32 Å. The minus sign for the distance means that the atom is on the same side of the plane as origo.

The shortest intermolecular distances are those between C_4—S_3 (3.13 Å) and C_5—S_3 (3.71 Å). The C_4S_3 distance is shorter than the generally accepted value of 3.4 to 3.7 Å between a methylene group and a sulphur atom[6,7]. Recently however, shorter CH_3—S intermolecular distances have been found *e.g.* in the crystal structure of metanethiosulphonates, a CH_2—S distance as low as about 3.1 Å was obtained [8] which is identical with the corresponding distance found in this structure.

CHEMICAL ASPECTS

On heating 2,3-dichloro-1,4-dioxan with 1,2-ethanedithiol in anhydrous benzene no compound was obtained which contained oxygen, but only bi-1,3-dithia*cyclo*pentyl(2). The first reaction mechanism mentioned by Furberg and Hassel and based on hydrogen bonds is of course not possible when ethylene glycol is replaced by the corresponding dithiol. The second alternative, however, also discussed by Proštenik and Balenović appears possible: 2,3-di-

Table 3.

h k l	F_{obs}	F_{calc}	*h k l*	F_{obs}	F_{calc}
0 2 0	43.9	43.8	0 0 4	32.7	41.7
0 4 0	5.2	7.9	0 1 4	10.6	−11.4
0 6 0	64.9	72.1	0 2 4	9.8	8.2
0 8 0	38.1	40.4	0 3 4	0	−1.7
0 10 0	5.0	5.1	0 4 4	4.4	2.9
0 12 0	9.8	10.4	0 5 4	7.5	6.3
0 14 0	16.0	18.3	0 6 4	20.3	26.4
			0 7 4	0	−3.8
			0 8 4	10.6	10.9
0 1 1	27.2	30.7	0 9 4	2.6	3.0
0 2 1	20.4	18.0	0 10 4	0	−0.8
0 3 1	27.4	35.7	0 11 4	0	−0.5
0 4 1	14.8	−14.8	0 12 4	5.8	6.5
0 5 1	7.1	2.4			
0 6 1	0	−1.1	0 1 5	0	−0.9
0 7 1	21.9	21.1	0 2 5	10.6	−8.1
0 8 1	6.0	−5.6	0 3 5	25.8	−36.6
0 9 1	11.0	−11.5	0 4 5	0	2.3
0 10 1	4.9	−2.4	0 5 5	12.1	13.4
0 11 1	9.6	−10.0	0 6 5	7.8	8.2
0 12 1	0	−0.4	0 7 5	0	2.0
0 13 1	7.3	7.6	0 8 5	3.7	−4.4
0 14 1	4.8	−5.8	0 9 5	10.1	−12.0
			0 10 5	3.5	−1.9
			0 11 5	9.9	−12.2
0 0 2	0	6.5			
0 1 2	9.1	−9.6	0 0 6	9.8	13.7
0 2 2	19.3	27.2	0 1 6	0	1.0
0 3 2	12.4	−9.1	0 2 6	0	−0.3
0 4 2	46.5	−53.8	0 3 6	0	−0.1
0 5 2	17.0	14.5	0 4 6	7.6	−6.5
0 6 2	0	−0.7	0 5 6	6.5	4.5
0 7 2	10.5	7.5	0 6 6	5.2	7.9
0 8 2	0	−0.1	0 7 6	2.5	2.6
0 9 2	6.8	−5.8	0 8 6	4.2	4.9
0 10 2	24.6	−30.7	0 9 6	0	2.2
0 11 2	0	3.9	0 10 6	4.2	−6.2
0 12 2	7.0	−6.4	0 11 6	5.1	5.8
0 13 2	5.0	5.1			
			0 1 7	8.8	13.9
			0 2 7	4.4	−6.8
0 1 3	46.1	54.5	0 3 7	5.6	5.7
0 2 3	4.2	−3.5	0 4 7	2.5	3.3
0 3 3	4.0	1.3	0 5 7	7.2	6.9
0 4 3	14.4	11.4	0 6 7	5.5	4.5
0 5 3	11.0	15.9	0 7 7	9.4	13.8
0 6 3	5.7	−1.0	0 8 7	3.9	−2.7
0 7 3	37.0	44.2			
0 8 3	0	2.3	0 0 8	0	3.9
0 9 3	8.0	8.6	0 1 8	4.5	−4.4
0 10 3	6.4	6.0	0 2 8	3.5	−4.4
0 11 3	0	−2.0	0 3 8	0	−0.4
0 12 3	6.8	2.6	0 4 8	5.8	−8.4
0 13 3	12.1	14.4	0 5 8	3.4	3.4

Table 4.

h	*k*	*l*	F_{obs}	F_{calc}.	*h*	*k*		F_{obs}	F_{calc}
0	0	2	0	1.59	3	0	$\bar{1}$	10.45	−10.52
0	0	4	13.35	11.57	3	0	1	0	0
0	0	6	4.82	4.28	3	0	3	9.24	−7.55
					3	0	5	1.98	−2.20
1	0	$\bar{5}$	0	−0.56	3	0	7	3.90	−2.79
1	0	$\bar{3}$	5.32	−1.82					
1	0	$\bar{1}$	15.90	20.05	4	0	$\bar{6}$	2.97	−3.34
1	0	1	10.80	−11.24	4	0	$\bar{4}$	2.86	2.03
1	0	3	10.35	11.00	4	0	$\bar{2}$	0	−0.12
1	0	5	2.21	2.04	4	0	0	5.12	7.95
					4	0	2	0	−0.80
2	0	$\bar{6}$	3.70	−5.27	4	0	4	2.87	2.94
2	0	$\bar{4}$	7.27	−4.14	4	0	6	0	0.04
2	0	$\bar{2}$	2.72	−3.38					
2	0	0	18.63	−19.78					
2	0	2	0	−1.17	5	0	1	3.53	6.58
2	0	4	8.40	−8.56	5	0	1	0	−0.54
2	0	6	0	−1.98	5	0	3	4.07	5.5
3	0	$\bar{5}$	0	0.74					
3	0	$\bar{3}$	2.58	−2.39	6	0	0	1.000	−2.99

chloro-1,4-dioxan by mild hydrolysis gives (besides glycol) a quantity of glyoxal equivalent to the amount of water originally present. Bi-1,3-dithia*cyclo*pentyl(2) will then be formed by the reaction between glyoxal and 1,2-ethanedithiol since this process again yields water, thus enabling the reaction to continue.

I am indebted to the *American Association of University Women* which gave me a fellowship thus making it possible for me to begin my work in the field of structure determination by X-ray diffraction. Thanks are due to professor M. Prošstenik and K. Balenović for sending to professor O. Hassel the compound to be determined. I will also express my gratitude to professor O. Hassel for suggesting this problem and giving me possibilities of working at his institute. Finally I will thank professor H. Haraldsen for giving me all possibilities to continue and finish the work at his institute.

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The Influence of the Solvent on Reaction Velocity

VII *. The Reaction between Triethylamine and Ethyl Iodide in Acetone-Benzene and Acetone-Dioxan Mixtures

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In all of the cases studied in the previous investigations of this series the solvent or one of its components takes part in the reaction. According to the results of these investigations, a common feature of all solvolytic reactions in mixed solvents seems to be that the parameters of the Arrhenius equation are greatly influenced by the medium; the plots of the activation energy and the frequency factor against the composition of the solvent are usually characterized by more or less pronounced maxima and minima. Their occurrence is, partly at least, accounted for by changes in the solvation of the reactants and the transition state [1]. In connexion with these observations it was found advisable to study a reaction in which the solvent does not take part, in mixed solvents in which the proportion of the components is continuously altered. As such a process the reaction between triethylamine and ethyl iodide was chosen:

$$(C_2H_5)_3N + C_2H_5I \rightarrow (C_2H_5)_4NI$$

The reaction is excellent for a kinetic study. It is quantitative, it proceeds at a conveniently measurable rate, and the amount of the amine present in the reaction mixture may be readily determined. To avoid every solvolysis of ethyl iodide, acetone-benzene and acetone-dioxan mixtures were chosen as solvents. In the former mixtures one of the components (acetone) is strongly polar, the other(benzene) non-polar; in the latter, the dipole moment of dioxan is practically zero, but in reality the dioxan molecule has two equal moments acting in opposite senses.

Menschutkin in his classical work[2] on the reaction between triethylamine and ethyl iodide measured its velocity in 22 different solvents, but at one temperature only, *viz.* at 100° C. He found that its velocity is greatly dependent on the solvent. Following the increasing order of the reaction velocity the

* VI. *Suomen Kemistilehti* **B 26** (1953) 79.

solvents can be arranged in the following row: hydrocarbons < ethers < esters < alcohols < ketones. The two last-mentioned must be written rather in the opposite order. The relative rate constants in some of the various solvents were: hexane 1, heptane 1.3, ethyl ether 4.2, xylene 15.9, benzene 38 .... ethyl alcohol 211, acetone 352, benzyl alcohol 769. Thus the rate constant increased almost 800-fold in passing from hexane to benzyl alcohol. The reaction is always faster in an aromatic solvent than in the corresponding aliphatic solvent. The parameters of the Arrhenius equation for the reaction were determined by Grimm, Ruf, and Wolff [3] in a number of pure solvents. According to their results, the general effect of changing the solvent is to bring about a change in both activation energy and frequency factor, in some cases the change in E, in some cases that in A being more important [4]. Moelwyn-Hughes and Hinshelwood [5] found that in gaseous phase the reaction takes place, at least partly, on the surface of the glass. They studied the reaction also in hexane. No information on this reaction in various mixtures of two liquids is available, but the reaction between triethylamine and ethyl bromide [6] has been investigated in various acetone-benzene mixtures and the results have been discussed [7,11]. Some other quaternary ammonium salt formation reactions have also been studied in mixed solvents, *viz.* the reactions of triethylamine and pyridine with benzyl bromide in benzene-nitrobenzene mixtures [8], and the formation of methylpyridinium iodide in various pure liquids [9] and binary mixtures [10]. The kinetics of the quaternary ammonium salt formation has been studied by many other investigators and it has been extensively discussed *e. g.* [4,11].

EXPERIMENTAL

Chemicals. All the substances used were commercial products of high purity. Triethylamine and ethyl iodide were fractionated to correct and constant boiling point, triethylamine after drying over potassium hydroxide. Merck's acetone 'pro analysi' was found to be dry enough and to require no further purification. The dioxan was purified by the method of Hess and Frahm [12]. Triophen-free benzene was refluxed several hours with sodium and distilled.

Preparation of the solvent mixtures. A quantity of the component the proportion of which in the mixture will be less than 50 % was measured with a pipette into a volumetric flask and made up to volume with the other component. The volume per cents given in the tables refer to these values. The proportion of the constituents in the solvent was measured also by weight. Each mixture was prepared in a quantity sufficient for all experiments in this solvent.

Equal volumes of 0.2 M solutions of triethylamine and ethyl iodide were mixed, so that the initial concentration of each was 0.1 M. From this mixture samples of 5 ml were pipetted into approximately 6 ml reaction tubes of Pyrex glass, the tubes sealed and immersed in the thermostats the temperature of which could be held constant to within about 0.02°. The exact concentration of the reactants at this moment was determined analytically as given below, for which purpose two or three of the tubes were used. After the reaction had proceeded for the desired length of time, the tubes were rapidly chilled in ice-water and the contents titrated with 0.02 N HCl, methyl red being used as indicator. The end-point of the titration was very sharp. No appreciable hydrolysis of ethyl iodide takes place during titration. In the case of the acetone-benzene mixtures, the contents of the tubes were washed into excess of 0.02 N HCl, vigorously shaken, and back-titrated with 0.1 N NaOH, a micro-burette being used in these titrations. Since the reaction times were fairly long (over one hour), the contents of the tubes small, and all operations could be carried out very quickly, correction for the time required for heating

up and analysing the mixtures could be neglected. — All thermometers were checked against a thermometer calibrated by the National Bureau of Standards (U.S.A.).

Satisfactory constants were obtained by applying the ordinary bimolecular formula, as exemplified by the following two runs:

1. Solvent: Acetone 7.28 % + dioxan 92.18 % by weight. a = 0.0988. 75.00°C.

t mins.	166	241	300	430	486	648	906	954	990
change, %	29.2	37.6	42.2	51.4	55.0	61.5	69.2	70.3	70.7
10^5k l mole^{-1} sec.$^{-1}$	41.9	41.4	41.0	41.5	42.4	41.5	41.9	41.8	41.2

2. Solvent: Acetone 37.56 % + benzene 62.44 % by weight. a = 0.0998. 75.00°.

t mins.	145	202	256	323	390
change, %	34.5	42.9	49.2	55.0	59.5
10^5k l mole^{-1} sec.$^{-1}$	61.7	62.1	63.2	63.3	62.8

All rate constants (means of the runs) were corrected for the thermal expansion of the solvents.

Since the object of the experiments was to measure small changes in activation energy and frequency factor as accurately as possible, the preparation of the solvent and all other operations were carefully standardised and the determination of the rate constants was carried out always at four temperatures. All the measurements were made by one of the writers (P.K.) in order to eliminate personal factors as much as possible.

RESULTS

Tables 1 and 2 summarise the values found for the rate constants, activation energies, frequency factors, and activation entropies. The activation energies and frequency factors were calculated by the method of least squares from the variation of log k with $1/T$. The entropy of activation, ΔS^*, was calculated from the frequency factor A by means of the equation [11]

$$A = e\ (kT/h)e^{\Delta S^*/R}$$

The Arrhenius equation is obeyed within the experimental error in all cases.

Estimated from the errors in the rate constants, the probable error in the activation energies is in the acetone-dioxan series $\pm$ 40 to $\pm$ 50 cal., in the acetone-benzene series $\pm$ 70 to $\pm$ 100 cal., and that in log A in the former series $\pm$ 0.040 to $\pm$ 0.050, and $\pm$ 0.050 to $\pm$ 0.070 in the latter, the probable error in the entropies of activation in the two series being thus $\pm$ 0.1 to $\pm$ 0.2 and $\pm$ 0.2 to $\pm$ to 0.3 entropy units, respectively. In view of this the variations of E and ΔS^* (or log A), to be discussed in the next section, are fully significant with respect to the experimental uncertainty, all the more if we take into account the fact that relative values of A and E are more accurate than their absolute values.

DISCUSSION

Our values for the pure acetone solution are in good agreement with those of Grimm, Ruf and Wolff (E 11.9 kcal., log A 4.4). The small difference is probably essentially due to the correction for the thermal expansion of the solvent, made by us. In benzene solution our values for E and A are much

Table 1. Acetone-dioxan mixtures.

D vol. %	D wt. %	x_D	10^5k liter mole^{-1} sec.$^{-1}$				E cal.	log A	ΔS^* E.U.
			40.00°	50.00°	60.00°	75.00°			
0	0	0	17.72	32.84	58.29	126.9	12 190	4.756	−38.8
5	6.40	0.0431	18.18	33.19	58.07	125.6	11 960	4.609	−39.4
10	12.72	0.0877	18.14	32.70	56.05	121.7	11 770	4.470	−40.0
12.5	15.74	0.1097	18.19	33.18	58.12	125.9	11 980	4.618	−39.4
15	18.74	0.1320	17.82	32.44	57.81	125.6	12 060	4.674	−39.1
20	24.62	0.1771	17.63	32.14	56.64	123.5	12 050	4.659	−39.2
30	35.84	0.2691	17.13	31.20	54.68	118.7	11 980	4.597	−39.5
45	51.58	0.4126	16.07	29.38	50.79	110.2	11 900	4.513	−39.9
55	61.54	0.5176	14.68	26.29	46.36	101.0	11 960	4.510	−39.9
60	66.36	0.5625	13.73	24.93	43.80	94.56	11 950	4.479	−40.0
65	70.79	0.6150	12.75	23.03	40.34	86.52	11 860	4.384	−40.5
70	75.26	0.6673	11.66	21.14	36.77	78.82	11 830	4.322	−40.8
75	79.71	0.7209	10.58	18.93	33.03	70.29	11 740	4.215	−41.2
80	83.97	0.7754	8.996	16.57	28.95	62.44	11 980	4.320	−40.8
80II	83.96	0.7753	9.089	16.53	29.23	63.11	12 010	4.340	−40.9
85	88.11	0.8301	7.731	14.11	24.93	54.36	12 080	4.319	−40.8
90	92.18	0.8860	6.225	11.45	20.08	44.41	12 160	4.281	−40.9
94.7	95.86	0.9386	4.978	9.220	16.39	36.63	12 350	4.316	−40.8
100	100	1.0000	3.343	6.318	11.52	25.88	12 680	4.374	−40.5

higher than those of these authors (E 11.4 kcal., log A 3.3). We are unable to explain this discrepancy. A possible explanation would be that Grimm, Ruf and Wolff carried out their experiments at higher temperatures than we did and that E and A are somewhat dependent on temperature. The reaction might also be very sensitive for impurities. In dioxan, where the reaction has not been previously investigated, its velocity is less than in acetone, but greater than in benzene. The value of the energy of activation is about the same as in benzene. On passing from acetone to benzene, or to dioxan, the reduction in reaction velocity is caused partly by an increase in E and partly by a

Table 2. Acetone-benzene mixtures.

B vol. %	B wt. %	x_B	10^5k liter mole^{-1} sec.$^{-1}$				E cal.	log A	ΔS^* E.U.
			40.00°	50.00°	60.000°	75.00°			
0	0	0	17.72	32.84	58.29	126.9	12 190	4.756	−38.8
10	10.99	0.0841	18.81	34.48	61.12	133.9	12 160	4.758	−38.8
20	21.85	0.1721	17.13	31.04	55.13	121.1	12 120	4.692	−39.1
40	42.66	0.3561	14.28	25.46	45.36	98.96	12 020	4.540	−39.8
60	62.44	0.5528	10.30	18.21	31.88	68.07	11 720	4.187	−41.4
80	81.61	0.7674	5.920	10.60	18.37	39.41	11 740	3.964	−42.4
90	90.93	0.8817	3.572	6.404	11.43	24.41	11 930	3.880	−43.8
95	95.48	0.9402	2.413	4.428	7.771	17.04	12 090	3.823	−43.0
100	100	1.0000	1.478	2.773	5.025	11.50	12 700	4.034	−42.1

decrease in A. The difference in reaction velocity between benzene and dioxan solutions is accounted for solely by changes in A.

The values of E and A are very low in all the three solvents, but this applies also to many other media [4]. For the low value of A the following explanation can be given [11]. In the reaction where the reactants are diatomic and for polyatomic molecules the formation of the transition complex is accompanied by the disappearance of three translational and three rotational degrees of freedom, and the formation of five new vibrational degrees of freedom and one along the reaction coordinate. The low values of A or the large negative values of the entropy of activation are clearly due to the restrictions in the energy transitions involved in the formation of the transition state. There is a considerable rearrangement of energy among the various degrees of freedom to accompany the formation of the transition state, *i.e.*, a small probability of formation of the transition state, and, therefore, the entropy of activation is negative the more elaborate the mechanical reorganization which the reaction involves. Two molecules possessing the necessary energy for reaction may approach but unless the energy can be transferred to the appropriate degrees of freedom the transition state cannot form. According to the statistical theory, the probability factor P in the equation $A = PZ$ (Z is the calculated collision frequency) is, for a reaction between two polyatomic molecules, $P = (f_V/f_R)^5$, where f_V and f_R denote the contributions of vibrational and rotational degrees of freedom to the total partition function. At ordinary temperatures, f_V is generally of the order of unity, while f_R is of the order of 10 to 100, and so the ratio f_V/f_R is 10^{-1} to 10^{-2} and $(f_V f_R)^5$ approximately 10^{-5} to 10^{-10}. However, that is so only for gas reactions; for the reactions in solution the precence of the solvent modifies the partition functions in a way which is not accurately known, and thus the calculations are more uncertain. The collision frequency Z is, also for the reactions in solution [4], about 2.8×10^{11}. The experimental values for P are thus 0.4×10^{-7} (benzene), 1.0×10^{-7} (dioxan), and 2.0×10^{-7} (acetone).

In the formation of quaternary ammonium salts the lone pair of electrons of the nitrogen atom in the amine is shared with carbon under splitting off the halogen. The product is a highly polar substance and also the transition state is highly polar. The process can be represented by

$$X + YZ \rightarrow \overset{+\delta}{X} \ldots . Y \ldots . \overset{-\delta}{Z} \rightarrow XY^+ Z^-$$

On the ground of the entropy data available [13] it has been shown [11] that for the reaction

$$C_6H_5N(CH_3)_2 + CH_3I \rightarrow C_6H_5N(CH_3)_3{+}I^-$$

the transition complex is very similar in structure to the quaternary ammonium salt obtained as product, and there is little doubt that this is the case also in the reaction between triethylamine and ethyl iodide.

In cases where the products are more polar than the reactants, there is a general tendency for A to be greater in a more polar solvent than in a less polar solvent [14]. It probably depends partly on an actual increase in Z with a change to a more polar solvent, and partly on an action of the latter in stabili-

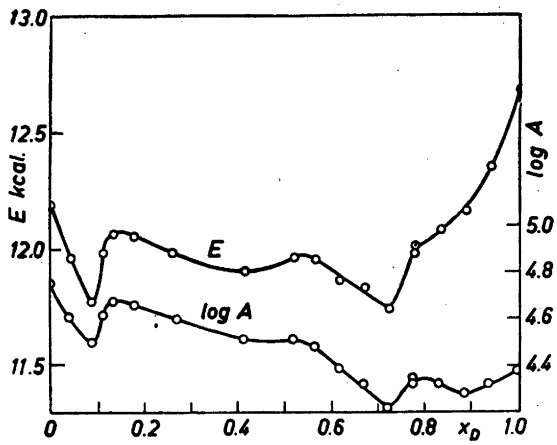

Fig. 1. Plots of E and log A against the mole fraction of dioxan in acetone-dioxan mixtures.

zing the products of the reaction. The increase in Z would be due to a kind of extrusion of solute molecules from the structural pattern of the solvent, as has been shown probable by the use of a mechanical model [15,16] and also inferred on the basis of chemical experiments [16]. According to Hinshelwood [14], "The stabilizing effect will only be in evidence in reactions, such as the formation of quaternary ammonium salts, where the product is a highly polar substance, and where the transition complex will have a great tendency to relapse into its original components unless a polar environment is provided to prevent it. If much reversion occurs, P will be small. Hence the increase of P with polarity of solvent." The same suggestion has been made by Soper and co-workers, who postulated that the solvent tends to disrupt the critical complex into those substances for which the solvent has the greater affinity [17]. From the standpoint of the theory of absolute reaction rates this means that the transmission coefficient, $\varkappa$, in the reaction rate equation

$$k = \varkappa \frac{kT}{h} \, e^{-\Delta G^*/RT}$$

is affected by the solvents.

However, there are also solvent effects of another kind, as can be estimated on the basis of activation energies. As highly polar, the transition complex no doubt is more solvated than the reactants and, accordingly, the large negative activation entropies found almost certainly are partly due to the entropy lost by solvent molecules frozen around the partially ionic transition states. Since this effect is the stronger the more polar the solvent, it operates contrary to that discussed above. The increase of solvation accompanied the formation of the transition complex decreases the value of the parameter A of the Arrhenius equation, but it also reduces the energy of activation. The former effect retards, the latter accelerates the reaction. Since a change from a less polar to a more polar solvent increases the organisation of the solvent molecules

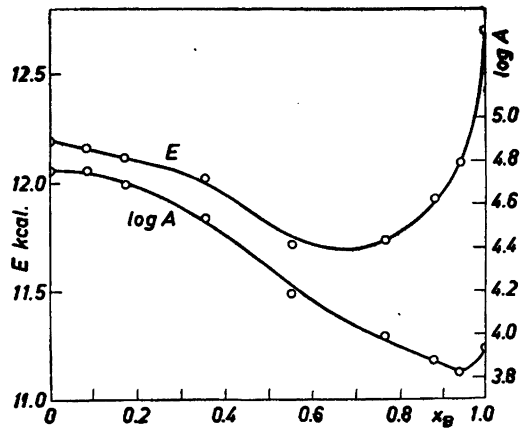

Fig. 2. Plots of E and log A against the mole fraction of benzene in acetone-benzene mixtures.

around the partially ionic transition complex more than around the less polar reactants, it will decrease the energy of activation and also decrease the frequency factor; the experimental result that a more polar solvent accelerates the reaction indicates that the energy change dominates the rate change. The solvation especially of the transition complex in a polar solvent is without any further consideration clear. Wynne-Jones, in a recent investigation [18], has assumed that also the benzene molecules are powerfully oriented by the strongly polar compounds and related his results to the mechanism of the Menschutkin reaction. Attention has also been focussed [19] to the fact that reactions in which the transition state is more highly polar, and hence more extensively solvated, than the initial state, are greatly accelerated by pressure, while those in which the transition state is less ionic than the initial state are retarded by pressure [20].

When we pass through a series of mixed solvents, the degree of the solvation in general will be changed, and hence it follows that E and A will be changed. The curves representing E and A as functions of the composition of the solvent may vary greatly from case to case. The experimental activation energies and frequency factors given in Tables 1 and 2, and related to the composition of the solvent in Figs. 1 and 2, show that for the reaction between triethylamine and ethyl iodide the state of affaires is simpler in acetone-benzene than in acetone-dioxan mixtures. The behaviour of E and A may highly probably principally be attributed to changes in the solvation of the transition state, and it is reasonable to assume that the solvation changes with the composition of the solvent more regularly in the case of acetone-benzene than in the case of acetone-dioxan.

The minima in E and A correspond to domains where the solvation of the transition state is particularly powerful; in the case of acetone-dioxan there are three such minima, two sharp and one flat; in the case of acetone-benzene there is only one minimum. It is obvious that in the regions of the minima the

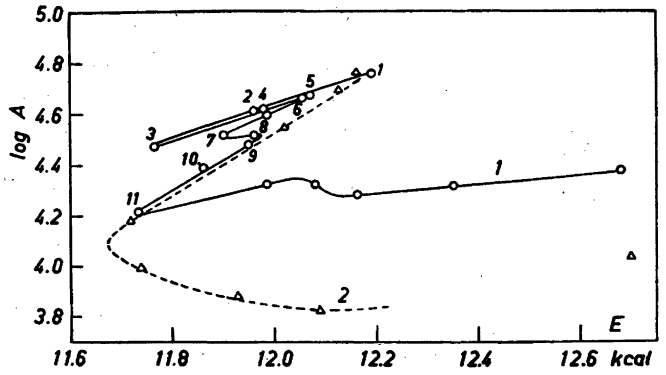

Fig. 3. Plot A log of against E. 1. Acetone-dioxan mixtures, 2. Acetone-benzene mixtures.

relative proportion of the components of the mixed solvent is suitable, so that both of the them can profitably take part in the formation of the solvation layer. Other factors, such as the internal structure of the solvent and the ability of the solvent to prevent the relapse of the transition complex into its original components, may also play a part in the matter. Especially the curves for acetone-dioxan mixtures show how important it is in the study of solvent effects in mixed solvents that a sufficient number of observations has been taken. In contrast to the behaviour of E and A the plots for the rate constants against the composition of the solvent are much simpler, certain deviations which are scarcely noticeable correspond to the irregularities in the E and log A curves.

In acetone-dioxan solvents the E and log A curves are close similar in form until the mixture contains 80 % of dioxan, and in acetone-benzene mixtures until the percentage of benzene is 70, after which A continuously decreases although there is a powerful rise in E. Fig 3, in which are given plots of log A against E for the both mixtures, shows linear relationships for all the regions between the different turning points in the curves in Figs. 1 and 2. Linear relationships between E and log A have been found in many other reactions, and various attempts to justify them have been made [1,10].

Much attention has been paid to the relationship between the dielectric constant of the medium and the rate of reaction. Unfortunately the dielectric constants of the mixtures used by us as solvent have not been measured.

In recent years, attention has been paid also to the discussion of the Arrhenius equation [4], and cases are known where the energy of activation changes with rise in temperature. Since our data at least for acetone-dioxan mixtures may be assumed to be of considerable accuracy, we have calculated the values of E for the temperature intervals 40—50°, 50—60°, and 60—75°. For the reaction in pure acetone the values are 12 400, 12 300, and 11 950 calories, respectively, but in the mixed solvents and in pure dioxan and in pure benzene the differences are too small to allow any definite conclusions. To save space the single values are not given here.

SUMMARY

The reaction between triethylamine and ethyl iodide has been investigated in various acetone-benzene and acetone-dioxan mixtures. The plot of E and log A against the composition of the solvent shows in the former case a minimum, in the latter one flat and two sharp minima. Between the turning points of the curves there exist linear relationships between log A and E. The results are discussed.

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An Automatic Conductivity Bridge for Chromatographic Analyses

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A recording device for the separation in chromatographic columns is always time-sparing and useful, especially if it admits quantitative conclusions. In that case it is a useful analytical tool and moreover gives information about the shape of the elution curve. Conductivity as a principle for measurement has been used by Wickbold [1], James, Martin and Randall [2], and Drake [3].

Wickbold uses a measuring cell and a comparison cell, fed from two separate transformer windings. The currents from the cells are rectified in a Graetz coupling and compared with each other by a recording millivoltmeter. The apparatus constructed by James *et al.* is built around a conventionel A. C. Wheatstone bridge. The amplified, unbalanced voltage is fed to a phase-discriminating circuit, the output voltage of which controls a strip chart recorder. The slidewire in the recorder is a part of the bridge and thus automatically corrects the adjustment of the bridge. The measuring device described by Drake is of high precision compared with the other two and has a sensitivity corresponding to about 0.02 % of the total resistance (cell: 1 600 ohms; bridge potentiometer: 50 ohms). In his apparatus the voltage from the discriminator controls a reversible motor which rotates two potentiometers. One of these is the slidewire in the bridge. A D.C. voltage over the second potentiometer is applied to a recorder. The conductivity-measuring cells have been differently constructed. James *et al.* have the cell in the column but give no details. As mentioned above, Wickbold has both a measuring and a comparison cell. The comparison cell is filled with the solvent used as elutriant. Drake has a micro cell (volume about 0.1 ml) immersed in a thermostated oil bath.

In order to obtain a less complicated apparatus than the one described by Drake we tried to improve the construction of James *et al.* However, it was found difficult to get full balance of the bridge due to the great capacity of the conductivity cell and electrical asymmetry against earth, which resulted in unsteadiness and no sharp zero point. These disadvantages were eliminated by inserting a Wagner earth and a comparison cell of the same geometrical shape as the measuring cell. A more detailed description and some applications are given in the following.

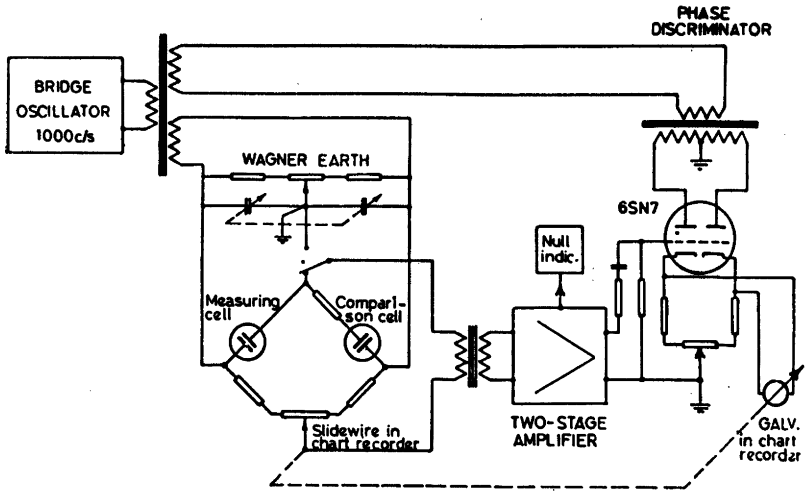

Fig. 1. Block scheme of the electronic device.

DESCRIPTION OF THE APPARATUS

Electronic equipment. A block scheme of the automatic conductivity bridge is shown in Fig. 1. Two opposite arms in the bridge contain the measuring cell and the comparison cell. By means of a switch a decade can be inserted in series with one of the cells. The regular slidewire in the chart recorder is also used as the continuously variable resistance in the bridge. It is mechanically coupled to the galvanometer. An unbalance in the bridge causes a deflection of the galvanometer pointer in one or the other direction. Then, by means of a special mechanism, this deflection is transformed to a winding movement which is such that it tends to restore the balance of the measuring circuit by moving the slidewire along the slider. The movement of the slidewire is also mechanically transferred to the pen, thus showing the difference in conductivity between measuring cell and comparison cell. The sensitivity of the bridge is determined by the resistance of the slidewire relative to the total resistance of the half of the bridge containing the slidewire. The sensitivity can consequently be chosen in arbitrary steps by giving suitable values to the resistors complementing the slidewire. A change of one ohm in the decade can be made to correspond to a certain fraction of the chart width. For instance with 500 ohms resistors in the arms containing the slidewire and a slidewire resistance of 2 ohms a change of 0.8 % of the total resistance in the cell corresponds to a deflection over the whole chart width. In order to compensate for the unbalance against earth the bridge is fed from the bridge oscillator over a Wagner earth. To eliminate the differences in capacity between the conductivity cells, the measuring device is supplied with a switch through which various condensers can be inserted in parallel with the measuring cell or the comparison cell. The voltage across the conductivity cells

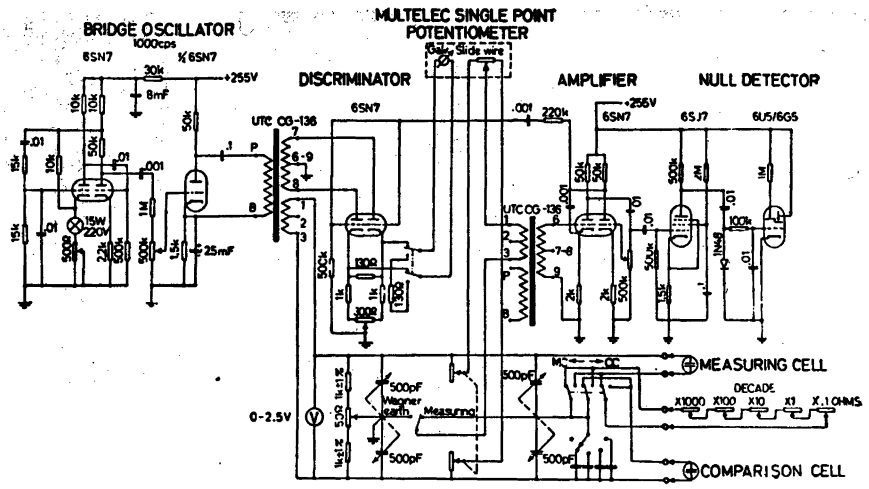

Fig. 2. Circuit diagram of the conductivity bridge.

can be varied between 0 and 1 volts in steps of 0.1 volts. The frequency of the oscillator is 1 000 c/s.

The unbalanced voltage from the bridge is amplified by a two-stage amplifier (gain about 350). The output voltage is then applied both to a phase discrimination circuit to which the galvanometer is connected and to a zero-point indicator. The purpose of this is to show when the bridge is sufficiently balanced so that the galvanometer circuit can be switched on without risk of ruining the galvanometer. The whole apparatus is fed from a voltage-stabilised rectifier. A more detailed circuit diagram is shown in Fig. 2.

The bridge oscillator is RC-coupled and is of conventional type. It is followed by a power amplifier with transformer output. The output-transformer has two secondary windings, one feeding the bridge, the other supplying the voltage of the discriminator anodes. The discriminator works as follows: when the bridge is in balance, the voltages over the cathode resistors are equal, *i.e.* the voltage to the galvanometer is zero. However, when the bridge is unbalanced the voltage is incoming over the grids of the discriminator. According to the phase relation of this voltage, the anode current rises in one of the triode-halves of the 6SN7. The result of this is a higher voltage over the corresponding cathode resistor and hence a voltage difference is indicated by the galvanometer. Of course, a rise of the anode current is only obtained when the phase relation is such that the grid voltage is growing more positive at the same time that the anode voltage is of positive direction.

The chart recorder connected to the conductivity bridge is a Kent multelec single point potentiometer, 0—10 mV. As already mentioned, the galvanometer and the slidewire in the recorder are the parts used. All the other electrical components and leads are disconnected. Certain precautions have been taken in order to avoid 50-cycle interferences from the motor and its cables. Therefore, all leads between the recorder and the brigde device have

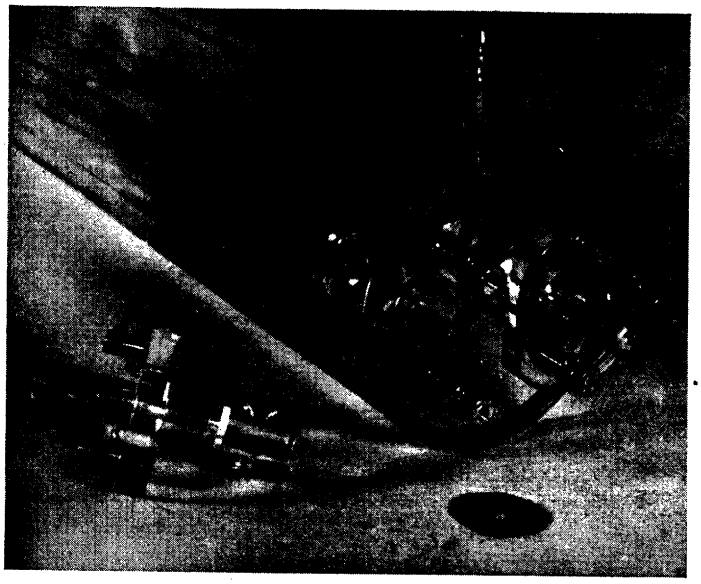

Fig. 3. Photograph of the conductivity cells.

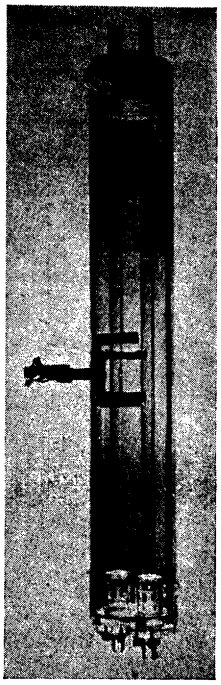

Fig. 4. Photograph of the columns and the jacket.

been carefully screened. This has also been done with the leads to the conductivity cells. A paper speed of 1 inch per hour is usually convenient.

Conductivity cell. In order to obtain a capacitive balance of the bridge two equal cells have been used. They are placed in the bottom of the columns and are filled with small glass beads (diameter 0.1 mm). Different constructions have been tried, and that found most suitable is shown in Fig. 3 and 4. The columns are made of "perspex" and the cells are easily removable. They are fastened to the columns by screws and rubber packing. The beads are held by a glass filter in the bottom of the cell. The electrodes are made of platinum and inserted in a niche in the wall. The surface facing the lumen is platinized and the wires from the electrodes are led through the walls down to the under-surface where terminal screws are placed. The columns mostly used have the dimensions 10 × 560 mm.

In these the conductivity cell constant is 0.20 cm^{-1}. A larger column with a diameter of 32.5 mm has been built for preparative work. To prevent temperature variations the columns are fitted with a jacket through which thermostated transformer oil is pumped. The temperature of the latter is kept constant to within $\pm$ 0.1° C. Greater temperature variations are also prevented in the room where the apparatus is located. The conductivity cell is placed in the bottom of the column largely in order to reduce the risk of mixing the fluid leaving the column. This might occur if the effluent were made to go through a capillary tube with an extension for the cell.

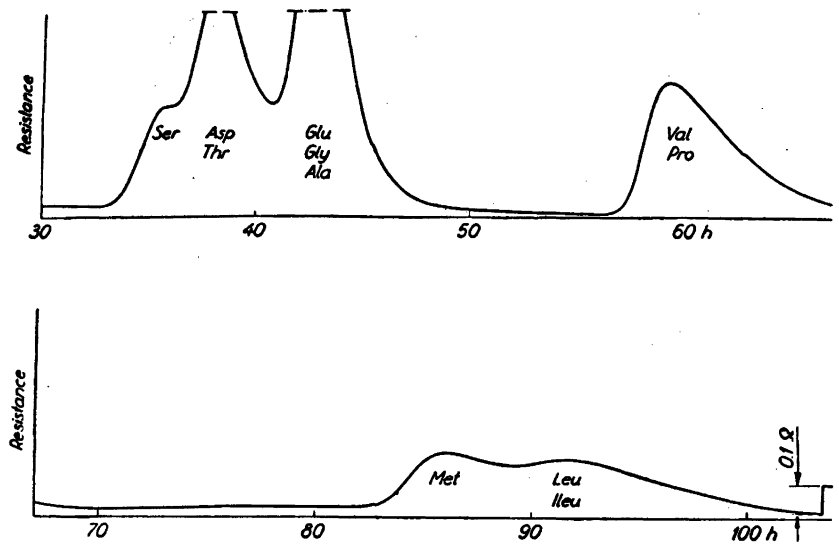

Fig. 5. Elution of amino acids from 1.0 ml of a casein hydrolysate (total nitrogen 44.8 mg) with 1.0 N HCl from a Dowex 50 column (2 % DVB).

Column dimensions: 10.3 × 387 mm. Flow rate: 1.36 ml/hr. Chart speed: 1 inch/hr. Abbreviations of amino acids according to Brand and Edsall [9].

EXPERIMENTS

The apparatus has been used for experiments with amino acids and phosphorus-containing amino acids and peptides.

Amino acids. The best procedures for chromatographic separations are those described by Stein and Moore [4,5]. The principle is elution chromatography on the strong cation exchanger, Dowex 50 (8 % cross-linking), by hydrochloric acid or various buffer mixtures. The last procedure gives the best resolution but works at a pH near the isoelectric point, making unsuitable the conductivity recording of the amino acids. Drake [3] has also used 0.1 *N* HCl for his separation of amino acids. He found that it was possible to elute serine, threonine, glycine, alanine and valine with that strength of hydrochloric acid although there was some overlapping and valine gave a low and broad peak. To reduce the adsorption to the resin, Dowex 50 with lower cross-linking (2 % DVB) and higher hydrochloric acid concentrations (1.0 *N*) have been tried. Fig. 5 shows such an experiment, but there is still some overlapping. For quantitative determinations a preliminary separation into groups is thus necessary. Using 1 *N* hydrochloric acid the cell resistance is 19.6 ohms. With 100 ohms in each of the arms containing the slidewire the apparatus easily detects 0.025 ohms corresponding to a sensitivity of 0.125 % of the total resistance.

Phosphorus-containing amino acids and peptides. The usefulness of the separation of these substances by elution with 0.01 *N* hydrochloric acid from a Dowex 50 column has been shown [6,7]. As a consequence of the low acid concentration employed, the compounds may be indicated with high sensitivity by measuring the conductivity. Investigations [8] in this laboratory indicate that elution with 0.5 *N* formic acid from a bed of a strong anion exchanger (Dowex 2, formate form, 10 % DVB) is also an excellent chromatographic system for this type of substances. Fig. 6 gives a photograph of a curve indicating phosphoserine and phosphothreonine in such an experiment. Because of the dissociation of the phosphoric acid group the peaks given correspond to a rise of the con-

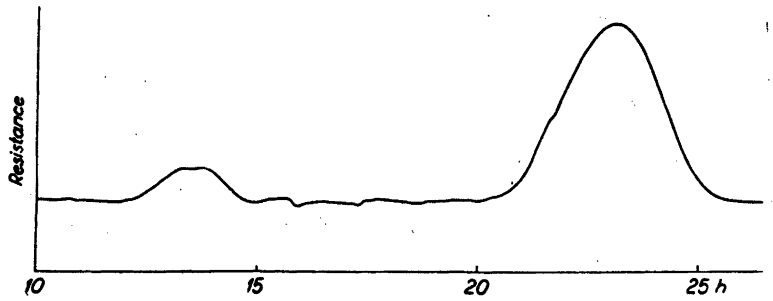

Fig. 6. Elution of phosphoserine (12.5 mg) and phosphothreonine (2.9 mg) with 0.5 N HCOOH from a Dowex 2 column (10 % DVB).
Column dimensions: 10.3 × 390 mm.
Flow rate: 12.1 ml/hr. Chart speed: 1 inch/hr.

ductivity, in contrast to the lowering of conductivity caused by the ordinary amino acids. The resistance of the cell with 0.01 *N* HCl and 0.5 *N* HCOOH is 1 270 and 1 540 ohms respectively. The sensitivity is about 0.05 % (100 ohms in each of the bridge arms containing the slidewire).

QUANTITATIVE DETERMINATIONS

Formulas for the estimation of amino acids by conductivity measurement are given by Drake [3].

To check the possibility of using the apparatus for quantitative determinations several curves were collected from elution of phosphoserine from Dowex 50 with 0.01 *N* hydrochloric acid. Different amounts and different flow rates were used.

The general formula for the determination of a substance in a flowing solution:

$$N_B = \int_{V_1}^{V_2} c_B \mathrm{d}V$$

(where N_B is the amount of a special substance B, c_B the concentration of the substance and V the passing volume) can be transformed to a form more suitable for calculations. Putting

$$c_B = k_1 R = k_1 k_2 b \text{ and}$$
$$V = k_3 t = k_4 a$$

(here R is the change in resistance in the conductivity cell due to the substance to be recorded, t is the time and a and b are the abscissa and ordinate respectively, expressed in cm) the formula can be transformed to

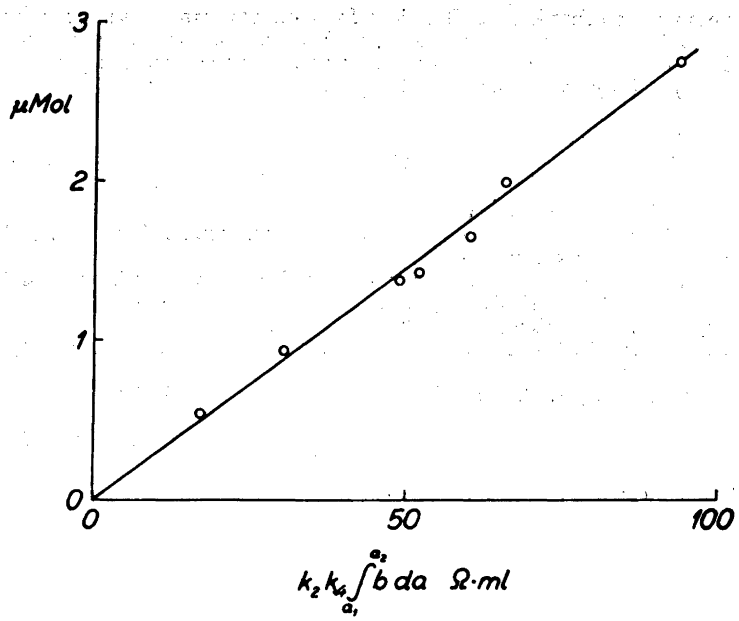

Fig. 7. Abscissa: $k_2k_4 \int_{a_1}^{a_2} b\ \mathrm{d}a$. *Ordinate: amount of phosphoserine (micromoles).*

$$N_B = k_1k_2k_4 \int_{a_1}^{a_2} b\ \mathrm{d}a$$

The area $\int_{a_1}^{a_2} b\ \mathrm{d}a$ can be determined with a planimeter, and k_2 and k_4 can be determined for each run. k_1 has to be determined for every system and measuring cell. Fig. 7 shows a curve, where N_B is plotted against $k_2k_4 \int_{a_1}^{a_2} b\ \mathrm{d}a$ indicating good linearity. k_1 for this system is 0.029 compared with $k_1 = 0.086$, when phosphoserine is eluted with 0.5 N formic acid through the same conductivity cell.

SUMMARY

A short discussion is given of earlier apparatus for automatic conductivity measurement of the effluent from chromatographic columns. A modification of an earlier design by James *et al.* is described. The conductivity bridge contains identical measurement and comparison cells. The modified apparatus has been of great value in the separation of ordinary amino acids and phosphorus-containing amino acids and peptides on ion exchange columns.

The authors wish to thank Prof. G. Ågren for valuable interest and criticism. We are also grateful to Messrs. Per Olsson and Axel Edenström for technical constructions. This investigation was sponsored by grants from *Statens medicinska forskningsråd* and *Riksföreningen för kräftsjukdomarnas bekämpande.*

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X-ray Crystallographic Data on Selenotrithionates

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Selenotrithionic acid, $Se(SO_3H)_2$, was discovered by Rathke [1] in 1865, two and a half decades after the preparation of the first salts of trithionic acid, $S(SO_3H)_2$, by Langlois [2].

Whereas crystal data on potassium, rubidium and cesium trithionate were reported by Mackenzie and Marshall [3] in 1908, and Zachariasen [4] in 1934 worked out the crystal structure of potassium trithionate by X-ray methods, the only crystallographic data available on selenotrithionates are from a morphological study of potassium selenotrithionate by Rathke [5,6] in 1870.

The chemical evidence concerning the constitution of selenotrithionic acid and selenotrithionates, *e.g.*, the formation reaction from selenium acetylacetonate and hydrogen sulphite [7]:

$$(C_5H_8O_2Se)_2 + 4\ HSO_3^- = 2\ Se(SO_3)_2^{--} + 2\ C_5H_8O_2 \qquad (1)$$

and the nucleophilic displacement equilibrium [8] involving selenotrithionate/selenopentathionate and thiosulphate/sulphite ions:

$$Se(SO_3)_2^{--} + 2\ S_2O_3^{--} \rightleftharpoons Se(S_2O_3)_2^{--} + 2\ SO_3^{--} \qquad (2)$$

leave little doubt that the selenotrithionate ion is built up of a divalent selenium atom to which are attached two sulphonate groups. Thus, selenotrithionate is derived from trithionate by substitution of selenium for the middle sulphur atom. Two organic analogues, *viz.*, sulphur and selenium dibenzenesulphinate, arise from the acids by substitution of phenyl for the hydroxyl groups. The benzenesulphinates form isomorphous crystals [9], and the detailed structures have been determined recently by Mathieson and Robertson [10] and Furberg and Öyum [11].

In the present study, crystallographic measurements have been made on ammonium, potassium, rubidium, cesium and barium selenotrithionate, with the purpose of establishing whether isomorphism exists between these crystals and those of the corresponding trithionates. No such isomorphism has been found. In view of the undoubtedly similar constitution of the

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trithionate and selenotrithionate anions, and the manyfold cases of isomorphism reported earlier for crystals of analogous sulphur, selenium and tellurium compounds, this result appears rather surprising and may be largely fortuitous. Thus, in addition to the numerous examples among inorganic sulphides, selenides and tellurides, and the sulphur and selenium dibenzenesulphinates mentioned above, isomorphism exists, *e.g.*, between selenium, diselenium and triselenium di-*p*-toluenesulphinate and the corresponding sulphur compounds [9,12], and between salts of pentathionic, selenopentathionic and telluropentathionic acid [13]. Likewise, crystals of sulphur, selenium and tellurium dimethanethiosulphonate are isomorphous [14], and so are those of the corresponding *p*-toluenethiosulphonates [12].

SYNTHESIS OF SALTS

The selenotrithionates were prepared from selenium acetylacetonate and aqueous solutions of the respective hydrogen sulphites, as done earlier by Morgan and Smith [7]. The cesium salt, and particularly the ammonium salt, are very soluble in water and were crystallized by addition of relatively large amounts of anhydrous ethanol to the concentrated aqueous solutions. Due to a lack of selenium acetylacetonate at the appropriate moment, rubidium selenotrithionate was prepared from the potassium salt by metathesis with an equivalent amount of sodium perchlorate, cooling and filtering, and addition of a slight excess of rubidium chloride.

The products were analyzed iodometrically according to Eq. (2) above [8], *viz.*, by the reaction with an excess of 0.1 *M* sodium thiosulphate, in phosphate buffer in presence of formaldehyde, and back-titration of the excess of thiosulphate with 0.1 *N* iodine. The purities of the recrystallized salts, used for X-ray measurements, ranged from 99.6 % to 99.85 %.

The selenotrithionates gradually liberate selenium on exposure to light and to X-rays. The crystals of barium selenotrithionate were found to be the more sensitive and those of cesium selenotrithionate the less sensitive ones in this respect.

Selenium acetylacetonate, required for the above syntheses, was prepared from acetylacetone and selenium tetrachloride suspended in anhydrous ether [7]. In working up the reaction mixture, it was found to be essential, in order to avoid an extensive decomposition of the product, to remove the ether rapidly in a vacuum and then, immediately, to shake the remaining crystalline mass with a relatively large amount of water. After filtering, the product was recrystallized from boiling glacial acetic acid.

Some measurements were also made on ammonium and barium trithionate, no crystallographic data on these salts being available in literature. Ammonium trithionate was prepared according to Kurtenacker and Laszlo [15]. The recrystallized salt was, by iodometric titrations, found to contain 0.17 % of ammonium thiosulphate as an impurity. Barium trithionate [2,16] was obtained from the potassium salt by metathesis with barium perchlorate.

CRYSTAL DATA

X-ray measurements (oscillation and Weissenberg photographs on small single-crystal specimens) were carried out using Cu$K\alpha$ radiation, $\lambda = 1.54$ Å, and in some cases Fe$K\alpha$ radiation, $\lambda = 1.934$ Å. The values recorded below for axial lengths are believed to be accurate to within 0.5 %, except in the case of rubidium selenotrithionate and ammonium trithionate, where approximate data were obtained from layer-line separations in oscillation photographs only.

In the following, for comparison, data on selenotrithionates and corresponding trithionates are listed consequtively. Those on potassium, rubidium and cesium trithionate are taken from the literature.

Ammonium selenotrithionate, $(NH_4)_2Se(SO_3)_2$. Monoclinic, $a = 6.74$ Å, $b = 8.96$ Å, $c = 14.00$ Å, $\beta = 102°$. Four molecules per unit cell; density, calc. 2.19, found 2.24 g/cm³. Absent reflections, $h0l$ when l is odd. The $0k0$ reflections for odd values of k are very weak and present only in a limited number. The habit of the crystals is monoclinic prismatic. They occur as prisms, elongated along the c axis and flattened along the b axis, and showing the forms $\{100\}$, $\{010\}$ and $\{012\}$. The space group is probably C_{2h}^4—$P2/c$, with an atomic arrangement close to that required by C_{2h}^5—$P2_1/c$.

Ammonium trithionate, $(NH_4)_2S(SO_3)_2$, was obtained as poorly developed prisms which showed a pronounced tendency of twinning. The crystals are probably triclinic, with four molecules per unit cell, and a repeat distance of 7.63 Å along the prism axis.

Potassium selenotrithionate, $K_2Se(SO_3)_2$. Monoclinic prismatic, $a = 9.54$ Å, $b = 5.97$ Å, $c = 15.34$ Å, $\beta = 110.4°$. Four molecules per unit cell; density, calc. 2.56, found 2.57 g/cm³. Absent reflections, $h0l$ when l is odd, $0k0$ when when k is odd. The space group is thus C_{2h}^5—$P2_1/c$. The crystals occur as prisms, elongated along the b axis and flattened along the c axis. The forms $\{100\}$, $\{001\}$ and $\{012\}$ were observed, the latter being less predominant. The crystals are described by Groth [6], on the basis of measurements by Rathke [5], as monoclinic prismatic, with $a' : b' : c' = 1.5050 : 1 : 0.6447$ and $\beta = 93° 26'$. With the transformations, $\mathbf{a} = \mathbf{a}' + \mathbf{c}'$ and $\mathbf{c} = -4\mathbf{c}'$, Groth's data give $a : b : c = 1.602 : 1 : 2.579$ and $\beta = 110.3°$, as compared with $a : b : c = 1.598 : 1 : 2.569$ from the X-ray data.

Potassium trithionate [4], $K_2S(SO_3)_2$, is orthorhombic bipyramidal, with $a = 9.77$ Å, $b = 13.63$ Å, $c = 5.76$ Å. There are four molecules per unit cell, and the space group is D_{2h}^{16}—$Pnam$.

Rubidium selenotrithionate, $Rb_2Se(SO_3)_2$. Orthorhombic, $a = 9.16$ Å, $b = 13.97$ Å, $c = 13.42$ Å. The morphology is like that of cesium selenotrithionate, described below, and this, together with the correspondence of axial lengths, makes it probable that the rubidium and cesium salts are isomorphous.

Rubidium trithionate [3] is isomorphous with potassium trithionate, referred to above.

Cesium selenotrithionate, $Cs_2Se(SO_3)_2$. Orthorhombic bipyramidal, $a = 9.21$ Å, $b = 14.45$ Å, $c = 14.11$ Å. Eight molecules per unit cell; density, calc. 3.55, found 3.53 g/cm³. The systematic absences, $0kl$ when k is odd, $h0l$ when l is odd, $hk0$ when h is odd, determine the space group as D_{2h}^{15}—$Pbca$. The crystals appear as flat prisms, elongated along the b axis and with $\{001\}$ predominant. The forms $\{102\}$ and $\{120\}$ also occur.

Cesium trithionate, according to Mackenzie and Marshall [3], may be obtained as a monohydrate and in the anhydrous state, the crystals in both cases being triclinic.

Barium selenotrithionate dihydrate, $BaSe(SO_3)_2 \cdot 2H_2O$. Orthorhombic, $a = 9.00$ Å, $b = 17.84$ Å, $c = 10.74$ Å. Eight molecules per unit cell; density, calc. 3.05, found 2.96 g/cm³. Absent reflections, hkl when $h + k$ is odd, $h0l$

when l is odd. These absences are characteristic for the centrosymmetric space group, D_{2h}^{17}—*Cmcm*, and for the space groups of lower symmetries, C_{2v}^{12}—$Cmc2_1$ and C_{2v}^{16}—*C*2*cm*. The crystals have an orthorhombic bipyramidal appearance. They occur as well developed prisms, bounded by {110} and usually also {100}, and terminated by {011}.

The Wilson ratio [17], calculated for 0*kl* and *hk*0 reflections corresponding to interplanar spacings smaller than [18] 2.0 Å, are in accordance with centrosymmetric distributions of intensities.

Patterson projections along the *a*, *b* and *c* axes have been made, and the results indicate the correctness of the centrosymmetric space group. The barium ion appears to lie in eightfold positions (*f*) of D_{2h}^{17}—*Cmcm*, as described in the *Int. Tables* [19], with $x = 0$, $y = 0.16$ and $z = 0.04$. On the basis of preliminary Fourier projections along the *a* and *c* axes, using signs of the reflections calculated from the barium contributions alone, the selenium and sulphur atoms have been tentatively assigned to two sets of fourfold positions (*c*) and two sets of eightfold positions (*g*), respectively. These positions are in the intersection-line of the two mirror planes and in the mirror plane normal to the *c* axis. The selenotrithionate ion would thus have the symmetry C_{2v}—*mm* in this salt.

Barium trithionate dihydrate, $BaS(SO_3)_2 \cdot 2H_2O$, gave poorly developed crystals which were invariably twinned. As far as could be ascertained, they are not isomorphous with those of barium selenotrithionate dihydrate.

INSTABILITY OF ANALOGOUS TELLURIUM COMPOUNDS

Tellurotrithionates, $Te(SO_3)_2^{--}$, are apparently not capable of existence. In reactions where such salts might be expected to be formed, from analogy with the selenium derivatives, elemental tellurium results. This is the case, *e. g.*, in the reaction of tellurous acid with sulphurous acid, and by the interaction of telluropentathionate with an excess of sulphite ions.

Likewise, attempts to prepare tellurium disulphinates, $Te(SO_2R)_2$, have met with no success. Thus, tellurium tetrachloride in benzene or chloroform does apparently not react with sodium benzenesulphinate or *p*-toluenesulphinate, but gradually, tellurium is set free. Furthermore, tellurium dimethanethiosulphonate and the aromatic analogues, in ethylacetate or chloroform solutions, immediately liberate tellurium on addition of methanol solutions of sodium benzenesulphinate or *p*-toluenesulphinate. This catalytic effect of sulphinate ions on tellurium thiosulphonates is, presumably, due to a displacement of thiosulphonate by sulphinate groups, to produce equilibrium amounts of rapidly decomposing tellurium disulphinates.

It thus appears that the grouping, divalent tellurium—sulphonate or sulphonyl sulphur, is unstable. The fact [8] that the selenotrithionate/selenopentathionate equilibrium (2) is displaced farther to the right than is the corresponding trithionate/pentathionate equilibrium:

$$S(SO_3)_2^{--} + 2\ S_2O_3^{--} \rightleftharpoons S(S_2O_3)_2^{--} + 2\ SO_3^{--}$$

together with the apparent non-existence of tellurotrithionates and tellurium disulphinates, suggests that the thermodynamic stabilities of the compounds, $X(SO_3)_2^{--}$ and $X(SO_2R)_2$, decrease progressively in the order X = S, Se and Te. The thio derivatives, $X(S_2O_3)_2^{--}$ and $X(S_2O_2R)_2$, show no corresponding variance of stability.

SUMMARY

Unit cell and space group data are reported for ammonium, potassium, rubidium and cesium selenotrithionate, and barium selenotrithionate dihydrate. No isomorphism appears to exist between the crystals of these salts and those of the corresponding trithionates.

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An Ion Exchange Study of the Formation of Anionic Complexes in the Cupric and Cadmium Sulphate Systems

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The complex formation within the cupric and cadmium sulphate systems is investigated by determinations of the distribution of copper and cadmium between an anion exchanger, saturated with sulphate ions, and the complex solutions. It is found that at small total concentrations of the central ion a pronounced maximum in the distribution is obtained for a sulphate ion concentration of 50—70 mC.

It is proved that the only possible explanation of the maximum is a formation of anionic complexes at high ligand concentrations. The values of the distribution indicate on the other hand that the complexes are very weak. The results are combined with those from previous measurements, and it is shown that in all probability the systems are fairly "normal" and built up stepwise.

Cupric and cadmium sulphate solutions have been the subject of a great number of investigations with the aim of deciding which complexes are formed. Among investigations of the cupric sulphate system which are not based on the use of a neutral salt medium a spectrophotometric study by Näsänen [1] may be mentioned. In this it was assumed that only the first mononuclear complex can be formed, and though the validity of this assumption was not proved, the results show clearly that the complexity constant is greatly dependent upon the ionic strength. Thus, for a closer quantitative study of the composition of this system it seems necessary to use a neutral salt medium for maintaining the activity coefficients as constant as possible.

Two potentiometric investigations of this kind have been performed previously by the present author. In the first [2] of these a copper amalgam electrode was used, while in the second one [3] the method of ligand displacement was applied. The results indicate that the strength of the first two mononuclear complexes is sligth, and consistent values of the first complexity constant were obtained. On the other hand, rather high and divergent values were calculated for the constant of the third complex. Thus it is probable that variations in the activity coefficients, influencing the two methods of measurement in

different ways, have not been sufficiently eliminated, and none of the high values of the third "constant" is reliable (*cf.* Fronæus [2], p. 63).

Recently Leden [4] has carried out two investigations of the cadmium sulphate system, using a neutral salt medium. Measurements with an amalgam electrode gave results very similar to those mentioned above for the cupric sulphate system.

On the other hand, from measurements by means of a silver electrode and the silver sulphate system as an indicator it was concluded that only one complex (or ion pair) can be formed in the cadmium sulphate system. However, necessary corrections for the decrease in ionic strength by the complex formation were of the same magnitude as the emf differences measured. Furthermore, as the cadmium concentrations were not very low, the possibility of an influence upon the complexity constants of the silver sulphate system cannot be precluded.

Thus it seems to the present author that the different measurements reported do not give a satisfactory answer to the question of whether anionic complexes can be formed in the cupric and cadmium sulphate systems, and for that reason this question is taken up in the present paper. The investigation is performed according to a method developed in a precious work [5]. It is based upon the use of an anion exchanger saturated with the ligand (here SO_4^{2-}), and though no quantitative calculations of complexity constants can be carried out, the method generally yields valuable information about the occurrence of anionic complexes.

THEORY OF THE METHOD

The equations that are used in the following but not deduced are to be found in the previous paper [5]. The central ion (Cu^{2+} or Cd^{2+}) is denoted M and the sulpate ion A. An index R indicates the resin phase.

For the ratio φ between the total concentrations of the central ion in the exchanger phase, C_{MR}, and in the solution, C_M, we have at equilibrium the relation

$$\varphi = l_1 \frac{\alpha_1}{\alpha_{1R}} \tag{1}$$

if we assume that the first, uncharged complex exists. l_1 is the Nernst distribution coefficient of this complex, and α_1 and α_{1R} denote the fractions $[MA]/C_M$ and $[MA]_R/C_{MR}$ respectively. In order to keep the ionic composition of the resin phase approximately constant it is necessary not to use a neutral salt medium, but then at increasing concentrations of sodium sulphate the activity coefficients of the different ionic species in the solution certainly vary considerably. Nevertheless, since l_1 refers to an uncharged complex this quantity can be assumed to be approximately constant.

Furthermore, if A is the only ligand in the resin phase, or if it forms strong complexes with M, then for a given system α_{1R} is also a constant, since $[A]_R$ is constant, and the variation in φ at increasing [A]-values gives a measure of the corresponding variation in α_1. In the present case we know in advance that A forms only weak complexes. Then we should take into consideration that the anion exchanger presumably contains fixed amine groups in small

concentrations in addition to the quaternary ammonium groups, and that these amine groups, which we will denote B, can form rather strong complexes with cupric and cadmium ions and thus are not displaced as ligands by A.

Then, if β denotes the complexity constant of MB and β_j that of MA_j, we have the following relation:

$$\alpha_{1R} = \frac{\beta_1 [A]_R}{1 + \beta [B]_R + \sum_{j=1}^{N} \beta_j [A]_R^j} \qquad (2)$$

Now, since the total concentration of B is constant, $[B]_R$ increases and α_{1R} decreases at decreasing values of C_{MR}. Thus, at small central ion concentrations the variation of φ is *increased* by the presence of the amine groups, and this is very valuable in those cases where the absolute values of φ are small owing to slight formation of anionic complexes with A.

If only the first complex can be formed, then α_1 is a monotonously increasing function of [A], even if varying activity coefficients cause a decrease in β_1. This is evident from the investigation by Näsänen[1].

On the other hand, if anionic complexes can also be formed in the system, then a maximum in α_1 may appear at a certain [A]-value within the concentration range investigated. For approximately constant β_j-values the maximum corresponds to the value 1 of the ligand number (*cf.* Fronæus [5]). At higher ligand concentrations, when the formation of anionic complexes is appreciable, α_1 decreases. It should be emphasized that the appearance of such a maximum cannot be explained in any other way. In those cases when C_{MR} is considerable, the sorption in the interior of the resin phase predominates, and then eq. (1) certainly is applicable without any correction. Consequently a maximum in φ indicates that anionic complexes can be formed.

However, since in the present case the complexes are weak, an adsorption on the surface of the resin, caused, for instance, by the coordination of M and its sulphate complexes to fixed amine groups, should be taken into consideration. If the surface adsorption decreases at increasing [A], it might be supposed that a combination of the two effects — the sorption in the interior and the surface adsorption — could produce a maximum in φ, even if only MA can be formed. Thus we will deduce a more correct expression for φ under the last-mentioned condition.

Then we may assume that the total concentration C_{MS} of the central ion adsorbed on the resin surface is a function of [M] and [MA] only. Denoting the values of $\partial C_{MS}/\partial[M]$ and $\partial C_{MS}/\partial[MA]$ at $C_M = 0$ (*i.e.*, $[M] = [MA] = 0$) by a_1 and a_2 respectively, we get:

$$\left(\frac{dC_{MS}}{d[M]}\right)_{C_M=0} = a_1 + a_2 \left(\frac{d[MA]}{d[M]}\right)_{C_M=0} \qquad (3)$$

Furthermore we have the relations:

$$\left(\frac{dC_M}{d[M]}\right)_{C_M=0} = 1 + \beta_1 [A] \; ; \; \left(\frac{d[MA]}{d[M]}\right)_{C_M=0} = \beta_1 [A] \qquad (4,5)$$

$$\lim_{C_M \to 0} \frac{C_{MS}}{C_M} = \left(\frac{dC_{MS}}{dC_M}\right)_{C_M=0} \qquad (6)$$

By combination of the relations (3)—(6) the following expression is obtained:

$$\lim_{C_M \to 0} \frac{C_{MS}}{C_M} = \frac{a_1 + a_2 \beta_1 [A]}{1 + \beta_1 [A]} \qquad (7)$$

Since the ratio of surface area and mass of the resin was constant at the measurements, C_{MS} and C_{MR} can be expressed in the same units. Then in the present case $(C_{MR}—C_{MS})/C_M$ is equal to the right member of eq. (1), and we have the final relation:

$$\lim_{C_M \to 0} \varphi = \frac{a_1 + (a_2 + a_{1R}^{-1} l_1) \beta_1 [A]}{1 + \beta_1 [A]} \qquad (8)$$

As a_1, a_2, a_{1R}, and l_1 are independent of [A], it is at once evident from (8) that if only the first complex can be formed, then lim φ for $C_M \to 0$ is always — like a_1 — a monotonous function of [A]. Thus if φ is determined experimentally, and a distinct, relative maximum is obtained, which persists or becomes more pronounced at decreasing values of the parameter C_M, the only possible explanation is that at least one anionic complex is also formed.

EXPERIMENTAL

Chemicals used. Stock solutions of radioactive cadmium perchlorate, containing some Cd^{115} (from A.E.R.E., Harwell, England), and cupric perchlorate were prepared according to the methods described in previous papers [5,2].

Sodium sulphate of analytical grade was used for the preparation of a 1 C solution, the concentration of which was determined by percolating samples through a H^+-saturated cation exchanger column and titrating with sodium hydroxide.

The anion exchanger, containing fixed quaternary ammonium groups, was Amberlite IRA-400. It was converted into the sulphate form and air-dried.

Measurements. It was possible to perform the experimental determination of the function φ with higher accuracy in the measurements on the cadmium sulphate system than in the measurements on the cupric sulphate system, since a radioactive cadmium tracer was used. Consequently the former system was investigated more closely. The cadmium concentrations of the different solutions were obtained by measuring the beta activity on a fixed volume with a G—M tube for liquids, solutions of known cadmium concentrations being used to provide calibration curves.

A complex solution of the volume v and of the initial composition: C'_M mC $Cd(ClO_4)_2$ + C'_A mC Na_2SO_4 was shaken in a thermostat at 20.0° C with the air-dried exchanger of the mass m. The value of $v \cdot m^{-1}$ was always constant (15.0 ml · g^{-1}), and C'_M had a constant value in one and the same measurement series.

At equilibrium between the two phases the anion exchanger was carefully separated from the solution, and the cadmium concentration, C_M, of the latter was determined. Then it was found that the values of $C'_M - C_M$ were rather small in comparison to C_M

Table 1. Ion exchange measurements on the cadmium sulphate system.
$M = Cd^{2+}$; $A = SO_4^{2-}$

C'_M mC	C_A mC	C_M mC	$C_{MR} \cdot 10^3$ mmole · g^{-1}	$\varphi \cdot 10^3$ l · g^{-1}
	1	1.02	1.20	1.18
	14	0.98	1.79	1.83
	28	0.98	1.76	1.80
1.10	54	0.97	1.88	1.94
	129	0.99	1.58	1.60
	400	1.02	1.25	1.23
	800	1.03	1.01	0.98
	10	9.7	10.8	1.11
	31	9.6	11.4	1.19
	52	9.6	12.2	1.27
10.4	100	9.6	12.0	1.25
	199	9.7	9.8	1.01
	400	9.8	8.9	0.91
	800	9.9	7.1	0.72
	21	19.7	14.9	0.76
	42	19.6	15.8	0.81
	63	19.5	17.3	0.89
20.7	100	19.6	17.1	0.87
	201	19.6	15.8	0.81
	400	19.7	14.3	0.73
	800	20.0	11.1	0.56

(see Table 1), and thus C_{MR} could not be obtained with great accuracy from this difference. For that reason the exchanger was eluted twice, each time with a 2 C sodium perchlorate solution of the volume v. After this elution the resin showed practically no radioactivity. Then the cadmium concentrations of the eluates were determined, and denoting their sum by C''_M, we can calculate C_{MR} from the relation: $C_{MR} = v \cdot m^{-1} \cdot C''_M$.

Since the resin could not be washed before the elution (*cf.* Fronæus [6]), the C_{MR}-value obtained in this way includes a quantity caused by the outer solution adherent to the resin particles after the separation. But the ratio of surface area and mass of the resin being the same in all the measurements, the quantity mentioned is of the form $k \cdot C_M$, where k is a constant. Thus the φ-values calculated differ by a constant term k from the true φ-values, but this circumstance is of no importance for the study of the variation in φ.

By anion exchange between the two phases the perchlorate ions, initially present in small concentrations in the outer solutions, were nearly completely exchanged for an equivalent amount of sulphate ions, but the relative variation in the high value of $[A]_R$ by this exchange is quite negligible. The sulphate concentrations C_A at equilibrium were then calculated from the approximate relation: $C_A = C'_A + C_M$.

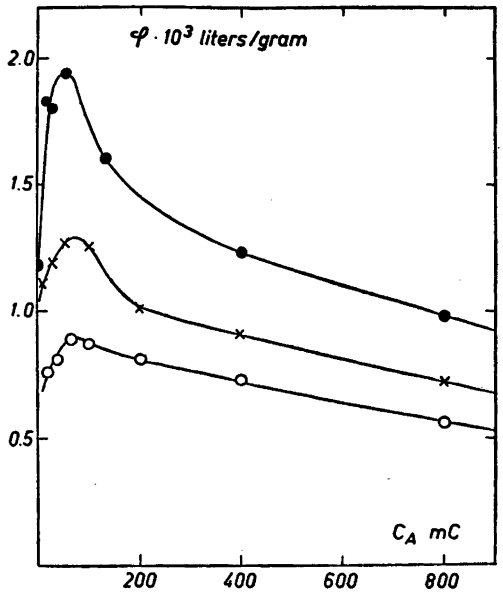

Fig. 1. The distribution φ as a function of C_A at different initial cadmium concentrations. 1. C'_M = 1.10 mC (●); 2. C'_M = 10.4 mC (×); 3. C'_M = 20.7 mC (○).

The data from the measurements on the cadmium sulphate system are collected in Table 1, and in Fig. 1 φ is plotted against C_A with C'_M as a parameter.

It is seen that the φ- values increase considerably at decreasing values of the parameter and thus of C_{MR}. According to the theory given this fact can be explained by the presence of amine groups, to which the cadmium in the resin is preferably bound. Then for $C_{BR} > C_{MR}$ we have $[B]_R = C_{BR} - C_{MR}$, and if eq.(1) is applicable, it is easily shown from eq.(1) and (2) that for a constant [A]-value φ should be a linearly decreasing function of C_{MR}. This is also found at higher C_A-values, *e.g.* C_A = 400 mC, where we can put $C_A \approx [A]$.

For very small cadmium concentrations and at [A] = 50—70 mC a pronounced maximum in the φ-curve is obtained, indicating that anionic complexes are also formed in appreciable amounts at higher sulphate concentrations.

In the investigation of the cupric sulphate system the method of measurement was the same, but the total cupric concentrations of the solutions were determinated polarographically, after ammonia in excess and some gelatin had been added. The small cupric concentrations of the eluates could not be determined with very high accuracy, and for that reason only one measurement series with a low value of the parameter C'_M was carried out. The data obtained from the measurements on this system are to be found in Table 2, and the function φ is graphically represented in Fig. 2.

Table 2. Ion exchange measurements on the cupric sulphate system.
$M = Cu^{2+}$; $A = SO_4^{2-}$

C'_M mC	C_A mC	C_M mC	$C_{MR} \cdot 10^3$ mmole · g^{-1}	$\varphi \cdot 10^3$ l · g^{-1}
1.67	2	1.61	0.93	0.58
	15	1.59	1.26	0.79
	28	1.58	1.33	0.84
	45	1.59	1.20	0.75
	60	1.59	1.15	0.72
	155	1.61	0.89	0.55
	300	1.62	0.78	0.48
	500	1.63	0.64	0.39

The φ-values are lower than the corresponding ones in the first measurement series on the cadmium sulphate system, though the difference in the C'_M-values is rather small. The explanation could be a small amount of free perchloric acid in the cupric perchlorate preparation, lowering the values of C_{BR}, a_1, and a_2. In any case the form of the φ-curve is very similar to that of the cadmium sulphate system, and no doubt the two systems are built up very analogously.

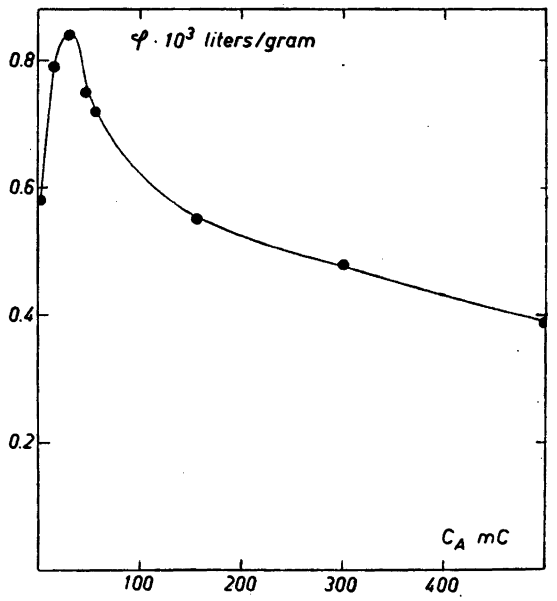

Fig. 2. The distribution φ as a function of C_A at the initial cupric concentration $C'_M = 1.67$ mC.

DISCUSSION

There are two effects that we have not hitherto taken into consideration. At increasing sulphate concentration in the outher solution the amount of diffusible non-exchange electrolyte in the resin also increases, and furthermore a de-swelling of the resin takes place.

In the exchanger with a very high value of $[A]_R$ and in the presence of the fixed amine groups the concentration $[M]_R$ is certainly quite negligible, and then, if only the first complex can be formed, we have $\alpha_{1R} \approx \beta_1[A]_R/(\beta[B]_R + \beta_1[A]_R)$. Assuming $[A]_R \geqq 3$ C for $[A] = 0$, we can calculate approximately by means of the Donnan equation that the increase in $[A]_R$ due to the former effect is less than 7 % at $[A] \leqq 0.5$ C and thus it cannot cause the pronounced maximum in the limit curve for φ.

On the other hand, since the ratio $[A]_R/[B]_R$ is independent of the de-swelling, the latter effect cannot noticeably influence the φ-values.

Then the only possible explanation of the appearance of the pronounced maximum is a formation of anionic complexes within both systems. In the previous paper[5] it was shown, however, that the maximum value of φ is a qualitative measure of the strength of the anionic complexes, and thus from a comparison with the φ-curve for the cadmium bromide system[5] we conclude that the anionic complexes in the sulphate systems are very weak.

If these results are compared with the quantitative data from the measurements[2,4] with amalgam electrodes, we find that the values $\beta_1 = 8$—10 C^{-1} and $\beta_2 = 10$—17 C^{-2}, determined at the ionic strength 1 C for the cupric and 3 C for the cadmium sulphate system with sodium perchlorate as a neutral salt medium, seem very probable. On the other hand, the high values obtained of β_3 (100 or 200 C^{-3}) are not supported by the present investigation. Consequently we must conclude that these sulphate systems are step systems with fairly "normal" values of the ratios between the stability constants $b_j = \beta_j/\beta_{j-1}$ ($\beta_0 = 1$) in a neutral salt medium. Here it should be mentioned that φ_{max} is attained at lower [A]-values than those calculated from the values of β_1 and β_2 above. This can be explained by the fact that in the absence of sodium perchlorate the value[1] of β_1 increases very much at decreasing sulphate concentration.

In the amalgam measurements the quantity determined is exactly $(1 + \sum_{j=1}^{N} \beta_j [A]^j) f^0_M/f_M$, where f^0_M denotes the value of the activity coefficient f_M for $[A] = 0$. From the investigation by Näsänen[1] we get for cupric sulphate $\beta_1 \approx 4$ C^{-1} at $[A] = 1/3$ C. Thus, if the ionic strength is kept constant at 1 C by means of sodium perchlorate, which is gradually exchanged for sodium sulphate, then β_1 does not increase. Now, in β_1 and β_2 are included the quantities $f_M f_A/f_{MA}$ and $f_M f_A^2/f_{MA_2}$ respectively. Since MA is uncharged and MA_2 and A have an equal net charge, f_{MA} and f_A/f_{MA_2} certainly can be treated as constants, and thus β_2 should vary in the same direction as β_1. Then, since the amalgam measurements formally give a high value of β_3, though in view of the present results the term $\beta_3[A]^3$ surely is negligible, it is evident that at greater exchanges of sodium perchlorate the factor f^0_M/f_M increases rapidly, making the calculated β_3-value illusory (*cf.* Leden[4]).

Finally it should be pointed out that the great changes in the extinction curve (Ref.[2], p. 104) at wave lengths < 325 mμ, that take place when sodium sulphate is added to a cupric perchlorate solution, make it probable that MA and MA_2 are "true" complexes, in which the sulphate ions are coordinated directly to the central ion as in the crystal structure of $CuSO_4 \cdot 5\,H_2O$, investigated by Beevers and Lipson [7].

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The Synthesis of Some Aliphatic Organosilicon Dicarboxylic Acids

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In order to prepare some organosilicon compounds containing several functional groups attached to carbon, the trimethylsilylmethylsuccinic, α-[trimethylsilylmethyl]-glutaric, and α-[trimethylsilylmethyl]-adipic acids have been synthesized in good yields *via* diethyl trimethylsilylmethylmalonate.

Relatively few aliphatic organosilicon compounds containing two or more functional groups (—OH, —NH_2, —COOH *etc.*) not directly attached to a silicon atom have been reported in the literature. This type of compound would be of considerable interest as starting materials in the formation of organosilicon polymers, in which the bonds between the single units consist not only of silicon and oxygen atoms but also of other atoms or groups.

Sommer and co-workers [1,2] have prepared several bifunctional organosiloxanes by utilization of the reaction between the $(CH_3)_3Si$-group and concentrated sulphuric acid. Noll, Speier and Daubert [3] have similarly synthesized organosiloxanes containing two amino groups attached to carbon.

In order to investigate some triorganosilyl-substituted aliphatic dibasic acids, the reaction between diethyl trimethylsilylmethylmalonate (I) in the form of its sodium compound and the ω-bromoesters of some fatty acids has been studied. In connection with this work the addition of I to acrylonitrile has been performed. The preparation of I from chloromethyltrimethylsilane and diethylmalonate was first reported by Sommer, Gold, Goldberg and Marans [4]. However, they never isolated the pure compound but hydrolysed and decarboxylated the crude product directly to form β-trimethylsilylpropionic acid. No attempts to introduce a second group into I have been described.

In this laboratory I was prepared from bromomethyltrimethylsilane, which was available in large quantities, and diethyl malonate in 80—85 % yield. The ω-bromoesters used were ethyl bromoacetate, methyl β-bromopropionate, and ethyl γ-bromobutyrate. They readily reacted with I to form the

corresponding triethyl trimethylsilylmethyl-ω-carboxyalkylmalonates * (II) in yields of 89, 89 and 80 %, respectively. Subsequent hydrolysis and decarboxylation gave good yields of the α-[trimethylsilylmethyl]-succinic, -glutaric, and -adipic ** acids (III)

$$(CH_3)_3Si \cdot CH_2 \cdot CH(COOEt)_2$$
I

$$(CH_3)_3Si \cdot CH_2 \cdot C(COOEt)_2 \cdot (CH_2)_n \cdot COOEt \quad (n = 1, 2, 3)$$
II

$$(CH_3)_3Si \cdot CH_2 \cdot CH(COOH) \cdot (CH_2)_n \cdot COOH \quad (n = 1, 2, 3)$$
III

An attempt to prepare the triester II, $n = 1$, from triethyl carboxymethylmalonate and bromomethyltrimethylsilane was not very successful, the yield being only about 30 %.

Addition of I to acrylonitrile gave a diethyl trimethylsilylmethylcyanoethylmalonate (IV or V) in good yield.

$$(CH_3)_3Si \cdot CH_2 \cdot C(COOEt)_2 \cdot CH_2 \cdot CH_2 \cdot CN$$
IV

$$(CH_3)_3Si \cdot CH_2 \cdot C(COOEt)_2 \cdot CH(CN)CH_3$$
V

After hydrolysis and decarboxylation there was obtained α-[trimethylsilylmethyl]-glutaric acid, identical with that synthesized from I and methyl β-bromopropionate, thus indicating that the formula IV must be assigned to the addition product.

The dibasic acids thus prepared gave, similarly to β-trimethylsilylpropionic acid [1], by treatment with concentrated sulphuric acid methane amounting to 95—100 % of the theoretically calculated volume (one mole of methane/one mole of acid). The nature of the products obtained after treatment of the reaction mixture with water is being investigated.

EXPERIMENTAL

All reagents used were carefully dried and fractionated in a glass-helix packed column of about ten theoretical plates. In the malonic ester syntheses precautions were taken to exclude moisture from the reaction vessels.

Diethyl trimethylsilylmethylmalonate (I). Several reactions of bromomethyltrimethylsilane with sodiomalonic ester gave yields of I ranging from 80 to 85 %. The following synthesis is typical.

In a 1-liter, three-necked flask equipped with a stirrer, a dropping funnel, a reflux condenser, and a thermometer reaching into the flask, sodium ethylate was prepared by the addition of 23.0 g (1.00 g.at.) of sodium to 300 ml of absolute ethanol. To this was added, during a few minutes, 160 g(1.00 mole) of diethyl malonate, followed by the dropwise addition of 167 g (1.00 mole) of bromomethyltrimethylsilane, while the temperature of the reaction mixture was maintained at 70—80°. The reaction mixture was stirred and heated under reflux for twenty-four hours, after which time its reaction was neutral

* In the case of methyl β-bromopropionate *trans*-esterification took place.

** The preparation of β-trimethylsilyladipic acid by oxidation of 4-trimethylsilylhexanol has been reported in the patent literature [5].

to litmus. After cooling, the reaction product was poured into 600 ml of water, the organic layer was separated and the aqueous solution extracted twice with ether. The combined extracts were washed several times with water and then dried over anhydrous calcium chloride. Fractionation gave 202 g (82 % yield) of compound I, b.p. 113—115°/10 mm, n_D^{20} 1.4313, d_4^{20} 0.9688. (Found: C 53.4; H 9.1; Si 11.4; r_D 0.2673. Calc. for $C_{11}H_{22}O_4Si$: C 52.6; H 9.0; Si 11.4; r_D 0.2672.)

Triethyl trimethylsilylmethyl-carboxymethylmalonate. A. From diethyl trimethylsilylmethylmalonate and ethyl bromoacetate. In a 500 ml, three-necked flask equipped with a stirrer, a dropping funnel, a reflux condenser, and a thermometer reaching into the flask, 4.6 g (0.200 g.at.) of sodium was dissolved in 60 ml of absolute ethanol. 49.2 g (0.200 mole) of I was added in one portion, followed by the addition of 33.4 g (0.200 mole) of ethyl bromoacetate during two hours. The temperature of the reaction mixture was maintained at 70—80°. The solution was then stirred and heated under reflux for one hour, after which time it reacted neutrally to litmus. Working up the reaction mixture in the usual manner gave 50.6 g (0.152 mole) of triethyl trimethylsilylmethyl-carboxymethylmalonate, b.p. 163—164°/11 mm, n_D^{20} 1.4443, d_4^{20} 1.0280. A small amount, 7.0 g, of compound I was recovered. The yield, based on unrecovered material, was 89 %. (Found: C 53.7; H 8.5; Si 8.4; r_D 0.2585. Calc. for $C_{15}H_{28}O_6Si$: C 54.2; H 8.5; Si 8.4; r_D 0.2588.)

B. *From triethyl carboxymethylmalonate and bromomethyltrimethylsilane.* Triethyl carboxymethylmalonate, 24.6 g (0.100 mole) was added to a well-stirred solution of 2.30 g (0.100 g.at.) of sodium in 30 ml of absolute ethanol. After the addition of 16.7 g (0.100 mole) of bromomethyltrimethylsilane, the reaction mixture was stirred and heated under reflux for forty-eight hours. Working up in the usual manner gave 10.3 g of triethyl trimethylsilylmethyl-carboxymethylmalonate. Yield 31 %, n_D^{20} 1.4443, d_4^{20} 1.0281.

Trimethylsilylmethyl-carboxymethylmalonic acid. 67.5 g (1.20 moles) of potassium hydroxide was dissolved in a small quantity of water and absolute ethanol was added until a homogeneous solution was obtained. To this was added 50.0 g (0.150 mole) of triethyl trimethylsilylmethyl-carboxymethylmalonate and the resulting mixture was refluxed for twenty-four hours. After cooling, the solution was neutralized with concentrated hydrochloric acid and evaporated to near dryness on a waterbath. Acidification with 20 % hydrochloric acid precipitated the tribasic acid which was filtered, washed with a small volume of water and dried over concentrated sulphuric acid in a vacuum desiccator. The yield of crude product was 33.5 g (90 %). A small quantity was recrystallised from hot water, m.p. 160° (decomp.). (Found: C 43.6; H 6.6; Si 11.4. Calc. for $C_9H_{14}O_6Si$: C 43.5; H 6.5; Si 11.3.)

Trimethylsilylmethylsuccinic acid. The crude trimethylsilylmethylcarboxymethylmalonic acid was decarboxylated by heating to 190—200° for ten minutes. After cooling, the oil solidified and the crystals were recrystallised from a mixture of benzene and petroleum ether. A 75 % yield of pure trimethylsilylmethylsuccinic acid, m.p. 106—107°. was obtained. (Found: C 43.7; H 6.6; Si 13.8; equiv. weight 102.7. Calc. for $C_8H_{16}O_4Si$: C 43.5; H 6.5; Si 13.7; equiv. weight 102.2.)

Triethyl trimethylsilylmethyl-β-carboxyethylmalonate. This compound was prepared from 4.60 g (0.200 g.at.) of sodium in 60 ml of absolute ethanol, 49.2 g (0.200 mole) of I and 33.4 g (0.200 mole) of methyl β-bromopropionate in the same way as the carboxymethyl derivative. There was obtained 59.0 g of triethyl trimethylsilylmethyl-β-carboxyethylmalonate, b.p. 175—177°/11 mm, n_D^{20} 1.4481, d_D^{20} 1.0289. 2.5 g of I was recovered. Yield 89 % based on unrecovered material. (Found: C 55.2; H 8.8; Si 8.1; r_D 0.2602. Calc. for $C_{16}H_{30}O_6Si$: C 55.5; H 8.7; Si 8.1; r_D 0.2617).

α-[*Trimethylsilylmethyl*]-*glutaric acid.* Triethyl trimethylsilylmethyl-β-carboxyethylmalonate was hydrolysed with alcoholic potassium hydroxide as before to yield 90 % of the corresponding tribasic acid. A small quantity was recrystallised from hot water, m.p.165° (decomp.). (Found: C 45.6; H 6.9; Si 10.9. Calc. for $C_{10}H_{18}O_6Si$: C 45.8; H 6.9; Si 10.7).

After decarboxylation, which was effected by heating at 190-200° for ten minutes, an oil was obtained which solidified after standing overnight in a vacuum desiccator over concentrated sulphuric acid. Recrystallisation from a mixture of ethyl acetate and petroleum ether gave α-[trimethylsilylmethyl]-glutaric acid in a pure state, m.p. 61—62°.

(Found: C 45.6; H 6.9; Si 12.8; equiv. weight 109.4. Calc. for $C_9H_{18}O_4Si$: C 45.8; H 6.9; Si 12.9; equiv. weight 109.2).

Diethyl trimethylsilylmethyl-β-cyanoethylmalonate. In a 100 ml three-necked flask equipped with a stirrer, a dropping funnel, a reflux condenser and a thermometer reaching into the flask, 0.1 g of sodium was dissolved in 20 ml of absolute ethanol. 24.6 g (0.100 mole) of I was added. Then 8.0 g (0.150 mole) of newly distilled acrylonitrile was added dropwise at such a rate that the temperature of the solution did not exceed 50°. The reaction mixture was stirred for one hour at room temperature. It was neutralised with glacial acetic acid and the solvent distilled off *in vacuo* at 30°. The remaining oil was taken up in ether, the ether solution was washed twice with water and dried over anhydrous magnesium sulphate. Removal of the ether and fractionation gave 24.3 g (81 % yield) of diethyl trimethylsilylmethyl-β-cyanoethylmalonate, b.p. 189—190°/19 mm, n_D^{20} 1.4525, d_4^{20} 1.0173. (Found: N 4.7; Si 9.4; r_D 0.2654. Calc. for $C_{14}H_{25}O_4NSi$: N 4.7; Si 9.4; r_D 0.2658.)

After hydrolysis with boiling alcoholic potassium hydroxide for 48 hours and decarboxylation of the tribasic acid as usual a 78 % yield of crude dibasic acid was obtained. Recrystallisation from a mixture of ethyl acetate and petroleum ether yielded the pure compound, m.p. 61—62°, mixed m.p. with the α-[trimethylsilylmethyl]-glutaric acid above, 61—62°.

Triethyl trimethylsilylmethyl-γ-carboxypropylmalonate. This synthesis was performed as in the case of the carboxymethyl derivative, with 4.60 g (0.200 g.at.) of sodium, 49.2 g (0.200 mole) of I and 39.0 g (0.200 mole) of ethyl γ-bromobutyrate except that the reaction mixture was stirred and refluxed for forty-eight hours before working up. There was obtained 36.0 g (0.100 mole) of the triester b.p. 184—185°/11 mm, n_D^{20} 1.4488, d_4^{20} 1.0165. 18.6 g of I was recovered. The yield was 80 % based on unrecovered material. (Found: C 56.6; H 8.9; Si 7.8; r_D 0.2638. Calc. for $C_{17}H_{32}O_6Si$: C 56.6; H 8.9; Si 7.8; r_D 0.2644.)

α-[Trimethylsilylmethyl]-adipic acid. Hydrolysis of triethyl trimethylsilylmethyl-γ-carboxypropylmalonate with alcoholic potassium hydroxide as before yielded 100 % of the corresponding tribasic acid.

A small quantity was recrystallised from hot water. It melted with decomposition at 177°. (Found: C 47.4; H 7.2; Si 10.3. Calc. for $C_{11}H_{20}O_6Si$: C 47.8; H 7.3; Si 10.2.)

Decarboxylation at 180° for ten minutes gave the crude dibasic acid in 85 % yield. Recrystallisation from a mixture of benzene and petroleum ether gave pure α-[trimethylsilylmethyl]-adipic acid, m.p. 54—55°. (Found: C 51.4; H 8.5; Si 12.0; equiv. weight 116.0. Calc. for $C_{10}H_{20}O_4Si$: C 51.7; H 8.7; Si 12.1; equiv. weight 116.2.)

The author's thanks are accorded to Professor Erik Larsson for his kind interest in this work.

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Anomalous Diffusion of Cellulose in Cupriethylenediamine Solution

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By using the ultracentrifuge method for molecular weight determinations on cupriethylenediamine solutions of cellulose it was found that while satisfactory results could be obtained from sedimentation velocity measurements [1] the diffusion behaviour of these solutions proved highly anomalous. Since complications of the same type have previously been noticed by others [2] it seems justified to give a brief preliminary account on the observed irregularities and attempts to deal with them.

The experimental material consisted of unfractionated hydrolyzed cotton cellulose. The diffusion experiments were performed in a cell designed by Claesson [3] using Lamm's scale method for observation. By using the conventional method of calculation the following deviations (Fig. 1) from a normal diffusion repeatedly appeared: 1) The (Z,x)-curve had a shape different from the Gaussian distribution curve by a pronounced compression of its lower part; 2) as a consequence of the foregoing the moment method of calculation gave a lower diffusion coefficient (D_m) than the area method (D_A); and 3) when σ^2 and A^2/H^2 (σ = the standard deviation, A and H the area under the curve and its maximal height respectively) were plotted against time straight lines were obtained but these cut the time axis at a positive value.

When cellulose is dissolved in cupriethylenediamine ($CuEn_2$) it undergoes complex formation and, owing to this, part of the $CuEn_2$ becomes chemically bound to cellulose. Thus the solution contains less free $CuEn_2$-ions than the initial solvent. In a diffusion experiment this will result in diffusion of $CuEn_2$-ions in a direction opposite to that of the cellulose complex. Since, however, this counter diffusion necessarily proceeds at a much higher rate than the diffusion of the cellulose complex its effect on the diffusion process was at first neglected. It was believed that at an early stage the diffusion of the low-molecular component would be completed and thus no appreciable error would occur in the evaluation of the diffusion coefficient of the main component.

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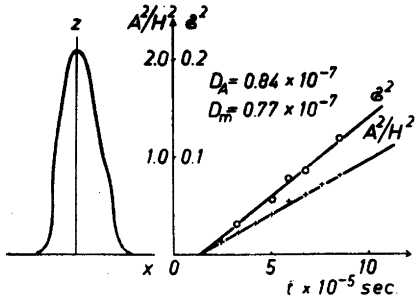

Fig. 1. The anomalous feature of the diffusion of cellulose in cupriethylenediamine. Shape of the (Z,x)-curve and the functions $(A^2/H^2,t)$ *and* (σ^2,t) *intersecting the time axis at a positive value.*

A theoretical analysis of the counter diffusion on the basis of a hypothetical system revealed a possible disturbance quite apart from the diffusion itself.

A system was assumed where two substances diffused in opposite directions. Diffusion coefficients D and concentrations c were chosen as: $D_1 = 1 \cdot 10^{-7}$, $D_2 = 16 \cdot 10^{-7}$, $c_1 = 100$, $c_2 = 50$. The concentration distribution was calculated according to the statistical distribution function

$$c = \frac{c_0}{2}\left(1 - \frac{2}{\sqrt{\pi}} \int_0^y e^{-y^2}\, dy\right) \qquad (1)$$

where $y = \dfrac{x}{2\sqrt{Dt}}$, c the concentration at distance x from the original boundary and c_0 the initial concentration.

As can be seen in Fig. 2, the concentration function of the total system represented by the curve $c = c_1 + c_2$ shows a maximum below the original boundary and a minimum above it. This would mean a heavier solution lying over a lighter one. Such a system, however, cannot persist and a conti-

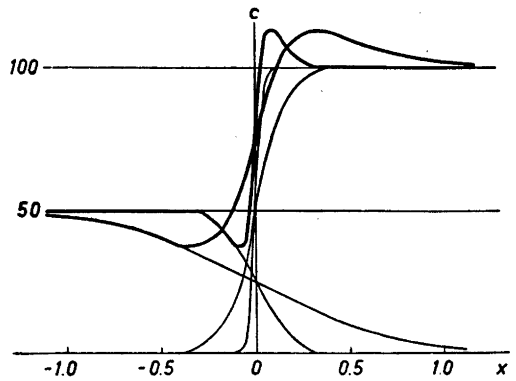

Fig. 2. Concentration function of the hypothetical system. Heavy line = total concentration.

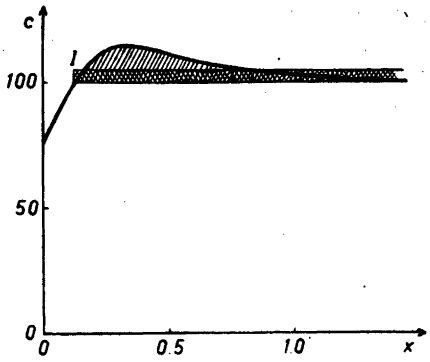

Fig. 3. Mathematical interpretation of the downward flow of substance.

nuous equalization must in some way occur. This was presumed to happen through a downward flow of substance. The mathematical interpretation of the lower half of the symmetrical concentration curve is illustrated in Fig. 3. The shaded area which represents growing concentration in the upward direction was substituted by a rectangular area of same size and length. The distance x from the boundary to the bottom and surface respectively was taken as 2 cm, corresponding to experimental conditions. The height of the rectangle determines a point I where the concentration curve breaks to become a horizontal line. When using the scale method the observed quantity is the concentration gradient $\frac{dc}{dx}$. Differentiation of equation (1) gives:

$$\frac{dc}{dx} = \frac{c_0}{2\sqrt{Dt}} \cdot \frac{1}{\sqrt{\pi}} \cdot e^{-y^2} \qquad (2)$$

If the gradient curves corresponding to the broken concentration curves are computed according to equation (2) they will have an appearance illustrated by Fig.4, which shows a resemblance to the experimental gradient curve of

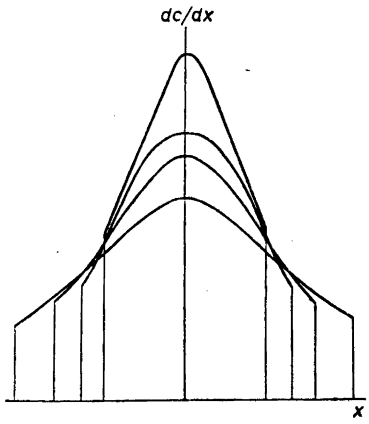

Fig. 4. Gradient curves corresponding to the corrected curves.

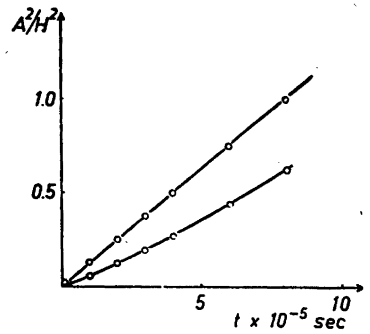

Fig. 5. The function $(A^2/H^2, t)$ *of the main component,* $D = 1 \cdot 10^{-7}$ *(straight line) compared with the same function of the total system.*

Fig. 1. Furthermore, the area A under these curves is equal to the concentration change between the two symmetrical points I. It is readily seen that for these curves A will not be constant which is the condition for the function $\left(\frac{A^2}{H^2}, t\right)$ to be a straight line. In Fig. 5 the function $\left(\frac{A^2}{H^2}, t\right)$ is compared with the line which gives the correct value $1 \cdot 10^{-7}$ for D. If an arbitrary series of points at time values for a normal diffusion experiment between 10^5 and $5 \cdot 10^5$ sec. are roughly combined by a straight line, this line will cut the time-axis at a positive point and have a slope that is definitely too low.

While it is evident that a calculation by the moment method on such abnormal curves is impossible, it can be concluded that the above speculative calculation reasonably explains all the anomalous features observed in the diffusion of cellulose in cupriethylenediamine. The conclusions thus made were further confirmed by the following experiments.

A 1 % solution of saccharose was run against a 0.5 % solution of potassium nitrate. The result is represented in Fig. 6. The compressed shape of the curve is again evident here and the calculated D-values obtained were $D_m = 13.3 \cdot 10^{-7}$ and $D_A = 19.5 \cdot 10^{-7}$ while the correct value for saccharose is $46 \cdot 10^{-7}$.

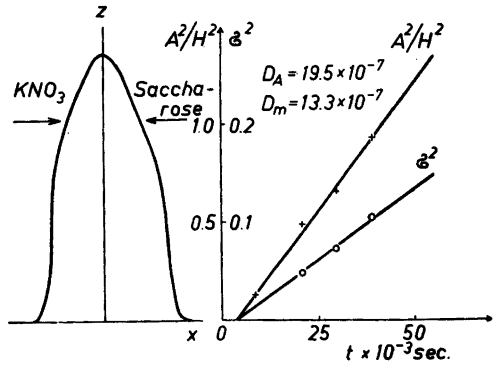

Fig. 6. The model experiment with saccharose diffusing against potassium nitrate. To be compared with Fig. 1.

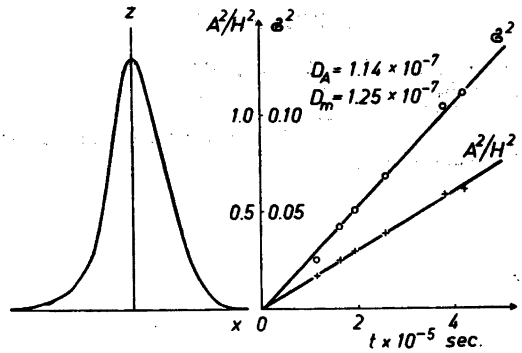

Fig. 7. Diffusion of cellulose in cupriethylenediamine when the counter diffusion is eliminated.

Finally some experiments were made with cellulose solutions in an attempt to eliminate the counter diffusion of free $CuEn_2$. As no suitable membranes for dialysis were available the compensation for bound $CuEn_2$ was effected according to Traube[5] by dissolving the cellulose in the presence of solid $Cu(OH)_2$. As is to be seen in Fig. 7 the results were indeed encouraging when compared with Fig. 1 which represents an experiment in which no steps were taken to exclude the counter diffusion. The values obtained for D from two experiments with the same material at equal concentrations were:

	compensated	not compensated
D_m	$1.26 \cdot 10^{-7}$	$0.81 \cdot 10^{-7}$
D_A	$1.14 \cdot 10^{-7}$	$0.89 \cdot 10^{-7}$

In a wider sense the results reported above indicate that a counter diffusion is bound to cause a disturbing effect due to transportation of substance independently of the diffusion itself and thus involves a grave source of error. In every system where a counter diffusion can occur this should be eliminated by dialysis or other suitable means.

The investigation to which the subject of this communication is attached has been carried out at the Institute of Physical Chemistry, Uppsala University, by benevolent consent of the head of the Institute, Professor Stig Claesson.

SUMMARY

The anomalous features of the diffusion of cellulose in cupriethylenediamine are described. An explanation is proposed connecting these to the counter diffusion of $CuEn_2$-ions. On basis of a hypothetical system is shown that a counter diffusion will lead to a disturbance of the system caused by a gravitational flow of substance. The conclusions drawn from the theoretical treatment of the question are finally confirmed by a model experiment on substances with known diffusion coefficients and by experiments on cellulose in cupriethylenediamine excluding the counter diffusion of free $CuEn_2$-ions.

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On the Correlation between Structure and Some Properties of Water

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Water is regarded as a mixture of an ice-like structure and a close-packed structure. This description is shown to make possible an approximate quantitative treatment of various properties of water. Such properties as heat of fusion, specific heat, magnetic susceptibility and X-ray diffraction data are found to be consistent with the theory developed. The directed forces responsible for association in water are probably rather weak. Applying the law of mass action, the bond energy is estimated to be within the range 1.3 to 2.6 kcal/mole. The larger part of the energy of vaporization is thus ascribed to forces which are only slightly dependent on orientation.

I. INTRODUCTION

The anomalous properties of water have frequently been treated by the introduction of a degree of association, defined by the average polymer $(H_2O)_n$ in the liquid. The magnitude and the temperature dependence of n reported from different sources, vary within considerable limits, however, which may be due partly to an unprecise meaning of n. Many properties will depend upon whether the molecules associate in linear polymers, in rings or in some three-dimensional structure. In the first two cases the number of association bonds per molecule of H_2O will approach unity when n increases, whereas in the latter case it may be much larger. To overcome such difficulties the concept of a distribution of different polymers with different properties was introduced in some papers. Eucken [1] postulated water to be composed of four distinct molecules, H_2O, $(H_2O)_2$, $(H_2O)_4$, $(H_2O)_8$. To each complex was attributed a specific volume and a dissociation energy consistent with the observed volume and thermal expansion of water. An objection could be raised, however, about Eucken's assumption that only some particular complexes should be stable.

The present point of view favours the interpretation of water as a distorted ice-like structure, with bonds constantly breaking and reforming and the association extending throughout the whole liquid. The major approach in this direction was started by the important work of Bernal and Fowler [2].

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The present point of view favours the interpretation of water as a distorted ice-like structure, with bonds constantly breaking and reforming and the association extending throughout the whole liquid. The major approach in this direction was started by the important work of Bernal and Fowler [2].

They assumed water to be composed of three structures. At low temperatures a dense quartz-like sturcture accounts for the high density compared with ice. At somewhat higher temperatures a tridymite-like structure (as in ice) is predominant. At even higher temperatures the usual closepacking of normal liquids prevails. As pointed out by Pople [3], the evidence for the quartz-like structure is poor, however.

Later workers have suggested that a large number of the bonds in water is broken, which would explain a collapse of the ice structure on melting and heating. This explanation has recently been rejected by Lennard-Jones and Pople [4]. Using the value 4.5 kcal/mole estimated by Pauling [5] for the hydrogen bond in ice, they argue that on thermodynamic grounds rather few bonds could be broken in water at room temperature. The structural changes were instead explained as a bending of the bonds, which of course would require less energy than their complete rupture.

II. THE RELATIONSHIP BETWEEN VOLUME AND BONDING IN WATER

A most striking evidence for the bonding in water is the temperature dependence of the volume. Obviously two effects counteract. The initial structure collapses, while the usual thermal expansion works in the other direction. Using this as a starting point, the following picture of water will be advanced as a hypothesis.

The water molecule, H_2O, is regarded as exerting on the surrounding molecules certain forces, which may be separated in two distinct parts. The first part gives rise to the major portion of the evaporation energy of water. It is assumed to be rather unsensitive to the orientation of the molecule. If no other forces were present, water would behave as a normal liquid. In addition another field of force is effective, responsible for a tetrahedral orientation around the oxygen atom in the water molecule. This force is supposed to be markedly dependent on direction, and associated with a definite, comparatively small energy. Whenever this force is effective between two molecules, we shall refer to it as a bond.

A water molecule has four places available for bond formation. If all places are occupied by the neighbours, the structure will be that of ice,[6] which for the sake of brevity will be called A. If no bonds exist, the structure will be that of the normal closepacked liquid, to be denoted by B in this paper.

The volume of water at higher temperatures can be shown to follow the empirical volume curves for a normal liquid quite closely, indicating that only few bonds are present. Below 170° C the observed volume of water diverges rapidly from the empirical curve. To obtain an idea about the number of bonds in water, the following assumption is introduced.

The volume of water at a given temperature will be altered if bonds are formed, the effect being proportional to the number of bonds. If two moles ($2N_{\text{Avogadro}}$) bonds per mole of water are formed, the volume will change to V_A which corresponds to the liquid A. Thus if x is the fraction of bonds (it is: the ratio of number of bonds to the possible total) and V is the observed volume of water, the following relationship is assumed:

$$V = V_B + x(V_A - V_B)$$

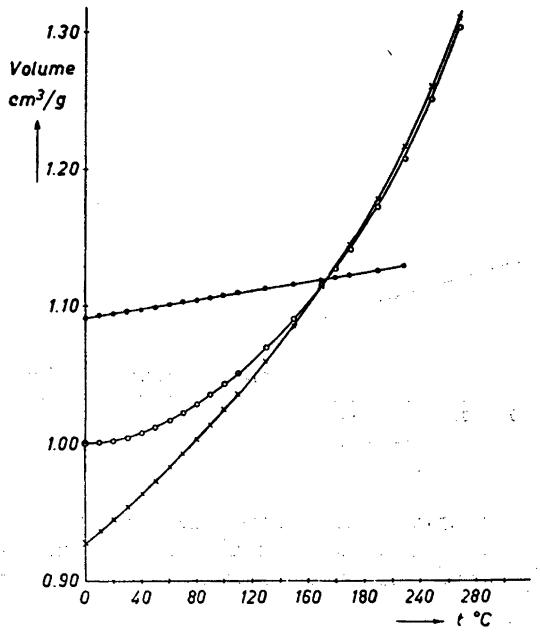

Fig. 1. O *Volume (orthobaric) of water as a function of temperature,* ● *Volume,* V_A, *of ice extrapolated.* × *Volume,* V_B, *of hypothetical normal water.*

This formula corresponds to the picture of x mole of liquid A being ideally mixed with 1—x mole of liquid B. In the temperature range from 0° to 150° C, where from 55 to 85 % of the bonds are broken, this approximation will probably hold.

Fig. 1 shows the volume of one g water as function of temperature. The volume of the same amount of liquid A is extrapolated from ice (data from Dorsey [7]):

$$V_A = 1.0911\ [1 + 0.000153\ (T—273)]$$

The volume of one g of B, V_B, is determined according to Jacobson and Heedman [8]. The critical temperature is taken as 374° C and the curve adjusted through the intersection of V_A and V, giving:

$$V_B = 0.7582\left[1.546—0.546\left(\frac{647—T}{647}\right)^{\frac{1}{3}}\right]^3$$

It is interesting to notice that the values for V_B correspond to a diameter of the B "molecule" of approximately 3.18 Å. (The estimate is based on the volume of B at 0° K, 0.7582 $cm^3 \cdot g^{-1}$ regarding the molecules as hexagonally closepacked).

The fraction of bonds as a function of temperature calculated from these equations is shown in Fig. 2. The extension of the curve above 150° C must

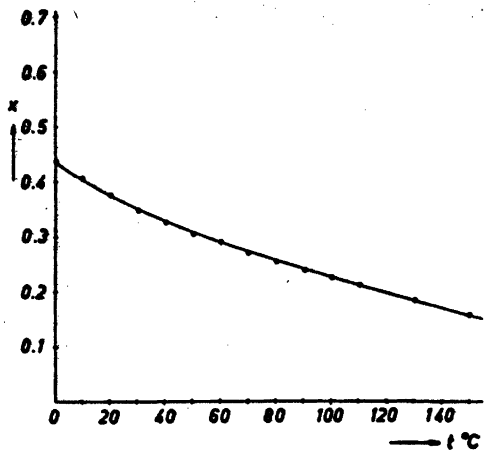

Fig. 2. Fraction of bonds in water as a function of temperature.

necessarily be rather uncertain. The curve itself is also to be regarded as approximate. Even if A and B mixes ideally, the volumes V_A and V_B are not known accurately, which causes an uncertainty in the determination of x.

III. THE MAGNETIC SUSCEPTIBILITY OF WATER

The specific susceptibility of water is likely to be dependent on the number of bonds by a simple relationship. Each bond alters the electron configuration of the molecule in a definite way and is assumed to reduce the susceptibility by a constant amount. As water is a diamagnetic substance, the increase in susceptibility with temperature is easily correlated with the bonding.

If $\chi_g \cdot 10^{-9}$ is the specific susceptibility at a given temperature, $2(1-x)$ the number of broken bonds at the same temperature we have:

$$\chi_g(\text{water}) = \chi_g(\text{ice}) + 2k(1-x)$$

where k is a constant, and corresponds to the rise in susceptibility due to the breaking of the total number of bonds.

For comparison with experiments we take χ_g(water) at 0° C to be 719.0 and using our previous values for x, determine the value of k to be 17.5. The results are given in Table 1. The calculated values are tabulated in the second column, showing a fair agreement with the observed values in the third column. For ice the experimental value is taken from *Int. Crit. Tables* [9]. A somewhat larger value has been reported by Cabrera and Fahlenbrach [10]. The values for water are given by Auer [11]. There is some spread between the values reported by the different authors, but according to Dorsey [7] the experimental results of Auer are among the most reliable ones.

Table 1. The magnetic susceptibility of water.

Temp.	$\chi_g \cdot 10^{-9}$ c.g.s		State
t°C	calc.	obs.	
0	699.4	699.0	ice
0	(719.0)		water
1		718.96	
10	720.2	720.67	
20	721.2	721.83	
30	722.1	722.58	
40	722.9	723.12	
50	723.6	723.61	
60	724.0	724.06	
70	724.7	724.54	

IV. X-RAY DATA FOR WATER

The picture given for water in this paper is in agreement with X-ray diffraction experiments. Morgan and Warren [12] have determined the radial distribution function for liquid water at various temperatures from 1.5° C to 83° C. Between these temperatures they find that the maximum position of the first peak increases from 2.90 Å to 3.05 Å. This is a considerably larger temperature depen dency than observed by normal liquids or crystalline solids. Thus the effect is unlikely to be due to an overall increase in the intermolecular bonds lengths.

A more likely explanation would be that the first distribution peak is caused by two different kinds of "molecules", A and B. The A-"molecules" have a diameter of 2.76 Å corresponding to the oxygen-oxygen distance in ice. The B-"molecules" have a diameter of approximately 3.18 Å as determined in the last part of section II. The two distances 2.76 Å and 3.18 Å are not resolved in the radial distribution curve owing to the large overlap of the two single contributions. The relative abundance of A and B will then determine the position of the first peak. A distance of 2.90 Å as observed in water just above the melting point, corresponds roughly to equal amounts of A and B, the distance increases when the amount of B rises.

A more quantitative treatment of the present X-ray data is questionable. The peak positions in the distribution function are quite reliable, but this does not hold for peak shapes and areas under the peak. The determination of the distribution curves introduces many sources of systematic errors which especially affects peak areas and shapes. A rewiew of these errors has been given by Finbak [13].

V. COMMENTS ON SOME OTHER PROPERTIES

Cross, Burnham and Leighton [14] have studied the Raman spectrum of water. Their results are discussed on the basis of a broken-down ice structure for liquid water. They conclude that water appears to have an average coordination number in the temperature interval from 25° to 90° C of slightly more

than two. This would mean that about half the possible number of intermolecular bonds were broken, a result in rough agreement with ours.

Experimental data based on irreversible phenomena such as viscous flow, have been used to determine the number of intermolecular bonds in water. Owing to the complexity of these phenomena, the data seem rather unsuitable. Ewell and Eyring [15] have determined the number of bonds from viscosity data. Their theory indicates the number of bonds to fall off more rapidly with temperature than the present work indicates. The ratio of energy of vaporization to activation energy of viscous flow increases with temperature. At 150° C the nearly normal value of unassociated polar liquids is attained.

Various authors apply measurements on the dielectric constant of water for a determination of the bonding [3,16]. A similar procedure is not well adapted to the present theory, because the unbonded B "molecules" are also supposed to have energies which are slightly dependent on orientation.

VI. THE ENERGY OF THE BONDING IN WATER

The total energy change associated with the transition from liquid A to B consists mainly of two parts. The major contribution is due to the breaking of bonds. In addition an energy change of structural rearrangement is effective. The latter contribution depends upon the number of nearest neighbours in liquid B, and is probably of minor importance at room temperature.

If some reaction scheme for the transition from A to B is known, the energy of the transition could be computed from the previous values of x. The reaction can be represented by several different schemes, however. Three particular cases shall be considered:

1) All four bonds originating from a given A-"molecule" are fully coupled.
2) No coupling exists between the four bonds.
3) There is a certain coupling between two and two of the four bonds.

The two first cases 1) and 2) represent hypothetical limiting cases and limit the possible transition energies to within a certain interval. The latter case is intermediate, giving an intermediate energy.

1) If a complete coupling between the four bonds from a A-"molecule" exists, all bonds must break or reform simultaneously. This reaction scheme is pictured schematically for the central molecule in Fig. 3a. The corresponding equilibrium constant is given by:

$$K_1 = \frac{1-x}{x}$$

A plot of the logarithm of this equilibrium constant against the inverse of the absolute temperature is shown in Fig. 4 to be a straight line. Using the Van't Hoff's equation:

$$\frac{\mathrm{d}\ \log\ K}{\mathrm{d}T} = -\frac{Q}{R'T^2}$$

an energy of 2.0 kcal/mole is determined. (Q in this case can be approximated by the energy change, $\triangle E$, as the volume work is negligible.) The corresponding entropy change is found to be 7.8 cal/mole·deg. If the energy of structural

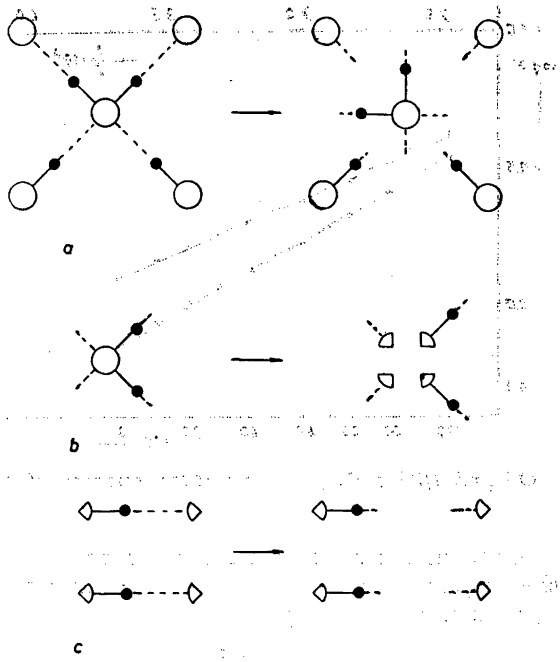

Fig. 3. O = *Oxygen.* ● = *Hydrogen.* ---- = *Hydrogen bond or possibility for bond formation.* ⌓ = *Hypothetical "subparticle". a) Reaction scheme when all bonds are coupled (case 1). b) Division into hypothetical "subparticles". c) Reaction scheme for uncoupled bonds (case 2).*

change is neglected, the mean energy of each bond must be 1.0 kcal/mole, half of the determined ΔE.

2) If there is no coupling between the bonds, the breaking or reforming of a bond will occur irrespectively of any bonds existing in advance. All bonds would then be equivalent. To picture the corresponding reaction scheme, a B-"molecule" with four bonding possibilities is divided into four hypothetical "subparticles" as shown in Fig. 3b. Each subparticle contains one bonding possibility. This gives a reaction scheme for the transition A → B as shown in Fig. 3c. The equilibrium constant is in this case:

$$K_2 = \frac{(1-x)^4}{x^2}$$

A plot of $1/2 \log K_2$ against $1/T$ is given in Fig. 4. The straight line obtained, corresponds to an energy of 5.2 kcal/mole for the reaction A → B, and 2.6 kcal for the breaking of one mole of bonds.

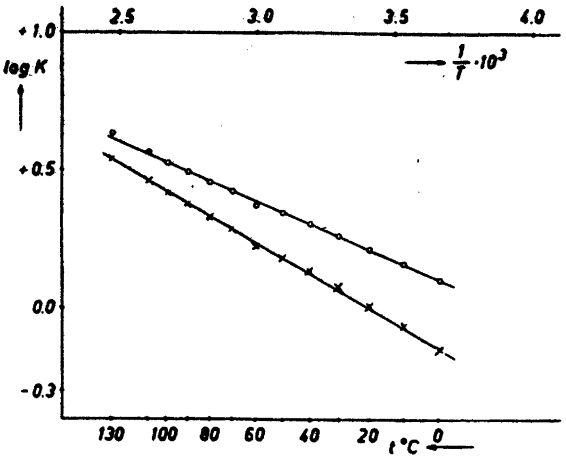

Fig. 4. Log K_1 (○) and 1/2 log K_2 (×) against the reciprocal of the absolute temperature.

3) If the bonds form and break in pairs, the reaction scheme will be analogous to the scheme just described. The equilibrium constant will be simply related to the previous constant by:

$$K_3 = \frac{(1-x)^2}{x} = K_2^{1/2}$$

As log K_3 is equivalent to 1/2 log K_2 we also here get a straight line with an energy change 2.6 kcal/mole (and an entropy change of 8.9 cal/mole.deg.) The mean energy for one mole of bonds in this case is 1.3 kcal.

The obtained plots of log K against the reciprocal absolute temperature does not suffice for a decision between different possible reaction schemes. The functions are not sensitive enough within a limited temperature interval which is evident from Fig. 4. Other experimental evidence involving energy changes should be considered, for instance heat of fusion and specific heat of water.

The energy of the reaction A → B as computed from case 3), 2.6 kcal/mole, is found to be consistent with the heat of fusion on the assumption that a negligible amount of bonds are broken in ice. According to Fig. 2, 0.56 moles of liquid B will be formed on melting one mole of ice. This gives a calculated heat of fusion $0.56 \times 2.6 = 1.46$ kcal/mole which compares favourably with the observed value of 1.44 kcal/mole.

The reaction energy of 2.6 kcal/mole is also consistent with specific heat data. The specific heat at constant volume, C_V, shows an anomality as it decreases by increasing temperatures. This is due to the break down of the A structure, (or breaking of bonds). The contribution to the specific heat of this break down is given by:

$$C_D = \Delta E \frac{dx}{dT}$$

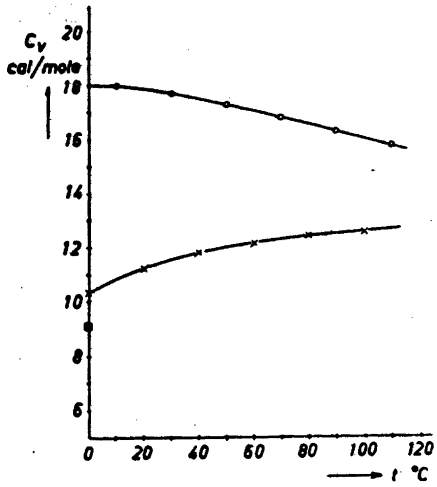

Fig. 5. O *Molar specific heat at constant volume,* C'_V, *for water.* × *Specific heat* C'_V *when heat of dissociation is subtracted.* ⊠ *Specific heat of ice at 0° C.*

where $\triangle E$ here is taken to be 2.6 kcal/mole. If this part of the specific heat is subtracted from the observed total, there remains a part:

$$C'_V = C_V - C_D$$

of the order of magnitude and with the temperature dependence to be expected if water were a normal liquid. C'_V (for one mole of water) as function of temperature is given in Fig. 5.

The agreement of these data does not prove the reaction scheme 3). If some bonds were broken in ice before melting, a larger bond energy than 1.3 kcal would be obtained from the heat of fusion. The bond energy is likely to be somewhere between 1.3 and 2.6 kcal. The correction for the energy of structural changes will perhaps increase the bond energy somewhat. Finbak [17] and later others [16] have used equations which incorporate the reaction scheme 2) given here.

The interaction between the molecules of ice is usually ascribed to hydrogen bonding. Various estimates of this hydrogen bond energy are to be found in the literature. The energy is commonly given as 5.8 kcal in water, half the sublimation energy of ice. Pauling [5] subtracts one part due to what is called ordinary van der Waals' forces, leaving 4.5 kcal for the hydrogen bond. This division of the total interaction seems rather arbitrary, however. Cross, Burnham and Leighton [14] have calculated the hydrogen bond energy from a simple Morse function model of the O—H · · · · O oscillator. They obtain energies from 1.6 to 2.8 kcal with different assumptions. From a more refined treatment they finally calculate 3.0 kcal for the hydrogen bond energy. The bond introduced in the present paper to explain the tetrahedral configuration in water could probably be compared with the hydrogen bond discussed by by Cross, Burnham and Leighton [14].

VII. DISCUSSION

The value 4.5 kcal/mole estimated by Pauling [5] for the hydrogen bond, allows few bonds to be broken at moderate temperatures. Much experimental evidence, however, indicates extensive structural changes in water by heating. Lennard-Jones and Pople [4] have tried to explain this by introducing a bending instead of breaking of the hydrogen bond. The picture given by them resembles the present to a certain extent. If the notion "bending of strong bonds" is substituted by "a breaking of weak bonds" the results would have some similarity. One would only have to impose on the treatment the condition that on reforming the broken bonds, the original configuration should be strongly preferred.

The idea of strong bonds connecting the water molecules seems to disagree with such properties as the rapid fall of viscosity with temperature, however. The general impression of the normal behaviour of water at moderately high temperatures indicates a smaller bonding energy than commonly accepted. This paper divides the total interaction between water molecules in a small energy responsible for tetrahedral orientation, and a larger energy not markedly dependent on orientation. A comparatively small energy of orientation seems adequate for a description of many properties of water.

The present picture of water is somewhat rough. When water is treated as an ideal mixture of liquid A and B, this could be only approximately true. The theories of water are not very advanced, however, and the introduction of such approximations at the present stage would thus be justified. Before any thorough treatment of water is attempted, a reliable general picture must be obtained. This paper indicates a possible approach which may perhaps be extended to other similar associated liquids.

Acknowledgment. The authors are indebted to professor Dr. H. Flood and dosent K. Sandved for reading and criticizing the manuscript.

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Studies on Antimetabolites

I. Synthesis of D,L-"*Neo*phenylalanine", D,L-"*Neo*tyrosine" and D,L-"*Neo*-3,5-diiodotyrosine", the β,β,-Dimethyl Analogues of optically inactive Phenylalanine, Tyrosine and 3,5-Diiodotyrosine

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The β,β-dimethyl ("*neo*") analogues of phenylalanine and tyrosine have been prepared by reduction of the oximinoacids. The intermediate α-ketoacids were obtained in a convenient way by alkaline permanganate oxidation of the corresponding 4,6-di*tert*alkylsubstituted pyrogallol derivatives, which were prepared in almost quantitative yields by the condensation of the α,α-dialkylbenzylalcohol or α-alkylstyrene with pyrogallol. β,β-Dimethyltyrosine was iodinated to 3,5-diiodo-β,β-dimethyltyrosine.

The fact, that the introduction of a *gem*dimethyl group at the α carbon atom of an α-aryloxyacetic acid [1], of 3-indoleacetic [1b,2] or of phenylacetic acid[3], possessing auxin (plant hormone) activity, invariably changed the compound into an auxin antagonist, whereas the introduction of only *one* α-alkyl group usually did not alter the type of activity, suggested that the introduction of such a *gem*dimethyl group on a carbon atom in the vicinity of a reactive centre of a physiologically active molecule might perhaps more generally produce compounds capable of antagonising the parent, unmethylated substance.

The introduction of a β-*gem*dimethyl group into straight chain α-amino-acids appeared to furnish a class of compounds, by means of which this hypothesis could be tested. Consequently the synthesis of a number of such acids was started, and in the present paper the preparation of the β,β-dimethyl analogues of optically inactive phenylalanine, tyrosine and 3,5-diiodotyrosine is described. For convenience they henceforth are named *neo*phenylalanine (I), *neo*tyrosine (II) and *neo*iodogorgoic acid (III) respectively. In these acids the amino and carboxyl groups are linked to a sterically highly hindered carbon atom, and it may be expected that the respective C—N and C—C bonds are rather invulnerable to attack. Compounds of this type exhibit abnormal reac-

tivity. For example *neo*pentylamine, $(CH_3)_3CCH_2NH_2$, is known to react abnormally with nitrous acid forming a relatively stable diazonium salt [4], and in "*neo*phylchloride", $C_6H_5C(CH_3)_2CH_2Cl$, the chlorine atom is replaced only with great difficulty [5]. Phenylalanine and tyrosine are believed to be involved as precursors in the *in vivo* synthesis of adrenaline and thyroxine. Analogues of the aminoacids may therefore be able to interfere with the metabolic processes leading to the hormones.

Several cases are known, where structural changes in the molecule of a natural metabolite produce compounds capable of antagonising the parent compound, and the literature on such antimetabolites has been excellently reviewed by Woolley [6]. The classical example is the antagonism between a sulphonamide and *p*-aminobenzoic acid. No attempts appear to have been made to produce antimetabolites by the introduction of a *gem*dialkyl group suitably situated in the molecule of an essential metabolite.

The only α-aminoacid containing a quarternary β-carbon atom, which seems to have been described, is D,L-α-amino-β-methyl*iso*valeric acid, *pseudo*leucine (IV), obtained in a very low yield by the ammonolysis of α-bromo-β-methyl*iso*valeric acid, or better by the Knoop synthesis from α-oxo-β-methyl-*iso*valeric acid [8]. A very good yield is obtained by the reduction of the oxime of the latter ketoacid [9].

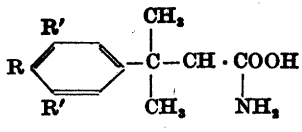
For the preparation of *neo*phenylalanine the Strecker synthesis from α-phenyl*iso*butyraldehyde and the reduction of α-oximino- or α-hydrazono-β-phenyl*iso*valeric acid appeared to be possible synthetic pathways. Ammonolysis of α-bromo-β-phenyl*iso*valeric acid and Knoop synthesis from the α-oxo-acid were considered impracticable, as the "sterical hindrance" would probably be even greater in these compounds than in the corresponding methyl derivative.

The α-phenyl*iso*butyraldehyde required for the Strecker synthesis was easily prepared by the oxidation of β-phenyl*iso*butanol, obtained as described by Whitmore, Weissgerber and Shabica [5]. The Strecker synthesis from this aldehyde failed, however, probably due to steric factors*.

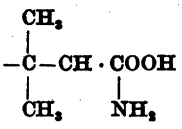
For the preparation of the intermediate oximino- or hydrazonoacid α-oxo-β-phenyl*iso*valeric acid was desired. Only a few α-ketoacids containing a quarternary β-carbon atom seem to have been described. Glücksmann [10a] obtained an acceptable yield of trimethylpyruvic acid and some trimethylacetic acid on the oxidation of pinacolone with alkaline permanganate, a reaction which was further studied by Bardhan [10b], and by similar oxidation of *p-tert*butylphenol and *p-tert*amylphenol Anschütz and Rauff [11] obtained mixtures of trimethylpyruvic and trimethylacetic acids and of ethyl-dimethylpyruvic and ethyl-dimethylacetic acids, respectively, in low yields.

Attempts to oxidize α,α-dimethyl-α-phenylacetone and its *p*-methoxy-derivative to the corresponding ketoacids with alkaline permanganate in aqueous solution at room temperature failed, probably due to the insolubility of the ketones. At the boiling point the only acid products isolated were the

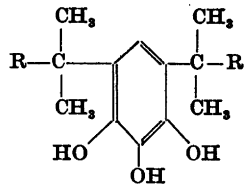
* Added in proof: In this connection it is interesting that it was recently reported that the cyanohydrin of diphenylglycolaldehyde could not be converted into the aminonitrile. (Favorskaya, T. A. and Remizova, L. A. *Zhur Obshchei Khim.* 23 (1953) 667, *Chem. Abstracts* 48 (1954) 7588.)



I R = R′ = H
II R = OH; R′ = H
III R = OH; R′ = I



VI



V R = C_6H_5
VI R = *p*-CH_3O-C_6H_4

α-phenyl*iso*butyric acids. Attempts to carry out the reaction in other solvents were also unsuccessful, as was an attempt to oxidize methyl α-phenyl*iso*valerate by refluxing it with selenium dioxide.

Application of the method of Anschütz and Rauff to *p*-(α,α-dimethylbenzyl)phenol afforded a very small yield of a mixture of α-oxo-β-phenyl-*iso*valeric and α-phenyl*iso*butyric acids, which was easily separated by selective hydrolysis of the ethyl ester mixture. Analogous oxidation of a mixture of 2,2-bis-(*p*-hydroxyphenyl)propane and its monomethyl ether, containing about 60 % monomethyl ether, prepared by partial methylation of the phenolic compound and removal of the dimethylderivative, afforded a small yield of a mixture of α-oxo-β-(*p*-methoxyphenyl)*iso*valeric and α-(*p*-methoxyphenyl)*iso*butyric acids, from which the pure acids were isolated after esterification and selective hydrolysis of the ester mixture.

A very good yield of ketoacid was obtained by permanganate oxidation of 4,6-di-(α,α-dimethylbenzyl)pyrogallol (V) [12]. This substance (V) was easily prepared in almost quantitative yield by the addition of α-methylstyrene to pyrogallol, a method analogous to that of Schulze and Flaig [13]. for preparation of 4,6-di*tert*butylpyrogallol.

In addition to the ketoacid and some α-phenyl*iso*butyric acid a substance, m.p. 174—175°, insoluble in sodium bicarbonate solution, and another, m.p. 185—186°, soluble in sodium bicarbonate, were isolated. These will be described in a separate paper.

The addition of α,α-dimethyl-*p*-methoxybenzyl alcohol, obtained from methyl anisate and methyl-magnesium bromide [14] to pyrogallol afforded a high yield of 4,6-di-(α,α-dimethyl-*p*-methoxybenzyl)pyrogallol (VI), permanganate oxidation of which gave α-oxo-β-(*p*-methoxyphenyl)*iso*valeric acid and a little α-(*p*-methoxyphenyl)*iso*butyric acid. No attempt was made to isolate other products from the reaction.

The ketoacids were converted to their oximes in almost quantitative yields, and reduction of the latter with 2 % sodium amalgam in absolute ethanol, lactic acid being added during the reaction to keep the solution faintly acid, afforded the aminoacids, which were characterized by their N-acetyl derivatives.

Iodination of *neo*tyrosine following the directions given by Block and Powell [15] for iodination of tyrosine afforded *neo*iodogorgoic acid (III).

EXPERIMENTAL *

α-Phenylisobutyraldehyde. Powdered potassium dichromate (19.9 g) was added in small portions over half an hour to a stirred mixture of β-phenyl*iso*butanol (30 g), conc. sulphuric acid (40 ml) and water (200 ml). The mixture was poured into water, and the resulting oil was taken up in ether, washed and dried. Removal of the ether and careful fractionation of the residual oil afforded a colourless liquid (20 g) boiling at 91—95°/10 mm Hg. It formed a *semicarbazone* melting at 168—170°. Tiffeneau [16] reports m.p. 172°. (Found: N 20.4. Calc. for $C_{11}H_{15}N_3O$: N 20.5.)

α,α-Dimethyl-α-phenylacetone. (The method described is a modification of that given by Suter and Weston [17].) A stock solution of 1 *M* potassium *tert*butoxide was prepared by dissolving potassium (39.1 g) in anhydrous *tert*butanol (400 ml) and diluting to 1000 ml. — Phenylacetone (26.8 g) was added to 1 *M* potassium *tert*butoxide solution (205 ml). The mixture was shaken and allowed to stand for ten minutes, and methyl iodide (29 g) was carefully added with shaking. After two hours the treatment with butoxide and methyl iodide was repeated, and the mixture set aside overnight. Most of the solvent was removed on the water bath, and water was added to dissolve the potassium iodide. The resulting oil was taken up in ether, washed and dried. The ether was removed and the residual oil fractionated yielding a colourless product (24 g) boiling at 98—101°/11 mm Hg. (Lit.[17] b.p. 99—99.5°/12 mm Hg).

A sample of the product (3 g) was oxidised with boiling alkaline aqueous potassium permanganate. α-Phenyl*iso*butyric acid (0.5 g), m.p. and mixed m.p. 78—78.5° was isolated.

α,α-Dimethyl-α-(p-methoxyphenyl)acetone. Anisylacetone was methylated by the method described above. The yield of carefully fractionated oil boiling at 138—141°/10 mm Hg was 65 %. (Found: CH_3O 16.0. $C_{12}H_{16}O_2$ requires: CH_3O 16.2). The *semicarbazone* melted at 184—185°. (Found: CH_3O 12.3. $C_{13}H_{19}N_3O_2$ requires: CH_3O 12.4). On oxidation with boiling permanganate α-*p*-methoxyphenyl*iso*butyric acid, m.p. 89°, identical with a specimen prepared from α-*p*-methoxyphenyl*iso*butyronitrile,[18] was formed.

Methylation of 2,2-bis-(p-hydroxyphenyl)propane, The phenol (1480 g, 6,5 moles) was dissolved in hot sodium hydroxide solution, prepared from sodium hydroxide (280 g, 7 moles) and water (4 l). Dimethylsulphate (882 g, 7 moles) was added with stirring over 2—3 hours, the temperature being kept at 30—40°. The mixture was stirred overnight and then refluxed for 2 hours, and, while hot, acidified with sulphuric acid. The aqueous layer was siphoned and washed with a little toluene, which was combined with the oily layer and washed twice with water. A solution of sodium hydroxide (300 g) in water (3 l) was added, and the mixture was rapidly stirred for one hour, the temperature being kept at about 60°. While hot the liquid was repeatedly extracted with toluene. (If allowed to cool the mixture solidified.) The combined toluene extracts were washed with 2 *N* sodium hydroxide, which was combined with the alkaline solution, and with water.

The alkaline solution was acidified with sulphuric acid and extracted with toluene. On distillation the extracts yielded a faintly yellow oil (970 g) boiling at 220—250°/10 mm Hg. It analysed 7.50 % CH_3O and thus contained about 60 % monomethyl ether. All attempts to separate the mixture by further distillation failed. On distillation the toluene extracts afforded 2,2-bis-(*p*-methoxyphenyl)-propane (305 g), b.p. 212—214°/10 mm Hg.

* All melting and boiling points uncorrected. Petrol refers to the fraction b.p. 40—60°.

4,6-Di-(α,α-dimethylbenzyl)pyrogallol (V). Pyrogallol (252 g) was dissolved in glacial acetic acid (600 ml) and conc. sulphuric acid (25 ml) in a 4 l beaker and cooled in ice water. A solution of α-methylstyrene (570 g, 20 % excess) in glacial acetic acid (600 ml) was added with stirring during about one hour, the temperature being kept below 15°. Stirring was continued for an additional half hour and the mixture allowed to stand overnight. The resulting solid cake was crushed, stirred with water (1 l), and the crystalline product collected. It was washed free from acid and air dried. Crystallisation from ligroin afforded an almost quantitative yield of glistening needles melting at 118—120°. Repeated crystallisations raised the melting point to 120—121°. (Found: C 79.7; H 7.06. $C_{24}H_{26}O_3$ requires: C 79.5; H 7.23.)

The *triacetate*, prepared with acetic anhydride in pyridine, crystallised in large, rhombic plates, m.p. 138—139°. (Found: C 74.0; H 6.56. $C_{30}H_{32}O_6$ requires: C 73.7; H 6.60.)

4,6-Di-(α,α-dimethyl-p-methoxybenzyl)pyrogallol (VI). Crude α,α-dimethylbenzyl alcohol was prepared [14] by refluxing methylmagnesium bromide (from 163 g of magnesium and 610 g of methyl bromide) in absolute ether (1 l) with methyl anisate (500 g) dissolved in absolute ether (1.5 l) for 24 hours, addition of saturated ammonium chloride solution and extraction of the carbinol with ether. After drying (Na_2SO_4) and removal of the solvent on the water bath, the last traces being removed under reduced pressure, a faintly yellowish oil (495 g) was obtained. It was dissolved in glacial acetic acid (500 ml) and added with stirring to a solution of pyrogallol (200 g) in glacial acetic acid (500 ml) and conc. sulphuric acid (50 ml) the temperature being kept below 15°. The mixture was set aside overnight and then treated with water (3 l) and the product collected by filtration and washed free from acetic acid. After drying it was crystallised from ethanol in needles, m.p. 158—161°. Yield 460 g (73 % calculated on methyl anisate). Repeated crystallisation raised the melting point to 163—163.5°. (Found: C 74.0; H 7.00; CH_3O 14.8. $C_{26}H_{30}O_5$ requires: C 73.9; H 7.16; CH_3O 14.7.) The *triacetate* was prepared with acetic anhydride in pyridine and crystallised from benzene-petrol (1 : 4). It separated in fine needles, m.p. 122—123°, or coarse rhombic prisms, the latter melting at 143—145°. (Found: CH_3O 11.2. $C_{32}H_{36}O_8$ requires: CH_3O 11.3.)

Ethyl α-oxo-β-phenylisovalerate from 4,6-di-(α,α-dimethylbenzyl)pyrogallol. The finely powdered pyrogallol derivative (300 g) was added to a stirred solution of sodium hydroxide (100 g) in water (3 l) immediately followed by powdered potassium permanganate (150 g). Additional permanganate (500 g) was added in portions over two hours. The temperature of the mixture rose to 60—70°, and further increase was prevented by addition of ice. Stirring was continued overnight, and excess permanganate destroyed with ethanol. The manganese sludge was removed by filtration (a layer of silicious earth nearest the filter paper effectively aided filtration) and thoroughly washed with hot water containing a little sodium hydroxide. The filtrate was acidified and extracted five times with ether. The combined ethereal extracts were in turn extracted with saturated sodium bicarbonate solution. The ether raffinate was evaporated and the residual solid crystallised from benzene-petrol (1 : 2) and from ethanol yielding a colourless compound (21 g) m.p. 174—175°. The bicarbonate extracts were acidified and the precipitated oil extracted with ether, dried (Na_2SO_4), and the ether removed yielding an oil (171 g). This was refluxed overnight with absolute ethanol (400 ml) containing sulphuric acid (25 ml). Most of the alcohol was removed on the water bath at reduced pressure, and the residual liquid poured into water. The oil, which separated, was taken up in ether, washed with water, with sodium bicarbonate solution and again with water, and dried. The ether was removed and the residual oil fractionated yielding a fore-run boiling at 60—140°/12 mm Hg, from which α-phenyl*iso*butyric acid, m.p. and mixed m.p. 78°, (24 g) was isolated after alkaline hydrolysis. The main fraction (118 g) boiled at 140—144°/12 mm Hg, and consisted of almost pure ethyl *α-oxo-β-phenylisovalerate.* (Found: C_2H_5O 20.2. $C_{13}H_{16}O_3$ requires: C_2H_5O 20.5).

The bicarbonate extract was acidified giving an oil, which crystallised. The product was recrystallised from benzene-petrol (1 : 1) and from ethanol. Colourless needles, m.p. 185—186° (15 g).

Ethyl α-oxo-β-phenylisovalerate and α-phenylisobutyric acid from p-(α,α-dimethylbenzyl)-phenol. p-(α,α-Dimethylbenzyl)phenol (212 g) was dissolved in water (2 l) containing sodium hydroxide (50 g), and oxidized with potassium permanganate (800 g), added in portions over 3 hours to the stirred solution. After stirring overnight the mixture was worked up as described for the oxidation of 4,6-di-(α,α-dimethylbenzyl)pyrogallol. The

yield of ethyl α-oxo-β-phenyl*iso*valerate was 18 g, and that of α- phenyl*iso*butyric acid 15 g.

α-Oxo-β-(p-methoxyphenyl)isovaleric acid from impure 2,2-bis-(p-hydroxyphenyl)-propane monomethyl ether. The ether mixture containing 60 % monomethylether (400 g) was dissolved in a solution of sodium hydroxide (100 g) in water (5 l) and oxidized with powdered potassium permanganate (2500 g), added in portions over 4 hours, the temperature being kept below 80°. The mixture was stirred overnight and worked up as described for the oxidation of 4,6-di-(α,α-dimethylbenzyl)pyrogallol. An ester mixture (78 g) boiling at 150—190°/12 mm Hg was obtained. It was shaken for 45 minutes with 2 *N* sodium hydroxide (250 ml), the undissolved oil extracted with ether, and the alkaline solution acidified with dilute sulphuric acid. The oil, which separated was taken up in ether, washed and dried. On removal of the ether an oil, which crystallised, was obtained (42 g). M.p. 70—74°. Crystallisation from benzene-petrol (1:1) raised the melting point to 79—79.5°. (Found: CH_3O 13.8; equiv. weight 222. $C_{12}H_{14}O_4$ requires: CH_3O 14.0; eguiv. weight 222.2.)

From the ether extracts containing unhydrolysed material α-(p-methoxyphenyl)-*iso*butyric acid (20 g) was isolated after further hydrolysis. M.p. 89—90° underpressed by a specimen prepared from α-p-(methoxyphenyl)*iso*butyronitrile [18].

α-Oxo-β-(p-methoxyphenyl)isovaleric acid from 4,6-di-(α,α-dimethyl-p-methoxybenzyl)-pyrogallol. 4,6-Di-(α,α-dimethyl-p-methoxybenzyl)pyrogallol (400 g) was suspended in a stirred solution of sodium hydroxide (100 g) in water (3,5 l) and oxidised with powdered potassium permanganate (770 g) added in portions at such a rate, that the temperature was kept below 70°. Stirring was continued for three hours after all the permanganate had been added, the solution was decolourised by addition of a little ethanol, and the mixture was worked up as described above for the unmethoxylated compound. Careful fractionation of the ester mixture yielded a fore-run from which α-(p-methoxyphenyl)-*iso*butyric acid (25 g) was obtained after hydrolysis, and a fraction b.p. 168—172°/12 mm Hg consisting of almost pure *ethyl α-oxo-β-(p-methoxyphenyl)isovalerate* (157 g). (Found: CH_3O + C_2H_5O 30.0. $C_{14}H_{18}O_4$ requires: CH_3O + C_2H_5O 30.4.) No attempt was made to isolate other products from the oxidation.

α-Oxo-β-phenylisovaleric acid. The above ethyl ester (73 g) was shaken with 2 *N* sodium hydroxide (250 ml) until the mixture became homogenous (15 minutes). The liquid was washed twice with ether and acidified. The precipitated oil was taken up in ether, washed and dried. Removal of the ether yielded an oil (62.3 g) which partially crystallised. A sample of the crystals were collected and carefully washed with very little, cold petrol. After drying they melted sharply at 27—28°. (Found: equiv. weight 197. $C_{11}H_{12}O_3$ requires: equiv. weight 192.2) Crystallised from very little petrol they melted sharply at 40.5—41°. (Found equiv. weight 195.) The low melting form on storing was converted to the high melting one and the former was not observed in subsequent preparations of the acid.

α-Oximino-β-phenylisovaleric acid. The crude keto acid (57.6 g) was dissolved in 7.5 % aqueous sodium hydroxide (150 ml). Hydroxylamine hydrochloride (28 g) in a little water was added followed by solid sodium carbonate (40 g) in small portions. The mixture was warmed on the water bath for 3 hours, diluted with water (200 ml) and acidified. After storing in a refrigerator the solid was collected and crystallised from benzene-petrol (1 : 1). Yield 90 %. M.p. 123—123.5°. (Found: N 6.73; equiv. weight 208. $C_{11}H_{13}NO_3$ requires: N 6.76; equiv. weight 207.2.)

α-Oximino-β-(p-methoxyphenyl)isovaleric acid. This substance was prepared exactly as described for the unmethoxylated compound and crystallised from aqueous ethanol. M.p. 151°. (Found: N 5.87; CH_3O 13.0; equiv. weight 239. $C_{12}H_{15}NO_4$ requires: N 5.90; CH_3O 13.1; equiv. weight 237.2).

D,L-*α-Amino-β-phenylisovaleric acid,* "D,L-*neophenylalanine*" *(I).* α-Oximino-β-phenyl*iso*valeric acid (5 g) was dissolved in absolute ethanol (80 ml) and acidified with very little lactic acid (sp.g. 1.24). Sodium amalgam (200 g, 2 %) was added in portions over one hour, the mixture being kept faintly acid to lithmus by addition of lactic acid. After four hours the reaction was considered complete. The clear liquid was decanted, the mercury was washed with hot ethanol, and the solution concentrated in a vacuum. It was then diluted with water to 75 ml and stored overnight in a refrigerator. The crystalline product was filtered and washed free from salts. Yield 3.3 g. The product was purified by dissolving it in dilute hydrochloric acid and adjusting the pH to 6 by the

addition of ammonia. For analysis it was crystallised from water. It decomposed without melting at about 240°. (Found: C 68.5; H 7.85; N 7.19. $C_{11}H_{15}NO_3$ requires: C 68.4; H 7.83; N 7.25). The *N-acetylderivative* was prepared with acetic anhydride in 2 *N* sodium hydroxide, and crystallised from water forming clusters of hard prisms, m.p. 178—179°. (Found: N 5.97; equiv. weight 233. $C_{13}H_{17}NO_3$ requires: N 5.95; equiv. weight 235.3.)

D,L-α-*Amino-β-(p-methoxyphenyl)-isovaleric acid.* The reduction was carried out as described for *neo*phenylalanine above. As the methoxyderivative was rather soluble in the aqueous solution of sodium lactate obtained, some additional material could be obtained from the mother liquor by acidification with sulphuric acid, continuous extraction of the lactic acid with ether and then adjustment of the pH to 6 with ammonia. Total yield of acid, purified by dissolution and reprecipitation, 45—55 %. For analysis it was crystallised from water. It decomposed without melting at about 220°. (Found: N 6.25; CH_3O 13.7. $C_{12}H_{17}NO_3$ requires: N 6.27; CH_3O 13.9.)

D,L-α-*Amino-β-(p-hydroxyphenyl)isovaleric acid,* D,L-"*neotyrosine*" *(II).* The methyl ether (8 g) was refluxed (oil bath) for three hours with 48 % hydrobromic acid (40 ml). Most of the hydrobromic acid was removed on the water bath at reduced pressure, and the aminoacid precipitated by adjusting the pH to 6 with ammonia. After storing overnight in a refrigerator the crystalline product was collected and washed free from inorganic material. It was purified by reprecipitation. The yield was almost quantitative. For analysis it was crystallised from water. It decomposed without melting at about 245°. (Found: C 63.0; H 7.11; N 6.69. $C_{11}H_{15}NO_3$ requires: C 63.2; H 7.23; N 6.69.) The *N-acetylderivative* crystallised in clusters of hard prisms, m.p. 181—181.5°. (Found: N 5.54, 5.57; equiv. weight 253. $C_{13}H_{17}NO_4$ requires: N 5.58; equiv. weight 251.2.)

D,L-α-*Amino-β-(3,5-diiodo-4-hydroxyphenyl)-isovaleric acid,* D,L-"*neoiodogorgoic acid*" *(III).* D,L-*Neo*tyrosine (7.6 g) was dissolved in acetic acid (30 ml). A solution of iodine monochloride (15 g) in acetic acid (30 ml) was added and the mixture heated on a water bath for half an hour. Most of the acetic acid was removed under reduced pressure, and water and a little sodium hydrogen sulphite added. After filtration the aminoacid was precipitated by adjusting the pH to 6 with ammonia. It was purified by dissolving it in ammonia, treating with decolorising carbon, and adjusting to pH 6 with acid. (Precipitated from acid with ammonia it tended initially to from an oil, which rapidly crystallised.) For analysis it was crystallised from water as a heavy faintly yellow, microcrystalline powder, which decomposed without melting at about 205°. (Found: I 54.7; N 3.12, 3.09. $C_{11}H_{13}I_2NO_3$ requires: I 55.0; N 3.04.)

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Studies on Antimetabolites

II. Synthesis of "*Neo*tyramine" and "*Neo*hordenine", the β,β-Dimethyl Analogues of Tyramine and Hordenine *

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The synthesis of the β,β-dimethyl analogues of tyramine and hordenine, *viz.* β-(*p*-hydroxyphenyl)*iso*butylamine and N,N-dimethyl-β-(*p*-hydroxyphenyl)*iso*butylamine, and some related compounds by the reactions shown on p. 1214 is described.

In connection with the investigation of the β,β-dimethyl analogues of phenylalanine and tyrosine, discussed in part I of this series [1], it was of interest to test the pharmacological activity of their decarboxylation products *viz.* β-phenyl- and β-(*p*-hydroxyphenyl)*iso*butylamine (I and II resp.). These compounds were prepared, and as the intermediates were considered to be valuable for the synthesis of the above mentioned amino acids *via* the corresponding acyl cyanides and α-ketocaids, a route which was abandoned when these acids became available by a different method [1], the syntheses were studied in some detail. In addition to the amines α-(*p*-aminophenyl)*iso*butyric acid (III) was prepared because of its interest as a potential *p*-aminobenzoic acid antagonist, as well as the ethyl (IV) and β-N,N-dimethylaminoethyl (V) esters of the former acid, which were of some interest because of their relation to the local anesthetics of the *p*-aminobenzoic acid ester type, and to the antispasmodics of α,α-dialkylphenylacetic acid aminoalkyl ester type studied by Domenjoz *et al.* and Jensen *et al.* [2].

It is the purpose of the present paper to describe the synthetical work, whereas the results of the physiological studies will be published elsewhere.

The principle steps involved in the syntheses are shown in Fig. 1.

The starting material, α-phenyl*iso*butyronitrile (VI), was obtained by methylation of benzyl cyanide according to Darzens and Lévy [3]. Nitration afforded a mixture of much *p*- and a little *o*-nitrophenyl*iso*butyronitrile, from which the pure *para*isomer was obtained by crystallisation and converted to α-(*p*-methoxyphenyl)*iso*butyronitrile as shown. The latter compound

* Part I. Acta Chem. Scand. 8 (1954) 1203.

was, however, more conveniently prepared from anisic alcohol or anisole *via* the benzyl chloride and the benzyl cyanide, methylation of which by the above method afforded the *iso*butyronitrile derivative. Reduction with lithium-aluminium hydride followed by demethylation with hydrobromic acid yielded β-(*p*-hydroxyphenyl)*iso*butylamine "*neo*tyramine", (II). The N,N-dimethylderivative, "*neo*hordenine" (VII) was obtained by the same route from the N,N-dimethylamide of α-(*p*-methoxyphenyl)*iso*butyric acid, available as a by-product from a different line of investigation. It was also obtained by methylation of *neo*tyramine by the Eschweiler-Clarke procedure [4].

Attempts to obtain *neo*tyramine by catalytic hydrogenation of α-(*p*-hydroxyphenyl)*iso*butyronitrile with palladium or platinum oxide catalyst in ethanol, ethanolic ammonia or ethanolic hydrogen chloride were unsuccessful.

The α-(*p*-aminophenyl)*iso*butyric acid esters were prepared by reduction of the corresponding nitro ester hydrochlorides with powdered iron.

EXPERIMENTAL *

α-Phenylisobutyronitrile was prepared in 60—70 % yield by the method of Darzens and Lévy [3] in runs using up to 580 g (5 moles) of commercial grade benzyl cyanide. The crude reaction product was freed from *iso*nitrile by washing with 20 % sulphuric acid followed by water, before distillation.

β-Phenylisobutylamine, "neophylamine". The above nitrile was reduced with lithium-aluminium hydride following the general directions given by Amundsen and Nelson [5]. The yield of product, b.p. 109—112°/20 mm Hg, was 65 %. It was converted to the *hydrochloride* m.p. 200—202°. (Lit. [6] 200—201.5°). (Found: Cl 19.1. Calc. for $C_{10}H_{16}ClN$: Cl 19.0.)

α-(p-Nitrophenyl)isobutyronitrile. α-Phenyl*iso*butyronitrile (300 g) was added drop by drop with stirring to fuming nitric acid (sp.g. 1.48 ; 1100 ml) below 0°. After the addition the cooling bath was removed, and the solution was set aside for 2 hours, poured onto ice, and the precipitated crystals collected, thoroughly washed with water and air dried. The crude product (90 % yield) was crystallised from ethanol (500 ml) and from a benzene-petrol, giving 200 to 225 g of faintly yellow needles, m.p. 67—68°. (Found: C 62.5; H 5.10. $C_{10}H_{10}N_2O_2$ requires: C 63.1; H 5.30.)

A sample (3 g) was refluxed for one hour with a solution of potassium dichromate (15 g) and conc. sulphuric acid (30 ml) in water (40 ml). *p*-Nitrobenzoic acid (0.8 g) m.p. and mixed m.p. 232—233° was obtained.

α-(p-Nitrophenyl)isobutyric acid. α-*p*-Nitrophenyl*iso*butyronitrile (35 g), conc. sulphuric acid (50 ml) and water (60 ml) were mixed, refluxed in an oil bath for about 20 hours, and poured into icewater. The precipitated acid crystallised from benzene-petrol (3 : 1) in coarse needles, m.p. 128—129°. Yield 31 g (80 %). (Found: N 6.73; equiv. weight 208. $C_{10}H_{11}NO_4$ requires: N 6.70; equiv. weight 209.2.)

α-(p-Nitrophenyl)isobutyryl chloride was prepared by refluxing the acid (one part) with thionyl chloride (two parts) for one hour on a water bath, and removing the excess thionyl chloride *in vacuo*. The product crystallised from petrol in faintly yellow prisms, m.p. 55—56° (Found: Cl 15.3 $C_{10}H_{10}ClNO_3$ requires: Cl 15.6).

α-(p-Nitrophenyl)isobutyramide formed coarse needles, m.p. 110—111° (from benzene). (Found: N 13.5 · $C_{10}H_{12}N_2O_3$ requires: N 13.5).

N,N-Dimethyl-(α-p-nitrophenyl)isobutyramide had m.p. 109—110°. (Found: N 11.8. $C_{12}H_{16}N_2O_3$ requires: N 11.9.)

α-(o-Nitrophenyl)isobutyric acid was isolated from the mother liquors from the crystallisation of the *p*-isomer when a hydrolysis was run with crude nitronitrile. Needles,

* All melting and boiling points uncorrected. Petrol refers to the fraction b.p. 40—60°.

m.p. 93—94°. (Found: N 6.75; equiv. weight 207. $C_{10}H_{11}NO_4$ requires: N 6.70 equiv. weight 209.2.) — Oxidation of a sample as described for the *p*-nitrophenyl*iso*butyronitrile yielded *o*-nitrobenzoic acid, m.p. and mixed m.p. 144—146°.

Ethyl α-(p-nitrophenyl)isobutyrate. α-*p*-Nitrophenyl*iso*butyronitrile (38 g) was refluxed for about 70 hours with 95 % ethanol (50 ml) and conc. sulphuric acid (20 ml). The solution was poured into water, the oil that separated was taken up in ether, washed with water and sodium bicarbonate solution and dried. Distillation afforded the ester, b.p. 174—176° C/10 mm, in 90 % yield. (Found: C_2H_5O 18.8. $C_{12}H_{15}NO_4$ requires: C_2H_5O 19.0.)

This ester was not aminolysed when heated in an autoclave for 24 hours with alcoholic ammonia, mono- or dimethylamine at 100°.

α-(p-Aminophenyl)isobutyronitrile. α-*p*-Nitrophenyl*iso*butyronitrile (38 g) was dissolved in ethanol (250 ml). A solution of anhydrous sodium dithionite (225 g) in hot water (200 ml) was carefully added, and the mixture was refluxed for 3 1/2 hours. The alcohol was removed, and the residual solution acidified by careful addition of conc. hydrochloric acid (200 ml) in water (200 ml). (Sulphur dioxide was generated!) The solution was refluxed for 3/4 hour, treated with decolourising carbon, cooled and made alkaline with 30 % sodium hydroxide. The amine was extracted with ether, washed with water, dried (K_2CO_3) and fractionated. The yield of colourless oil boiling at 173—174°/10 mm was 21 g (66 %). (Found: N 17.6 $C_{10}H_{12}N_2$ requires: N 17.5.)

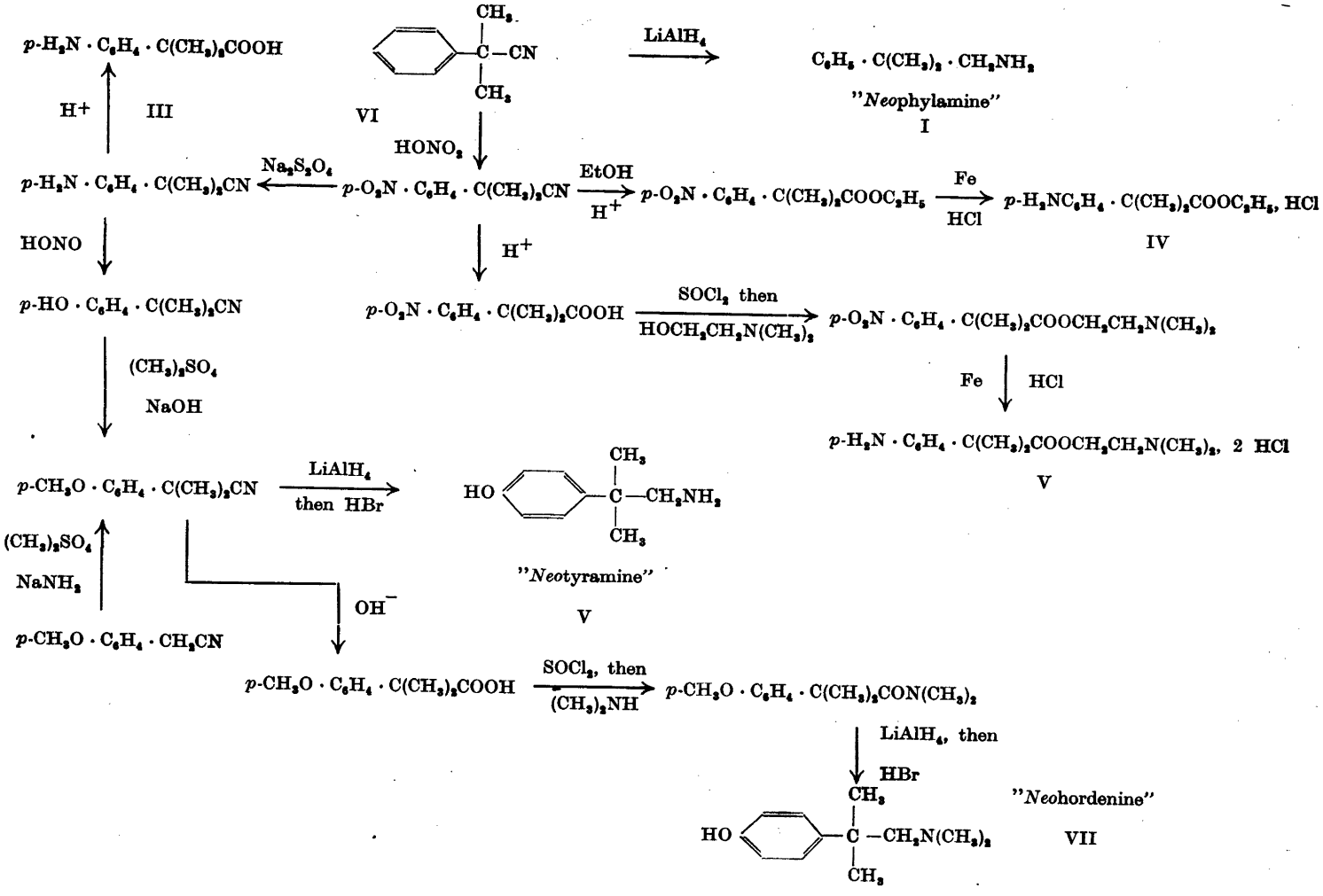
Picrate, yellow needles, m.p. 179—180° (decomp.). (Found: C 49.1; H 3.90. $C_{16}H_{15}N_5O_7$ requires: C 49.4; H 3.88.)

α-(p-Aminophenyl)isobutyric acid. α-*p*-Aminophenyl*iso*butyronitrile (7 g) was heated with conc. hydrochloric acid (20 ml) in a sealed tube at 100° for 8 hours, poured into water (75 ml) and treated with decolourising carbon. Ammonia was added to pH 6, and the amino acid isolated by filtration after cooling overnight in a refrigerator, and washed free from ammonium chloride. Needles, m.p. 217—218° (decomp.). Yield 7 g. (Found: N 7.8; equiv weight 178. $C_{10}H_{13}NO_2$ requires: N 7.8, equiv. weight 179.2.) — The *hydrochloride* was obtained by dissolving the acid in absolute ethanol saturated with hydrogen chloride and precipitating the chloride by careful addition of ether. It crystallised from ethanol-ether in needles, which decomposed at 210—215°. (Found: Cl 6.44. $C_{10}H_{14}ClNO_2$: requires Cl 6.49.)

The *N-acetylderivative,* m.p. 166—167° was prepared from the amino acid and acetic anhydride in pyridine, and crystallised from water. (Found: N 6.38; equiv. weight 223. $C_{12}H_{15}NO_3$ requires: N 6.33; equiv. weight 221.2.)

Ethyl α-(p-aminophenyl)isobutyrate hydrochloride. Ethyl α-*p*-nitrophenyl*iso*butyrate (15 g) was diluted with ethanol (50 ml) and conc. hydrochloric acid (15 ml). Powdered iron (20 g) was added in small portions with occasional shaking and cooling in water so that the temperature did not exceed 50°. When the reduction was complete (disapperance of the yellow colour) the reaction mixture was made slightly alkaline by careful addition of aqueous ammonia. The ferrous hydroxide sludge was filtered off and washed with ethanol. The filtrate and washings were concentrated to a small volume at reduced pressure. Absolute ethanol (100 ml) was added and the solution again concentrated to about 25 ml. The solution was saturated with dry hydrogen chloride, and the aminoester hydrochloride precipitated by careful addition of much ether. Yield 8 g. The compound crystallised from an ethyl acetate-petrol in colourless needles, m.p. 131—133° (decomp.) (Found: Cl 14.5; N 5.83. $C_{12}H_{16}ClNO_2$ requires: Cl 14.6; N 5.75.)

β-(N,N-Dimethylamino)ethyl α-(p-nitrophenyl)isobutyrate hydrochloride. α-*p*-Nitrophenyl*iso*butyryl chloride was prepared by refluxing the acid (20.9 g) with thionyl chloride (25 ml) for one hour on a water bath and removing the excess reagent *in vacuo.* The crude chloride was dissolved in dry benzene (50 ml) and refluxed for two hours with a solution of dimethylaminoethanol (12 g) in dry benzene (50 ml). After cooling the ester hydrochloride was extracted from the benzene solution with 2 *N* hydrochloric acid. The combined extracts were washed twice with ether, made alkaline with potassium carbonate, and the free aminoester was extracted with ether and dried (Na_2SO_4). The ether was removed leaving a light yellow oil (22 g), that did not crystallise. It was dissolved in a little boiling anhydrous ethanolic hydrogen chloride, from which the ester hydrochloride separated on storing. For analysis it was crystallised from dry ethanol. M.p. 148—149°. (Found: Cl 11.1. $C_{14}H_{21}ClN_2O_4$ requires: Cl 11.2.)


p-H2N · C6H4 · C(CH3)2COOH
H+
III
p-H2N · C6H4 · C(CH3)2CN
HONO
p-HO · C6H4 · C(CH3)2CN
(CH3)2SO4
NaOH
p-CH3O · C6H4 · C(CH3)2CN
LiAlH4
then HBr
(CH3)2SO4
NaNH2
p-CH3O · C6H4 · CH2CN
OH−
VI
HONO2
Na2S2O4
p-O2N · C6H4 · C(CH3)2CN
EtOH
H+
H+
p-O2N · C6H4 · C(CH3)2COOH
SOCl2 then
HOCH2CH2N(CH3)2
p-O2N · C6H4 · C(CH3)2COOCH2CH2N(CH3)2
Fe
HCl
p-H2N · C6H4 · C(CH3)2COOCH2CH2N(CH3)2, 2 HCl
V
p-CH3O · C6H4 · C(CH3)2COOH
SOCl2, then
(CH3)2NH
p-CH3O · C6H4 · C(CH3)2CON(CH3)2
LiAlH4, then
HBr
HO
CH2NH2
"Neotyramine"
V
LiAlH4
p-O2N · C6H4 · C(CH3)2CN
C6H5 · C(CH3)2 · CH2NH2
"Neophylamine"
I
Fe
HCl
p-H2NC6H4 · C(CH3)2COOC2H5, HCl
IV
HO
CH2N(CH3)2
"Neohordenine"
VII

β-(N,N-Dimethylamino)ethyl α-(p-aminophenyl)isobutyrate dihydrochloride. Crude β-dimethylaminoethyl α-*p*-nitrophenyl*iso*butyrate hydrochloride (14.2 g) was stirred with water (10 ml). Powdered iron (20 g) was added in small portions. The reaction started almost immediately and the mixture was cooled in water so that the temperature did not exceed 50°. When the reaction was complete, the mixture was repeatedly extracted with ether. The extracts were combined, dried (Na_2SO_4) and evaporated to a small volume. Dry hydrogen chloride was introduced, and the precipitated ester hydrochloride collected. It crystallised from ethanol in needles, m.p. 209—211° (decomp.). (Found: Cl 21.6; N 8.61. $C_{14}H_{24}Cl_2N_2O_2$ requires: Cl 21.9; N 8.67.)

α-(p-Hydroxyphenyl)isobutyronitrile. α-*p*-Aminophenyl*iso*butyronitrile (16.0 g) was dissolved in conc. hydrochloric acid (25 ml) and diazotised at 0 to 5° with an aqueous solution of sodium nitrite (6.9 g). The reaction mixture was immediately poured into a violently boiling mixture of conc. sulphuric acid (50 ml) and water (500 ml). As soon as the evolution of nitrogen ceased, the flask was cooled in ice water. The solution was extracted with ether, the combined ethereal extracts repeatedly shaken with 2 *N* sodium hydroxide and the combined alkaline extracts acidified with dilute sulphuric acid. The hydroxynitrile set free was taken up in ether and dried (Na_2SO_4). The ether was removed and the residual oil distilled *in vacuo*. The colourless oil boiled at 177°/11 mm and crystallised on storing, m.p. 45—48°. Yield 60—70 %. To obtain a good yield, the sulphuric acid must be boiling *violently* during the addition of the diazonium salt solution. (Found: N 8.67. $C_{10}H_{11}NO$ requires: N 8.69.)

The *benzoic acid ester* was prepared in almost quantitative yield from the hydroxynitrile and an equivalent amount of benzoyl chloride in pyridine. Crystallised from a benzene-petrol (1 : 1) it formed colourless needles m.p. 103—104°. (Found: N 5.27. $C_{17}H_{15}NO_2$ requires: N 5.28.)

α-(p-Hydroxyphenyl)isobutyric acid. A sample (5 g) of α-*p*-hydroxyphenyl*iso*butyronitrile was refluxed with 48 % hydrobromic acid (25 ml) for 8 hours, poured into water (75 ml) and cooled. The colourless needles that separated were collected and repeatedly crystallised from small amounts of water. M.p. 152—153°. (Found: C 66.9; H 6.70; equiv. weight 178. $C_{10}H_{12}O_3$ requires: C 66.6; H 6.71; equiv. weight 180.2).

α-(p-Methoxyphenyl)isobutyronitrile: a) *From α-p-hydroxyphenylisobutyronitrile.* α-*p*-Hydroxyphenylisobutyronitrile (8.2 g) and sodium hydroxide (2 g) were dissolved in methanol (50 ml) and with occasional shaking methylated by the simultaneous dropwise addition of dimethyl sulphate (10 ml) and 40 % sodium hydroxide (10 ml). After one hour the mixture was diluted with water (200 ml) and repeatedly extracted with ether. The product distilled as a colourless oil (8.0 g) b.p. 278—280° at atmospheric pressure. (Found: CH_3O 17.6. $C_{11}H_{13}NO$ requires: CH_3O 17.7.)

b) *From p-methoxyphenylbenzyl cyanide.* *p*-Methoxybenzyl chloride was obtained in 40—50 % yield by chloromethylation of anisole by the method of Sommelet and Marszak [7] and in 65—70 % overall yield by reduction of anisaldehyde by the crossed Cannizzaro reaction with formaldehyde by the method of Livshits *et al.*[8] followed by chlorination by shaking for 2 hours with ice cold concentrated hydrochloric acid (5 parts per 1 part anisalcohol).

The chloride was converted to the cyanide in 85 % yield by the method of Lee *et al.*[9].

p-Methoxybenzyl cyanide (76.5 g) was diluted with dry benzene (250 ml) and added to a stirred mixture of sodium amide (70 g, commercial, May & Baker product) and dry benzene (500 ml). Evolution of ammonia started immediately and was completed by heating the mixture for two hours on a water bath. Without cooling dimethylsulphate (198 g) diluted with an equal volume of dry benzene was added drop by drop, with continuous stirring, at such a rate that the mixture refluxed gently. After the addition the mixture was heated for one hour on the water bath, and cooled. Aqueous ethanol (100 ml; 50 %) was carefully added with stirring to destroy any residual amide, followed by conc. aqueous ammonia (100 ml) and water (500 ml). After stirring for half an hour, the benzene layer was separated, the aqueous layer extracted with benzene, and the combined benzene solutions thoroughly washed with water, dilute sulphuric acid, water and dried (K_2CO_3). The solvent was removed using a short column, and the residual oil fractionated *in vacuo*. A colourless liquid (157 g, 63 %) boiling at 140—142°/10 mm, 276—278° at atmospheric pressure, was obtained. (Found: CH_3O 17.6.)

α-(p-Methoxyphenyl)isobutyric acid. α-*p*-Methoxyphenyl*iso*butyronitrile (10 g) potassium hydroxide (10 g) dissolved in water (10 ml) and ethanol (25 ml) were mixed and heated in a stainless steel autoclave at 140° for 20 hours. The liquid was poured into water (150 ml) and acidified. After some hours the crystalline product (10.1 g) was collected. Colourless plates, m.p. 84–86°, raised by crystallisation from water to 89–90°. (Found: CH_3O 16.1; equiv. weight 195. $C_{11}H_{14}O_3$ requires: CH_3O 16.0; equiv. weight 194.2.)

The *amide* was prepared *via* the acid chloride (thionylchloride). It crystallised from water in needles, m.p. 133°. (Found: N 7.22; CH_3O 15.8. $C_{11}H_{15}NO_2$ requires: N 7.25; CH_3O 16.1.) The *N,N-dimethylamide* was obtained as a colourless oil, which distilled at 170–172°/10 mm Hg and did not crystallise. (Found: N 6.23; CH_3O 13.7. $C_{13}H_{19}NO_2$ requires: N 6.33; CH_3O 14.0.)

β-(p-Methoxyphenyl)isobutylamine. α-*p*-Methoxyphenyl*iso*butyronitrile (57 g) dissolved in absolute ether (100 ml) was slowly added to a stirred suspension of lithium- aluminium hydride (15 g) in absolute ether (400 ml) at such a rate that the liquid refluxed rapidly. The mixture was stirred for further 15 minutes, and excess hydride was destroyed by careful addition of moist ether, followed by water. The precipitated aluminium hydroxide was dissolved by addition of 40 % sodium hydroxide solution, the ether layer was separated and the aqueous layer extracted with ether. The combined ethereal solutions were washed with water, and extracted with 2 *N* hydrochloric acid. The amine was liberated with sodium hydroxide solution and taken up in ether. After drying (K_2CO_3) the ether was removed and the residual oil fractionated *in vacuo*. Yield 43 g (74 %) of a colourless oil b.p. 124–126°/10 mm. (Found: CH_3O 16.8. $C_{11}H_{17}NO$ requires: CH_3O 17.3.)

The *hydrochloride* was prepared by dissolving the amine in a little anhydrous ethanolic hydrogen chloride and precipitation of the amine hydrochloride by the addition of much ether. It crystallised from ethanol-ether in needles, m.p. 155–156°. (Found: Cl 16.5; CH_3O 14.2. $C_{12}H_{18}ClNO$ requires: Cl 16.4; CH_3O 14.4.)

The *picrate* was prepared in aqueous methanol, m.p. 215–216°. (Found: CH_3O 7.66. $C_{17}H_{20}N_4O_8$ requires: CH_3O 7.60.)

β-(p-Hydroxyphenyl)isobutylamine, "neotyramine". β-*p*-Methoxyphenyl*iso*butylamine (5 g) and 48 % hydrobromic acid (30 ml) were refluxed for 3 hours. The excess acid was removed on the water bath at reduced pressure, the salt cake dissolved in water (30 ml) and treated with decolourising carbon. The *neo*tyramine was precipitated by adjusting the pH to 9–10 by careful addition of ammonia. The needles, that slowly separated, were collected and washed free from inorganic material. The yield was almost quantitative. M.p. 152–153° (decomp.). (Found: C 72.4; H 9.21; N 8.43. $C_{10}H_{15}NO$ requires: C 72.7; H 9.15; N 8.48.)

The *hydrochloride* was prepared in the usual way and crystallised from an ethanol-ether. Needles, which decomposed at 219–220°. (Found: Cl 17.4; N 6.91. $C_{10}H_{16}ClNO$ requires: Cl 17.6; N 6.94.)

N,N-Dimethyl-β-(p-methoxyphenyl)isobutylamine. N,N-Dimethyl-α-(*p*-methoxyphenyl)*iso*butyramide (30 g) was reduced with lithium aluminium hydride as described for the preparation of β-*p*-methoxyphenyl*iso*butylamine. The yield of amine, b.p. 128–130°/10 mm was 18 g (65 %). (Found: N 6.83; CH_3O 14.8. $C_{13}H_{21}NO$ requires: N 6.95; CH_3O 15.0.)

The *hydrochloride* crystallised from ethanol-ether, m.p. 195–96°. (Found: Cl 14.5; N 5.73. $C_{13}H_{22}ClNO$ requires: Cl 14.6; N 5.75.)

N,N-Dimethyl-α-(p-hydroxyphenyl)isobutylamine, "neohordenine", was prepared in almost quantitative yield by the method described for *neo*tyramine. Needles, m.p. 116–117° (decomp.). (Found: N 7.19. $C_{12}H_{19}NO$ requires: N 7.25.)

The *hydrochloride* crystallised from ethanol-ether in colourless needles, m.p. 200–202° (decomp.). (Found: Cl 15.3; N 6.06. $C_{12}H_{20}ClNO$ requires: Cl 15.4; N 6.10.)

A product identical with the above amine (mixed m.p.) was obtained, when a sample of *neo*tyramine was methylated by the Eschweiler-Clarke procedure [4]. The reaction was carried out as described by Decker and Becker for β-phenethylamine [10].

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Das Gleichgewicht zwischen Äthoxyl- und Hydroxyl-Ionen in wasserhaltigem Äthanol

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Bei der Untersuchung der Basenkatalyse wurde meine Aufmerksamkeit auf das in alkalischem Alkohol-Wasser-Gemisch vorliegende Gleichgewicht

$$OH^- + ROH \rightleftharpoons OR^- + H_2O \qquad (1)$$

gerichtet.

In einer kürzlich erschienenen Arbeit haben J. Hine und M. Hine [1] für die Gleichgewichtskonstante

$$K = \frac{[EtO^-]\,[H_2O]}{[OH^-]\,[EtOH]} \qquad (2)$$

in Isopropanol-Lösung bei 25° C den Wert 0,8 gefunden. Caldin und Long [2] erhielten in Äthanol-Wasser-Gemisch bei derselben Temperatur den Wert $0{,}7 \pm 0{,}2$. Beide Messungen wurden mittels des Indikator-Verfahrens ausgeführt. Ich habe dasselbe Gleichgewicht bei einer Temperatur von 78° C mittels eines anderen Verfahrens untersucht. Die Versuche wurden im Jahre 1951 ausgeführt.

Die Gleichgewichtskonzentrationen können wegen der Störung der Gleichgewichtslage nicht mittels üblicher analytischer Methoden gemessen werden. Die Summe der Konzentrationen der Ionen lässt sich jedoch durch Säure-Basen-Titrierung ermitteln. Auch die Summe der Konzentrationen des Wassers und der Hydroxyl-Ionen lässt sich messen, z.B. nach dem Smith'schen Esterhydrolyse-Verfahren. Die Konzentration des Wassers lässt sich mit Hilfe des Verteilungsgesetzes ermitteln, wobei als zweite Phase aus Löslichkeitsgründen nur die feste (z.B. Salzhydrat) oder die Dampfphase in Betracht kommt. In der vorliegenden Arbeit wurde die Dampfphase gewählt. Grundsätzlich wird mittels des Verteilungsgesetzes der Aktivitätsbruch zwischen Wasser und Äthanol anstatt des Konzentrationsbruches gemessen. Bei Abnahme der Alkalikonzentration nähern sich dieselben asymptotisch und erhalten bei der Alkalikonzentration Null ein und denselben Wert.

Wir bezeichnen die messbaren * Grössen

$$\frac{EtO^- + OH^-}{EtOH} = a, \quad \frac{H_2O + OH^-}{EtOH} = b, \quad \left(\frac{H_2O}{EtOH}\right)_h = f \cdot \left(\frac{H_2O}{EtOH}\right)_n = c, \quad (3)$$

wobei die Ausdrücke H_2O, OH^- usw. die Anzahl der Mole bzw. Grammionen, und die Indizes h und n die Dampf- bzw. Flüssigkeitsphase angeben. Dann ist

$$\frac{OH^-}{EtOH} = b - \frac{c}{f}, \quad \frac{EtO^-}{EtOH} = a - b + \frac{c}{f}, \quad \left[\frac{H_2O}{EtOH}\right]_n = \frac{c}{f} \quad (4)$$

$$K = \frac{[f(a-b) + c]c}{(f \cdot b - c)f} \quad (5)$$

Bei Abnahme des Alkaligehalts nähert sich f dem Wert f_0 des reinen Äthanol-Wasser-Gemisches. Schreiben wir

$$K' = \frac{[f_0(a-b) + c]c}{(f_0 \cdot b - c)f_0}, \quad (6)$$

so nähern sich die K'-Werte bei Abnahme des Alkaligehaltes dem von K. Ist der Salzeffekt des Alkalis auf das Flüssigkeit-Dampf-Gleichgewicht gering, so ist $K' = K$.**

VERSUCHE

Die Äthanol-Lösungen, die wenig Wasser und variierende Mengen von Natriumäthylat enthielten, wurden bei gewöhnlichem Druck aus einem 300 ml fassenden Kolben destilliert. Der Kühler war direkt mit dem Kolben verbunden und der Oberteil des Kolbens bis zum Kühler mittels Asbest wärmeisoliert. Die Lösungsproben wurden durch eine Öffnung im Oberteil des Kolbens entnommen. Die Destillatproben wurden direkt in Ampullen aufgenommen. Der Destillationskolben wurde möglichst voll gehalten und zwar in allen Destillation gleich voll. Die Destillationsgeschwindigkeit betrug 3 Tropfen pro Sekunde und wurde in allen Destillationen konstant gehalten. Zunächst wurde *ca.* 10 min. destilliert, um das stationäre Wärmegleichgewicht zu erreichen und um das in den Destillationsvorstössen eventuell vorhandene Wasser in die Flüssigkeit zu spülen. Unter fortgesetztem Erhitzen wurde das Destillat in den Kolben zurückgegeben. Nach *ca.* 5 min. weiterem Destillieren wurden vom Destillat und vom Destillationsgut die Proben entnommen (etwa 3 ml) die unmittelbar in über Phosphorpentoxyd vakuumgetrocknete Ampullen eingefüllt wurden. Die Menge der Proben wurde durch Wägen bestimmt, das Gesamtalkali durch Titrieren mit Salzsäure, die Summe des Wassers und Hydroxyls durch ein dem Smith'schen [3] Esterhydrolyse-Prinzip ähnliches Verfahren, das in einer folgenden Veröffentlichung [5] beschrieben wird. Zur Verminderung der Versuchsfehler waren die

* Streng genommen sind diese Grössen nur dann messbar, wenn die Menge des Alkalis im Verhältnis zur Menge des Äthanols verschwindend gering ist. Da in dieser Arbeit die Molzahl des Äthanols in Bezug auf diejenigen des Alkalis und Wassers 50—200-fach ist, und da das Alkali hauptsächlich als Natriumäthylat vorliegt, so lässt sich die Äthanolmenge genügend exakt durch Abziehen des auf Natriumäthylat umgerechneten titrierten Alkalis vom Wägeergebnis ermitteln.

** Bei konstantem Druck ist der Siedepunkt der Lösung von deren Zusammensetzung abhängig. Die Siedepunkte sowohl des alkalischen als des alkalifreien Äthanol-Wassergemisches in dem hier vorliegenden Konzentrationsgebiet liegen einander derart nahe dass die Werte von K' bzw. K fast konstant bleiben. Auf die Herleitung der Gleichung sind diese Umstände ohne Einfluss. In diesem Zusammenhang sei bemerkt, dass die Angabe von Noyes [4], laut der eine *ca.* 1 molare äthanolische Lösung von Natriumäthylat auf dem Wasserbade nicht siedet, ein Irrtum ist, der auf Siedeverzug beruhen dürfte. Nach einer von mir ausgeführten groben Messung übersteigt die Temperatur des Dampfes nicht das Normale.

Proben möglichst gleich gross, und bei der Messung des Wassergehalts wurden stets gleiche Mengen Alkalialkoholat-Esterlösung genommen. Zur Kontrolle wurden mitunter auch das Wasser des Destillats und des Destillationsguts gemeinschaftlich gemessen. Dabei wurde in Parallelversuchen eine Genauigkeit von 1 % erreicht.

Da die Destillatproben nur *ca.* 1 % des Destillationsguts betrugen, und da die Kurve des Flüssigkeits-Dampfdiagramms der Gleichgewichtsdestillation ziemlich in Richtung der Diagonale und nahe derselben verläuft, so ist die Veränderung des Wassergehalts während der Probenahme verschwindend gering.

Vom Standpunkt der in dieser Arbeit ausgeführten Versuche ist es nicht wichtig, dass die Destillation eine vollkommene Gleichgewichtsdestillation ist; dagegen ist es unumgänglich, dass die Destillationskurve durch Gleichhalten der Bedingungen unverändert gehalten wird. Die Gleichgewichtsdestillations-Ergebnisse anderer Forscher [6] liegen nahe denjenigen dieser Arbeit.

Das Äthanol zu den Versuchen wurde durch Kochen von gewöhnlichem absolutem Alkohol mit wenig Natriumhydroxyd, Destillieren und Behandlung des Destillats mit Aluminiumamalgam erhalten [7]. Zur Bereitung des Natriumäthylats wurde reines Natrium verwendet.

VERSUCHSERGEBNISSE

Tabelle 1 gibt die Zusammensetzung der Flüssigkeit und des entsprechenden Dampfs beim Destillieren von alkalifreiem, wässerigem Äthanol. Es zeigt sich, dass das Verhältnis der Zusammensetzung von Flüssigkeits — und Dampfphase innerhalb der Fehlergrenzen in diesem kleinen Konzentrationsgebiet konstant ist. Auf Grund der Form der Flüssigkeit-Dampf-Gleichgewichtskurve wäre zu erwarten, dass in diesem Gebiet die Konzentration in der Dampfphase ein wenig langsamer als in der Flüssigkeitsphase abnimmt.

Da die Wassergehalte bei der Destillation der alkalischen Lösungen von gleicher Grössenordnung wie bei derjenigen der alkalifreien sind, so kann zur Berechnung der Gleichgewichtskonzentrationen der Mittelwert der Werte der Tabelle 1 verwendet werden. Die Bezeichnung $\frac{H_2O}{EtOH}$ betrifft das Verhältnis der Anzahl Mole.

Tabelle 1. Zusammensetzung von Flüssigkeits- und Dampfphase in alkalifreier Lösung.

$\frac{H_2O}{EtOH}$		$\frac{\text{Zusammensetzung des Dampfes}}{\text{Zusammensetzung der Flüssigkeit}} = f_0$
Flüssigkeit	*Dampf*	
0,01119	0,01222	1,094
0,01230	0,01340	1,090
0,01491	0,01635	1,096
0,01497	0,01642	1,096
0,01528	0,01669	1,092
	Mittelwert	1,094

Durch Vergleich der Versuchsergebnisse verchiedener Forscher habe ich für das Verhältnis $\frac{\text{Zusammensetzung des Dampfes}}{\text{Zusammensetzung der Flüssigkeit}}$ in der Gleichgewichtsdestillation auf gleichem Konzentrationsgebiet und bei gleichem Druck

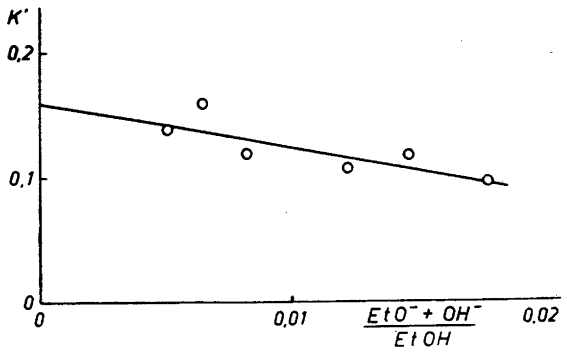

Fig. 1. Abhängigkeit von K' von der Gesamtalkalikonzentration $\frac{EtO^- + OH^-}{EtOH}$

den Wert 1,09 erhalten. Wegen einem geringen Wärmeverlust ist der in dieser Arbeit gefundene Wert etwas grösser, nämlich 1,094.

Tabelle 2 zeigt die Destillation des alkalischen wässerigen Äthanols. Sie enthält auch die nach den Gleichungen 3—6 berechneten Grössen $\left(\frac{H_2O}{EtOH}\right)'$, $\left(\frac{OH^-}{EtOH}\right)'$, $\left(\frac{EtO^-}{EtOH}\right)'$ und K', die sich bei Abnahme des Alkaligehalts den entsprechenden Molverhältnissen, bzw. der Gleichgewichtskonstante K nähern.

Tabelle 2. Zusammensetzung von Flüssigkeits- und Dampfphase in alkalischer Lösung.

Dampf $\frac{H_2O}{EtOH}$	Flüssigkeit					
	$\frac{EtO^- + OH^-}{EtOH}$	$\frac{H_2O + OH^-}{EtOH}$	Rechengrössen			
			$\left(\frac{H_2O}{EtOH}\right)'$	$\left(\frac{OH^-}{EtOH}\right)'$	$\left(\frac{EtO^-}{EtOH}\right)'$	K'
0,0134	0,0051	0,0127	0,0123	0,0004	0,0047	0,14
0,0151	0,0065	0,0143	0,0138	0,0005	0,0059	0,16
0,0146	0,0082	0,0141	0,0133	0,0008	0,0074	0,12
0,0117	0,0123	0,01175	0,0107	0,00105	0,0113	0,11
0,0155	0,0147	0,0157	0,0142	0,0015	0,0132	0,12
0,0099	0,0179	0,0105	0,0091	0,0014	0,0165	0,10

Fig. 1 zeigt die Abhängigkeit von K' von der Gesamtalkalikonzentration $\frac{EtO^- + OH^-}{EtOH}$. Lineare Extrapolation auf die Alkalikonzentration Null gibt für die Gleichgewichtskonstante $K = \frac{[EtO^-]\,[H_2O]}{[OH^-]\,[EtOH]}$ den Wert 0,16 bei 78° C.

BESPRECHUNG DER ERGEBNISSE

Nach Tabelle 2 und Fig. 1 ist der Wert der Hilfsgrösse K' nicht besonders von der Alkalikonzentration abhängig, d.h. er weicht nur wenig von dem der Konzentrations-Gleichgewichtskonstanten K ab. Da das betr. Gleichgewicht symmetrisch in Bezug auf Anzahl und Ladung der Ionen ist und da das Natriumhydroxyd sowohl in Wasser als in Alkohol leicht löslich ist, so ist schon zu erwarten, dass die Salzwirkung nicht gross ist.

Der erhaltene Wert der Gleichgewichtskonstanten bei der Temperatur 78° C ist nur ein Drittel bis Fünftel des von Caldin und Long [2] bei 25° C erhaltenen Wertes. Der Zusammenhang zwischen Gleichgewichtskonstante, Reaktionswärme und Temperatur wird durch die van't Hoffsche Gleichung ausgedrückt. Die Reaktionswärme ist nicht bekannt. Aus den Flüssigkeit-Dampf-Gleichgewichtskurven [7] geht hervor, dass in dem Konzentrationsgebiet der vorliegenden Arbeit (*ca.* 99,5 % Äthanol) die Aktivität des Wassers mit der Temperatur schneller zunimmt als die des Äthanols. Wenn die Wirkung der Temperatur auf die Aktivitätskoeffizienten der Ionen EtO^- und OH^- gleichgross wäre, so würde sich wegen der schnelleren Zunahme der Aktivität des Wassers das Gleichgewicht (1) zugunsten des Hydroxyl-Ions und des Äthanols verschieben — in Übereinstimmung mit dem in dieser Arbeit erhaltenen Ergebnis.

Die Genauigkeit des erhaltenen Wertes der Gleichgewichtskonstante ist etwas schwierig zu beurteilen, da sich der Fehler vor allem in der in Fig. 1 ausgeführten Extrapolation verbirgt. Es ist anzunehmen, dass der relative Fehler 30 % nicht übersteigt.

ZUSAMMENFASSUNG

1. Ein Verfahren zur Messung der Lage des Gleichgewichts $OH^- + ROH \rightleftharpoons OR^- + H_2O$ ist entwickelt worden.

2. Die Gleichgewichtskonstante $K = \frac{[EtO^-][H_2O]}{[OH^-][EtOH]}$ für wenig Wasser enthaltende Äthanol-Lösungen bei 78° C ist bestimmt worden.

3. Die Temperatur- und Salzwirkung auf das vorliegende Gleichgewicht ist erörtert worden.

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Die alkalische Hydrolyse von einfachen Estern in absoluten Alkoholen. I. Die Ordnung der Reaktion

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Zahlreiche Untersuchungen bezeugen, dass die Geschwindigkeit der alkalischen Hydrolyse in Wasser-Alkoholgemischen stark vom Wassergehalt des Lösungsmittels abhängt[1]. In wasserfreien Alkoholen verursacht Alkalialkoholat keine Salzbildung. Dagegen findet eine Umesterung statt, wenn die Alkoxygruppe des Esters verschieden ist von derjenigen des als Lösungsmittel dienenden Alkohols.

In Lösungen von Alkalihydroxyd in Wasser-Alkoholgemischen herrscht das Gleichgewicht

$$OH^- + ROH \rightleftharpoons OR^- + H_2O \qquad (1)$$

woraus

$$[OH^-] = \frac{[OR^-][H_2O]}{K[ROH]} \quad ; \; K = \text{Gleichgewichtskonstante} \qquad (2)$$

Es wird allgemein angenommen, dass die Hydrolyse durch das Hydroxyl-Ion katalysiert wird. Seine Konzentration ist in absoluten Alkoholen nach Gleichung (2) proportional der zweiten Potenz der Konzentration des gelösten Alkalihydroxyds. Es ist nämlich anzunehmen, dass die Reaktion der Gleichung (1) in verdünnten Lösungen ziemlich quantitativ von links nach rechts verläuft, wobei dann die Konzentrationen des Alkalialkoholats und des Wassers ebenso gross wie die stöchiometrische Alkalikonzentration sind. Man kann also schreiben

$$[OH^-] = \frac{c^2}{K[ROH]} \qquad (3)$$

wo c die stöchiometrische Konzentration des Alkalis ist.

Wenn also das Hydroxyl-Ion die die Hydrolyse katalysierende Partikel ist, so ist die Geschwindigkeit der Hydrolyse in absoluten Alkoholen proportional zur zweiten Potenz der Konzentration des gelösten Alkalis, und die Gesamtordnung der Hydrolyse ist 3.

Wenn wiederum die Geschwindigkeit der Hydrolyse proportional dem Produkt der Konzentrationen des Totalalkalis und des Wassers ist, d.h. wenn

das Wasser an der Reaktion in deren geschwindigkeitsbestimmenden Stufe teilnimmt, so ist auch dann die Ordnung der Hydrolyse in absoluten Alkoholen gleich 3.

Bei früheren Hydrolyseversuchen in absoluten Alkoholen ist die Alkalikonzentration gering gewesen, meistens 0,05 *N*. Die Wasserkonzentration lässt sich nur mittels effektiver Trockenmittel und unter sorgfältiger Behandlung beträchtlich erniedrigen. Darauf ist es zurückzuführen, dass die bisherigen Versuche kein richtiges Bild über die Kinetik der Esterhydrolyse in absoluten Alkoholen ergeben haben. Wegscheider [2] hat jedoch festgestellt, dass die gewöhnliche Gleichung zweiter Ordnung keine Konstanz für *k* gibt.

Es war deshalb wünschenswert, die Esterhydrolyse in absoluten Alkoholen erneut zu untersuchen, und zwar in Methanol, Äthanol, *n*-Propanol und *iso*-Propanol, mit dem Acetat des jeweiligen Alkohols als Esterkomponente und mit Natriumhydroxyd als Katalysator.

VERSUCHE

Die Methyl-, Äthyl-, *n*-Propyl- und *iso*-Propylacetate wurden aus Essigsäureanhydrid und den entsprechenden Alkoholen dargestellt. Nach 2—3facher Destillation enthielt der Ester weder Essigsäureanhydrid noch Essigsäure. Durch vollständige Hydrolyse wurde festgestellt, dass der Reinheitsgrad der Ester wenigstens 99 % betrug.

Methanol und Äthanol waren gewöhnliche absolute Handelswaren, *n*- und *iso*-Propanol von May & Baker für Laboratoriumszwecke. Vor dem Trocknen wurden die Alkohole mit wenig Natriumhydroxyd gekocht, um eventuell vorhandene verseifbare oder saure Substanzen zu entfernen. Das Destillat wurde mit Aluminiumalkoholat [3] getrocknet, im Falle Methanol mit Magnesiummethylat. [4]

Das analysenreine Natriumhydroxyd wurde im Exsikkator über Phosphorpentoxyd im Vakuum getrocknet.

Der Wassergehalt der getrockneten Alkohole wurde nach dem folgendermassen modifizierten Verfahren von Smith [5] bestimmt. Aus Natrium und (in der Regel) *n*-Propanol wurde eine *ca.* 0,1 *N* Alkoholatlösung bereitet, die mit Propylacetat bis zu einem Gehalt von *ca.* 0,4 *N* versetzt wurde. Die Lösung wurde zur Beseitigung des Wassers während einer Stunde auf 70—80° erhitzt und, durch Calciumchlorid- und Natronkalkrohre gegen Feuchtigkeit und Kohlendioxyd geschützt, in einem Gefäss mit Mikrobürette aufbewahrt.

Zur Bestimmung des Wassergehalts wurden 1—4 ml Alkohol in eine exsikkatortrokkene Glasampulle einpipettiert, aus der der Luftsauerstoff durch Stickstoff verdrängt worden war. Aus der Mikropipette wurde 1 ml der obengenannten Natriumalkoholat-Esterlösung zugefügt. Nach dem Zuschmelzen wurde die Ampulle je nach dem Alkohol und dessen Wassergehalt 10—50 Min. in siedendem Wasser gehalten, wonach sie in eine dem ursprünglichen Alkali entsprechende Salzsäuremenge entleert wurde. Der Alkalischwund gibt die Wassermenge an. Oft wurde zur Beseitigung von Fehlern ein Parallelversuch mit Natriumalkoholat-Estergemisch ausgeführt. Die Genauigkeit des Verfahrens beträgt beim Arbeiten mit oben beschriebenen Mengen 0,002—0,004 Mol Wasser pro Liter. Mit grösseren Substanzmengen dürfte eine grössere Genauigkeit erzielbar sein.

Die Alkalihydroxydlösungen wurden durch Lösen von Natriumhydroxyd in Alkohol oder aus Natrium und Alkohol bereitet, in diesem Fall unter Zusatz der äquivalenten Menge Wasser. Sowohl die Alkali- als die Esterlösungen wurden wie die oben beschriebene Natriumalkoholat-Esterlösung in Gefässen mit Mikrobürette aufbewahrt.

Als Reaktiongefässe wurden Glasampullen (2—10 ml) benutzt. Verlief die Reaktion bei Zimmertemperatur schnell, so wurde bei der Bereitung des Reaktionsgemisches und beim Zuschmelzen die Ampulle in Eiswasser gehalten. Die Reaktionsdauer wurde dabei von dem Augenblick an gerechnet, in dem die Ampulle in den Thermostaten eingeführt wurde. Nach besonderen Versuchen verlief die Temperierung dieser kleinen Flüssigkeitsmengen durch Schütteln der Ampulle derart schnell, dass der Fehler nicht 1 % überschritt. Die Reaktion wurde durch Eingiessen des Reaktionsgemisches in eine dem ur-

sprünglichen Alkali entsprechende Menge Salzsäure unterbrochen. Die Titration wurde teils mit karbonatfreiem Natriumhydroxyd, teils mit Bariumhydroxyd und Phenolphthalein oder Kresolrot als Indikator ausgeführt. Am Äquivalenzpunkt betrug der Wassergehalt im allgemeinen über 70 %.

Die Thermostaten waren durch Toluolschalter geregelt, die Temperaturschwankungen betrugen 0,02°. (Die Thermometer waren mit einem Standardthermometer verglichen.)

VERSUCHSERGEBNISSE

Die Ergebnisse sind in den Tabellen 1—3 wiedergegeben. Die Reaktionsgeschwindigkeitskonstanten sind aus den bekannten Geschwindigkeitsgleichungen für Reaktionen 3. Ordnung berechnet worden,

$$k = \frac{1}{t}\left(\frac{1}{(a-x)^2} - \frac{1}{a^2}\right) \tag{4}$$

$$k = \frac{1}{t(a-b)^2}\left[\frac{(b-a)x}{a(a-x)} + \ln\frac{b(a-x)}{a(b-x)}\right] \tag{5}$$

wobei Gleichung (4) benutzt wurde, wenn die Konzentrationen von Ester und Alkali gleich, Gleichung (5), wenn sie verschieden waren. Die Grösse a ist die Ausgangskonzentration des Alkalis, b die des Esters. Als Konzentrationseinheit dient 1 Mol/l, als Zeiteinheit 1 s. Eine Korrektur für die Wärmeausdehnung ist nicht angebracht worden; die Konzentration ist diejenige bei der Bereitungstemperatur der Lösungen (20° C.)

In den Tabellen bezeichnet w den gemessenen Wassergehalt der Lösungen (nicht herrührend von der Reaktion nach Gleichung (1)), p Umsatz in Prozenten während der Zeit t.

Tabelle 1. Beispiele von Reaktionsgeschwindigkeitskonstanten bei gleicher Konzentration von Alkali und Ester.

Methanol
$a = 0{,}1$; $w<0{,}003$; 50° C

$10^{-3}t$	18	58	149	186	407
p	17,4	36,0	54,1	59,2	70,6
10^3k	1,29	1,24	1,25	1,34	1,29

n-Propanol
$a = 0{,}125$; $w\approx0{,}003$; 25° C

$10^{-3}t$	3	5	6,3	10,8	19,5	43,2	110
p	18,1	25,7	30,8	40,8	51,1	65,6	77,7
10^3k	5,2	5,2	5,5	5,1	5,2	5,5	5,5

Äthanol
$a = 0{,}2$; $w<0{,}003$; 25° C

$10^{-3}t$	3,6	6,6	23,4	46,2	92,4	172	262
p	13,3	21,3	43,7	56,6	67,1	75,0	79,1
10^3k	1,15	1,16	1,15	1,17	1,12	1,09	1,05

iso-Propanol
$a = 0{,}075$; $w\approx0{,}003$; 25° C

$10^{-3}t$	1,8	3,6	5,1	10,2	13,2	40,5
p	12,1	22,0	27,1	40,7	45,6	64,8
10^3k	14,7	15,9	15,4	16,1	16,0	15,5

BESPRECHUNG DER ERGEBNISSE

Aus Tabelle 1 geht hervor, dass die Reaktion gute Geschwindigkeitskonstanten für eine Reaktion 3. Ordnung liefert. Aus Tabelle 2 wieder ist ersichtlich, dass die Reaktion in Bezug auf Alkali von 2. Ordnung und in Bezug auf Ester von 1. Ordnung ist, denn nur in diesem Falle gibt die entsprechende

Tabelle 2. Beispiele von Reaktionsgeschwindigkeitskonstanten bei verschiedener Konzentration von Alkali und Ester. 25° C.

Äthanol

	$a = 0{,}4$; $b = 0{,}2$; $w<0{,}003$				$a = 0{,}2$; $b = 0{,}4$; $w<0{,}003$			
$10^{-3}t$	1,57	3,18	4,8	12	2,69	7,8	16,8	32,4
p	23,4	38,9	49,5	74,3	19,2	39,4	56,7	69,3
10^3k	1,24	1,22	1,21	1,20	1,16	1,18	1,20	1,16

n-Propanol

	$a = 0{,}125$; $b = 0{,}0625$; $w \approx 0{,}003$				$a = 0{,}0625$; $b = 0{,}125$; $w \approx 0{,}003$			
$10^{-3}t$	3	10,7	22,2	28,9	5,81	14,8	58	87
p	19,1	47,0	68,0	74,3	18,3	35,2	66,8	72,2
10^3k	5,1	5,1	5,4	5,3	5,3	5,2	5,8	5,2

iso-Propanol

	$a = 0{,}1$; $b = 0{,}05$; $w \approx 0{,}003$				$a = 0{,}05$; $b = 0{,}1$; $w \approx 0{,}003$			
$10^{-3}t$	1,53	3,9	10,5	13,4	2,54	3,9	5,4	11,2
p	19,2	40,7	66,4	71,7	16,4	29,5	34,7	52,0
10^3k	15,3	16,8	16,7	16,1	16,4	22,6	21,7	23,2

Tabelle 3. Anfangskonzentrationen und Reaktionsgeschwindigkeitskonstanten. $a = b$.

Methanol				Äthanol			*n*-Propanol			*iso*-Propanol			
a	10^4k			a	10^4k		a	10^4k		a	10^4k		
	50°	40°	25°		50°	25°		25°	0°		50°	25°	0°
0,5	16,8	6,8	1,61	0,4	144	13,2	0,125	52	3,8	0,075	1 090	156	15,0
0,25	14,0		1,36	0,2		11,6	0,0833	52		0,0375	1 110		
0,125	12,8			0,125		11,0	0,0625	52					
0,1	12,5			0,08		10,7	0,0416	47					
0,05	12,6			0,04		10,3	0,0208	49					

Geschwindigkeitsformel (5) konstante Werte. Wie Tabelle 3 zeigt, wächst die Geschwindigkeitskonstante 3. Ordnung wenigstens in Methanol- und Äthanollösungen mit dem Anwachsen der Anfangskonzentration des Alkalis, d.h. die Reaktion hat eine positive Salzwirkung. Unter Berücksichtigung der Wirkungsweise des Wassers, die in der folgenden Arbeit beschrieben wird, wäre eher zu erwarten, dass die Geschwindigkeitskonstante mit dem Abnehmen der Anfangskonzentration anwüchse.

Wie erwähnt, ist die Hydrolyse in absoluten Alkoholen von 2. Ordnung in Bezug auf Alkali sowohl in dem Falle, dass die geschwindigkeitsbestimmende Stufe von einem Estermolekül und einem Hydroxyl-Ion geliefert wird, als auch in dem Falle, dass diese Stufe von einem Ester-und einem Wassermolekül geliefert wird, katalysiert durch das Totalalkali. Sogar eine Reaktion zwischen Estermolekül, Wassermolekül und Hydroxyl-Ion in der geschwindigkeitsbestimmenden Stufe führt zu demselben Ergebnis.

Wie aus den Tabellen ersichtlich ist, wächst die Geschwindigkeit der Hydrolyse in der Reihenfolge Methanol, Äthanol, *n*-Propanol, *iso*-Propanol. In wässriger Lösung sind die Hydrolysengeschwindigkeiten der Acetate dieser Alkohole bei 25° : [6]

Methylacetat,	$k = 0{,}189$	k = Geschwindigkeitskonstante
Äthylacetat,	$k = 0{,}113$	zweiter Ordnung.
n-Propylacetat,	$k = 0{,}094$	
i-Propylacetat,	$k = 0{,}0350$	

In absoluten Alkoholen sind die Geschwindigkeiten bei 25°, ausgedrückt als Geschwindigkeitskonstanten dritter Ordnung:

Methylacetat in Methanol,	$k = 0{,}000128$
Äthylacetat in Äthanol,	$k = 0{,}00110$
n-Propylacetat in *n*-Propanol,	$k = 0{,}0052$
i-Propylacetat in *i*-Propanol,	$k = 0{,}0156$

Die Hydrolysengeschwindigkeiten dieser Ester unterscheiden sich in absoluten Alkoholen viel mehr als im Wasser, z.B. Methanol verzögert die Hydrolyse des Methylacetats etwa 600 mal mehr als *iso*-Propanol diejenige des *iso*-Propylacetats. Die hydrolysengeschwindigkeitsverzögernde Wirkung der verschiedenen Alkohole auf die entsprechenden Acetate verhalten sich wie die Zahlen

600	48	8	1
Methanol	Äthanol	*n*-Propanol	*iso*-Propanol

Die Stärken von Methanol, Äthanol, *n*-Propanol und *iso*-Propanol als Säuren verhalten sich wie die Zahlen 53, 12,5, 6,6, und 1 [7]. Umgekehrt verhalten sich die Hydroxyl-Ion-Konzentrationen in Alkohollösungen, deren Alkalihydroxydkonzentration in Molbrüchen die gleiche ist. In Volumenkonzentrationen ausgedrückt sind die Zahlen 100, 17, 6,6 und 1. Es existiert also eine bemerkenswerte Korrelation zwischen den Säurestärken der Alkohole und deren verzögernder Wirkung auf die Hydrolysengeschwindigkeit. Auf Grund hiervon könnte man annehmen, dass die Esterhydrolyse eine Reaktion zwischen nur Estermolekül und Hydroxyl-Ion ist.

ZUSAMMENFASSUNG

Die Geschwindigkeit der alkalischen Hydrolyse von Methyl-, Äthyl-, *n*-Propyl- und *i*-Propylacetat in Methanol, Äthanol, *n*-Propanol bzw. *i*-Propanol bei verschiedenen Anfangskonzentrationen und Temperaturen ist gemessen worden.

Es wurde gefunden, dass die Geschwindigkeit der Hydrolyse in absoluten Alkoholen proportional der zweiten Potenz der Konzentration des Alkalihydroxyds und der ersten der des Esters ist, womit die Hydrolyse von dritter Ordnung ist. Die Geschwindigkeitskonstante dritter Ordnung wächst mit der Zunahme der Anfangskonzentration des Alkalis, wenigstens in Methanol und Äthanol.

Das Verfahren von Smith zur Bestimmung des Wassergehalts ist modifiziert worden. Der Wassergehalt der in den Versuchen benutzten Alkohole wurde gemessen.

Der Einfluss der verschiedenen Alkohole auf die Geschwindigkeit der Esterhydrolyse in Abhängigkeit der Säurestärken der Alkohole ist erörtet worden.

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Die alkalische Hydrolyse von einfachen Estern in absoluten Alkoholen. II. Die Wirkung von Alkalialkoholat und Wasser

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In der vorangehenden Arbeit [1] wurde festgestellt, dass die Esterhydrolyse in absoluten Alkoholen eine Reaktion 3. Ordnung ist, wobei die Geschwindigkeit proportional dem Produkt aus der zweiten Potenz der Konzentration des Alkalihydroxyds und der ersten Potenz der Konzentration des Esters ist. Unter der üblichen Annahme, dass die Hydrolyse in der geschwindigkeitsbestimmenden Stufe eine Reaktion zwischen Estermolekül und Hydroxyl-Ion ist, beeinflusst das Wasser die Geschwindigkeit der Hydrolyse (über das Gleichgewicht):

$$OH^- + ROH \rightleftharpoons OR^- + H_2O \qquad (1)$$

Auch in dem Falle, dass die Hydrolyse in ihrer geschwindigkeitsbestimmenden Stufe eine Reaktion zwischen Estermolekül und Wassermolekül ist, ist sie in absoluten Alkoholen von 3. Ordnung. Als Katalysator würden dabei Lösungsmittelanionen wirken.

Aus der Gleichgewichtsgleichung (1) erhält man für die Konzentration des Hydroxylions

$$[OH^-] = \frac{[OR^-]\,[H_2O]}{[ROH] \cdot K}, \qquad (2)$$

wonach sie direkt proportional der Konzentration sowohl des Alkalialkoholats als des Wassers ist. Es wurde deshalb untersucht, ob die Einwirkung von Alkalialkoholat und Wasser auf die Geschwindigkeit der Hydrolyse der Gleichung (2) entspricht, unter der Annahme, dass die Esterhydrolyse in der geschwindigkeitsbestimmenden Stufe eine Reaktion zwischen Estermolekül und Hydroxyl-Ion ist.

VERSUCHE

Die Versuche wurden mit Methyl-, Äthyl- und *n*-Propylacetat ausgeführt mit den entsprechenden Alkoholen Methanol, Äthanol bzw. *n*-Propanol als Lösungsmittel. Der Katalysator bestand aus einem Gemisch von Natriumhydroxyd mit Natriummethylat,

-äthylat bzw. -propylat. Die Alkalialkoholate wurden aus Natrium und den Alkoholen dargestellt. Die Darstellung der Ester, die Trocknung der Alkohole und die Messung ihres Wassergehalts geschah wie in der vorangehenden Arbeit [1] beschrieben worden ist. Die Geschwindigkeit wurde wie früher gemessen.

VERSUCHSERGEBNISSE

Die Versuchsergebnisse sind in den Tabellen 1—4 angegeben. Die Versuche der Tabellen 1 und 2 beschreiben die Wirkung des Wassers auf die Reaktionsgeschwindigkeit, die der Tabelle 3 die Wirkung des Alkalialkoholats. Die Salzwirkung ist in den Versuchen der Tabelle 4 untersucht worden.

Betrachtet man die Alkoholat-Ionen-Konzentration in einer absoluten oder nur wenig Wasser enthaltenden, verdünnten alkoholischen Lösung als approximativ die Summe der Konzentrationen des zugefügten Alkalialkoholats und Alkalihydroxyds, sowie entsprechend die Wasserkonzentration als die der Summe der Konzentrationen des zugefügten Wassers und Alkalihydroxyds, so erhält man aus Gleichung (2) bei konstanter Alkoholkonzentration

$$[\mathrm{OH}] = K' \cdot (a + b)(b + c)$$

Die Grösse a bezeichnet die stöchiometrische Konzentration des zugefügten Wassers, b die des zugefügten Alkalihydroxyds und c die des zugefügten Alkalialkoholats. Bezeichnet man die Konzentration des Esters mit d so erhält man für die Geschwindigkeit der Hydrolyse die Gleichung

$$\frac{\mathrm{d}x}{\mathrm{d}t} = k \cdot (a + b - x)\,(b + c - x)\,(d - x).$$

Wenn zwei der Konzentrationen gleich sind, erhält man durch Integrieren die Geschwindigkeitskonstante für Reaktionen 3. Ordnung

$$k = \frac{1}{t(c_1 - c_2)^2}\left[\frac{(c_1 - c_2)x}{c_2(c_2 - x)} + \ln\frac{c_1(c_2 - x)}{c_2(c_1 - x)}\right]$$

In den vorliegenden Versuchen sind im allgemeinen zwei der Konzentrationen gleich gewählt worden, entweder Gesamtalkali und Ester oder Gesamtwasser und Ester.

Die übrigen Bezeichnungen der Tabellen bedeuten:

t = Reaktionsdauer in Sekunden

p = Umsatz in Prozenten in der Zeit t.

Die Konzentrationseinheit ist 1 Mol/l. Bei der Berechnung sind die Konzentrationen bei der Bereitungstemperatur der Lösungen (20°) benutzt worden.

Die in Tabelle 2 angeführten Geschwindigkeitskonstanten sind aus der Formel

$$k = \frac{1}{t}\left\{\frac{10^4}{2b^2}\left(\frac{1}{(100\text{-p})^2} - \frac{1}{100^2}\right) - \frac{1}{a^2}\left[\ln\frac{1 + \dfrac{100f}{100\text{-}p}}{1 \times f} - \frac{1}{2}\left(\frac{100f}{100\text{-}p} - f\right)\left(2 - \frac{100f}{100\text{-}p} - f\right)\right]\right\}$$

Tabelle 1. Beispiele von Reaktionsgeschwindigkeitskonstanten.
Methanol; 25° C.

	$a = 0{,}567$; $b = d = 0{,}25$				$a = 2{,}80$; $b = d = 0{,}25$	
t	5 400	12 600	55 800	1 410	3 600	18 100
p	12,0	23,5	55,7	14,1	29,5	68,3
10^4k	1,35	1,33	1,30	1,51	1,55	1,54

Äthanol; 25° C.

	$a = 0{,}4$; $b = d = 0{,}2$			$a = 0{,}045$; $b = d = 0{,}2$			
t	2 100	9 300	39 900	1 300	2 400	30 500	94 800
p	22,6	53,5	81,0	8,5	11,6	54,7	73,0
10^3k	1,21	1,16	1,12	1,17	1,18	1,16	1,07

	$a = 0{,}4$; $b = d = 0{,}08$			$a = 0{,}08$; $b = d = 0{,}0444$		
t	12 000	37 320	84 000	54 300	143 100	467 000
p	27,6	53,0	70,4	23,3	40,2	67,4
10^3k	1,03	1,02	0,98	0,95	0,93	0,94

	$a = 0{,}5$; $b = d = 0{,}02$		
t	37 800	75 600	271 000
p	24,6	39,2	70,1
10^3k	0,83	0,82	0,85

oder durch graphische Integrierung berechnet worden.

Die angeführte Formel kann benutzt werden, wenn in einer Reaktion 3. Ordnung zwei der Konzentrationen gleich sind und die dritte von diesen nur wenig abweicht.

b = zwei gleiche Konzentrationen, die des Esters und die des Natriumhydroxyds
a = Konzentration des zugesetzten Wassers
$f = \frac{a}{b}$
p = Umsatz in Prozenten

Die Geschwindigkeitskonstanten der letzten Versuchsreihe in Tabelle 3 sind durch graphische Integration über den Zeitabschnitt t_2—t_1 aus folgender Gleichung bestimmt worden:

$$\frac{dx}{dt} = k(c_{NaOH} + c_{H_2O} - x)(c_{NaOR} - x)(c_{Ester} - x)$$

BESPRECHUNG DER ERGEBNISSE

Aus den Tabellen 1, 2 und 3 geht hervor, dass die berechneten Geschwindigkeitskonstanten ziemlich konstant sind und somit die Brauchbarkeit der verwendeten Gleichungen beweisen. Zum Vergleich sind in der Tabelle 5 einige Geschwindigkeitskonstanten zusammengestellt. Die Geschwindigkeitskonstanten der mit Natriumhydroxyd allein katalysierten Hydrolyse in wasserfreien Alkoholen sind in der vorigen Arbeit [1] angegeben. Zur Vermeidung der Salzwirkung sind vergleichshalber möglichst solche Fälle herangezogen worden, bei denen die Gesamtalkalikonzentration die gleiche ist.

Tabelle 2. Beispiele von Reaktionsgeschwindigkeitskonstanten. n-Propanol; 25° C.

$a = 0{,}017$; $b = 0{,}125$

t	2 640	5 220	9 420	15 600	33 780	51 000	84 840
p	19,4	30,3	41,8	51,5	65,6	72,7	78,9
10^3k	5,1	5,1	5,2	5,2	5,2	5,3	5,3

$a = 0{,}012$, $b = 0{,}0416$

t	18 600	42 000	74 700	138 900	430 800
p	16,2	28,0	39,2	51,8	72,8
10^3k	5,0	4,5	4,7	4,6	4,8

Tabelle 3. Wirkung des Natriumalkoholats auf die Reaktionsgeschwindigkeit. Methanol; 50° C.

$c = 0{,}0484$; $b = d = 0{,}074$ $\qquad$ $c = 0{,}0805$; $b = d = 0{,}057$

t	53 100	164 400	400 500	51 600	144 000	321 900
p	34,4	57,2	73,8	33,3	56,2	73,3
10^3k	1,23	1,17	1,17	1,32	1,35	1,40

Äthanol; 25° C.

$c = 0{,}62$; $b = d = 0{,}2$ $\qquad$ $c = 0{,}413$; $b = d = 0{,}266$

t	1 330	2 580	7 450	1 230	2 220	6 000
p	27,2	40,2	65,9	24,0	36,1	57,8
10^3k	1,77	1,73	1,81	1,49	1,53	1,48

$c = 0{,}393$; $b = d = 0{,}2$

t	2 220	5 880	5 700	9 900
p	29,3	51,5	51,2	63,8
10^3k	1,46	1,46	1,44	1,43

Methanol; 25° C.

$c_{NaOH} = 0{,}333$; $c_{NaOR} = 0{,}333$; $c_{H_2O} = 0{,}041$; $c_{Ester} = 0{,}333$

t	3 900	5 400	12 000	20 880	27 000	64 800	96 000	159 960	258 000
p	13,6	18,5	31,3	43,3	48,8	67,6	75,2	82,0	89,3
10^4k	1,63	1,60	1,58	1,64	1,49	1,44	1,50	1,17	1,61

Tabelle 4. Einfluss von Salz auf die Reaktionsgeschwindigkeit; 25° C.

Lösungsmittel	$c_{NaOH} = c_{Ester}$	Salz	10^4k
Methanol	0,25		1,35
»	0,25	0,25 *N* Natriumacetat	1,53
»	0,25	0,25 *N* Kaliumjodid	1,55
Äthanol, $c_{H_2O} = 0{,}5$	0,02		8,2
»	0,02	0,02 *N* Natriumjodid	9,3
»	0,02	0,07 *N* »	10,2
»	0,02	0,14 *N*	10,9
»	0,02	0,34 *N* »	11,2

Tabelle 5.

Lösungsmittel	c_{NaOH}	c_{H_2O}	c_{NaOR}	$10^4 k_{25°}$
Methanol	0,25	—	—	1,36
»	0,25	0,57	—	1,32
»	0,25	2,8	—	1,55
»	0,057	—	0,080	1,36
Äthanol	0,2	—	—	11,6
»	0,2	0,4	—	11,6
»	0,092	—	0,099	11,7
n-Propanol	0,125	—	—	52
»	0,0833	0,192	—	51

Nehmen wir an, dass die Geschwindigkeit der Esterhydrolyse proportional der Volumenkonzentration des Hydroxyl-Ions ist und dass die Verminderung der Geschwindigkeit in Alkohol-Wasser-Gemischen hauptsächlich von der, durch die Zunahme des Alkoholgehalts hervorgerufenen, Verminderung der Hydroxyl-Ionenkonzentration herrührt, so erhält man aus der Gleichung $[OH^-] = \frac{[OR^-]\,[H_2O]}{[ROH]}\,K$ für die Konstante K folgende Werte: Methanol 62, Äthanol 6,0, *n*-Propanol 1,37, *i*-Propanol 0,17. Die in *iso*-Propanollösung mittels des Indikatorverfahrens erhaltenen Werte sind: Methanol 3,3, Äthanol 0,79, *n*-Propanol 0,42, *i*-Propanol 0,063 [2]. Für Äthanol-Wasser-Gemisch ist der Wert $0,7 \pm 0,2$ [3] erhalten worden. Besonders beim Methanol unterscheiden sich die erhaltenen Werte recht beträchtlich. Andrerseits hängt die relative Stärke der Elektrolyte vom Lösungsmittel ab. Die mittels der erhaltenen Werte für die Konstante K berechneten Geschwindigkeitskonstanten für verschiedene Gemische von Wasser und Alkoholen weichen von den gemessenen [4] nur um Faktoren 2—3 ab, obgleich die Reaktionsgeschwindigkeiten selbst sich um Faktoren von Zehntausenden unterscheiden können. Dies spricht für die Berechtigung der Verwendung der genannten Gleichungen.

Die Geschwindigkeit der Esterhydrolyse nimmt schwach ab beim Übergang von wässriger Lösung zu Wasser-Dioxan-Gemischen [3]. Dies dürfte nicht auf Grund des Vorigen verständlich sein, ist doch Dioxan ein schlechter Protonendonator. Zweitens variiert der Lösungsmitteleffekt, obwohl in den Hauptzügen von gleicher Art, etwas bei den verschiedenen Estern. Es ist möglich, dass die Esterhydrolyse in der geschwindigkeitsbestimmenden Stufe eine Reaktion zwischen Ester- und Wassermolekül ist, deren Geschwindigkeit proportional der Basenstärke des Lösungsmittelanions ist. Es ist festgestellt worden, dass die Geschwindigkeit der sauren Hydrolyse von Methylacetat in wenig Wasser enthaltendem Aceton proportional der Konzentration des Wassers ist. [5]

ZUSAMMENFASSUNG

1. Die Einwirkung kleiner Wassermengen auf die Geschwindigkeit der alkalischen Hydrolyse von Methyl-, Äthyl- und *n*-Propylacetat in Methanol-, Äthanol- bzw. *n*-Propanollösungen ist gemessen worden.

2. Die Einwirkung von Alkalialkoholat auf die Hydrolysengeschwindigkeit von Methyl- und Äthylacetat in Methanol- bzw. Äthanollösungen ist gemessen worden.

3. Es wurde gefunden, dass die Geschwindigkeit der Hydrolyse durch die Gleichung

$$\frac{dx}{dt} = k(c_{NaOH} + c_{H_2O} - x)(c_{NaOH} + c_{NaOR} - x)(c_{Ester} - x)$$

wiedergegeben wird.

4. Der Einfluss einiger Neutralsalze auf die Hydrolysengeschwindigkeit von Methyl- und Äthylacetat in Methanol bzw. Äthanollösungen ist gemessen worden.

5. Es wird die Möglichkeit besprochen, dass das Lösungsmittel die Geschwindigkeit der Esterhydrolyse beeinflusst, indem es die Volumenkonzentration des Hydroxyl-Ions bestimmt.

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Empirical Relationships of the Minor Bands in the Absorption Spectra of Polyenes

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A study of published electronic absorption spectra of polyenes has led to the simple rule that all minor bands at short wave lengths correspond in location with the main band of shorter chromophores of lengths equal to integral fractions of the chromophore considered. The relative intensities of these "overtone" bands are shown to be influenced by stereochemical factors (compare Zechmeister's *cis*-peak). Some new ideas, pertaining to the distribution of extinction value on the various bands, are developed and applied in a discussion of poly-*cis* carotenoids and Karrer's sterically hindered *cis*-compounds. The spectra of retro-polyenes and vitamins A_1 and A_2 are also discussed, and an explanation of the difference between "retro" and "normal" spectra is proposed. The more general validity of the principles established is demonstrated by the spectra of diphenylpolyenes, symmetrical azines, polyazobenzenes, and polyphenyls.

It is well known that the absorption spectra of polyenes in addition to the main band (with its vibrational fine structure) show minor bands (with or without fine structure) at shorter wave lengths. The main band is explained in terms of classical theory as being due to oscillations of the π-electrons from one end of the chromophore to the other, resulting from a transition from the stable ground state to the first excited state of the total conjugated chromophore. (From a quantum mechanical viewpoint the same electronic transition is described as an electron jump from the highest occupied π-orbital, or energy level, to the lowest vacant π-orbital.) The minor bands, however, can be explained as being due either to transitions to higher excited states of the total chromophore, or to electronic transitions of independent partial or localized chromophores. Evidence is put forward here to show that bands of partial or localized oscillation are of importance only in rare cases when special structural features are present, and possibly also in the case of very short systems where the resonance is not very pronounced. The general indications are that the minor bands are to be attributed to higher order transitions of the total chromophore, and that this is always the case with aliphatic polyenes. Such bands have been called overtones [1,2], or fundamental

bands of higher order [3], but will here mostly be referred to specifically as the λ_2-band, the λ_3-band, and so on, the main band being then the λ_1-band.

Only two of these overtones have so far been recognized as such: the λ_2-band, (fundamental band of second order, or first overtone, which represents an electronic transition that is forbidden in the linear all-*trans* compound, but can take place in *cis*-isomers with an angular shape, and hence has been termed "*cis*-peak" by Zechmeister and Polgár [4,15]), and the λ_3-band (second overtone, which is observable in the all-*trans* compound and most *cis*-isomers [1,6]). However, many authors have, from theoretical considerations, predicted the existence of further overtones [7-9]. Among these only bands of odd order (λ_5, λ_7, *etc.*) represent transitions that are allowed in the essentially one-dimensional all-*trans* molecule, while bands of both odd and even order are to be expected in the spectra of two-dimensional *cis*-isomers [8-9].

A survey of published absorption spectra of polyenes reveals that until recently further bands which may be interpreted as higher overtones (λ_4- and occasionally λ_5-bands) have only been observed in the region accessible for solution spectroscopy in the spectra of a few carotenoids [10,11,1,6]. It is clear that by extending the length of the conjugated system, whereby the absorption bands are displaced towards longer wave lengths, more overtones should become observable. In fact the spectra of the higher homologues of carotenoids synthesized recently by Karrer and co-workers [12-14], show several short

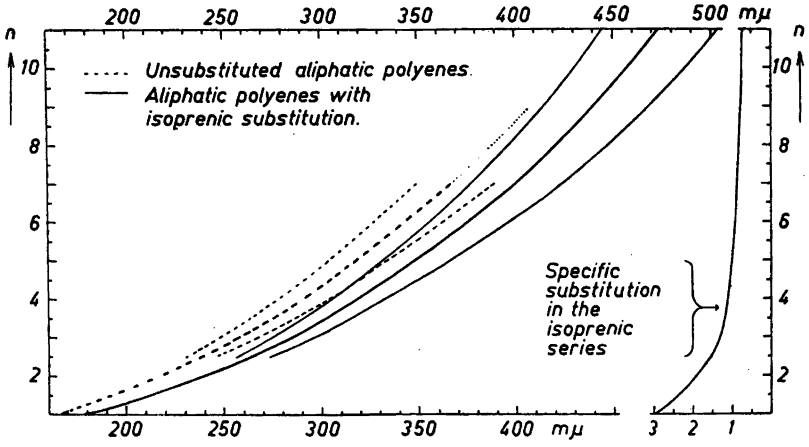


Fig. 1. Wave length positions of the three first peaks in the main absorption band of aliphatic polyenes plotted against number of conjugated double bonds.

The following data were used in plotting the curves: Unsubstituted series: Ethylene, 163, vapor [58], butadiene, 217 in hexane [10], hexatriene 247, 256, 266 in hexane [59], octatetraene, 278, 291, 304 in *cyclo*hexane [60], decapentaene, 304, 318, 334, in *iso*octane [61], tetradecaheptaene, 350, 368, 390 in *iso*octane [61].

Isoprenic series: Trimethyl-ethylene, 178, vapor [62], phytoene, 275, 285, 297 [63], phytofluene, 331, 348, 367 [64], ξ-carotene, 378, 400, 425 [65], 5,6-dihydro-α-carotene, 415, 438, 469 [4], lycopene, 445, 473, 504 [1], all in hexane.

The single curve to the right represents number of substituents per double bond as a function of n in the isoprenic series for use when interpolating between the two curves.

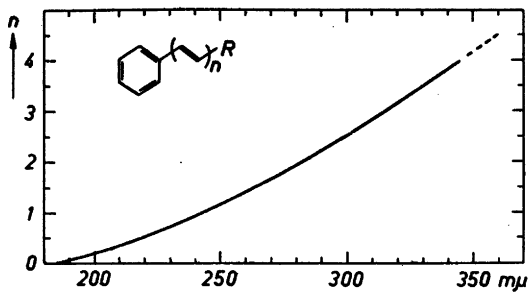


Fig. 2. Wave length position of the main absorption band of α-phenylpolyenes plotted against number of conjugated double bonds in the chain.

The following data were used in plotting the curve: Benzene, 183 in heptane [30], styrene, 244 in ethanol [66], 1-phenylbutadiene, 280 [67], 1-phenyl-3-methyl-octatriene-7-ol, 319 in ethanol [68], 1-phenyl-3,7-dimethyl-nonatetraene-9-ol, 343 in ethanol [68].

wave length bands. In the case of dodecapreno-β-carotene with 19 conjugated double bonds, as many as seven bands can be recognized [14].

With regard to the wave length position of the overtones it was observed by Zechmeister and Polgár [4] that the difference in wave length between the first peak (o-o vibrational transition) in the main band and the *cis*-peak amounted to about 140 mμ for all carotenoids. In shorter conjugated systems this difference was much smaller.

A simple and exact relationship between the positions of the various absorption bands does not seem to have been demonstrated, although a theoretically

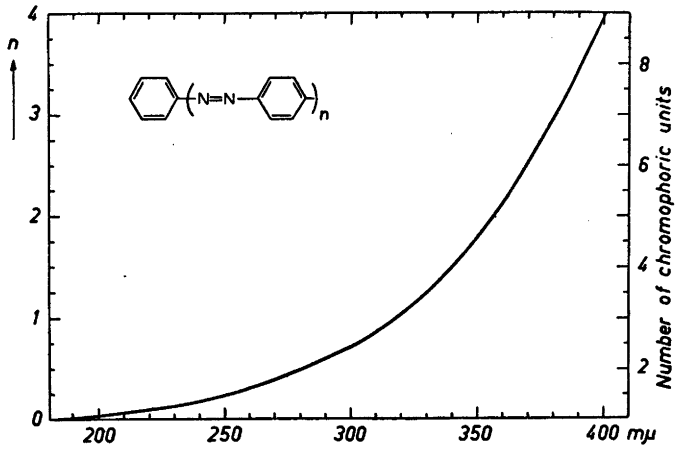


Fig. 3. Wave length position of the main absorption band of "polyazobenzenes" plotted against total number of benzene nuclei and azo groups.

The following data were used in plotting the curve: Benzene, 183 in heptane [30], azobenzene, 318 [53], disazobenzene, 352—359 [55,58], trisazobenzene, 380 [53], tetrakisazobenzene, 400 [53], all in ethanol.

derived formula involving two empirical parameters has been proposed by W. Kuhn [9]. However, this formula fails badly for the higher overtones (see footnotes to Table 1), and thus cannot be used to distinguish reliably between bands belonging to a given all-*trans* polyene and bands due to the presence of *cis*-isomers or to foreign impurities; neither can it be used to determine whether true partial chromophores are active in addition to the main chromophore.

It will be shown here that *if an aliphatic polyene contains n conjugated double bonds, the wave length maximum of the* λ_s *-band will lie very close to the wave length maximum of the main band of a corresponding polyene with n/s conjugated double bonds* (where *n/s* need not be an integer). Since alkyl substituents shift the absorption maximum to longer wave lengths compared with the unsubstituted chromophore, due consideration must be given to the degreé of substitution. The calculation of the wave lengths (λ_s) is most easily carried out graphically (Fig. 1) by plotting λ_1 against n for two polyene series with different degree of substitution and evaluating λ_s by proper interpolation between the values found for *n/s* on the two curves.

A similar procedure can be adapted for α,ω-diphenyl-polyenes and symmetrical azines to estimate the position of the λ_2-band (Fig. 2), for "polyazobenzenes" (Fig. 3), and for "polyphenyls".

The results are compared with observed bands in Tables 1, 2, 3, 4, and 5.

DETAILS OF CALCULATING PROCEDURE

In Fig. 1 the wave length maxima of the first three peaks in the main absorption band are plotted against the number of conjugated double bonds for aliphatic polyenes. Reliable data for a sufficient number of compounds was found in the literature for two series only; unsubstituted polyenes, and polyenes with isoprenic substitution (carotenoids *etc.*). In this last series only the points for polyenes with an odd number of double bonds were used in drawing the curve in order to obtain a continuous curve with a continuous variation in substitution per double bond. This specific substitution is comparatively lower in the even members, and they consequently absorb at slightly shorter wave lengths than evaluated from the curve.

As far as possible only spectra taken in petrol ether, hexane or similar solvents were included. This becomes especially important at longer wave lengths, since the shift of λ_{max} due to change of solvent has been found to be proportional to the number of double bonds n in the conjugated chain [15].

The curve representing the isoprenic series migth have been extended [14] to $n = 19$, but this has not been done here for several reasons. Firstly, most of the compounds reported in this region contain β-ionone rings and because of steric hindrance to coplanarity with resulting decreased resonance in the excited state would be expected to absorb at considerably lower wave lengths than a corresponding true aliphatic polyene, (the shift in λ_{max} due to one ring closure being known to correspond to a reduction of around 2/3 of a double bond at $n = 11$). Secondly, published spectrophotometric curves are not taken up in hexane or petrol ether. Thirdly, there is much evidence to show that the curves given represent mixtures of *cis-trans*-isomers rather than the true all-*trans*-form (see below), and the shift to shorter wave lengths resulting from isomerization is also known to increase with n.

The curves obtained have been used for a graphical calculation of the data given in Table 1. In the present work only the second peak (heavy line) has been used, since in most cases this represents the peak of highest extinction and, accordingly, would be expected to represent λ_{max} in cases where the vibrational fine structure is not discernible. A curve representing the degree of substitution per double bond on each point of the

"isoprenic" curve has been introduced into Fig. 1 to allow easy interpolation for obtaining a correction for differences in substitution.

The same procedure can be applied with satisfactory results to carotenoids containing β-ionone rings when a correction in n is made for the effect of ring closure.

Table 1 also contains in the footnotes λ_{max} values for the same bands for lycopene * and dodecapreno-β-carotene as calculated from Kuhn's formula [9]

$$\lambda_s = \frac{157 \text{ m } \mu}{\sqrt{1-0.922 \cos\frac{\pi \cdot s}{n+1}}}$$

where n is the number of conjugated double bonds and s the band order. A comparison will show the large deviations from observed values at short wave lengths.

Only hydrocarbons with aliphatic or β-ionone-ring chromophores have been included in Table 1. When separated from the chromophore the presence of functional groups containing oxygen *etc.* in the molecule has no influence on the spectra. Examples can be found in a paper by Karrer and Würgler [11], but these have not been included in Table1.

When carboxyl and carbonyl groups are in conjugation it would appear that such groups can be counted as equivalent to slightly less and slightly more than a conjugated C=C double bond, respectively. From Table 2 it can be seen that the agreement between calculated and observed values is not perfect, but fairly good when the heterogeneity of the systems and the sensitivity of λ_{max} to change of solvents is considered.

A further discussion is given below, where the effect of more unsaturated ring structures conjugated with the chain is also discussed.

GENERAL DISCUSSION

How is it possible then to be sure that the bands observed actually represent overtones of the calculated order? No direct proof is given here **, but to date no feature has been found that does not fit in with the postulate. In the following some indications of the correctness of the correlations made will be mentioned.

Following Pauling's semi-classical treatment of the *cis* peak phenomenon [1,88], in which it is assumed that the λ_1-band represents electronic oscillations throughout the whole length of the chromophore; the λ_2-band coupled oscillations in the two halves; the λ_3-band oscillations in the three thirds, *etc.*; it does not seem unreasonable to expect these overtone oscillations to correspond in wave length to similar λ_1-band oscillations in shorter chromophores of comparable length and specific substitution.

Furthermore, in all spectra of carefully purified all-*trans* isomers only bands of odd order have been observed, while spectra of established *cis*-isomers also show bands of even order [5]. Whenever bands have been observed other than those predicted from the calculations, it can be shown that an exceptional structural feature has caused a displacement of, what it seems, regular overtone bands, or more rarely has given rise to independent partial chromophores. (See below.)

In cases where it is more or less tacitly assumed that an all-*trans* form has been isolated but no proof of stereochemical homogeneity has been presented,

* In the original paper n for lycopene is set equal to $11 + 2 \cdot 1/2 = 12$. It is not clear why isolated double bonds should be considered as adding to the effective length of the cromophore. Rather, cyclization would reduce the effective length because of steric hindrance to coplanarity [16,17].

** Further studies, based on the quantum mechanical picture of polyenes, have led to some interesting results, the discussion of which will be postponed to a later date.

Table 1. Calculated and observed wave length maxima for overtone bands in the absorption spectra of hydrocarbon carotenoids and aliphatic polyenes.

Compound	n	Eff. n	Spec.[a] subst.	Solvent	λ_2[b] (n/2) calc.	obs.	λ_3(n/3) calc.	obs.	λ_4 (n/4) calc.	obs.	λ_5 (n/5) calc.	obs.	Ref.
Dodecapreno-β-carotene [c]	19	18[d]	0.63	*cyclo*-hexane	437	444[e]	366	372	323	328	292	288[f]	14
Decapreno-β-carotene	15	14[d]	0.67	» »	394	405[e]	329	328	290	~290	253	245	12
Decapreno-ε_1-carotene	13	13	0.61	» »	378	(392)[e,g] 374	318	321	280	280	252	246	13
C_{42}-Homo-β-carotene	12	11[d]	0.67	ether	354	355	296	296					25
Lycopene [h]	11	11	0.73	hexane	355	(362)[g] 348	297	296	260	255	235	234	1,6
γ-Carotene	11	10.3	0.73	» »	345	349	288	282	253	245			1
β-Carotene	11	9.3	0.73	» »	337	339	280	272					4
7,7'-Bis-desmethyl-β-Carotene	11	9.7	0.55	—	330	330	276	270					22
δ-Carotene	10?	10	0.70	*iso*-octane	340	~345	285	280	249	~245			69
α-Carotene	10	9.3	0.70	hexane	330	331	275	267					4
5,6-Dihydro-β-carotene	10	9.3	0.70	» »	330	331	275	268					4
5,6-Dihydro-α-carotene	9	9	0.67	» »	324	(329)[g] 316	270	266					4
ε_1-Carotene	9	9	0.67	*cyclo*-hexane	324	332[e]	270	267					18
1,1'-Dihydro-β-carotene	8	8	0.75	ether	309	(312)[g] 302	258	254					70
"Dehydro-phytofluene"	7?	7	0.86	hexane	294	(297)[g] 287							71
Phytofluene	5	5	1.00	» »	256	(259)[g] 252							72
Tetradecaheptaene	7	7	0	*iso*-octane	275	265							61
1,12-Dimethyl-dodecahexaene	6	6	0.33	chloro-form	264	~265							73
Decapentaene	5	5	0	*iso*-octane	238	235							61
Hexatriene	3	3	0	vapor			163	175					74
Butadiene	2	2	0	» »	163	170[i]							75
*Cyclo*hexadiene [j]	2	2.5	1.00	» »	185	199[i]							75

[a] Because of the minor contribution from ring double bonds, cyclization is not counted as extra substitution.

[b] All wave lengths given in mμ.

the bands of odd order are always the major ones, while bands of even order are usually less developed. Particularly interesting examples are offered by Karrer's higher homologues of carotenoids. Probably, in most cases isomerization has taken place after dissolution of the substance. Thus the absorption curves of ε_1-carotene [18] in ethanol and in cyclohexane show very nearly the same extinction values in the λ_1- and in the λ_3-bands, but widely different values in the λ_2-band. Dodecapreno-β-carotene [14] affords an even better example. Here all bands from λ_1 to λ_7 are present, and it is evident even in the main band that the solution must have contained *cis*-isomers. The first "vibrational" peak (0—0 transition) is lower than the third peak (0—3 transition), while it is an experimental fact that in the all-*trans* form the first peak is always the higher one and decreases more rapidly upon isomerization to become sometimes even lower than the third peak. Also the degree of fine structure is diminished and would likewise be explained as a consequence of isomerization *. The relatively low melting point ** and good solubility might indicate that the crystallized compound is a *cis*-isomer, but this need not be the case. According to calculations by Coulson [19,20] the double bond character of especially the central bonds becomes very much reduced in such long systems, and consequently the ease of isomerization should increase with chain length [1,21]. The all-*trans* crystal might well isomerize spontaneously and rapidly upon dissolution, and also during melting point determination.

In a discussion of the relative intensities of the various bands of a polyene one is limited to systems where the steric configuration of isomers has been established beyond doubt by the synthesis, *e. g.*, the synthetic carotenoids prepared by Inhoffen and co-workers [22–26]. For the all-*trans* isomer the intensity of bands of odd order has been predicted by W. Kuhn [9] to be inversely proportional to the square of the band order; thus the λ_3-band $= 1/3^2 \times \lambda_1$,

* There is no apparent reason to believe as do Karrer and Eugster [14] that the fine structure should be less pronounced for this all-*trans* form than for all-*trans*-β-carotene. All added double bonds belong to types already present in β-carotene and should not introduce new stretching frequencies or vibrational levels to effect a smearing out of the fine structure [28].

** 190—91° as against 192° for decapreno-β-carotene and 180° for β-carotene.

c Calculated from Kuhn's formula: $\lambda_2 = 445$, $\lambda_3 = 371$, $\lambda_4 = 311$, $\lambda_5 = 266$, $\lambda_6 = 232$, $\lambda_7 = 206$.

d In lack of comparative data ring double bonds are here tentatively assumed equivalent to 0.5 of a chain double bond.

e Because of solvent shift and proximity to the main band, the real $\lambda_{2\max.}$ is expected to be at shorter wave lengths.

f Two additional bands are observed, one at 265 (calc. 267 for λ_6), and one at 240 (calc. 252, 237, and 225 for λ_7, λ_8, and λ_9). (See discussion in text.)

g The first vibrational peak (o—o transition) is here higher than the second one on which the calculation is based.

h Calculated from Kuhn's formula: $\lambda_2 = 349$, $\lambda_3 = 266$, $\lambda_4 = 214$, $\lambda_5 = 180$.

i The assignment of this band as a λ_2-band is supported by the fact that in butadiene (straight) it is very weak compared with the λ_1-band, whereas in *cyclo*hexadiene (bent) the λ_2-band is strong and the λ_1-band is rather weak.

j Included for comparison.

or 11 % of the λ_1-band. This is equivalent to Pauling's statement [1,27] that since the light absorption is proportional to the square of the regularly reversing electric dipole moment, the intensity of the λ_1-band is proportional to the square of the distance between the ends of the conjugated system, hence will be lower for a bent *cis*-isomer. Similarly, the extinction of the λ_2-band in *cis*-isomers should be proportional to the square of the distance between the middle of the conjugated system and the middle of the line connecting the ends of the conjugated system, while λ_3 is supposed by Pauling to vary roughly proportionally to λ_1. This is demonstrated clearly in the spectra of 7,7'-bis-desmethyl-β-carotene [22] and β-carotene [24] synthesized by Inhoffen *et al.* where the central double bond assumes a *cis*-configuration resulting from partial hydrogenation of an acetylenic bond. It is especially noteworthy that this mono-*cis*-β-carotene, as would be expected, has a higher *cis*-peak than the mono-*cis*-isomer (*neo* U) isolated by Zechmeister and Polgár and assigned *cis*-configuration around a more peripheral double bond [29], and is in fact higher than any other *cis*-isomer of this set. Inhoffen's C_{42}-homo-β-carotene synthesis [25] provides both a central di-*cis* and a mono-*cis* isomer. The mono-*cis* behaves as expected, but the di-*cis* does not. It is true that it shows no λ_2-band or *cis*-peak which, being essentially linear, it should not, but the λ_1-band has fallen to about 75 % instead of 93 % as calculated from a model, and the λ_3-band has increased with about 50 % instead of decreasing slightly.

Obviously there must also be other factors than the possibility and estimated magnitude of a reversing dipole moment, connected with electronic oscillations, which determine the probability of light absorption taking place. One might imagine that the two central *cis*-bonds, because they are twisted slightly out of the plane (*cf. Pauling* [27]), constitute a partially insulating group having a certain blocking effect for oscillations through the whole length of the chromophore, thereby causing an additional decrease in the λ_1-band. On the other hand the λ_3-band may increase, since in two of the three thirds of the chromophore there are still no blocking groups, and the overtone oscillation may gain in importance in conformity with the theoretically derived requirement that the total extinction, or oscillator strength (f-value), summed over all electronic absorption bands shall be constant and determined only by the number of double bonds or π-electrons [9,30].

Ferguson [31] has depicted the *cis*-peak as due to partial oscillations on each side of a blocking *cis*-bond out to the ends of the system. This cannot be true since in a non-central mono-*cis* compund one would then expect two *cis*-peaks at different wave lengths. There is ample material [5] to show that the *cis*-peak has very closely the same location in all types of *cis*-isomers of the same compound and hence must be regarded as an overtone band (λ_2-band). The wave length location of all overtones seems to be determined purely as a function of the total conjugated system in the manner shown above, and in no way by the individual positions of any *cis*-bonds. However, as already pointed out, *cis*-bonds may constitute a hindrance for some of the oscillations even when they represent allowed transitions, thus making other (allowed) transitions more probable. This would especially be the case with oscillations of lowest possible order for which the positions of the *cis*-bonds coincide with the borders between any of the imagined "overtone chromopho-

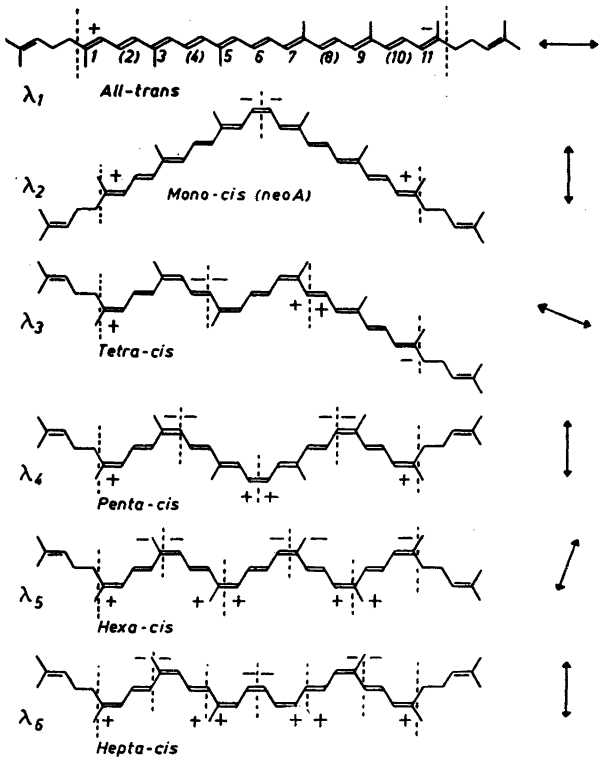


Fig. 4. Polarization diagrams for the electronic transitions responsible for the main absorption band and overtone bands of polyenes, exemplified by some possible "unhindered" isomers of the lycopene set [1,88].

res", thereby providing an optimal possibility for transitions without the need for the oscillating electrons to "pass through" blocking *cis*-bonds.*

It might be speculated as to how much the angle of twist at the *cis*-bonds has to be increased before such "overtone chromophores" change to become true partial chromophores taking over the total extinction of longer wave length bands. Even the quite considerable angle of twist at a sterically hindered carotenoid double bond in *cis*-configuration (*cf.* Pauling [27]) does not seem to be sufficiently large to prevent π-electron interaction and to cause a splitting up of the main chromophore into partial chromophores [32-36].

POLY-*CIS* CAROTENOIDS AND STERICALLY HINDERED DOUBLE BONDS

The ideas just outlined find an excellent illustration in the spectra of several poly-*cis* lycopenes isolated from plant material by Zechmeister and

* This principle can be more clearly visualized by likening the chromophore to a vibrating string fastened at both ends. Then the preferred overtone vibrations will be those for which the nodes of the associated standing wave coincide with any loosely clamped position (or *cis*-bond).

co-workers [1,6]. If the double bonds are enumerated as in Fig. 4, then according to Pauling [27,1,21] only 1, 3, 5, 6, 7, 9 and 11 will be reasonably free of steric hindrance in a *cis*-configuration and would be expected in a poly-*cis* compound. Fig. 4 shows that the four poly-*cis* isomers depicted are essentially linear, and according to Pauling [1] should show an intensity in the λ_1-band around 80 % of that of the all-*trans*. The measured intensities, however, are considerably lower, in some cases even below 60 %. The geometry of the tetra-*cis* and the penta-*cis* models in Fig. 4 should allow λ_2-bands of low intensity as seen from the polarization diagrams. None of the isolated poly-*cis* lycopenes has any distinct λ_2-band, and the reason would be that the *cis*-bonds decrease an already low probability of transition.

Likewise, the λ_3-band is mostly absent except in a few poly-*cis* isomers with fewer *cis* double bonds, where the intensity is still rather weak. These isomers might be of the type illustrated by the tetra-*cis* in Fig. 4, where one of the three thirds of the conjugated chain is free of blocking *cis*-bonds. The λ_4- and λ_5- bands, however, are mostly very well developed, and this is more pronounced the more *cis*-bonds the chain contains. It might be imagined that an isomer of the penta-*cis* type shown in Fig. 4 would give a very intense λ_4-band and a somewhat lower λ_5-band, while in the hexa-*cis* model shown the λ_5-band would be more intense than the λ_4-band. (Similarly it might be inferred from Fig. 4 that a λ_6-band, calculated at 217 mμ, would be absent or weak in poly-*cis* isomers.) It remains to be emphasized that in the all-*trans* λ_5 is weak and λ_4 absent, while in 6-mono-*cis* lycopene (*neo* A) both λ_4 and λ_5 can be observed as weak bands.

The good agreement found between the models and the spectra of these remarkable poly-*cis* lycopenes would not have been possible if both types of double bonds were able to assume a *cis*-configuration, thus providing a confirmation of the validity of the concept of steric restrictions proposed by Pauling. Certainly, from this it does not follow that isomers with *cis*-configuration at "hindered" double bonds cannot exist, even if Pauling has shown that they are very unlikely to arise in an isomerization mixture [21]. It is, for example, possible to prepare such isomers by making use of the fact that acetylenic bonds can be partially hydrogenated to yield *cis*-ethylenic bonds.

Very recently Garbers and Karrer [35] have in this way succeeded in preparing isomers of lycopene in which bond 4, or 4 and 8, are believed to be in *cis*-configuration (possibly also one or more "unhindered" bonds). From a similarity between the degraded main bands in the spectra of these isomers and in the spectra of the poly-*cis* lycopenes the authors find support for the suggestion previously put forward by them [33] that the natural poly-*cis* lycopenes might have *cis*-configuration at one or two "hindered" double bonds rather than at several unhindered bonds. However, they completely disregard the conspicuous fact that their "hindered" isomers show a considerable extinction in the λ_2-band region as compared with a very low extinction in poly-*cis* lycopenes (see above). Unfortunately, their published spectra do not extend far enough into the ultraviolet to make possible a comparison of higher overtones, but by using arguments similar to those applied in the case of the poly-*cis* lycopenes, one would expect a strong λ_3-band (*cf.* Fig. 4), while the λ_4- and λ_5-bands, although of no special preference, might have to take over some of the extinc-

tion of the degraded longer wave length bands. The presence of strong λ_3-bands is actually shown in corresponding hindered *cis*-isomers of the β-carotene set [36]. In fact these λ_3-bands are much stronger than those of any other known *cis*-isomers of β-carotene [4].

A further confirmation of the correctness of these assumptions is provided by the spectra of hindered and unhindered *cis*-isomers of 1,18-diphenyl-3, 7, 12, 16-tetramethyl-octadeca-nonaene prepared by Eugster, Garbers and Karrer[37,33,34]. If the phenyl groups of this compound are assigned the chromophoric value of one double bond, the total chromophore is nearly equivalent to that of lycopene. Furthermore, the aliphatic chain carries exactly the carotenoid pattern of methyl substituents, and the spectrum of the all-*trans* form * is very similar to that of all-*trans* lycopene (see wave length locations in Table 1). Some unhindered *cis*-isomers which have been isolated [37] show the normal spectral changes when compared with the all-*trans* form. Among the hindered isomers prepared discussion will be confined to the two isomers with established configuration, the 5,6-mono-*cis* and the 5, 6, 13, 14-di-*cis*. The 5,6-mono-*cis* [34] has the chromophore "divided" into two parts approximately 1/3 and 2/3 in length, respectively. As might be predicted, the λ_1-band (with the third and second vibrational peaks the highest) is here lower than in any unhindered isomer, the λ_2-band is higher, the λ_3-band is even higher than that of the all-*trans*, and both λ_4 and λ_5 are moderately developed. In the 5,6,13,14-di-*cis* [33] the chromophore is "divided" into three approximately equal parts, and the spectrum is accordingly further degraded. The λ_1-band has decreased relative to the 5,6-mono-*cis*, the fine structure is practically absent, and it seems that the third and fourth vibrational peaks are now the highest. A λ_2-band should not be allowed (no dipole moment) and appears only as a low bump squeezed in between the displaced λ_1-band and the λ_3-band, which latter, as expected, has increased relative to the 5,6-mono-*cis*, The λ_4-band is absent (not allowed), but λ_5 has gained in importance.

Thus it seems that the spectra of these "hindered" *cis*-isomers also conform to the principles now developed, and it will be realized that it is hard to reconcile the overtone part of their spectra with the typical overtone spectra of the natural poly-*cis* lycopenes.

Unfortunately, the published spectrum of the natural poly-*cis* compound pro-γ-carotene [1] does not extend far enough into the ultraviolet to reveal more than the λ_4-band, but approximately the same relations as found in the lycopene set should be valid also in this stereoisomeric set. However, in possible poly-*cis* isomers of higher or lower homologous carotenoids it is easily seen that if the correlations proposed above are correct, then other bands would have a favoured absorption probability similar to that of the λ_5-band in the lycopene set, *e.g.*, the λ_7-band in dehydrolycopene ($n = 15$), the λ_9-band in dodeca-preno-β-carotene ($n = 19$), *etc.* Since the length of the corresponding "overtone chromophore" is practically identical in all these cases, *i.e.*, the distance between successive tertiary carbon atoms, the wave length position of this "poly-*cis* peak" should be approximately constant, at about 230 mμ. Simi-

* A strong λ_2-band in *cyclo*hexane solution indicates that isomerization has taken place upon dissolution, inasmuch as the band is rather weak in benzene solution [37].

larly, when the central double bond is also in *cis*-configuration, the favoured absorption of the λ_4-band in poly-*cis* lycopenes might be taken over by λ_6- and λ_8-bands respectively.

This corollary may give a clue to the problem of the remarkable height of the assumed λ_7-band in the isomer mixture of dodecapreno-β-carotene [14], and also explain the discrepancy between its calculated and observed position at 252 and 240 mμ (see Table 1). It is very probable that the observed band is actually a fusion of the λ_7-, λ_8- and λ_9-bands, calculated at 252, 237 and 225 mμ, respectively, the λ_9-band and possibly the λ_8-band contributing relatively strongly to the observed extinction, since the corresponding transitions for reasons suggested above should become somewhat more probable in *cis*-isomers, although not of the intensity expected for poly-*cis* compounds. Bands beyond λ_9 should be of no special preference, either in *cis*-isomers or in the all-*trans* isomer, and accordingly the extinction drops very sharply beyond this "fused" band.

NON-ALIPHATIC AND OXYGENATED POLYENES. VITAMINS A_1 AND A_2

It has already been mentioned that although the construction of the curve in Fig. 1 is based on data for aliphatic polyenes, overtone bands can also be estimated from it with reasonable accuracy for carotenoids containing β-ionone rings and for systems with a carboxyl or carbonyl group in conjugation. However, there are types of compounds where the quantitative agreement is not quite as satisfactory, although the qualitative picture is the same.

A complication arises, for instance, with the polyene-dicarboxylic acids. It was observed early by Hausser *et al.*[38] that a second carboxyl group in the opposite end can be neglected when estimating the wave length positions of λ_1-bands for polyene-carboxylic acids. This rule incidentally seems to apply to polyene carbonyl — polyene dicarbonyl pairs as well [39]. However, when calculating overtone bands it seems that in some cases both C=O bonds have to be taken into account in order to reach good agreement with observed values (see Table 2). This peculiarity can be compared with the abnormal location of the λ_1-band in azines as against the normal position of the λ_2-band (see below).

A similar abnormality can be observed in polyenes with "retro"-β-ionylidene ring structures, *i.e.*, where the conjugated system has moved one step into the ring relative to the β-ionylidene compounds. Oroshnik, Karmas and Mebane [32] have prepared two members of this series with the vitamin A skeleton but containing four and five conjugated double bonds ending in a "retro" structure (probably similar to anhydrovitamin A). These compounds oddly enough do not seem to be sterically hindered at the ring, and both λ_1- and λ_2-bands correspond completely with aliphatic polyenes (see Table 3). However, in longer systems with "retro" structures in both ends the position is more complicated. Thus in dehydro-β-carotene [40,46] and eschscholtzxanthin [42] with 12 conjugated double bonds the λ_1-band corresponds in location to 11 only, whereas the overtone bands must be calculated on the basis of $n = 12$ (Table 3). In bisdehydro-β-carotene [43] with 13 conjugated double bonds where the unsaturation most probably extends further into one of the rings,

Table 2. Calculated and observed overtone bands of polyenes conjugated with carbonyl and carboxyl groups.

Compound	n	Eff. n	Spec. subst.	Solvent	λ_2 calc. obs.	λ_3 calc. obs.	λ_4 calc. obs.	Ref.
Capsanthin	10 + C=O	11	0.54	hexane	350 355	294 284		76
α-Apo-2-carotenal	8 + C=O	9.5	0.56	» »	327 (330) 314	274 265		11
α-Citraurin	8 + C=O	9.5	0.56	» »	327 (330) 315	274 265		11
Hexadecaheptaenal	7 + C=O	8.5	0.13	dioxane	302 305	255~260		51
Tetradecahexaenal	6 + C=O	7.5	0.14	» »	286 285			51
Dodecapentaenal	5 + C=O	6.5	0.17	» »	270 264			51
C_{23}-Ketone (vitamin A homologue)	6 + C=O	7	0.71	*cyclo*-hexane	290 269	240 237		14
Astaxanthin	11 + 2C=O	11	0.61	hexane	351 353	295 289	259 249	11
Bixindialdehyde	9 + 2C=O	12	0.36	ethanol	356 372	300 294		11
β-Carotenone	9 + 2C=O	11	0.55	hexane	350 (367) 352	295 295		11
Methylbixin	9 + 2C=O	10.5	0.36	» »	336 340	282 277	249~245	10cf.77
Bixin	9 + 2C=O	10	0.36	ethanol	330~360	277 286	244 244	11
Apo-3-norbixinal-methyl-ester	7 + 2C=O	8.5	0.33	» »	307 310			11
Crocetin-dimethyl-ester	7 + 2C=O	8.5	0.44	petrol ether	310 314	257 255		26
Corticrocin	6 + 2C=O	8	0	chloro-form	291 (295) 287	245~235		78
2,7-Dimethyl-octatriene-dial	3 + 2C=O	4.5	0.40	petrol ether	233 234			79

the λ_1-band corresponds to $n = 12$ and overtone bands to $n = 14$. In the case of anhydro-eschscholtzxanthin [42,43] with 14 conjugated double bonds (both rings dehydrogenated) the λ_1-band corresponds to $n = 13$ and overtone bands to $n = 15$—17. Rhodoxanthin [11] also has a "retro" structure, but the unsaturated system is terminated by a keto-group in both rings. A similar spectral irregularity is evident here as well.

The most surprising feature is that the λ_2-band, although present in sterically hindered *cis*-isomers of the retro-vitamin A type compounds, is of very low intensity in the dehydro-β-carotene type polyenes even in cases where all other polyene spectra simultaneously published, and obviously representing isomer mixtures (see above), exhibit very distinct λ_2-bands. According to Zechmeister and Wallcave [41,43] isolated *cis*-isomers have λ_2-bands (or rather

Table 3. Calculated and observed overtone bands of polyenes with "retro-ionylidene" structure.

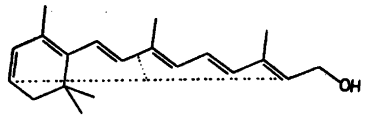
Compound	n	Spec. subst.	λ_2 calc. obs.	λ_3 calc. obs.	λ_4 calc. obs.	λ_5 calc. obs.	λ_6 calc. obs.	Ref.
1 ring: "Tetra-retro" vitamin A methyl ether	4	1.00	230 232					32
"Penta-retro" vitamin A methyl ether	5	1.00	256 255					32
2 rings: Dehydro-β-carotene	12	0.67	367 (382) 366	308 309	271 269		225 (218)	40, 41
Eschscholtzxanthin	12	0.67		308 310	271 272	244 240		42
Bisdehydro-β-carotene	13	0.62	378 (404) 386	316 333				43
Anhydro-esch-scholtzxanthin	14	0.57	389 (425) 405	326 353	288 310	261 272	240 245	42, 43

bumps) as low as the all-*trans* form. It is not easy to understand why λ_2-transitions should be forbidden in such compounds. It might be connected with the fact that the λ_1-band for some reason (see below) retains its extinction value almost completely in *cis*-isomers of these compounds, thus leaving little over for overtone bands.

The spectrum of vitamin A_1 has been the subject of much speculation because of the abnormally short wave length position and lack of fine structure. Oroshnik *et al.*[44] have been able to show that this is not due to the allylic hydroxyl group, but rather to the β-ionylidene structure. The explanation put forward is that because of steric conflict between the *gem*-dimethyl group and a hydrogen atom of the chain, a partial chromophore is set up involving only the four double bonds of the chain [32], and the resulting observed band is thought to be a fusion of this band and a band due to the complete chromophore.

Farrar *et al.*[45] similarly resort to the postulation of partial chromophores to explain the confusing (*cf.* Cama *et al.*[46]) subsidiary absorption band in vitamin A_2 and analogous compounds. They suggest that this band represents the combined absorption of two partial chromophores, one consisting of the two double bonds in the ring, and the other of the side chain double bonds only. An estimation of the mean λ-value for the subsidiary band has been made for vitamin A_2 and for several homologues with the same ring systems, assuming the ring chromophore band to absorb at 270 mμ and the chain chromophore band at appropriately varying wave lengths. The justification of such a calculation is very doubtful when it is considered that to explain the band at 315 mμ in retinene$_2$ it has to be regarded as a combination of one band at 270 mμ and another at 360 mμ! No evidence of band splitting can be found in any case. Furthermore, the main band of vitamin A_2 (and homologues) shows the same lack of fine structure and is located at abnormally short wave lengths, in the same way as vitamin A_1 (and corresponding homolo-

Fig. 5. Model of vitamin A_1 showing how the subsidiary band can be explained as a λ_2-band.



gues). The explanation given by Oroshnik *et al.*[32] concerning the spectrum of vitamin A_1 is accordingly also made unacceptable, as it is very difficult to understand how the chain partial chromophore band in vitamin A_2 can at the same time combine with both the main band and the subsidiary band, still leaving a minimum between them, and furthermore, why a difference of two double bonds between the partial chain chromophore and the complete chromophore in vitamin A_2, compared with a difference of one in vitamin A_1, should not become manifest in the shape of the main band when compared with the main band of vitamin A_1.

It seems that a more plausible interpretation can be arrived at by simply regarding the subsidiary band in the vitamin A_2 series as a λ_2-band (forbidden in the vitamin A_1 series). Even without assuming *cis*-configuration at any chain double bond it is easily seen from Fig. 5 that the added double bond in the ring will create a twodimensional chromophore (*cis* single bond) which would make a λ_2-transition allowed. From Fig. 5 it will also be realized that the λ_2-band will become relatively less important as the length of the unsaturated chain increases, in accordance with the observed fact that it appears more like an inflection in retinene$_2$ and similar compounds[45]. It can also be inferred that in mono-*cis* isomers this band should have increased intensity. That the main band is relatively weaker in the A_2 series than in the A_1 series would be a consequence of the distribution of extinction value on two bands instead of one. Also, one half of the system is free of steric hindrance, thus increasing the relative probability of the λ_2-transition.

A calculation of wave lengths of λ_2-bands in the A_2 series based on the curve in Fig. 1 gives as a result values which on the average are 20 mμ too short, even when the true unreduced n-values are used. This can be compared with the results for bis-dehydro-β-carotene and anhydro-eschscholtzxanthin, which likewise contain two double bonds in the ring, and for which calculated overtone bands are at shorter wave lengths (see Table 3).

In Table 4 the results from overtone calculations are presented for compounds both in the A_2 and A_1 series. The A_1 compounds can of course show λ_2-bands only as a consequence of isomerization.

To sum up, it seems safe to state that the spectra of all polyenes that contain the β-ionylidene structure (vitamin A_1,β-carotene, *etc.*) or the dehydro-β-ionylidene structure (vitamin A_2) indicate a spatial conflict between the *gem*-dimethyl group of the ring and the opposing hydrogen of the chain, resulting in a non-coplanar conjugated system. The marked features of the main band in such spectra, compared with the spectra of corresponding aliphatic systems, are: low extinction value, less or complete lack of fine structure,

Table 4. Calculated and observed overtone bands of vitamins A_1 and A_2 and related compounds (in ethanol).

Compound	n	Spec. subst.	λ_2 calc.	λ_2 obs.	λ_3 calc.	λ_3 obs.	Ref.
A_1 series:							
C_{20} Alcohol	5	1.00	254 ~ 250		210 ~ 208		80
C_{20} Aldehyde	6	0.67			225 ~ 245		81
C_{20} Acid	6	0.67	271 ~ 260		225	233	82
C_{23} Ketone	7	0.71	290	272	240	238	14
A_2 series:							
C_{17} Alcohol	5	0.80	251	269			45
C_{17} Acid	6	0.50	268	287			45
C_{18} Ketone	6	0.67	271	304			45
C_{20} Alcohol	6	0.83	275	288			45
C_{20} Aldehyde	7	0.57	289	315	240	260	45, 83
C_{20} Acid	7	0.57	289	305			45

and displacement to shorter wave lengths. In contrast, all polyenes that contain the "retro"-β-ionylidene structure (anhydrovitamin A_1, Oroshnik's retro compounds, dehydro-β-carotene, *etc.*) or the dehydro-"retro"-β-ionylidene structure (anhydro-eschscholtzxanthin, *etc.*) retain a high extinction value, sharp fine structure, normal location of the main band and a surprisingly low extinction of overtone bands (in particular the λ_2-band). Evidently, "retro"-polyenes manage in some way to keep the whole conjugated system coplanar.

Oroshnik *et al.*[32] have examined molecular models and state that these cannot show that "retro" compounds are less sterically hindered than "normal" compounds. However, the assumption that the *gem*-dimethyl group behaves as a single grouping (*cf.* Farrar *et al.*[45]) is hardly valid. In fact, inspection of such models actually shows that if the ring is planar, one of the methyl groups will lie above and the other below the plane, thus leaving room between them just sufficient to accommodate the opposing hydrogen atom on carbon 8 in the chain (see Fig. 6, c and d), whereby the hydrogens of the methyl groups might have to assume fixed positions. The resistance of the methyl groups against giving up their free rotation is, of course, more than outweighed by the gain in stability upon the attainment of coplanarity of the unsaturated system. On the other hand, if the ring is not planar, one of the methyl groups will collide with the chain hydrogen (Fig. 6, a and b), making it impossible for the

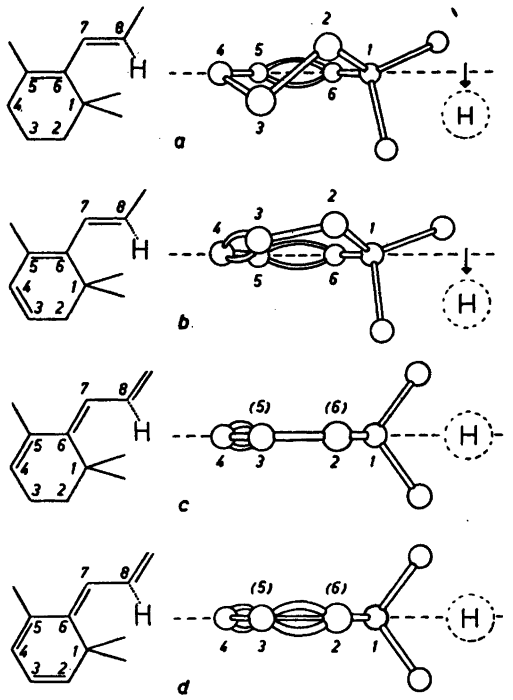


Fig. 6. Sketches of ring systems occurring in vitamins A and in carotenoids showing that "normal" rings (a and b) are skew or puckered, so that one of the 1-methyl groups will repel the opposing chain hydrogen, whereas "retro" rings (c and d) can become planar and accommodate the chain hydrogen between the two methyl groups (see text). The drawings to the right are as viewed in front of the 2—3 bond and parallel to the plane of the paper.

ring double bond (or bonds) to lie in the same plane as the double bonds of the chain.

The models also show just what should then be expected, *viz.*, that "retro" rings can easily become planar, whereas "normal" rings tend to be puckered. The drawings in Fig. 6 will demonstrate this more clearly. In the *cyclo*hexenyl ring (Fig. 6a) the carbon atoms 1, 6, 5 and 4 necessarily lie in one plane, while carbons 3 and 2 will lie below and above this plane, respectively, in order to satisfy the four tetrahedral angles [47,89]. One might at first expect the *cyclo*hexadienyl ring (Fig. 6b) to be planar because of the double bond character of bond 4—5. However, such a ring would involve considerable strain, which can easily be relieved by a slight rotation about bond 4—5 in order to lift carbon 2 sufficiently relative to carbon 1 to establish both required tetrahedral angles [48]. That the two double bonds are thus not wholly conjugated is also indicated by the abnormally small resonance energy found for 1,3-*cyclo*hexadiene [49]. In the *cyclo*hexenylidene ring (Fig. 6c) nothing can be gained by a similar rotation about bond 5—6, and not much by lifting carbon 2 out of the plane; hence this ring can more easily become planar. For the *cyclo*hexadien-

ylidene ring (Fig. 6d) no other configuration than a planar one can be envisaged *. Also, it should not be overlooked that a "retro" system would have to rotate a greater amount about the single bond 7—8 to move the hydrogen at carbon 8 away from the dimethyl group, than is required when rotation occurs about the single bond 6—7 in a "normal" system, thus making the planar structure still more favourable.

The great decrease in energy connected with the attainment of coplanarity in a conjugated system should lead one to expect "retro" compounds to be more stable than the "normal" ones. It is therefore interesting to note that Oroshnik *et al.*[50] have pointed out the great ease with which the "retro" system is formed and the fact that "the only really successful synthesis of vitamin A started with a carbinol in which "retro"-ionylidene formation was structurally impossible". Similarly, the various dehydrogenation processes of β-carotene evidently lead exclusively to "retro" configurations [43].

One would also expect that since the chain hydrogen atom, as proposed here, is thought to be squeezed in between the two 1-methyl groups of the ring, the coplanar system should be quite fixed in these compounds and more rigid than an aliphatic coplanar system. One consequence would then be that the "loose bolt effect" of Lewis and Calvin [3] would be reduced; hence the spectra of "retro" compounds show sharper fine structure than might be expected in such heterogeneous double bond systems. The suggestion is also made that the low extinction of overtone bands even in *cis*-isomers of the "retro" carotenoids can be ascribed to this stiffening effect, since the reverse effect, *viz.*, the loosening up of the rigid molecule by introduction of steric hindrance (at *cis*-bonds, *etc.*) is known to favour a high extinction of overtone bands.

DIPHENYLPOLYENES AND SYMMETRICAL AZINES

The curve in Fig. 1 can be used to estimate overtone bands for diphenylpolyenes if each phenyl group is assigned some arbitrary chromophoric value. However, satisfactory agreement for all overtones is achieved only when the aliphatic chain is predominant, since the phenyl groups become more significant in short systems.

A more direct estimation, involving no choice of chromophoric value, of only the λ_2-bands in the spectra of α,ω-diphenylpolyenes is possible using the same principle, *i.e.*, a comparison is made with the λ_1-bands of compounds with chromophores corresponding to half the molecule. Thus in Fig. 2 the wave length maxima of the main band for α-phenylpolyenes have been plotted against number of conjugated double bonds in the chain. The resulting curve can then be used to estimate the wave length positions of λ_2-bands in diphenylpolyenes. Because of limited selection of data substitutional effects have had to be neglected. Nevertheless, the values calculated from Fig. 2 and given in Table 5 show a good coincidence with observed values. Data for some isomorphic symmetrical azines have also been included assuming a C=N-bond equivalent to a C=C-bond. Although the λ_1-band of the azines lies at abnormally short wave lengths [3,51], the λ_2-band is in perfect agreement.

* Both these planar rings have to be somewhat strained. A practically strainless planar structure, related to 1,4-*cyclo*-hexadiene [90], is obtained when one double bond is moved out of conjugation, and is possibly present in anhydrovitamin A_2 and *iso*anhydrovitamin A_1 [91]:

Table 5. Calculated and observed λ_2-bands of α,ω-diphenylpolyenes and symmetrical azines.

Compound	Aliphatic double bonds (n)	$\lambda_2(n/2)$ calc.	obs.	Ref.
Stilbene	1	220	222 [a]	84
Diphenylbutadiene	2	244	240 [b]	85, 86
Diphenyloctatetraene	4	282	283	4
Diphenyldodecahexaene	6	315	315	28
Diphenyl-tetramethyl-octadecanonaene	9	~360 [c]	365	37
Cinnamalazine	4	280	277	52
Phenylpentadienalazine	6	315	311	52

[a] This band is also present in the *trans* form, and is possibly combined with a partial [54].
[b] Possibly a fused λ_2 and λ_3-band.
[c] By extrapolation.

Another interesting relationship, which has already been noted by Dale and Zechmeister [52], is brought out by the data given in Table 6 for the aldehydes from which the azines are made. Their λ_1-bands lie very close to the λ_2-bands or *cis*-peaks of the corresponding azines.

POLYAZOBENZENES AND POLYPHENYLS

To test the more general validity of the ideas put forward it was of considerable interest to examine the spectra of mixed aromatic-aliphatic polyenes, where *para*-disubstituted aromatic links might be expected to maintain a linear chain, whereas *ortho*-disub-

Table 6. Comparison of λ_2-bands of polyenal-azines with λ_1-bands of the parent aldehydes.

Compound	Azine		Parent aldehyde		Ref.
	Aliphatic double bonds	λ_2 obs.	Aliphatic double bonds	λ_1 obs.	
Cinnamalazine	4	277	2	279	52
Phenylpentadienalazine	6	311	3	308	52
Hexadienalazine *	6	~260	3	270	51
Octatrienalazine	8	304	4	312	51
β-C_{19}-Aldazine	8	310	4	311	87

* A series of α-furyl-polyenal azines all show inflections at approximately the same wave lengths as the main band of the corresponding α-furyl-polyenals.

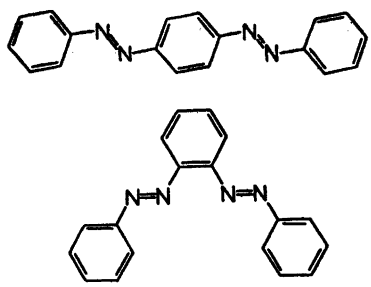


Fig. 7. Models demonstrating the linear, trans-like shape of p-disazobenzene and the angular, cis-like shape of o-disazobenzene.

stituted aromatic links should cause a bend of the chain similar to the bend at *cis*-bonds in aliphatic polyenes (see Fig. 7). As is well known, *meta*-disubstituted links break up the conjugation and are of no interest here.

Unfortunately, the only series for which comparable spectral data have been given for *para*- and *ortho*-compounds are the socalled "polyazobenzenes" described by Ueno [52] and by Dahn and Castelmur [53]. Their spectra are not too easy to analyze because the bands are compressed in a narrow region, causing much overlap. In addition to the regular bands that shift position with chain length, there are also two interfering bands with about constant location for all members. It is generally agreed [53,54] that the weak long wave length band at around 440 mμ is due to the azo group, and the constant band at 228 mμ to some transition in the benzene ring, since the extinction of these partial bands is fairly proportional to the number of the respective groups in the molecule.

The migrating band of highest extinction is supposed to involve the total chromophore. In order to estimate wave length positions of overtone bands, one has to choose arbitrary relative chromophoric values of the azo group and the benzene ring, and to plot the wave length maxima against number of chromophoric units. The plot in Fig. 3 has been made by simply assuming them equivalent.

Considering the *para*-series first, λ_2-bands should of course be forbidden, and no evidence of such bands can be found. λ_2-bands are estimated at around 265 mμ for disazobenzene against 275 mμ observed for a "band shoulder" [55]; at 295 mμ for trisazobenzene, which may explain the broadening of the λ_1-band into this region [53]; and at 318 mμ for tetrakisazobenzene against a distinct inflection observed at 308 mμ [53].

In the *ortho*-series only disazobenzene has been prepared by the same authors [52,53], who feel very confused by the peculiar shape of the main band with a peak at 305 mμ and inflection at around 350 mμ. Now the λ_1-band of *para*-disazobenzene lies at 359 mμ (or 352 mμ), and if the inflection at 350 mμ is regarded as a λ_1-band of decreased intensity, the band at 305 mμ must be a λ_2-band or *cis*-peak (estimated from the curve at 303 mμ) *.

Another series for which a more limited number of spectra are available are the "polyphenyls". The benzene nuclei are here directly attached to each other, so that *ortho*-linked chains cannot become coplanar because of steric hindrance. The main bands should therefore become very much degraded in the *ortho*-series.

Published spectra of *para*-polyphenyls include only the very near overtone region [56] where λ_2-bands should not and do not occur. The only member of the *ortho*-series, *o*-diphenylbenzene [57], shows a rudimentary band in the region where p-diphenyl-benzene has its intense λ_1-band ($\lambda_{max.}$ at 276 mμ), thus indicating severe inhibition of resonance. However, a new and strong band has grown up at 232 mμ where the *para*-compound does not absorb. The location is in the region anticipated for $n/2 = 1.5$ (between benzene, 183 mμ, and diphenyl, 246 mμ). It might be argued that this band is a partial band and no overtone. It would then seem natural to expect partial oscillations to involve two benzene rings as evidenced in *m*-diphenylbenzene [57], where conjugation through all three rings is not possible and the main band has exactly the same

* Recent unpublished investigations by the author on the spectra of *o*- and *p*-distyrylbenzene support these assignments.

location as in diphenyl (246 mμ). That the strong band in *o*-diphenylbenzene corresponds in location neither to one nor two benzene nuclei, lends support to the belief that a true overtone is involved.

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Ionization of Acetic Acid in Electrolytic Solutions

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A study of the salt solutions of weak electrolytes presents as good an opportunity for reaching conclusions regarding the dependence of ion activity on the other ions present as the investigation of the solubility of sparingly soluble salts. For this reason the writer has investigated the ionization of acetic acid in solutions of salts of alkali and alkaline earth metals. On the solutions of the former salts detailed assays have been published elsewhere [1], for which reason only a brief summary will be given here. Comments and further conclusions drawn from these have, however, been added.

MEASUREMENTS PERFORMED ON SOLUTIONS OF SALTS OF ALKALI METALS

The measurements were performed on solutions of 11 different salts and at two different ionic strengths. The conclusion was that the following equations are at least approximately true:

$$\begin{aligned} &pK_{KCl}-pK_{NaCl} = pK_{KBr}-pK_{NaBr} = pK_{KNO_3}-pK_{NaNO_3} = pK_{KClO_4}-pK_{NaClO_4} \\ &pK_{NaCl}-pK_{LiCl} = pK_{NaBr}-pK_{LiBr} = pK_{NaNO_3}-pK_{LiNO_3} = pK_{NaClO_4}-pK_{LiClO_4} \end{aligned} \quad (1)$$

and

$$\begin{aligned} &pK_{NaClO_4}-pK_{NaBr} = pK_{KClO_4}-pK_{KBr} = pK_{LiClO_4}-pK_{LiBr} \\ &pK_{NaBr}-pK_{NaCl} = pK_{KBr}-pK_{KCl} = pK_{LiBr}-pK_{LiBr} \end{aligned} \quad (2)$$

where $pK = -\log K$.

The conformity presented by the equations can be expressed as follows: *If, in solutions of equal ionic strength one ion of the electrolyte is substituted for another, this always causes the same change in the* pK *value for acetic acid, no matter what ion of the same charge the other remaining ion is. For this reason the* pK *value for acetic acid is an additive quantity separately dependent on each species of ion present.*

The additivity phenomenon and Brønsted's theory

Brønsted [2] was the first to note the additivity phenomenon when he investigated the solubility of sparingly soluble salts in electrolyte solutions. These observations made with dilute solutions led Brønsted to present his

theory of specific ionic interaction [3]. Applying Brønsted's theory to the ionization of acetic acid the influence of the activity of the unionized part on the equilibrium must also be taken into consideration. Larsson [4] was the first to note that the logarithm of the activity coefficient of the unionized part of benzoic acid is additive in the same way as the pK shown above. From Sugden's[5] measurements it can be proved that the same is also true for acetic acid. Applying Brønsted's theory to the case in question we have, for example,

$$pK_{KCl} - pK_{NaCl} = pf_{Ac(K)} + pf_{(K)} - pf_{Ac(Na)} - pf_{(Na)} \quad (3)$$

It can be observed that the difference indicated is independent of the anion of the salt added. In the equation Ac stands for the anion of acetic acid. This result is the same as that expressed by equations (1). Similarly it can be shown that equations (2) are also in conformity with Brønsted's theory. Consequently the observations reported here and those of Brønsted are similar.

Harned [6] has examined whether Brønsted's theory is applicable to concentrated solutions and found that it cannot be used for solutions stronger than 0.1 m. Harned's results and the measurements presented for acetic acid are apparently in disagreement, which prompted us to more detailed examination.

Brønsted's theory can be represented by two postulates [7a]:

1. Ions of unlike sign react differently upon one another, while ions of like sign react identically with one another.

2. Each ion has a characteristic "salting out effect" which acts upon all other in the solution.

The first postulate evidently corresponds to the concept of the structure of the solution given by Debye-Hückel's theory [8]. The same cannot be said about the second postulate. According to this each ion has a salting out and salting in effect upon another ion irrespective of the specific properties of the latter. Randall and Failey [9], Harned and Owen [7b] and Long and McDevit [10] have collected data relating to the salting out and salting in effect upon neutral molecules. These data indicate that the specific properties of the particle upon which the effect is focussed must be taken into consideration. For this reason a corresponding correction should be made in the second postulate. Thus the activity coefficient of, for instance, hydrochloric acid in a solution of potassium chloride may be written as follows:

$$f_{HCl(KCl)} = f_{H(K)} f_{H(Cl)} f_{(H,K)} f_{(H,Cl)} f_{Cl(K)} f_{Cl(Cl)} f_{(Cl,K)} f_{(Cl,Cl)} \quad (4)$$

where $f_{(H,K)}$, $f_{(H,Cl)}$, $f_{(Cl,K)}$ and $f_{(Cl,Cl)}$ represent the specific salting out and salting in terms obtained after applying the corrections to Brønsted's theory.

The following equations are obtained for the activity coefficients of potassium chloride in hydrochloric acid, hydrochloric acid in hydrochloric acid and potassium chloride in a solution of potassium chloride, respectively.

$$f_{KCl(HCl)} = f_{K(H)} f_{K(Cl)} f_{(K,H)} f_{(K,Cl)} f_{Cl(H)} f_{Cl(Cl)} f_{(Cl,H)} f_{(Cl,Cl)} \quad (5)$$

$$f_{HCl(HCl)} = f_{H(H)} f_{H(Cl)} f_{(H,H)} f_{(H,Cl)} f_{Cl(H)} f_{Cl(Cl)} f_{(Cl,H)} f_{(Cl,Cl)} \quad (6)$$

$$f_{KCl(KCl)} = f_{K(K)} f_{K(Cl)} f_{(K,K)} f_{(K,Cl)} f_{Cl(K)} f_{Cl(Cl)} f_{(Cl,K)} f_{(Cl,Cl)} \quad (7)$$

If the specific salting out and salting in terms are taken into consideration when an equation corresponding to equation (3) is derived, we have

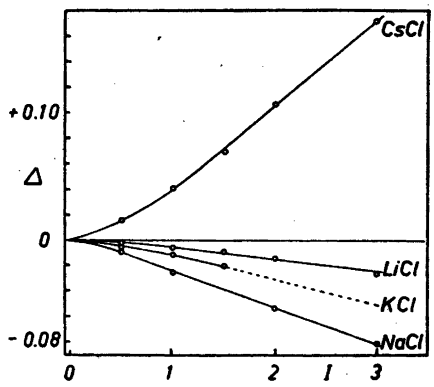

Fig. 1. Plots of Δ (equation 10) against ionic strength.

$$pK_{KCl} - pK_{NaCl} = pf_{Ac(K)} + pf_{(Ac,K)} + pf_{(H,K)} - pf_{(HAc,K)} - pf_{Ac(Na)} + pf_{(Ac,Na)} + pf_{(H,Na)} - pf_{(HAc,Na)} \quad (8)$$

which, like equation (3), indicates that the difference is dependent only on the cation of the electrolyte added.

From Brønsted's theory in its original form it follows directly that the following equations are valid:

$$\log \frac{f_{AgCl(HCl)}}{f_{AgCl(MCl)}} = \log \frac{f_{RCl(HCl)}}{f_{RCl(MCl)}} = \log \frac{f_{HCl(HCl)}}{f_{HCl(MCl)}} \quad (9)$$

Harned and his co-workers [11], using the potentiometric method, have investigated the activity coefficient of hydrochloric acid in aqueous solutions of alkali chlorides. On the basis of the values calculated from these measurements by Harned [6], it is possible to ascertain whether equation (9) is valid for different cases. For this purpose equation (9) can be written in the following form

$$\log \frac{f_{HCl(HCl)}}{f_{HCl(MCl)}} - \log \frac{f_{MCl(HCl)}}{f_{MCl(MCl)}} = \Delta \quad (10)$$

where MCl denotes alkali chloride. In Fig. 1 the value of Δ is presented as a function of the ionic strength.

In all cases the value of Δ seems to approach zero as the ionic strength approaches zero. The requirement $\Delta = 0$, which is prescribed by the original theory of Brønsted, is almost fulfilled in a 0.1 molal solution indicating that this theory is almost true when $0 < I < 0.1$. On approaching greater ionic strengths, however, the value of Δ increases or decreases rapidly from zero.

If, on the other hand, equations (4), (5), (6), and (7) are inserted in equation (10), it is observed that

$$\log \frac{f_{(H,H)}}{f_{(H,M)}} - \log \frac{f_{(M,H)}}{f_{(M,M)}} = \Delta \quad (11)$$

where Δ may have values differing from 0, as in Fig. 1. According to Harned and Owen [7c] it can easily be shown that the theory modified as suggested no longer assumes that at constant ionic strength the osmotic coefficient in a

solution of two electrolytes is a linear function of the concentration of both components. Owen and Cooke [12] have been able to prove by isopiestic measurements that the osmotic coefficient does not increase linearly, but that the relation is of higher order.

Debye-Hückel's formula and Brønsted's theory

When Brønsted's original theory is applied to the ionization of acetic acid in a solution of potassium chloride, we get the equation:

$$pK_{KCl} = pK_o - pf_{H(K)} - pf_{H(Cl)} - pf_{Ac(Cl)} - pf_{Ac(K)} - pf_{(K)} - pf_{(Cl)} \qquad (12)$$

Assuming that Brønsted's first postulate corresponds to the quotient term of Debye-Hückel's extended semi-empirical equation and that the second postulate corresponds to the linear term of the equation, the dependence of the pK value for acetic acid on the concentration of potassium chloride can be represented by the following equation:

$$pK_{KCl} = pK_o - \frac{A'\sqrt{I_-}}{1 + a_{H(-)}\sqrt{I_-}} - \frac{A'\sqrt{I_+}}{1 + a_{Ac(+)}\sqrt{I_+}} + BI, \qquad (13)$$

where

$$\frac{A'\sqrt{I_-}}{1 + a_{(H-)}\sqrt{I_-}} = pf_{H(Cl)} + pf_{H(K)} \qquad (14)$$

$$\frac{A'\sqrt{I_+}}{1 + a_{Ac(+)}\sqrt{I_+}} = pf_{Ac(K)} + pf_{Ac(Cl)} \qquad (15)$$

$$BI = pf_{(K)} + pf_{(Cl)} \qquad (16)$$

In the equations, I_+ represents the strength of the positive ions and I_- that of the negative ions. They are defined as $I_+ = \Sigma z_+^2 c_+$ and $I_- = \Sigma z_-^2 c_-$, where z and c are the charge and the concentration of the ions concerned; I has its old designation. This was deduced from the assumption that according to Brønsted's theory $pf_{H(K)} = pf_{Ac(Cl)}$, and thus they have no specific properties. For this reason only ions of opposite charge give specific properties to equations (14) and (15), their ionic strength also being crucial. For the same reason the a parameter is given a designation which indicates what specific interaction between the ions is referred to.

In the following a method for evaluating these parameters is presented. This seems to give at least approximately reliable results and it lends support to the ideas presented. It differs from the method of calculation proposed earlier by the writer [13], which later proved to be incorrect.

Debye-Hückel's equation expressing the negative logarithm of the activity coefficient of, for instance, hydrochloric acid is written as a function of the ionic strength in a form corresponding to equation (13),

$$pf_{HCl(HCl)} = -\frac{A'\sqrt{I_+}}{1 + a_{Cl(H)}\sqrt{I_+}} - \frac{A'\sqrt{I_-}}{1 + a_{H(Cl)}\sqrt{I_-}} + BI + DI^2 \qquad (17)$$

The method of calculation is based on the plausible assumption that $\alpha_{Cl(H)} = \alpha_{H(Cl)}$. Since $f_{Cl(Cl)} = f_{H(H)}$, it follows that $f_{Cl(H)}f_{Cl(Cl)} = f_{H(Cl)}f_{H(H)}$. The same assumption is naturally valid for all solutions of single electrolytes. Where these are concerned, the quotient term of equation (17) can be combined into one, leaving three unknowns in the equation, which can be solved if at least three points on the curve are known.

Even if the α parameters concerned were of different magnitude, the quotient terms could be combined without involving any great error. The α parameter of the combined quotient term is approximately the mean value of the α parameters of the original quotient terms, because the graph of the equation obtained is similar to the original one within the limits of experimental error [13]. This is the case when a mixture of two electrolytes is examined. If equation (17) is used to represent the dependence of the mean activity coefficient of hydrochloric acid on the concentration of potassium chloride the α parameters will be $\alpha_{H(Cl)}$ and $\alpha_{Cl(K)}$, which are not equal. When the quotient terms are combined, the value of the parameter becomes approximately $(\alpha_{Cl(K)} + \alpha_{H(Cl)})/2$. The value of this can be calculated directly from the experimental data. On the other hand, $\alpha_{H(Cl)}$ and $\alpha_{Cl(K)}$ can be obtained from the measurements which were performed with hydrochloric acid and potassium chloride. Inserting these values into equation (17), the sum of the quotient terms can be calculated and from this backwards the value of the α parameter of the combined quotient term, which should be the same as that obtained by direct calculations from the experimental data, if the assumption that $\alpha_{+(-)} = \alpha_{-(+)}$ and Brønsted's first postulate are valid.

Harned and Owen [7d] have calculated the values of Debye-Hückel's parameters for solutions of hydrochloric acid and hydrobromic acid and for the corresponding alkali salts. From Harned's [6] and his co-workers' measurements, which deal with the mean activity coefficient of hydrochloric and hydrobromic acid in solutions of the corresponding alkali metal salts, the values of the corresponding parameters can be calculated. Harned and Hickey [14] have studied the ionization of acetic acid in lithium, sodium and potassium chloride

Table 1.

Solutions	$\alpha_{obt.}$	$\alpha_{calc.}$	Solution	$\alpha_{obt.}$	$\alpha_{calc.}$
HCl(HCl)	1.41		HBr(HBr)	1.45	
NaCl(NaCl)	1.38		NaBr(NaBr)	1.38	
KCl(KCl)	1.27		KBr(KBr)	1.27	
CsCl(CsCl)	0.82		HBr(NaBr)	1.36	1.41
HCl(NaCl)	1.34	1.39	HBr(KBr)	1.38	1.35
HCl(KCl)	1.28	1.33	* HCl(HCl)	1.44	
HCl(CsCl)	1.03	1.07	* LiAc(LiAc)	1.35	
NaCl(HCl)	1.39	1.39	* NaAc(NaAc)	1.30	
KCl(HCl)	1.15	1.33	* KAc(KAc)	1.44	
CsCl(HCl)	1.05	1.07	* HAc(LiCl)	1.51	1.40
			* HAc(NaCl)	1.40	1.37
			* HAc(KCl)	1.25	1.44

* Has not been converted into mole fractions.

solutions. Harned and Owen [7c] report the mean activity coefficients of the corresponding acetates. The same calculation can also be made from these data. In the foregoing table the results are listed. In the third column are found the values of the α parameters obtained for mixed solutions by means of the method of calculation presented above, utilizing the values of the α parameters for pure solutions. The values obtained and those calculated are in good agreement in all cases except for KCl (HCl), HAc (LiCl) and HAc(KCl).

If the value 1.41 is used for the parameter $\alpha_{H(Cl)}$, the corresponding values for the α parameter of acetic acid in all the cases investigated [1] can be calculated The values are listed in Table 2.

Table 2.

$\alpha_{Ac(Li)} = 1.08$	$\alpha_{Ac(Na)} = 1.515$	$\alpha_{Ac(K)} = 1.70$	
$\alpha_{H(Cl)} = 1.41$	$\alpha_{H(Br)} = 1.29$	$\alpha_{H(ClO_4)} = 1.59$	$\alpha_{H(NO_3)} = 2.03$

When these values are compared to those in Table 1 we notice that the three first are in opposite order in respect of their size. This may be caused by the acetate ion.

Olson and Simonson [15] have studied equilibria in which only ions of like sign take part. They noticed that in the cases investigated only those ions of the added electrolytes which were of the opposite charge to the ions of the system affected the equilibrium and the activity of the ions taking part in it. These experiments were performed in dilute solutions, in which the quotient term of Debye-Hückel's equation is the determining factor. Thus, Olson's and Simonson's observations are completely in agreement with those presented above. Kilpi and his co-workers [16] investigated similar equilibria, but in more concentrated solutions. They studied the hydrolysis of carbonate and phosphate ions and found that Olson's and Simonson's observations also hold approximately for more concentrated solutions. In both systems, however, the influence of ions of like sign is also clearly noticeable. According to the investigators mentioned these differences are certainly of almost the same magnitude as the probable error, but since in both cases the order of the experimental data is the same with respect to the anion of the added electrolyte, the difference must, in writer's opinion, be real. This is also to be expected on the basis of Brønsted's original theory as well as on that of the modification suggested here.

Measurements performed on mixed solutions of alkali metal salts

By means of numerous measurements on mixed solutions of alkali metal salts it was found that at constant ionic strength, the pK is a linear function of the concentration of the components. Some of the measurements are illustrated in Fig. 2. The diameter of the circles representing the experimental data is 0.004 pK units. A direct consequence of the linear relation is the additivity of pK, since by mixing, for instance, sodium chloride and potassium bromide in equivalent proportions the same solution is obtained as by dissolving sodium bromide and potassium chloride in the same proportions. Thus both

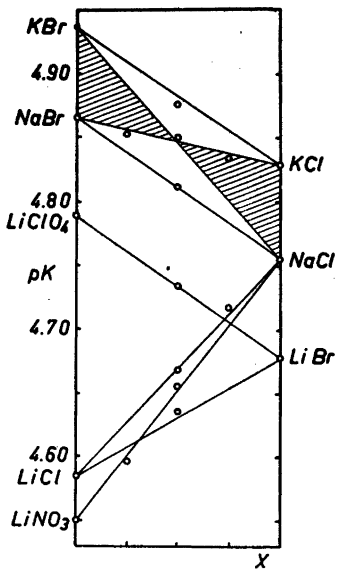

Fig. 2. pK of acetic acid in some mixtures of 2.7 molar total concentration plotted gainst the composition x of the solution.

mixed systems have this point in common, and consequently two coincident triangles are formed, from which it further follows that $pK_{KBr}—pK_{NaBr} = pK_{KCl}—pK_{NaCl}$.

MEASUREMENTS PERFORMED ON SOLUTIONS OF SALTS OF ALKALINE EARTH METALS

In view of the results obtained for solutions of alkali metal salts it seemed interesting to investigate the ionization of acetic acid in aqueous solutions of salts of the alkaline earth metals. It was assumed that in the case to be investigated the additivity should be valid just as for 1—1 salts, and solutions were used in which the concentration of the anion was the same as in the solutions of the 1—1 salts.

Comparison of the results with earlier measurements and Debye-Hückel's parameters

The ionization of acetic acid in electrolytic solutions has been investigated by several investigators. Only Harned and Hickey [17], and Larsson and Adell[18] have, however, performed measurements on solutions of 2—1 salts, the former only on solutions of barium chloride, the latter both with barium and strontium chloride. In Table 3 the results of these investigators are compared with those of the present author.

Since the measurements were performed at two different ionic strengths and since the value of the thermodynamical ionization constant is known, the parameters of Debye-Hückel's equation can be evaluated. When the value

Table 3. The pK *of acetic acid in some 2—1 salt solutions according to various authors.*

Concentration in molalities			Concentration in molarities				
I	$BaCl_2$		I	$BaCl_2$		$SrCl_2$	
	Harned Hickey	Ellilä		Larsson Adell	Ellilä	Larsson Adell	Ellilä
0.11	4.540	4.529	0.16	4.516	4.502	4.510	4.489
0.51	4.442	4.418	0.76	4.406	4.397	4.385	4.371
1.01	4.420	4.387	1.36	4.391	4.385	—	—
2.01	4.434	4.386	1.51	—	—	4.362	4.359
			3.01	—	—	4.414	4.417

4.757, determined for pK_o of acetic acid by Harned and Ehlers [20] and by Kilpi and Meretoja [19], is employed, the parameters obtain the values listed in Table 4.

Table 4. The a and B parameters of the Debye-Hückel formula.

Anion	Cl		NO_3		ClO_4	
Cation	a	B	a	B	a	B
Ba	1.286	0.0738			1.215	0.1206
Sr	1.089	0.0887	1.310	0.0607	1.125	0.1188
Ca	1.045	0.0575	1.140	0.0426	1.035	0.0933

The parameters in the table follow the expected sequences with respect to their magnitudes with the exception that in the chloride column the order of the B terms for strontium and calcium is improbable. According to the theory, pK values compared to mole fractions should have been used in the calculations of these parameters. This, however, was not done. Instead the values of the last column in Table 7 were used directly, as the aim was not to compare the values of the parameters with the theory but to facilitate an interpolation and extrapolation by means of them.

The additivity of the pK of acetic acid in solutions of 2—1 salts

In Table 5 the experimental results are listed as pK values. The differences of the values for different anions are also calculated.

In the fifth column of Table 5 the mean value of the differences is given. The error is ca. $\pm$ 0.004 pK units when $I = 1.5$. This is almost the same as for solutions of 1—1 salts. When $I = 4.05$ the error is ca. $\pm$ 0.008 pK units, which is slightly smaller than that for 1—1 salts. Thus, the additivity holds for 2—1 salts with approximately the same accuracy as for 1—1 salts.

The accuracy of the measurements is insufficient to allow of a reliable comparison between the differences listed in Table 5 and those observed for the corresponding 1—1 salts. For 1—1 salts it is observed [1] that if $I = 1.0$, it follows that $pK_{ClO_4}-pK_{Cl} = 0.084$, $pK_{ClO_4}-pK_{NO_3} = 0.078$ and pK_{NO_3}

Table 5. Differences due to different anions between the pK values of acetic acid measured in solutions of 2—1 salts.

$I = 1.5$

Anion	Ca	Sr	Ba	mean of difference	2/3 of diff. of 1—1 salts
ClO_4	4.350	4.414	4.439		
Cl	4.300	4.359	4.386		
diff.	0.050	0.055	0.053	0.052	0.056
ClO_4	4.350	4.414			
NO_3	4.304	4.377			
diff.	0.046	0.037		0.041(5)	0.052
NO_3	4.304	4.377			
Cl	4.300	4.359			
diff.	0.004	0.018		0.011	0.004

$I = 4.05$

ClO_4	4.474	4.614	4.653		
Cl	4.333	4.478	4.488		
diff.	0.141	0.136	0.165	0.147	0.136
ClO_4	4.474	4.614			
NO_3	4.311	4.442			
diff.	0.163	0.172		0.167(5)	0.163
Cl	4.333	4.478			
NO_3	4.311	4.442			
diff.	0.022	0.036		0.029	0.027

$-pK_{Cl} = 0.006$. If $I = 2.7$ we have correspondingly 0.202, 0.244 and 0.042. These values multiplied by two thirds are appended in the sixth column of Table 5. Within the limits of experimental error it seems to be true that, if the concentration of the anion is the same, the differences in pK for the anions in solutions of 2—1 salts equal two thirds of the values which they have in solutions of 1—1 salts. In the light of Brønsted's theory this means that the effect of the anion in a solution of a 2—1 salt is only two thirds of its effect in a solution of a 1—1 salt. Apparently the effect of the anion in a solution of a 3—1 salt would be only half of its effect in a solution of a 1—1 salt, *etc.* This means that, depending on the properties of the dissolved salt that affect the pK value of acetic acid, the corresponding Debye-Hückel equation also attains different forms. For 2—1 salts equation (13) should be written as follows:

$$pK_{2\text{-}1} = pK_o - \frac{2/3\ A' \sqrt{I_-}}{1 + a_{H(\cdot)} \sqrt{I_-}} - \frac{4/3\ A' \sqrt{I_+}}{1 + a_{Ac(+)} \sqrt{I_+}} + BI \qquad (18)$$

and for 3—1 salts

$$pK_{3\text{-}1} = pK_o - \frac{1/2\ A'\sqrt{I_-}}{1 + a_{H(-)}\sqrt{I_-}} - \frac{3/2\ A'\sqrt{I_+}}{1 + a_{Ac(+)}\sqrt{I_+}} + BI \qquad (19)$$

It should be noted that the sum of the ratios which represent valence factors must equal two in order that the limiting slope of the combined equation may obtain a correct value which is in conformity with the experimental data. Consequently, if the effect of the anion in a solution of a 2—1 salt is two thirds of its effect in a solution of a 1—1 salt, the effect of a bivalent cation on the anion of acetic acid must be correspondingly greater than that of an equivalent quantity of univalent cation. For this reason the coefficient must be equal to 4/3.

MEASUREMENTS ON MIXED SOLUTIONS OF 2—1 AND 1—1 SALTS

In mixed solutions of 1—1 salts it was observed that the pK of acetic acid at constant ionic strength was a linear function of the concentration of the components. With a few measurements it was found that the same is also true for mixed solutions of 2—1 salts.

In investigations of mixtures of 2—1 and 1—1 salts it was found [21] that the logarithm of the mean activity coefficient increases or decreases linearly at constant ionic strength. Similar investigations were performed with acetic acid in mixed solutions of sodium chloride and barium chloride. In Fig. 3 the results of the measurements are illustrated. It is observed that the pK increases almost linearly with the increase of the ionic strengths of the components. Each point in the figure represents the mean of two measurements. The mean of the differences between the parallels for six points is ca. 0.005 pK units. The diameter of the circles which represent the results is 0.005 pK units, which can be considered the probable error of the measurements. As all the points measured lie below the straight line between the limiting values, it is, however, to be considered probable that the relationship investigated is not completely linear, but that the pK is dependent on the ionic strengths of the components according to an equation of higher order. The deviation from the linear relation is not great, but apparently real. Randall and Breckenridge[22] have made a similar observation at high concentration when they investigated the change of the mean activity coefficients of hydrochloric acid in solutions of barium chloride and hydrochloric acid at constant ionic strength.

Just as the concentration of the 2—1 salts was chosen so that the anion concentration was the same as that used for 1—1 salts, a series of measurements was performed on a mixture where the anion concentration was constant. The salts in the solution were sodium perchlorate and barium perchlorate. The results are illustrated in Fig. 4. Two measurements were made in each of the two pure solutions and the mean values of these was taken as the actual result. On the other hand, in the mixtures only one measurement was made for each point. The diameter of the circles representing the experimental results is 0.005 pK units, as in the former case.

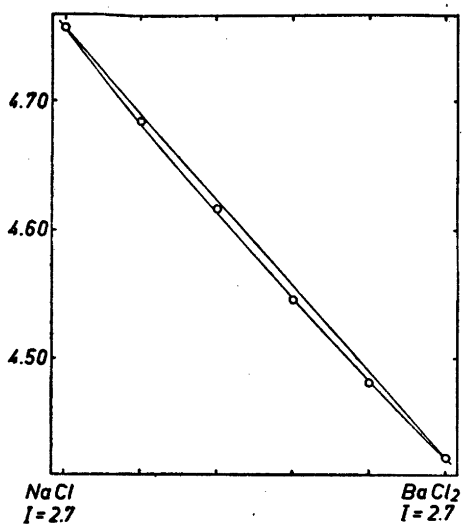

Fig. 3. pK of acetic acid in mixtures of sodium chloride and barium chloride. Ionic strength 2.7. The circles represent the measurements. The curve has been calculated utilizing equation (23).

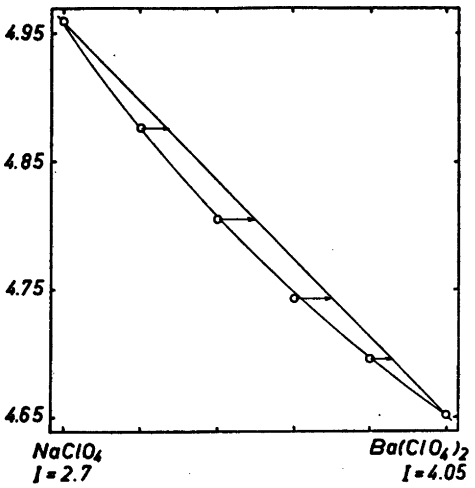

Fig. 4. pK of acetic acid in mixtures of sodium perchlorate and barium perchlorate at constant anion concentration. The circles represent the measurements. The curve has been calculate utilizing equation (23).

Figure 4 shows clearly that the relationship between the pK and the composition of the mixture is of a higher order if the anion concentration is maintained unchanged. On the basis of the measurements concerning solutions of 1—1 salts the conclusion was drawn that the cations and the anions of the electrolyte affect the equilibrium separately. Accordingly, the curvature of the graph should depend only on the difference in the effect of the univalent and bivalent cations. For a study of the effect of the equivalent ratios of these ions the cases in question are exceedingly interesting.

The study is best undertaken by attempting to find an equation which holds for the experimental data by suitable applications or corrections of Debye-Hückel's equation. The equation must consequently be built up of terms which have been obtained from measurements of both components in pure solutions. The most significant attempts are presented here:

1. The case is examined in the light of the concept of ionic strength. At each point of the curve (Fig. 4) the ionic strength is different. This fact must be taken into consideration and the values of the points should be calculated on this basis. The equation to be used in the calculations has the form

$$\mathrm{p}K = \frac{I_{\mathrm{NaClO_4}}}{I}\,\mathrm{p}K_{\mathrm{NaClO_4}} + \frac{I_{\mathrm{Ba(ClO_4)_2}}}{I}\,\mathrm{p}K_{\mathrm{Ba(ClO_4)_2}} \qquad (20)$$

which represents a straight line.

2. The following equation leads to a good result:

$$pK = \frac{I_{NaClO_4}}{I} pK_{NaClO_4,\, I=2.7} + \frac{I_{Ba(ClO_4)_2}}{I} pK_{Ba(ClO_4)_2,\, I=5.4} \qquad (21)$$

Here, the ratio of the ionic strengths was used. Thus the measured points in the graph move through distances which are represented by the arrows. They then come to lie, within the accuracy of measurements, on the straight line that connects the limiting values. As regards the concept of ionic strength this calculation method is incorrect, since according to Debye-Hückel's theory the ionic strength of the solution to be investigated must be used. In equation (21) the ionic strengths of the limiting values are considered, whereas at the point examined the ionic strength has a value which lies between these values.

Equation (21) has, however, an interesting significance of its own. According to it the pK value is linearly dependent on the relation between the ionic strengths if the anion concentration is constant. For instance of sodium chloride and barium bromide the same solution is obtained as of sodium bromide and barium chloride when they are mixed in equivalent proportions. For this reason it follows directly from equation (21) that

$$2/3(pK_{NaBr} - pK_{NaCl}) = pK_{BaBr_2} - pK_{BaCl_2} \qquad (22)$$

This means that for the anions the difference between the values of pK is equal to 2/3 of the correspoding differences in solutions of 1—1 salts. This provides evidence in favour of the observation mentioned in connection with Table 5.

3. For acetic acid in a solution of sodium perchlorate, equation (13) is valid. Likewise equation (18) is valid in a solution of barium perchlorate. When these equations are combined, an equation is obtained for the case illustrated in Fig. 4.

$$pK = pK_o - \frac{4/3\ A'\sqrt{I_+}}{1 + a_{Ac(Ba)}\sqrt{I_+}} - \frac{A'\sqrt{I_+}}{1 + a_{Ac(Na)}\sqrt{I_+}} - \frac{2/3C_{Ba} + 1/2C_{Na}}{I_-} - \frac{2\ A'\sqrt{I_-}}{1 + a_{H(ClO_4)}\sqrt{I_-}} + I_{NaClO_4}B_{NaClO_4} + I_{Ba(ClO_4)_2}B_{Ba(ClO_4)_2} \qquad (23)$$

This equation is derived on the assumption that the effect of the different ions is proportional to their ionic strength. If the observations and the conclusions drawn from them are correct, then equation (23) should correspond to the measurements illustrated in Figs. 3 and 4. Before this the values of the corresponding a parameters must be calculated. These can be calculated by the insertion in equation (18) of 1.59 from Table 2 in place of $a_{H(ClO_4)}$ and correspondingly from Tables 4 and 5 the values of pK and $I_{Ba(ClO_4)_2}$. If $I = 4.05$, the result is $a_{Ac(Ba)} = 1.10$. Inserting these values and 0.2481 for B_{NaClO_4}[1] and 1.515 for $a_{Ac(Na)}$ [1] into equation (23), the curve in Fig. 4 is obtained. The agreement between the calculated and observed values is good, as the mean value of their differences is 0.0035 pK units.

Correspondingly the case illustrated in Fig. 3 can be calculated. As the experiments do not yield the ideal results displaying additivity, the correspond-

ing α parmaeters do not have constant values either. For this reason in the case in question the value of $\alpha_{Ac(Ba)}$ must be calculated again. The result is 1.27. Utilizing this and 0.1813 for B_{NaCl}[1] values are obtained which are represented by the curve in Fig. 3. The agreement is good again. The corresponding case observed by Randall and Breakenridge, which was mentioned earlier, can apparently be explained on the same basis.

The α parameters for multivalent ions

In Table 1 it has been shown that the assunptions that $\alpha_{+(-)} = \alpha_{-(+)}$ and that the α parameter is a function of the sum of the ionic radii of two interacting ions of unlike sign are not in disagreement with the experimental data for 1—1 salts and acids. A similar investigation may be made for ions of higher valences. For this purpose the equation representing the dependence of the activity coefficient of 2—1 salts on their concentrations is written in the following form:

$$\mathrm{p}f = \frac{10/3\ \mathrm{A}'\sqrt{I_-}}{1 + \alpha_{+(-)}\sqrt{I_-}} + \frac{8/3\ \mathrm{A}'\sqrt{I_+}}{1 + \alpha_{-(+)}\sqrt{I_+}} - \mathrm{B}I - \mathrm{D}I^2 \qquad (24)$$

It should be noted concerning the valence factors in the equation that the activity coefficient for barium chloride, for instance, is in the form $f_{Ba} \cdot f_{Cl}$. Earlier it was found (eq. (18)) that univalent ions, on which bivalent ions act, have a valence factor equal to 4/3. Since in this case there are two of them, we have 8/3. On the other hand, according to Debye-Hückel the value of the limiting slope is $6\ \mathrm{A}'\sqrt{I}$. Of the factor 6, 8/3 have already been used, leaving 6—8/3 = 10/3 for the barium ion.

The dependence of the mean activity coefficient of hydrochloric acid on this factor is expressed by equation (18), to which for the sake of conformity a second-order term DI^2 is to be added. Robinson [23] has measured the mean activity coefficient for strontium chloride at different concentrations, whereas Vance [24] has determined the mean activity coefficient of hydrochloric acid in solutions of strontium chloride. When $\alpha_{H(Cl)} = 1.41$ is inserted into the earlier mentioned equation (18), which represents the dependence of the mean activity coefficient of hydrochloric acid on the concentration of strontium chloride, and when the parameter $\alpha_{Cl(Sr)}$ is calculated, the result is 1.61. When this value in turn is inserted into equation (24) and the parameter $\alpha_{+(-)}$ is evaluated, we have $\alpha_{+(-)} = 1.12$, which should be equal to $\alpha_{Cl(Sr)}$. The agreement is not good. Harned and Geary [25] have determined the mean activity coefficient of hydrochloric acid in solutions of barium chloride, and Robinson [23] that of barium chloride. If their experimental data are treated in the above way, the result is also unsatisfactory.

The dependence of the activity coefficient of 3—1 salts on concentration is expressed by equation

$$\mathrm{p}f = \frac{9/2\ \mathrm{A}'\sqrt{I_+}}{1 + \alpha_{-(+)}\sqrt{I_+}} + \frac{15/2\ \mathrm{A}'\sqrt{I_-}}{1 + \alpha_{+(-)}\sqrt{I_-}} - \mathrm{B}I - \mathrm{D}I^2 \qquad (25)$$

Correspondingly equation (19) represents the activity coefficient of hydrochloric acid in a solution of a 3—1 salt.

Robinson has corrected the values of the mean activity coefficient of cerium chloride obtained by Mason [21]. When the a parameters calculated from these values and from those of Mason and Kellam [26] for hydrochloric acid in a solution of cerium chloride are compared in the manner described above, it is found that even in this case the agreement is not satisfactory.

These deviations may partly depend on the inaccuracy of the measurements but apparently other factors are also involved. The limiting slopes of the equations deviate somewhat from the original values of Debye-Hückel. For instance, in normal solutions of 2—1 salts

$$\begin{aligned} 4/3\ A'\sqrt{I_+} + 2/3\ A'\sqrt{I_-} &= 2.552\ A' \\ 2\ A'\sqrt{I} &= 2.450\ A' \end{aligned}$$

and

$$\begin{aligned} 8/3\ A'\sqrt{I_+} + 10/3\ A'\sqrt{I_-} &= 7.103\ A' \\ 6\ A'\sqrt{I} &= 7.350\ A' \end{aligned}$$

If in the value of A' a correction corresponding to the difference is made, the result is no better. A great number of cases are, however, known in which the limiting slope does not completely correspond to the theoretical one, for which reason it is difficult to judge this effect. On the other hand, it should further be noted that the curves represented by the equations are not of entirely the same form as those represented by Debye-Hückel's original equation. The difference is not great, but at small ionic strengths, used in order to give the same results, the a parameters have values which differ considerably from each other. For instance, in a 0.01 M solution of a 2—1 salt the following requirement is set for the quotient term of a 1—1 salt, if it is assumed that $a_{-(+)} = a_{+(-)} = 1$.

$$\frac{4/3\ A'\sqrt{0.04}}{1+1\sqrt{0.04}} + \frac{2/3\ A'\sqrt{0.02}}{1+1\sqrt{0.02}} = \frac{2\ A'\sqrt{0.03}}{1+0.8\sqrt{0.03}} \qquad (26)$$

whereas for a 1 molar solution the requirements are as follows:

$$\frac{4/3\ A'\sqrt{4}}{1+1\sqrt{4}} + \frac{2/3\ A'\sqrt{2}}{1+1\sqrt{2}} = \frac{2\ A'\sqrt{3}}{1+0.99\sqrt{3}} \qquad (27)$$

Thus, for ionic strengths the equations become identical with Debye-Hückel's equation. This again indicates that the values of the a parameters should be written as functions of the concentration, their value being greater at low concentrations than at high concentrations. Taking these facts into consideration, it is difficult to determine the validity or non-validity of the theory for multivalent ions on the basis of these calculations.

If, however, the calculations are treated in the way suggested in connection with Table 1, we obtain the results summarized in Table 6.

Table 6.

* Solution	$a_{obt.}$	$a_{calc.}$	Solution	$a_{obt.}$	$a_{calc.}$
HCl(HCl)	1.44		HCl(HCl)	1.41	
$BaCl_2$($BaCl_2$)	1.31		$SrCl_2$($SrCl_2$)	1.50	
HCl($BaCl_2$)	1.42	1.38	HCl($SrCl_2$)	1.51	1.46

* Has not been converted into mole fractions.

It was earlier mentioned that Olson and Simonson have investigated the hydrolysis of the ferric ion. In this case the equilibrium may be expressed according to the law of mass action as $[FeOH^{++}][H^+]/[Fe^{+++}] = K$. According to Debye-Hückel's theory, the limiting slope should obtain the value $-4\ A'\sqrt{I}$. The investigators mentioned observed, however, that the value of the limiting slope remained unchanged independent of the charge on the cation of the electrolyte added. Thus, in this case the limiting slope can be written in the form $-4\ A'\sqrt{I_-}$. If the ideas presented are applied to this case, the values of the limiting slopes are as follows:

Salt	
1—1	$(1 + 4—9)\ A'\sqrt{I_-} = -4\ A'\sqrt{I_-}$
2—1	$(2/3 + 10/3—8)\ A'\sqrt{I_-} = -4\ A'\sqrt{I_-}$
3—1	$(1/2 + 3—15/2)\ A'\sqrt{I_-} = -4\ A'\sqrt{I_-}$

The terms 1, 4 and 9 in the sum at the left of the first equation follow directly from Debye-Hückel's theory. 2/3 and 10/3 are obtained from equation (18) and (24), and 1/2 and 15/2 from equations (19) and (25). 8 can be derived by considering that the ferric ion and the anion of the added salt, which is affected by the bivalent cation of this salt, act upon each other. The activity coefficient of this couple is consequently $f_{Fe^{+++}} \cdot f_-^3$ and the corresponding valencee factor according to Debye-Hückel $9 + 3 = 12$. As, however, the amount of anions of the added salt is large in comparison to the amount of ferric ions, the former is the deciding factor. Since it is affected by a bivalent anion, its valence factor is 4/3, leaving $12—3 \times 4/3 = 8$ for the ferric ion. The valence factor of the $FeOH^{++}$ ion can be calculated according to the same principle, yielding $6—2 \times 3/2 = 3$.

In the same way the values of the limiting slopes corresponding to equilibria investigated by Kilpi and his co-workers could be calculated, whereby it should be noted that they also have obtained values which differ from Debye-Hückel's limiting slopes. The equilibria investigated by them do not, however, make it possible to control this matter experimentally, because according to Kilpi [27] the titrability vanishes in so dilute solutions as should be used in the investigation.

EXPERIMENTAL

The investigation was carr ed out at 25° C according to a method based on potentiometric titration and developed by Kilpi [28]. As electrode a quinhydrone electrode was mostly used. The accuracy of the potentiometer readings was 0.01 mV, which corresponds

Table 7. Experimental results obtained in aqueous solutions of 2—1 salts at 25°.

Electrolyte	I	P · 10^3	pK	pK_{mean}
$CaCl_2$	1.5	6.320	4.300	4.300
		6.320	4.300	
	4.05	6.115	4.330	
		6.105	4.332	4.333
		6.070	4.336	
$Ca(NO_3)_2$	1.5	6.320	4.300	
		6.255	4.309	4.304
		6.310	4.302	
	4.05	6.275	4.307	
		6.255	4.309	4.311
		6.200	4.317	
$Ca(ClO_4)_2$	1.5	6.000	4.348	4.350
		5.970	4.352	
	4.05	5.220	4.472	4.473
		5.205	4.475	
$SrCl_2$	1.5	5.890	4.364	
		5.935	4.357	4.359
		5.935	4.357	
	4.05	5.185	4.478	4.477
		5.195	4.477	
$Sr(NO_3)_2$	1.5	5.770	4.382	4.377
		5.835	4.372	
	4.05	5.405	4.441	4.442
		5.400	4.442	
$Sr(ClO_4)_2$	1.5	5.565	4.414	4.414
	4.05	4.465	4.612	4.614
		4.445	4.616	
$Ba(ClO_4)_2$	1.5	5.430	4.437	4.439
		5.405	4.441	
	4.05	4.265	4.652	4.652
		4.260	4.653	
$BaCl_2$	1.0	5.730	4.389	4.387
		5.750	4.385	
	2.7	5.525	4.422	4.422
		5.515	4.423	

to the accuracy with which the hydroxide can be added during the titration. For the addition of the latter a micro syringe, designed and constructed by the writer, was used, the accuracy being ± 0.1 % for addition of *ca.* 0.1 ml.

In Table 7 only those measurements are listed which were carried out in solutions of salts of alkaline earth metals, because the results of the measurements relating to solutions of alkali salts have been presented earlier. The solutions of the salts of alkaline earth metals were prepared by dissolving a weighed quantity of carbonate in the acid and diluting in a volumetric flask to the proper volume. In the first column of Table 7 is the electrolyte, in the second the concentration of the salt, expressed as ionic strength at the startingpoint of the titration. This is *ca.* 0.2 % higher than that at the inflexion point of the titration curve from which the ionization constant is calculated. In the third column is the buffer capacity, in the fourth the correesponding pK value and in the fifth the mean value of the pK values of parallel measurements. In all cases the concentration of acetic acid at the point of inflexion is 0.04044. The ionization constant was not extrapolated to zero concentration of acetic acid.

SUMMARY

The object of the investigation here reported was to study the ionization of acetic acid in colutions of 1—1 and 2—1 salts and in mixtures of these. Of the salts investigated, 11 were 1—1 salts and 8 were 2—1 salts. The temperature was in all cases 25° C.

The results obtained may be summarized as follows:

1. It was found that the value of pK for acetic acid in solutions of 1—1 salts is an additive quantity dependent separately on each species of ion in the solution.

2. This observation is compared to Brønsted's theory and the confirmity is found to be good.

3. Brønsted's theory is examined in the light of the results of other investigators and a correction made in the second postulate, the modified theory being applicable to concentrated solutions also.

4. Brønsted's theory and Debye-Hückel's equation are compared and a method is suggested for the calculation of the a parameter, which is dependent on the sum of the radii of the two interacting ions. This method seems to be at least approximately valid within the scope of the data presented.

5. The value of pK for acetic acid at constant ionic strength was found to be a linear function of the concentration of the components of the mixture. From this the additivity phenomenon follows directly.

6. Measurements on solutions of 2—1 salts indicated that in these solutions also the additivity phenomenon occurs.

7. In solutions of 2—1 salts it appears that the anion only exerts two-thirds of its effect in a solution of a 1—1 salt, if the anion concentration is the same as in the solution of the 1—1 salt. This observation is examined and generalised to include solutions of salts of still higher valences. The corresponding equations are derived from Debye-Hückel's equation.

8. The results obtained in mixtures of 1—1 and 2—1 salts are discussed. If the ionic strength is constant, pK in an almost linear function of the ionic strengths of the components. When the anion concentration is kept constant, pK is a function of higher order of the concentrations of the components. The corresponding equations are derived.

9. Measurements in which the anion concentration was maintained constant indicate that from them also additivity follows and that the effect of the anions in a solution of a 2—1 salt is 2/3 of its effect in a solution of a 1—1 salt.

10. The possibility of calculating the values of the a parameter, which is dependent on the sum of the radii of the interacting ions, for ions of higher valences also, is examined.

11. It is concluded that, on the basis of the suggestions presented here, it is possible to explain some observed deviations from Debye-Hückel's limiting slope.

Miss *A-L. Mukula*, fil. mag., who made a part of the experimental work, is gratefully acknewledged.

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Studies on Absorption Spectra

I. Results of Calculations on the Spectra and Configuration of Copper(II) Ions

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Various theories for absorption spectra are considered, particularly the theory of Hartmann and Ilse. In the present paper this theory is applied to the spectra of the ammine cupric ions in aqueous solution. The calculations performed show that the chemical and spectroscopical facts can be explained by assuming, with Orgel, a configuration with four planar and two more distant neighbours perpendicular to the plane. This explanation is based upon the crystal field theory of Bethe and van Vleck without taking into account electronic exchange between the cation and the ligands. The distances and dipole moments determined from the spectra in the red and near infrared show that the configuration deviates a few per cent from regular octahedral configuration. The spectra of the ammine complexes can be resolved into at least two bands in agreement with the theory. The theory can further predict the bands of the intermediate complex ions, and in the diammine spectrum allow the characterization of the *cis* band as well as a band belonging to the *trans*-diammine ion. A band in the near ultraviolet discovered by Winther and by Ley is shown to be caused by hydroxo-ammine complexes in small concentrations.

Many investigators have studied the absorption spectra of ions and complexes of the elements in the first transition group, and various theories have been proposed to explain the general appearance and origin of the different bands. After the picture offered by Bohr's atomic theory, it was generally assumed that the spectra were produced by electron transitions between different energy levels, but the difficulty was to decide which electrons were involved and between which levels they were jumping.

To account for the variety of colours observable for the same transition element with different ligands, Luther and Nikolopulos [1] — even before the theory of Bohr — proposed that the spectra were due to "die atomare Struktur der Elektrizität, und die elektrische Natur der chemischen Bindung".

Ten years later Fajans [2] suggested that the colour was caused by the reciprocal influence of the electron system of the metal ion on the electrons of the ligand, paying much attention to the blue colour of the aquo cupric ion and the colourless anhydrous $CuSO_4$. These early suggestions were developed into two theories. Mead [3] was of the opinion that it was the ligands' co-ordinated electrons that were the causes of the bands, while Kato [4] assumed that the spectra in solution were the spectra of the naked ion deformed only by the environment. An explanation on similar lines was set forth by Bose and Mukherji [5], and they proposed a formula for the mean frequencies of the absorption bands which did not correspond well with the experimental facts, however. Ideas from both schools were incorporated in Shibata's theory [6], which was further developed by Tsuchida [7]. The spectra of $[Co(NH_3)_{6-n}(NO_2)_n]^{(3-n)+}$, for example, were divided into three bands, the first originating from the central ion, the second being due to the coordination electrons, and the third caused by anions in *trans*-position. If, in other compounds, there were more bands, they were denoted "special bands" and were considered to originate from different causes.

All these theories were rather *ad hoc* and indeed none of them could predict more than the experimental facts from which they were deduced. Tsuchida's theory was even proved wrong by the more elaborate work of Lindhard and Weigel [8].

Such was the confusing state of affairs until Hartmann and Ilse [9] came forth with their theory for the spectrum of titanium(III) ions, utilizing ideas developed by Bethe [10] and in the school of van Vleck [11]. The hexaquo titanium-(III) ion was assumed to have octahedral configuration. In that case Bethe [10] has proved that under the influence of a cubic crystal field, such as that provided by the six water dipoles, the energy level of the 3d electron will split up into two levels, transition of the 3d electron between which produces one band. The calculation makes use of the quantum-mechanical "perturbation theory", and as the ground system they considered the 3d electron moving in the field originating from the kernel, the field resulting from the water dipoles being treated as the perturbation. The calculations determine the frequency of the maximum of the band as a function of various parameters. Briefly: $\nu_n = \mu \cdot f(R,Z)$, where μ is the point dipole moment of water, and $f(R,Z)$ is a rather complicated function of R, the distance between the point dipole and the metal ion, and Z, the "effective" charge of the cation.

Putting Z for $Ti^{+++} = 22-18 = 4$, $\mu = 1.84$ Debye, *i. e.*, the permanent dipole moment of water, and $R = 1.58$ Å, Hartmann and Ilse found that the maximum absorption of $Ti(H_2O)_6^{+++}$ was placed at 570 mμ; the experimental results [9] show it to be about 490 mμ. Later Hartmann and Ilse [12] extended the theory to the more complicated system of 2 electrons, *viz.* V^{+++}, and also here the theory seems to be tolerably consistent with the experimental facts. But their interpretation of the theory predicts two bands for which the ratio of wavenumbers is 2.25, invariable with the chosen parameters, while the experimental ratio is about 1.4. Other features of the vanadium(III) spectra also deserve further investigation.

This application of the theory suggested to us that quantitative results might be obtained with Cu^{++}, an ion whose complexes and spectra are well

known. The Cu(II)-ion has the electron configuration $1s^2 2s^2 2p^6 3s^2 3p^6 3d^9$, *i. e.* nine d-electrons outside the argon configuration. A relation originating from Pauli and proved for this special case by van Vleck [13] states that $d^n \equiv d^{10-n}$. This means that nine d-electrons give the same number of terms as one d-electron. The system is therefore treated analogously to the Ti(III)-ion. The complete calculations will be published elsewhere by C. J. Ballhausen, but the results are put to use and quoted here. Before we mention the results of the calculations, we shall first discuss the present state of the coordination chemistry of Cu(II).

COORDINATION CHEMISTRY OF COPPER(II) IONS

In the nomenclature of J. Bjerrum [14] the copper(II) ion has the characteristic coordination number 4. This is a way of expressing the fact that the solvated cupric ion, *e. g.* the aquo ion, when exchanging the water molecules consecutively with a ligand of not too different an interference volume [15,16], takes up all of the first 4 ligands with about the same affinity and thereafter has only a slight affinity to take up more ligands.

Transition metals normally have the characteristic coordination number 6, but in some cases they have the characteristic coordination number 4, as *e. g.*, in the nickel(II)-cyanide and cobalt(II)-halide systems. If in such systems the metal ion has residual affinity to bind more than four ligand groups the maximum coordination number is usually 6, as demonstrated in the auric thiocyanate system, where the very stable $Au(SCN)_4^-$ ion nearly statistically takes up two more ligands at relatively high thiocyanate concentrations [17]. For the cupric ammonia system Rosenblatt [18] and one of the authors [19] have shown independently that the rather stable tetrammine cupric ion is converted into a pentammine complex at higher ammonia concentrations. This was proved by spectrophotometric as well as by potentiometric methods [20]. Further, it was found that the pentammine spectrum estimated from mixed tetrammine-pentammine spectra in dilute ammonia solutions is but little different from the spectrum of the cupric ion in liquid ammonia [20]. For this reason it has hitherto not been possible to decide with certainty whether or not the cupric ion in solution is able to exceed the coordination number 5.

The conditions are different in the solid state where cupric salts besides tetrammines and pentammines — occasionally containing 2 and 1 water molecules, respectively — in many cases also form hexammoniates [14,p.98]. Peyronel [21] found by X-ray analysis that $Cu(NH_3)_6Br_2$ has face centered cubic structure and that the 6 ammonia molecules are octahedrally arranged around the copper ion, but this does not agree well with the fact that the crystals are mentioned to be optically anisotropic. The experimental evidence for an undistorted octahedral configuration is more certain for $Ni(NH_3)_6Br_2$, and for $Zn(BrO_3)_2$, 6 H_2O[22], where it was shown directly from evidence of the precise positions of the ligands. It is also remarkable that according to West [23], $Cu(ClO_4)_2$, 6 H_2O has monoclinic symmetry, while the series of analogous salt hydrates of zinc and the transition metals exhibits hexagonal symmetry. The hexacoordinated cupric compounds therefore cannot have a regular octahedral configuration, and it is very reasonable to assume, with Orgel [24], a con-

figuration, with four planar and two more distant neighbours perpendicular to the plane. This configuration is also found in $CuSO_4$, 5 H_2O[25], where only four of the water molecules are co-ordinated to the copper ion, and two oxygen atoms from the sulphate ions are placed in *trans* positions to the plane formed by the copper ion and the 4 water molecules. A special configuration was recently[26] found for cupric acetate monohydrate in which the copper ion is coordinated to 5 oxygen atoms, and is directly linked to another copper atom at a distance of 2.64 Å.

It is interesting that the anhydrous pentammines and hexammines of the copper(II) halides form an isomorphous mixture[27] which has the same symmetry as the hexammine[21], and therefore it cannot be excluded that in solution complex ions may exist with coordination number 5. The pentacyano nickel(II) ion was recently discussed as a possible case of this kind[28].

Considering the fact that tetracoordinated cupric compounds according to X-ray analysis always seem to have a planar configuration, one of the authors[14,p.108] has postulated that the four groups corresponding to the characteristic coordination number are also co-planar in solution. The suggestion that the 5th ammonia molecule occupies an exceptional position is supported by the fact that the absorption band is displaced evenly toward blue by the uptake of the first four molecules of a monamine, or two molecules of a diamine, after which additional binding of one more amine molecule displaces the absorption maximum in the opposite direction.

In later publications J. Bjerrum *et al.*[29,30] and Klixbüll Jørgensen[31] gave more experimental evidence on the problem, and the results published here seems to permit a distinction to be made between the various possible configurations and to furnish a complete understanding of the stereochemistry of copper(II).

RESULTS OF CALCULATIONS OF SPECTRA OF COPPER(II)-COMPLEXES WITH DIPOLE MOLECULES

Four configurational possibilities are *a priori* considered in the calculations, *viz.* the square planar, the tetrahedral, the square pyramidal and the octahedral (*cf.* Fig. 1). In the models point dipoles are placed at the different sites of the configuration at a distance R from the centre of the metal ion, which is situated at the centre of the regular polyhedron; in the square-pyramidal configuration the copper ion is assumed to be placed at the midpoint of the square.

As already mentioned, the quantum-mechanical calculations lead to expressions for the wavenumber of the maximum of the bands. When the R-values are all equal, these functions are of the form:

$$\Delta E_n = hc\nu_n = \mu \left(\frac{Z}{3}\right)^2 \left\{ K_M \left(\frac{3}{ZR}\right)^M - \sum_N P_N \cdot e^{-\frac{2ZR}{3}} \right\} \qquad (1)$$

where K_M is a constant and P_N polynomials of integral degrees of $\left(\frac{3}{ZR}\right)$. Of the parameters, the effective charge Z on the cupric ion is taken as 7.85 a.u., after Slater's screening theory for hydrogen-like wave functions[32]. The distance R is usually known from X-ray analyzed structures of the solid com-

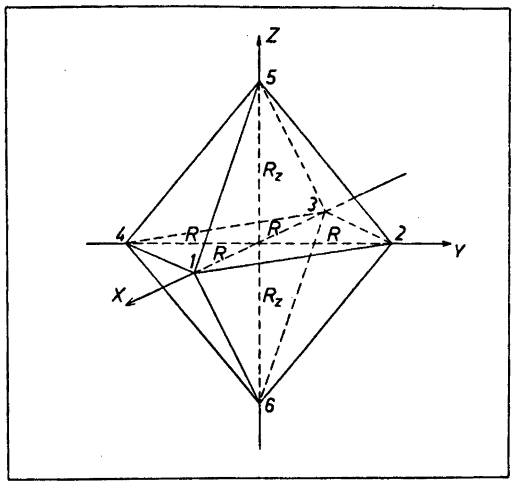

Fig. 1. Model of various types of complexes. The positions labelled 1—4 represent the the square, 1—5 the square pyramid, and 1—6 the octahedron (or tetragonal bipyramid).

plex salts, and it is reasonable to assume that the same values for R can be used for the distances of the metal ions to their nearest neighbours in solution. In the crystalline salt hydrates of the divalent metal ions in the first transition group R-values are usually found to be very nearly 2 Å[33]. Thus Beevers and Lipson [25] determined the distance Cu-H_2O in the plane to be 1.96 Å in $CuSO_4$, $5H_2O$. By studying the influence of the refractive index on the light absorption Schuyer [34] estimates the same distance in aqueous solution to be 2.01 Å. In the analogous ammoniates the distances are perhaps a little higher [21,23], but are not known with the same accuracy [33], and in the calculations reported here distances Cu-H_2O and Cu-NH_3 are put equal. The least known of the parameters is μ, the effective dipole moment of the ligand. This quantity is considered to be the sum of the permanent dipole moment and the induced dipole moment. The permanent dipole moment for water has the value 1.84 D., and for ammonia the value 1.44 D.; the induced dipole moment, on the other hand, cannot be estimated from the polarizability of the ligand because of the very strong electric fields within the complex.

Under the influence of the outer fields the five-fold degenerate ^{2}D-state of the cupric ion splits up into a number of energy levels, the actual number depending on the symmetry of the field. Of the four models considered the regular tetrahedron and octahedron give two energy levels, while the less symmetrical square and square pyramid give 4 levels. The bands arise because of transitions between the ground level and the higher ones. Thus the regular tetrahedral and octahedral configurations both give one band ($\gamma_5^6\gamma_3^3 \rightleftharpoons \gamma_5^5\gamma_3^4$, *cf.* Santen and Wieringen [35]), whilst the two other configurations should theoretically give 3 bands.

Table 1. Results of calculations of models of copper (II)-ion dipole complexes with equal distance to all ligands.

The wavelength of the band of highest frequency λ_1 (in mμ) is given as function of the parameters: R in Å, μ in Debye.

a. The square planar case

R	μ = 1.84	2.00	3.00	4.00	5.00	6.00
1.72	810	740	500	370	300	250
1.84	1 100	1 000	670	500	400	340
2.00	1 620	1 490	990	740	590	500
2.20	2 380	2 190	1 450	1 090	880	730
2.40	3 450	3 180	2 110	1 590	1 270	1 060

b. The tetrahedral case

R	μ = 1.84	2.00	3.00	4.00	5.00	6.00
1.72	3 200	2 940	1 960	1 470	1 180	980
1.84	4 590	4 220	2 810	2 110	1 690	1 410
2.00	7 180	6 600	4 390	3 300	2 640	2 200
2.20	13 500	12 400	8 270	6 220	4 960	4 150
2.40	22 700	20 900	13 900	10 400	8 320	6 960

c. The square pyramidal case

R	μ = 1.84	2.00	3.00	4.00	5.00	6.00
1.72	1 040	960	640	480	380	320
1.84	1 440	1 320	880	660	530	440
2.00	2 200	2 020	1 350	1 010	810	670
2.20	3 460	3 190	2 120	1 590	1 280	1 060
2.40	5 320	4 890	3 260	2 450	1 960	1 630

d. The octahedral case

R	μ = 1.84	2.00	3.00	4.00	5.00	6.00
1.72	1 430	1 320	880	660	530	440
1.84	2 060	1 900	1 270	950	760	630
2.00	3 390	3 110	2 070	1 550	1 250	1 040
2.20	5 750	5 290	3 520	2 640	2 120	1 760
2.40	9 860	9 080	6 050	4 540	3 620	3 030

The wavelength λ_1 of the maximum of the band of highest frequency in the various cases, calculated by insertion in the functions (1), are given in Table 1. The maximum for the aquo cupric ion is situated at 790 mμ, the maximum for the cupric ion in liquid ammonia at 640 mμ, and that for the tetrammine cupric ion at 590 mμ (see Figs. 2 and 3). It is readily seen from the table that these maxima are impossible to explain assuming tetrahedral, and difficult to explain assuming regular octahedral configuration, since R cannot be very different from 2 Å and there must also be a limit to the size of μ. In the two other configurations considered, the ratio of the wavenumbers of the maxima of the possible bands has definite values for a given value of R

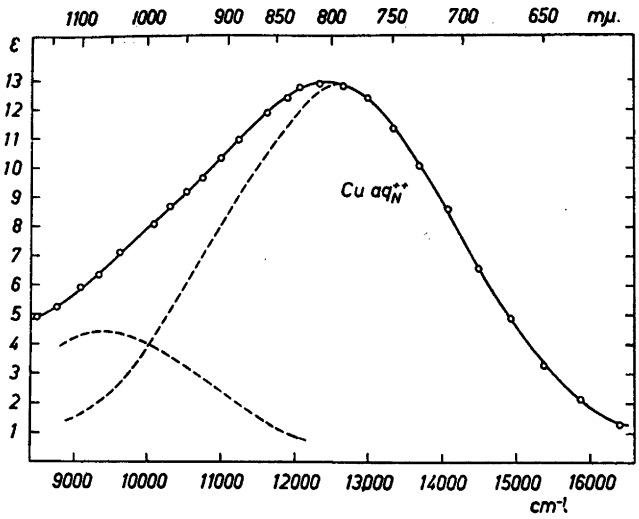

Fig. 2. Absorption spectrum of an aqueous cupric nitrate at 25° C ($C_{Cu(NO_3)_2}$ = 0.01 M, C_{HNO_3} = 0.02 M, $C_{NH_4NO_3}$ = 2.00 M), measured with a Beckman DU spectrophotometer. Analysis by the principle of symmetrical partial bands. o—o Experimental points, — — — analyzed band.

according to (1). The values of λ_2/λ_1 corresponding to the square planar and square pyramidal configurations for selected values of R are given in Table 2. Table 2 also gives the dipole moment μ for hypothetical complex ions of the configurations mentioned, and the wavelengths of the two possible bands of

Table 2. Wavelength ratios, dipole moments and wavelengths of possible bands (in mμ) according to the theory for square planar, square pyramidal and regular octahedral models of Cu(II)-ion dipole complexes. (R in Å, μ in Debye).

Formula	Configuration	R	$\nu_1/\nu_2 = \lambda_2/\lambda_1$	μ	λ_1	λ_2	λ_3
Cu aq_4++	square planar	1.8	1.87	2.30	(790)	1 480	$>10^4$
»	» »	2.0	2.07	3.75	»	1 635	»
»	» »	2.2	2.39	5.50	»	1 890	»
Cu am_4++	» »	1.8	1.87	3.09	(590)	1 110	»
»	» »	2.0	2.07	5.04	»	1 220	»
»	» »	2.2	2.39	7.40	»	1 410	»
Cu aq_5++	square pyramid	1.8	1.67	3.00	(790)	1 320	1 380
»	» »	2.0	1.66	5.06	»	1 310	1 330
»	» »	2.2	1.64	8.11	»	1 300	1 290
Cu am_5++	» »	1.8	1.67	3.72	(640)	1 070	1 100
»	» »	2.0	1.66	6.27	»	1 060	1 070
»	» »	2.2	1.64	10.04	»	1 050	1 040
Cu aq_6++	octahedral	1.8	1.00	4.35	(790)	—	—
»	» »	2.0	»	7.83	»		
»	» »	2.2	»	13.6	»		
Cu am_6++	» »	1.8	»	5.39	(640)	—	—
»	» »	2.0	»	9.70	»		
»	» »	2.2	»	16.85	»		

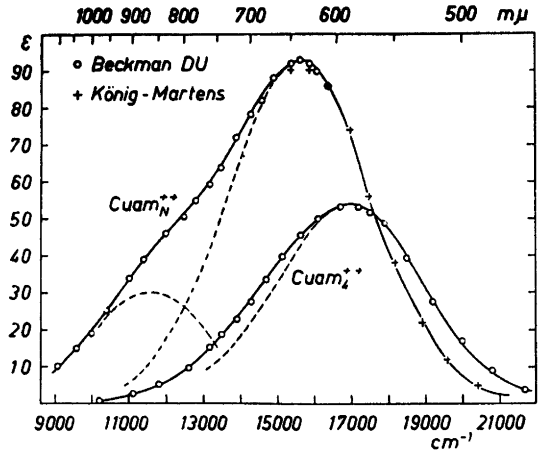

Fig. 3. Analysis of the absorption curve of cupric nitrate in liquid ammonia[20] ($Cu\,am_N^{++}$), *and of tetrammine cupric ion* ($Cu\,am_4^{++}$) *in water. o—o New measurements, — — — analyzed band.*

lower frequency corresponding to the measured values of λ_1. For comparison the values of μ corresponding to λ_1 in the cases of octahedrally built aquo and ammine complexes are also given.

Cupric ions in aqueous solution apparently have only one band, but a closer examination of the absorption curve given in Fig. 2 shows that this spectrum is a superposition of more than one band. According to one of us (Klixbüll Jørgensen, to be published) there is a great deal of experimental evidence that absorption curves (the extinction coefficient as function of the wavenumber) follow a simple Gaussian error curve, and consequently are symmetrical around their maximum value if the band is arising from a single transition. If this principle is used on the spectrum of the aquo cupric ion the experimentally determined absorption curve can be resolved into at least two symmetrical absorption curves with maxima at 790 and 1060 mμ, corresponding to the ratio of wavelengths $\lambda_2/\lambda_1 = 1.34$.

Fig. 3 shows the curve for cupric nitrate in liquid ammonia ($Cu\,am_N^{++}$). The second maximum is here directly noticeable, and it is especially noteworthy that the ratio of wavelengths is very nearly the same as that found for cupric ions in aqueous solution. Analyzed as above we find $\lambda_1 = 640$, $\lambda_2 = 855$ mμ, giving $\lambda_2/\lambda_1 = 1.33$.

The experimentally determined wavelength ratio gives direct information about the configuration of the complex ions. This is easily seen in the following way: in going from the square to the square pyramidal configuration by gradually moving a point dipole along the Z-axis from infinity to $R_5 = R$ (see Fig. 1), the ratio of wavelengths λ_2/λ_1, according to the theory, should decrease steadily from ~ 2 to ~ 1.6 (see Table 2). Now if the saturated aquo and ammine cupric ions have square pyramidal configurations with $R_5 > R$, one should expect $\lambda_2/\lambda_1 > 1.6$. The opposite is found, and as far as we

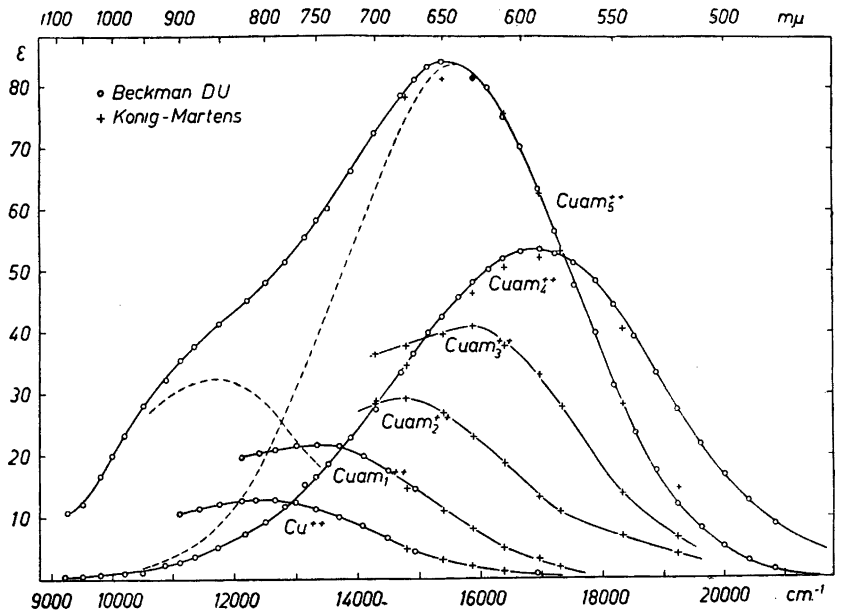

Fig. 4. Absorption spectra of copper(II)-ammonia complexes in 2 M ammonium nitrate at 20—25° C. Curves for Cu++ *(see Fig. 2), and for the intermediate ammine cupric ions*[19]. *The curve for* Cu am_1++ *in the infrared as well as the curves for* Cu am_4++ *and* Cu am_5++ *are computed from new measurements with a Beckman DU spectrophotometer. Analysis by the principle of symmetrical partial bands (— — analyzed band for* Cu am_5).

can see the only way to explain the observed ratio ~ 1.3 in water as well as in liquid ammonia is to assume, with Orgel [24], a tetragonal bipyramidal configuration for the copper(II) ions.

According to the theory, point dipoles placed along the Z-axis (μ_5 as well as μ_6) contribute independently to the wavenumber, and a model with $R_Z > R$ and $\mu_Z < \mu$ (using an index Z as a common term for both of the positions 5 and 6), is therefore in complete agreement with the wavelength ratio found experimentally.

According to this model the tetrammine cupric ion in aqueous solution should contain 2 water molecules. The absorption curve for this ion is more symmetrical than for the homogeneous complexes (see Fig. 3), and the analysis of the spectrum in this case does not permit conclusions to be drawn about the second band, the intensity of which seems to be small.

When the di-aquo tetrammine ion exchanges one of its water molecules with an ammonia molecule there is an anomalous shift of λ_1 towards the red (the so-called "pentammine effect"), but a similar effect is not found with the uptake of the 6th ammonia molecule. It is very remarkable that the pentammine spectrum — estimated from rather dilute ammonia solutions — is but little different from the spectrum of cupric nitrate in liquid ammonia (compare the absorption curve for Cu am_N^{++} in Fig. 3 with the curve for Cu am_5^{++} in Fig.

4). How can this be explained? J. Bjerrum [14,20] has maintained that it does not give much evidence for the existence of a hexammine complex, and later [29,30] has tried to prove that the cupric ion in solution cannot exceed the coordination number 5. However, in the picture of the theory developed here the missing hexammine effect can be explained by assuming that the effective dipole moments for the last water molecule and the 6th ammonia molecule are nearly equal, and for this reason give the same contribution to the wave number. The permanent dipole moment for water is higher than that for ammonia, but the polarizability of the latter molecule is higher, and this causes the average effective dipole moments to be essentially higher for ammonia than for water ligands (*cf.* Table 2). For the 6th ammonia molecule we may also assume for thermodynamic reasons that $\mu_6(NH_3) \sim \mu_6(H_2O)$, as seen by the following consideration. The decrease in free energy resulting from the uptake of the 6th ammonia molecule is formally given by

$$-\Delta G_6 = \frac{q \cdot \Delta \mu_6}{R^2 \cdot D}, \qquad (2)$$

where q is the charge of the cupric ion, $\Delta \mu_6 = \mu_6\,(NH_3) - \mu_6\,(H_2O)$, and D the effective dielectric constant.

The intensity of the absorption band in liquid ammonia is about 10 % higher than that of the pentammine band (see Fig. 4), and it can be estimated that the average ligand number $\bar{n} = 5.5$ is first reached when the mole fraction of ammonia is about 0.35 — 0.40. This shows directly that water and ammonia compete on equal terms for the 6th and last position in the configuration assumed, thus giving thermodynamic support for $\Delta \mu_6 \sim 0$. Referring to molar concentrations in water ($a_{H_2O} \lesssim 1$), the 5th consecutive formation constant K_5 is about 1/3, and it was earlier postulated [14,p.109] that K_6 is at least 200 times smaller. A closer consideration of the spectra previously recorded makes it probable that we have $K_5/K_6 \sim 100$, but this ratio is still considerably higher than the statistically expected value of 4.

It is possible to get some information on the extent to which the tetragonal bipyramidal model deviates from the regular octahedral configuration. The stronger the electric field the closer a given ligand is pulled nearer to the central ion and the greater must be the fraction of μ due to the polarization; we must therefore expect that if $R < R_Z$, then $\mu > \mu_Z$. Using this postulate a closer examination of the model shows that in order to obtain the experimentally determined wavenumbers for the hexaquo and hexammine spectra, $\nu_1/\nu_2 = 1.33$ and $R \sim 2$ Å, R_Z cannot exceed R by more than about 8 % if the dipole moments of the ligands bound at the four positions in the square (see Fig. 1) are to be higher than the dipole moments of the ligands placed at the positions 5 and 6 along the Z-axis. This result is utilized in Table 3 where μ and μ_Z are calculated from the wavenumbers of the hexaquo and hexammine ions for $R = 1.95$ and 2.00 Å, respectively, selecting values for R_Z a few per cent higher than R. The ratios $\frac{\Delta\mu}{R^2}$ and $\frac{\Delta\mu_Z}{R_Z^2}$ are similarly tabulated, giving a rough measure of the tendency of water to exchange with ammonia in the square and perpendicular to the square. The pentammine effect defined

Table 3. Results of calculations with the tetragonal bipyramidal model of copper (II)-ion dipole complexes.

Dipole moments and pentammine effect $\triangle\lambda_1 = \lambda_1(\text{Cu am}_5) - \lambda_1(\text{Cu am}_4)$ as function of the distances R and R_Z. $\triangle\lambda_1(\text{exp.}) = 50\text{m}\mu$.

Ligand	R (Å)	R_Z (Å)	μ(Debye)	$\frac{\triangle\mu}{R^2}$	μ_Z (Debye)	$\frac{\triangle\mu_Z}{R_Z^2}$	$\triangle\lambda_1$(mμ)
H_2O	1.95	2.00	5.12	0.324	3.74	0.222	55
NH_3	»	»	6.35		4.63		
H_2O	1.95	2.05	5.07	0.328	4.05	0.231	55
NH_3	»	»	6.32		5.02		
H_2O	2.00	2.05	5.79	0.396	4.35	0.242	85
NH_3	»	»	7.37		5.37		
H_2O	2.00	2.20	5.65	0.338	6.23	0.304	35
NH_3	»	»	7.00		7.70		

as $\triangle\lambda_1 = \lambda_1(\text{Cu am}_5) - \lambda_1(\text{Cu am}_4)$ is also given, and Table 3 shows that the calculations made give a pentammine effect of the right order of magnitude ($\sim 50\ m\mu$). This is a remarkable result since an effect of this kind will manifest itself only in the cases where the ligands are not equivalently bound. The final choice of the parameters was taken as $R = 1.95$ Å, and $R_Z = 2.00$ Å, a pair of distances giving nearly the right pentammine effect, and for which the ratio $\frac{\triangle\mu}{R^2} : \frac{\triangle\mu_Z}{R_Z^2}$ is found to be ~ 1.5. The ratio of the affinities estimated from the complexity constants are considerably higher, but a quantitative agreement cannot be expected.

The interesting result that the Cu(II)-complexes seem to deviate only slightly from regular octahedral configuration agrees well with X-ray diagrams of crystalline hexammine cupric bromide taken at room temperature with a 19 cm Bradley-Jay camera using CuKα-radiation in Haldor Topsøe's Laboratories by J. Rathlev. In complete agreement with Peyronel [21] this salt was found to have a cubic face centered structure.

The reflection spectra of crystalline $CuSO_4$,5 H_2O and of anhydrous $CuSO_4$ have been measured for us in the laboratory of F. L. Smith and Co. by K. A. Simonsen; the spectra are plotted in Fig. 5. It will be seen that the colourless $CuSO_4$ has a reflection spectrum in the near infrared only slightly displaced relative to the spectrum of the blue hydrate. The oxygen in the sulphate groups has nearly the same polarizability as water, and this explains why the maximum of reflection of the crystalline hydrate is situated at the same wavelength as the absorption maximum of the hexaquo cupric ion in solution (*cf.* Fig. 2). The structure of anhydrous $CuSO_4$ is unknown, but it is assumed that the spectrum is due to the influence of the oxygen groups.

Thus the chemical facts and the theoretical considerations seem to verify the proposed constitution of the hexaquo cupric ion and analogous Cu(II)-complexes.

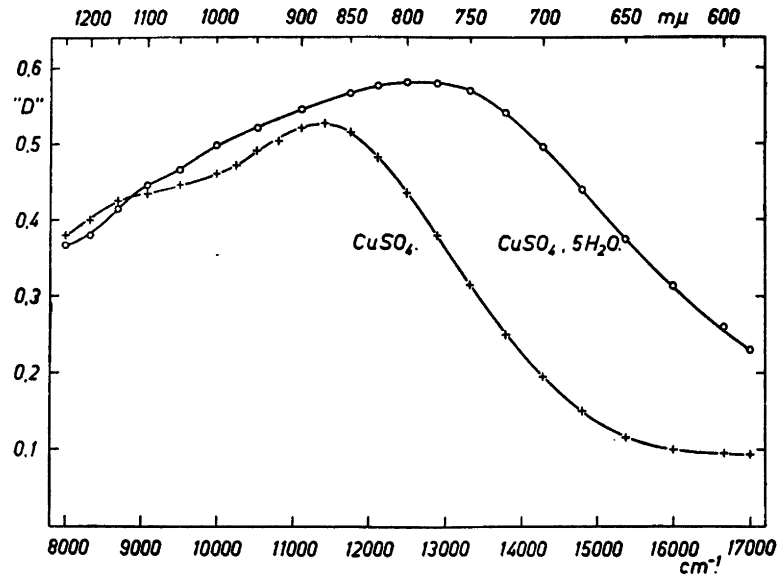

Fig. 5. Reflection spectra of cupric sulphate, anhydrous and pentahydrate. Measurements with the reflection spectrum attachment of the Beckman DU spectrophotometer by K. A. Simonsen (the optical density 'D' is only a relative measure for the light absorbed).

RESULTS WITH MORE UNSYMMETRICAL COMPLEXES

J. Bjerrum [19] has estimated the spectra of the intermediate complexes Cu am_n^{++} for $n = 1, 2, 3$ and 4 by resolving the mixed spectra of equilibrium solutions for values of $\bar{n}$ varying from 0 to ~ 5. Using the ideas outlined in the preceding sections it is also possible to calculate the wavelength of the band maxima of these complexes. The calculations are more tedious to perform than in the previous cases, since the symmetry of the field is lower. This causes the original five-fold degeneracy of the ground level to be removed to a higher degree. The mixed complexes are, therefore, supposed to have three or four bands, the first two of which, according to the theory, are placed adjacently. For statistical reasons (*cf.* Ref. 14, pp. 43, 109) the first four ammonia molecules must occupy the positions 1—4 in Fig. 1, and as before it was assumed that the distances Cu-NH_3 and Cu-H_2O are equal. The complete calculations will be published by C. J. Ballhausen and only the results are quoted here. If the chosen values of the parameters R and R_Z, μ and μ_Z, are inserted in the derived expressions we get remarkable agreement with the experimental values for the bands of highest frequency, as shown in Table 4.

Only one of the intermediate complexes, the diammine cupric ion, should exist in a *cis* and a *trans* form, and it is of considerable interest that the computations give some information on this point. The theory implies that the

Table 4. Comparison of calculated and experimental values of the wavelength of the band of highest frequency in the series of cupric ammine complexes.

Values of the parameters used in the calculation:

$R = 1.95$ Å, $\mu(H_2O) = 5.12$ D., $\mu(NH_3) = 6.35$ D.
$Rz = 2.00$ Å, $\mu_Z(H_2O) = 3.74$ D., $\mu_Z(NH_3) = 4.63$ D.

Formula of complex	λ_1 (mμ) calculated	λ_1 (mμ) experimental
$Cu(H_2O)_4$, $(H_2O)_2$++	—	790
$Cu(NH_3)(H_2O)_3$, $(H_2O)_2$++	710	745
cis-$Cu(NH_3)_2(H_2O)_2$, $(H_2O)_2$++	670	680
trans-$Cu(NH_3)_2(H_2O)_2$, $(H_2O)_2$++	620	~600
$Cu(NH_3)_3(H_2O)$, $(H_2O)_2$++	660	645
$Cu(NH_3)_4$, $(H_2O)_2$++	(590)	590
$Cu(NH_3)_4$, $(NH_3)(H_2O)$++	—	640
$Cu(NH_3)_4$, $(NH_3)_2$++	—	640

maximum of the experimental curve for the diammine complex at 680 mμ is a *cis* band by analogy with the band of the mono-ethylenediamine complex (see Fig. 6), which necessarily is that of a *cis* spectrum. The theory further predicts the situation of a *trans* band for the diammine ion at ~620 mμ. After the calculations had been performed, evidence for such a band was found near 600 mμ by a careful examination of Bjerrum's [19] original curve for the diammine spectrum. The equilibrium is probably displaced strongly in favour of the *cis* ion in this case (*cf.* Bjerrum and Rasmussen [36]). The *trans* band is more pronounced in the computed spectrum of the dipyridino complex, the three spectra discussed being drawn for comparison in Fig. 6.

The spectra so far discussed are all atomic. In addition to their atomic spectra in the red and infrared many acido copper(II) complexes, *e. g.* the chloro cupric complexes [37], have very high absorption bands in the ultraviolet,

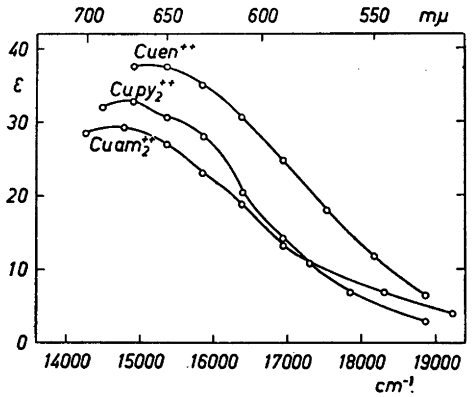

Fig. 6. Comparison of spectra of diammine cupric ions. Curves for the diammonia complex (see Fig. 4), for the mono-ethylenediamine complex [29], and for the dipyridino complex (unpublished result [29]). The mixed spectra of cupric nitrate pyridine solutions in 0.5 M *pyridinium nitrate were resolved by the method of least squares.*

which are probably molecular spectra due to polarization of the ligands. Winther [38] and Ley [39] have demonstrated ultraviolet absorption by $CuSO_4$-ammonia solutions, especially if they are poor in ammonia. The intensity of the band is approximately inversely proportional to the concentration of ammonium ions (at least in the range 0.1—0.8 *M* NH_4ClO_4 for solutions having $[NH_3] \sim 0.1$ *M*). Further, Kirson [40] has shown that the catalytic influence of cupric ammonia solutions on the decomposition of hydrogen peroxide also varies inversely with the ammonium concentration. The ultraviolet absorption disappears in strong ammonium salt solutions, and we must therefore conclude that it is due to the formation of hydroxo complexes, as *e. g.* $[Cu(NH_3)_3(OH)(H_2O)_2]^+$. The concentrations of hydroxo complexes cannot be very high, since they do not noticeably disturb the ammine formation in 0.5—5 *M* ammonium nitrate solutions [14,p.125].

REMARKS ON THE THEORY

It is rather unexpected that a first order approximation using but few parameters is able to describe the spectra of such complex systems. In the foregoing sections the parameters have been chosen in such a way that the experimental facts are explained. But we wish to point out that the parameters (the effective charge Z and the dipole moment μ) used here do not necessarily correspond directly to any physical reality, and they should not be used uncritically in other connections.

The theory predicts sharp lines, but in fact, bands are observed. An explanation of this fact may be found in the oscillations of the dipole ligands in the potential hole of the equilibrium positions. These oscillations affect the energy of the system, which is very sensitive to changes in R. The energy differences between the levels are, therefore, not very constant, and as the frequency of the electron is about 1 000 times the frequency of the oscillating dipole, it is reasonable to assume that the broadening of the bands is a statistical effect due to light absorption by all the complex ions in the solution. The total broad absorption curves thus produced are nearly symmetrical and, as experiments show, have approximately the shape of an error-curve. The bands are also sensitive to variations in temperature; by lowering the temperature they all show hypsochromic effects. This is perhaps explained as a consequence of temperature lowering drawing the ligand closer to the cation thus increasing the perturbation.

In those cases where the energy differences are not very dependent on the oscillations of the ligands, the bands do not broaden much. This is the case with the absorption spectra of the rare earths (when the crystal field has nearly no influence at all), and *e. g.* with a narrow band of the chromium(III) ion near 670 mμ [41].

It is a result of the above discussion that the spectra of the copper(II)-ammines in aqueous solution can be described without assuming overlap forces between the ligand and the cation. However, in the total description of a complex ion we must take electron exchange into account; the number and sites of the ligands is at least partly due to such an effect. On the other hand, our calculations show that when we take, for example, a cupric ion, and sur-

round it with four ligands, then the ground energy level of the complex resulting both from the perturbations and the electrostatic forces will be lower in a planar configuration than in a tetrahedral one. Thus both the crystal-field theory and the hybridization theory predict that in a cupric complex with coordination number 4 the ligands will be placed square planar.

In later papers it will be shown that the spectra of all the transition group elements can be explained along these lines using the theories of Bethe [10] applied to an intermediately strong crystal field. It also seems possible that the magnetic and kinetic properties of, *e. g.*, the hexammine cobalt(III) ion and other complex ions, can be explained by this theory, as has been pointed out in special cases by Orgel [24].

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Short Communications

5-Hydroxy-Piperidine-2-Carboxylic Acid in Green Plants

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70 % alcoholic extracts of different *Acacia* species and *Rhapis flabelliformis* gave on a two-dimensional chromatogram (solvents: butanol-acetic acid and phenol-NH_3), after treatment with ninhydrin a very strong blue spot C (Fig. 1) which did not disappear after hydrolysis with 6 *N* HCl.

The fluorescence of the blue spot was very similar to that of the corresponding spot of pipecolic acid (piperidine-2-carboxylic acid). When treated with isatin the colour of the spot C is sky blue, and that of pipecolic acid greenish blue. Both pipecolic acid, proline, and the unknown C react with *p*-nitrobenzoylchloride, the amino group in compound C is accordingly secondary. This is also shown by the fact that compound C does not become deaminated when treated with oxides of nitrogen.

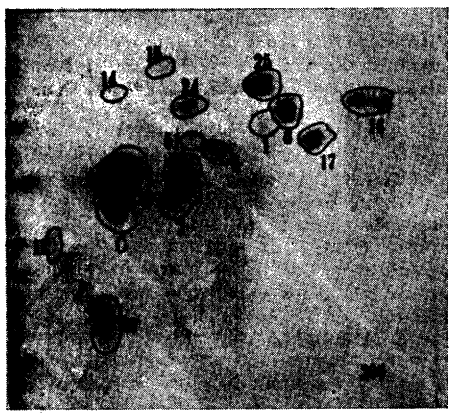


Fig. 1. Two-dimensional paper chromatogram of the unhydrolyzed 70 % alcohol extract of Rhapis flabelliformis. 1 = gly, 2 = ala, 8 = ser, 9 = threo, 11 = pro, 14 = arg, 15 = lys, 24 = glu-NH, 25 = asp-NH, 29 = α-aminobut. acid, 51 = homoser., C = unknown spot.

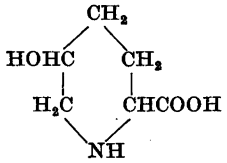
All these findings suggested that the substance C is a derivative of piperidinecarboxylic acid. By comparing the chromatograms of piperidine-2-, 3- and 4-carboxylic acids with that of substance C it could be noticed that these carboxylic acids moved with butanol-acetic acid appreciably faster than substance C. It seemed therefore most likely that the unknown compound was a hydroxy-piperidine-carboxylic acid.

The substance C was isolated from the alcohol extract of *Rhapis flabelliformis* (650 g fresh material = 290 g dry substance) using an Amberlite IR—120 column. Amino acids were displaced with 1 *N* ammonia taking 16 fractions of 16—17 ml. Fractions 8—12 were mixed and evaporated to 2 ml. The syrup crystallized partly in an icebox, and the crystals washed with abs. alcohol appeared to represent the compound C.

The syrup with crystals was dissolved in 25 ml of 1.5 *N* HCl, and using a Dovex 50 column 276 fractions of 10 ml were taken. Fractions 181—210 and 211—233 were concentrated. Upon cooling beautiful crystals (prisms) were formed. They were filtered off and washed with 70 % alcohol. The substance gave on the paper chromatogram only spot C. M. p. 225—230° (decomp.). (Found: C 40.14; H 6.81; N 7.58. Calc. for $C_6H_{11}O_3NHCl$: C 39.68; H 6.66; N 7.71.)

A comparison of our unknown compound with synthetic 5-hydroxy-piperidine-2-carboxylic acid showed that both substances moved with the same velocity on the paper chromatogram (butanol-acetic acid

and phenol-NH_3). Because we do not know to what extent the position of the OH group in the ring influences the R_F-value we do not consider the identical chromatogram given by compound C and 5-hydroxy-piperidine-2-carboxylic acid a conclusive proof for the identity of these substances. Oxidation with permanganate in 20 % sulphuric acid solution at 10° C gave mainly glutamic acid and aspartic acid in smaller amounts. On the basis of this we may conclude that in the hydroxy-piperidine-carboxylic acid which we have isolated the OH group actually is in the position 5 and the carboxyl group in the position 2. The formation of glutamic acid cannot be explained otherwise.



It is possible that the new amino acid is formed from δ-hydroxylysine.

We are very grateful to Professor T. J. King, Nottingham, for a preparation containing some 5-hydroxy-pipecolic acid, to Dr. G. Curzon, Stanmore, England, for the *iso*nipecotic acid (piperidine-4-carboxylic acid), to Mr. O. Oja for helping us in the preparation of nipecotic acid (piperidine-3-carboxylic acid), and to Professor A. Kalela for the plant material we got from the University Botanical Garden.

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Triterpenoids in Lichens
II. Taraxerene, a Naturally Occurring Triterpene

TORGER BRUUN

Institutt for Organisk Kjemi, Norges Tekniske Högskole, Trondheim, Norway

During our researches on lichens a sample of *Cladonia deformis* Hoffm. was investigated. The origin of the sample has been reported previously [1]. The general procedure for extraction with ether and working up of the extract has been reported in part I of this series [2].

The neutral part of the ether extract, when chromatographed on alumina furnished a petroleum (b. r. 40—70°) eluate, which contained a hydrocarbon mixture (0.3 g) that did only partly melt on the boiling water-bath. The high-melting part of the fraction was much less soluble in petroleum than the low-melting one, and by taking advantage of this fact a sample of m. p. 230—233°, $[\alpha]_D$ +4° (*c*, 0.83) was obtained *. A much better separation, however, was achieved by careful chromatography: The hydrocarbon, dissolved in petroleum, was brought on a column of 30 g of alumina, 17 cm high, 1.5 cm diameter, and fractions of 5 ml were collected. The high-melting hydrocarbon was retained more tenaciously than the low-melting one, and thus a satisfactory separation could be obtained. For further purification the hydrocarbon was treated with petroleum, and finally the substance was crystallised from ether, m. p. 237—238°, $[\alpha]_D$ + 1° (*c*, 0.81). The total amount of hydrocarbon was about 15 mg from 2.9 kg of dry lichen.

The manner in which the substance was obtained seemed to justify the assumption that it was a hydrocarbon, and the high m. p. suggested a polycyclic one. A triterpene ** was not ruled out, and inspection of the literature indicated that identity with skimmiene IIa[3] might exist. The chemistry of taraxerol (skimmiol) is presently being investigated by Dr. C. J. W. Brooks, compare [4]. Dr. Brooks incidentally learned of this possible identity through Dr. P. de Mayo, and very kindly provided a sample of taraxerene, which, although, strictly speaking, it has not been proved, is very likely identical with skimmiene IIa,*** and which he had recently prepared by Wolff-Kishner reduction of taraxerone, and for which he reported m. p. 238—240° (strong sublimation), $[\alpha]_D$ + 3°, very sparingly soluble in chloroform, in agreement with our observations on the hydrocarbon from *Cl. deformis*. Taken at the same time the hydrocarbon from the lichen,

* All rotations in chloroform in a 1 dm tube.

** For nomenclature compare Barton, D.H.R. in Simonsen, John, *The Terpenes*, Vol. III, Cambridge 1952, p. 328.

*** Note added in proof: Weissenberg diagram of skimmiene II a, very kindly provided by Dr. K. Takeda, showed identity with taraxerene.

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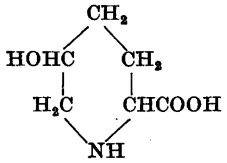

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Fig. 1. A. Hydrocarbon from Cladonia deformis. B. Taraxerene.

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So far 5 triterpenoids are known to occur in lichens: ursolic acid [7], zeorin [8], leucotylin [9], friedelin and *epi*friedelinol [2] and now taraxerene. Of these only ursolic acid and taraxerene have fairly well established constitutions, the former being related to α-amyrin [10], the latter a stereoisomer of germanicene, with the C/D ring junction *cis* instead of *trans* [4], *i. e.* it belongs in the β-amyrin series.

* The triterpene squalene is of the alifatic type.

The author is glad to have this opportunity to express his gratitude to Professor N. A. Sörensen for help in collecting the lichen and for the interest he has taken in the work.

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8. *Elsevier's Encyclopedia of Organic Chemistry*, Vol. **14** Suppl. London 1952, p. 1197s.
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The Biosynthesis of Riboflavin in *Eremothecium ashbyii*

LEIV KLUNGSOYR

Biochemical Laboratory, University Clinic of Bergen, Norway

The hypothesis of MacLaren [1] that a substance with purine structure is utilized for the synthesis of riboflavin by *E. ashbyii*, is supported by recent work by Goodwin and Pendlington [2] and by Klungsöyr [3].

Previous studies have shown that the carboxyl group of acetic acid is incorporated into the position 2 of the *iso*alloxazine part of the riboflavin molecule [3]. This observation was taken as indirect evidence of the utilization of a purine intermediate



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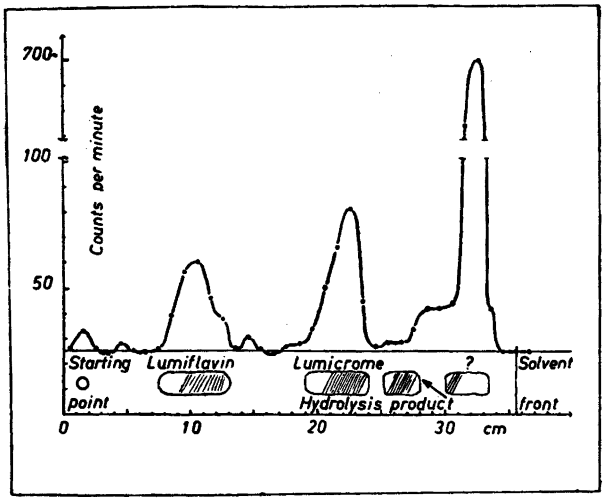

Fig. 1. C^{14} distribution curve and spots visible in ultraviolet light. Counted along the intact paper. The spots represent degradation products of riboflavin, formed by E. a s h b y i i incubated in a phosphate buffer containing C^{14} formate.

in riboflavin synthesis. The conclusion was based on the assumption that a formyl-compound may be formed from the carboxyl group of acetic acid in *E. ashbyii*[4]. If this assumption is valid C^{14} formic acid should label the riboflavin molecule in the same position as does 1-C^{14} acetic acid. Experiments were therefore made to study the incorporation of formic acid into riboflavin.

Eremothecium ashbyii was collected from a liquid medium, washed, and resuspended in phosphate buffer. To 20 ml suspension 40 microCurie C^{14} sodium formate was added, and the suspension was shaken at 28° C for 48 hours. At the end of the incubation period the riboflavin was degraded, and the degradation products were isolated by extraction with chloroform and separated by paper chromatography. The positions of the spots were marked off in ultraviolet light, and the C^{14} distribution curve was determined by counting along the paper, one cm at a time. (The details of the technique have been described previously [3]). The results are presented in Fig. 1.

Fig. 1 shows that some C^{14} activity is present in lumiflavin and in lumicrome, but none in the hydrolysis product of lumiflavin. Positions 1(N), 2(C) and 3(N) are split off from lumiflavin by hydrolysis, and C^{14} from formic acid is therefore present in position 2 in lumiflavin.

The findings that C^{14} from 1-C^{14} acetic acid and from C^{14} formic acid are both incorporated into position 2 of riboflavin show that the carboxyl group of acetic acid may be converted into a formyl-compound by *E. ashbyii*, in accordance with the scheme suggested by Krebs *et al.*[4]

The C^{14} sodium-formate was purchased from The Radiochemical Centre, Amersham, England, and the 1-C^{14} acetic acid from Nyegaard & Co. A/S, Oslo. (The acetic acid was prepared by the reaction between carbon dioxide and methyl-magnesium-bromide and should be free from formic acid.)

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Bromopodophyllotoxin

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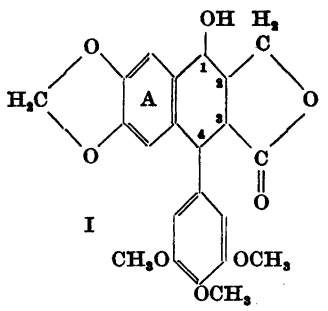
It has been postulated [1] that podophyllotoxin (I) is unreactive toward bromine, and this appears to be generally believed. In recent X-ray work, for instance, during which the introduction of a bromine atom in the molecule was desirable, this has been done indirectly by bromoacetylation of the hydroxy-group [2].

We find, however, that a solution of podophyllotoxin in chloroform virtually consumes approximately 2 equivalents of bromine per molecule, when shaken with an acidified aqueous bromide-bromate solution.



Podophyllotoxin $C_{22}H_{22}O_8$
trans 1:2, trans 2:3, cis 3:4.

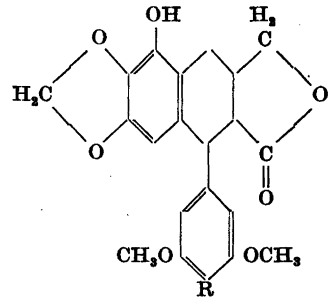
(Hartwell *et al.*)

From the reaction mixture a product has been isolated in good yield as colourless needles (from benzene) melting at 182–183° C or at 155° C. The substance is dimorphous, the lower melting form is slowly converted into the higher melting one when heated to its melting point. It analyzed as monobromopodophyllotoxin, $C_{22}H_{21}O_8Br$ (493.3): Calc. C 53.5; H 4.3; Br 16.2; $3OCH_3$ 18.7.
Found. 53. 6, 53.8; 4.4, 4.5; 16.3, 16.7; 18.7.

The bromine is firmly bound. The substance gave no precipitate of silver halide with alcoholic silver nitrate even on heating, and the bromine was not hydrolyzed by boiling alcoholic sodium hydroxide, which indicates that substitution has taken place in an aromatic ring. The nature of the carbon-bromine bond and the analyses prove the non-identity with the podophyllotoxin bromide $C_{22}H_{21}O_7Br$, m. p. 157.5–159°, (the hydroxy-group at C_1 replaced by bromine) prepared by Hartwell and Schrecker [3] by the action of phosphorus tribromide on podophyllotoxin. That the hydroxy-group is intact in our compound was finally proved by hydrogenation with Raney-nickel catalyst. This process, because simultaneous epimerization occurred in the alkaline medium, yielded picropodophyllin, identified by its melting point and by paper chromatography [4].

Degradation studies with the aim of locating the bromine substituent are in progress. Paper-chromatographic examination of the oxidation products seems to indicate that the bromine atom occupies one of the two free positions in ring A.

Picropodophyllin, which according to Hartwell *et al.*[3] is an epimer of I with the configuration *trans 1 : 2, cis 2 : 3, trans 3 : 4*, also consumes *ca.* 2 equivalents of bromine under similar conditions. The configurations of this and the foregoing bromoderivative are being examined. The peltatins (II, III) consume approximately 4 equivalents of bromine per molecule. This too is contrary to previous reports [1].



II R = OH α–Peltatin
III R = OCH_3 β–Peltatin

(Hartwell *et al.*)

Analyses by Mr. P. Hansen, Chemical Laboratory, University of Copenhagen, and by Chr. Jørgensen.

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Simplified Preparation of 6-Hydroxytropinone

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6-Hydroxytropinone (IV) has been prepared by Sheehan and Bloom [1] from 2,5-dimethoxy-2,5-dihydrofuran(I)through a four-step reaction (yield 7 %) and by Stoll, Becker and Jucker [2] from the corresponding ethoxy compound through a three-step reaction (yield probably about 15 %).

If an acid solution of dimethoxydihydrofuran is left standing for some time at room temperature, neutralized and added to a buffered solution of methylamine and acetonedicarboxylic acid, 6-hydroxytropinone is formed in a 55 % yield. Apparently malealdehyde (II), which is formed very rapidly by hydrolysis of dimethoxydihydrofuran, adds one mole of water whereby malaldehyde (III) is formed, which then condenses with methylamine and acetonedicarboxylic acid to hydroxytropinone.

Experimental. (Microanalyses by E. Boss.) *6-Hydroxytropinone (IV).* I[3] (0.65 g, 0.005 mole) was dissolved in hydrochloric acid (3 *N*, 12.5 ml) and the mixture left standing for 18 hours. The yellowish-brown solution was neutralized with a solution of sodium hydroxide (6 *N*, 6.2 ml) and added to a solution of acetonedicarboxylic acid (1.47 g, 0.01 mole), methylamine hydrochloride (0.68 g, 0.01 mole) and sodium acetate (3.4 g) in water (80 ml). The mixture (pH 4.3) was left standing for 2 days, whereby the acidity decreased to pH 4.9. Potassium carbonate (25 g) and sodium chloride (25 g) were dissolved in the light-brown reaction mixture and the solution continuously extracted with ether. The etheral extract was evaporated in a vacuum, leaving white crystals of hydroxytropinone embedded in a brown oil. By addition of a hot solution of picric acid (1.0 g) in ethanol (9 ml) the picrate of hydroxytropinone was obtained. The yield was 1.05 g (55 %), m. p. 199—200° (dec.) (Hershberg app., corr.), previously found [2] 199° (dec.). (Found: C 43.9; H 4.1; N 14.6. Calc. for $C_{14}H_{16}O_9N_4$ (384.3): C 43.8; H 4.2; N 14.6).

The free base was prepared from the picrate (250 mg) in the usual way and purified by sublimation. The yield of sublimed hydroxytropinone was 82 mg (82 %), m. p. 121—122°, previously found [1] 122.5—123.5°. (Found: C 61.7; H 8.5; N 8.8. Calc. for $C_8H_{13}O_2N$ (155.2): C 61.9; H 8.4; N 9.0).

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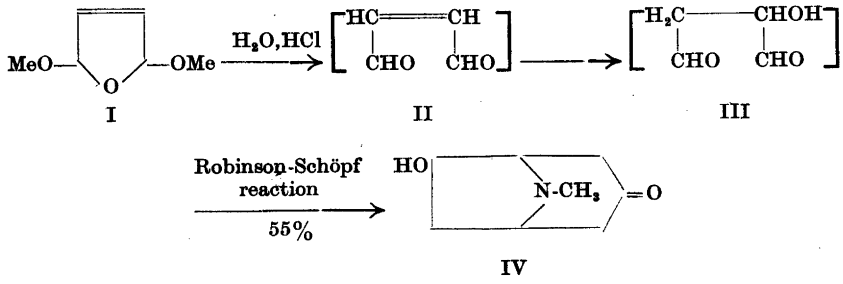

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Simplified Preparation of 6-Hydroxytropinone

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If an acid solution of dimethoxydihydrofuran is left standing for some time at room temperature, neutralized and added to a buffered solution of methylamine and acetonedicarboxylic acid, 6-hydroxytropinone is formed in a 55 % yield. Apparently malealdehyde (II), which is formed very rapidly by hydrolysis of dimethoxydihydrofuran, adds one mole of water whereby malaldehyde (III) is formed, which then condenses with methylamine and acetonedicarboxylic acid to hydroxytropinone.

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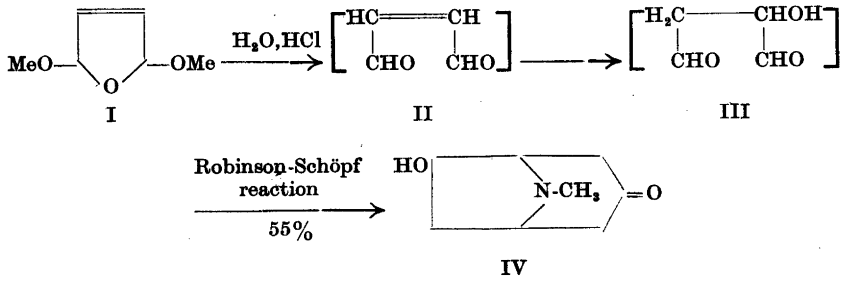

The Depolymerization of Dextran in Non-aqueous Solutions

ESKIL HULTIN AND LENNART NORDSTRÖM

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

It is previously known [1] that dextran is successively depolymerized in aqueous solutions in the presence of acids, enzymes or alkalies. The depolymerization by acids and by enzymes has the character of a hydrolysis. Methods have also been described for the depolymerization of dextran by heat and by ultrasound.

We have found that dextran can also be successively depolymerized by alcoholysis in organic solvents with free hydroxyl groups. The monovalent aliphatic alcohols are poor solvents for dextran. However, at temperatures above 140° C dextran dissolves rapidly in polyvalent alcohols, *e. g.* ethylene glycol, glycerol, or sorbitol. In such a solution dextran slowly reacts with the solvent, but the reaction proceeds more rapidly, if the solution is heated to a higher temperature (up to about 200° C) or if a small quantity (about 0.1 to 1 %) of an acid catalyst (phosphoric acid, trichloroacetic acid or acid sodium sulphate) is added. In the same way dextran reacts with phenols, *e. g.* resorcinol.

This type of reaction was first carried out with starch by Zulkowsky [2]. Berner found in his investigations on lichenin [3], inulin [4] and starch [5] that the depolymerization of polysaccharides in glycerol solution is an alcoholysis.

The depolymerization reaction can easily be followed by measuring the viscosity of samples taken out at intervals, as the intrinsic viscosity of dextran is approximately proportional to its average molecular weight [6].

By controlling temperature, catalyst and time in carrying out the alcoholysis, it is thus possible to obtain dextran glycosides with any desired average molecular weight.

The best way to isolate the reaction products from the solution is to add several volumes of water and then to precipitate them by adding a water-soluble organic liquid in which dextran is almost insoluble, *e. g.* ethyl alcohol or acetone. As the solubility of the dextran glycosides in this mixture decreases with increasing molecular weight of the reaction product and increasing amount of precipitating agent, it is possible to make a fractionated precipitation and thereby obtain preparations with different average molecular weights.

If a partial depolymerization of dextran is carried out in glycerol solution and the reaction product is subsequently fractionated to give a preparation with an average molecular weight of about 70 000, a product is obtained which has proved to be excellent for the manufacture of blood plasma substitute.

1. Jeanes, A. *Dextran — A Selected Bibliography.* Northern Regional Research Laboratory, Peoria, Ill., 1952.
2. Zulkowsky, K. *Ber.* **13** (1880) 1395.
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Dehydropodophyllotoxin, a New Compound Isolated from *Podophyllum peltatum L.*

HELMER KOFOD AND CHR. JØRGENSEN

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In his first report on the isolation of α-peltatin from podophyllin Hartwell [1] described the substance as exhibiting a strong blue fluorescence in solution, contrary to podophyllotoxin, which was reported to be but weakly fluorescent. The statement was amended in a subsequent paper [2], and none of the natural lignan-derivatives so far isolated from *Podophyllum peltatum L.* is in fact fluorescent when sufficiently purified.

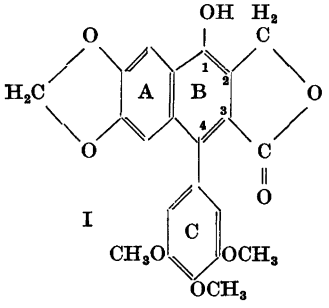
During a systematic reinvestigation of podophyllin by chromatography using sorbed formamide as a stationary phase and benzene as an eluent we noticed an intense blue fluorescence of certain fractions of the eluate between those containing podophyllotoxin and those containing α-peltatin.

Paper-chromatographic analysis of these and of other fractions by the method previously reported by us [3] revealed five,

possibly six different spots detectible by their more or less pronounced blue fluorescence in ultra-violet radiation. Traces of these compounds may well have been responsible for the apparent fluorescence of Hartwell's first preparations of α-peltatin and podophyllotoxin.

The substance corresponding to the largest spot on the paper-chromatograms has been isolated as colourless needles (from ethanol). It melted at 272–274° C (corr.). (Found: C 63.6; 63.3; H 4.8; 4.8; 3(OCH_3) 22.0. Calc. for $C_{22}H_{18}O_8$ (410.1): C 64.4; H 4.4; 3(OCH_3) 22.7).

Only minute quantities can be isolated from podophyllin, approximately 0.1 %.



The structure (I), 1-hydroxy-4-(3,4,5-trimethoxyphenyl)-6,7-methylenedioxy-2-hydroxymethyl-3-naphthoic acid lactone, is proposed for the substance, which appears not to have been described before. Formally it is podophyllotoxin or — equally well — picropodo- phyllin dehydrogenated at 1,2 and 3,4. Since podophyllotoxin is the better known, and since the dehydrogenation of this compound to (I) has been accomplished experimentally (*vide infra*) we propose the name dehydro-podophyllotoxin for the new substance.

The fluorescence and a striking similarity of the absorption spectrum in ultra-violet to that of dehydro-anhydropicropodophyllin (I less the hydroxy-group), prepared according to Späth *et al.*[4] is strong evidence for the fully aromatized naphthalene nucleus. The possibility that ring B contains only two formal double bonds can be ruled out with fair certainty, although it must be admitted that this structure would fit better in with the elementary analysis. In that case the substance would be a 1-hydroxy-derivative of one of the isomeric apo-picropodophyllins. These are, however, non-fluorescent, and their UV-spectra [5] are fundamentally different from that of our substance. A decisive proof of the naphthalene nucleus would be provided by demonstrating the failure of further dehydrogenation or by degradation. This is being attempted.

Although the substance does not give coloured coupling products with diazobenzene-sulphonic acid and does not respond to ferric chloride, we nevertheless propose a phenolic structure. The analytical data leave little doubt that the substance contains one oxygen atom more than dehydro-anhydro-picropodophyllin (calc. for $C_{22}H_{18}O_7$: C 67.0; H 4.6; 3(OCH_3) 23.6). Further evidence for the phenolic hydroxy-group is the location of the substance on the paper chromatograms close to other phenolic podophyllin constituents (the peltatins). The relation of the R_F to the number of hydroxy-groups has been established in previous work [3]. Dehydro-anhydro-picropodophyllin has a much larger R_F-value. It almost follows the benzene front in accordance with its lack of a hydroxy-group.

The position of the hydroxy-group in ring B is consistent with the failure of the coupling reaction, since this ring is then fully substituted. In ring A, on the other hand, there would be a para-position free for coupling as in the peltatins, which form strongly coloured azo-compounds with diazobenzenesulphonic acid [3]. The absence of the colour reaction with ferric chloride is unimportant; several substituted phenols fail to react. The presence and the proposed position of the hydroxy-group is finally supported by the fact that podophyllotoxin upon dehydrogenation with lead tetra-acetate produces a spot on the paper-chromatograms which is identical with that of the natural compound (I).

Further details will appear in this journal at a later date. Attempts to isolate the remaining four or five fluorescent substances are in progress.

This work is part of investigations supported by *Statens almindelige Videnskabsfond.*

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Hydroxyproline and Stability of Collagens

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From investigations of the hydrothermal stability and the reactivity of various collagens from the corium of mammals and fishes (*Teleostei*), listed in a recent paper [1], it has been concluded that the interchain cross-linking by hydrogen bonds is mainly responsible for the intermolecular cohesion of mammalian collagen. The collagen of the skin of cold-water teleosts, for instance that of the cod fish, appears to have this cross-linking by hydrogen bonding less developed. The collagen of skins of warm-water fish, that of the pike for instance, is indicated to represent an intermediate degree of cross-linking of the protofibrils by hydrogen bond bridges. Neuman and Logan [2] found later that the principal difference between the amino acid composition of the two extreme types of collagens was the very much lower hydroxyproline (= hypro) content of fish collagen (halibut) than that of bovine collagen. There is somewhat more of aliphatic hydroxyamino acids and methionine in the teleost collagen also [3]. In the comprehensive investigation of Takahashi[4], a direct correlation between the hypro content of the three types of collagen mentioned and their degree of hydrothermal stability, measured by the temperature of instantaneous shrinkage of the fibers, *Ts*, has been demonstrated independently. The present author had earlier proved such a correlation for these collagens [1]. It appears as if the hypro residue of collagen should play the same governing role for its stability as the cystine residue does for keratin.

The values for hypro listed in the literature have been obtained by the method of Neuman and Logan [2]. The accuracy and reproducibility of this method is not as great as could be desired, and since indirect values only are obtained by this technique, a direct estimation of this imino acid was imperative. By a combination of the chromatographic procedures of Moore and Stein [5], and of Stein and Moore,[6] it has been possible to separate the hypro and proline (= pro) fractions of the hydrolysates from collagens of native bovine skin, skins of cod fish (*Gadus morrhua*) and of pike (*Esox lucius*). The first fractionation was accomplished on a column of starch, followed by the final separation on Dowex 50, operating in strongly acid solution, according to Åqvist's modification [7]. The estimation was kindly carried out by Dr. S. Åqvist. The collagens were in the native state (*not* alkali treated). The purification of the skins and the preparation of the collagens have been described in earlier publications [1]. For the final estimation of the pure imino acids, the micro-Kjeldahl method was applied. The data in the following table are given as g pro and hypro on 100 g collagen (fat- and ash-free dry substance) and as % of total nitrogen.

The hypro content of the collagen of the skin of cod, a typical cold-water fish, is appreciably lower than the figure of 9 % reported for the skin of halibut with T_s 40° [2]. However, Beveridge and Lucas [8] found a still lower value for the collagen (ichthyocol) from the swim bladder of hake, or about 4 %. The lowest hypro value in Takahashi's list [4] is 7.0 % obtained for the skin of the rockfish (*Sebastodes*) which also has the lowest T_s ever recorded for collagens, or 33—34 °C.

The sum of the pyrrolidine residues for the codskin collagen is hardly more than half of the value for the mammalian collagen. The great variations of the content of these imino acids in various collagens cannot be dismissed in framing of structural models of collagen. Approximately

Table 1.

Type of skin	T_s in°C	% total N	Pro		Hypro		Pro + Hypro	
			% on protein	% of total N	% on protein	% of total N	% on protein	% of Total N
Cod fish (*Gadus morrhua*)	40	18.3	10.8	7.2	5.8	3.4	16.6	10.6
Pike (*Esox lucius*)	55	18.4	13.3	8.8	7.9	4.6	21.2	13.4
Calf (*Bos taurus*)	65	18.3	14.1	9.5	12.7	7.4	26.8	16.9

every third amino acid residue of collagen is generally considered to be a pyrrolidine residue. This proportion is an integral part in recent models of collagen commencing with Astbury's classical model [9], and up to the helical structure principle of Pauling and Corey [10], although they have recently suggested occasional units of one residue of prolines of four residues [11].

The effect of the hypro content of collagen on its stability and reactivity was first examined in the light of Klotz's concept [12], according to which the hydroxy groups of the residues of aliphatic amino acids form hydrogen bonds of the type: $-OH\cdots\overline{O}CO-$ in some globular proteins. This linking should affect the reactivity of the carboxyl ion. The reactions of bovine and cod skin collagen with the cupric ion and with a number of cationic, anionic and non-ionic complex compounds of chromium, dyestuffs and non-electrolytes have been studied in the isoelectric zone. The results could not readily be reconciled with an extension of the Klotz concept to the collagens. Moreover, according to Bear [13], the electron-optical researches suggest the presence of the prolines in the interbands of the protofibrils, whereas the bands should contain the residues of the dicarboxylic amino acids. Hence, formation of the hypro-hydroxy—carboxyl bond appears improbable for steric reasons. Bear's concept has recently received strong experimental support chemically by Zahn's [14] isolation of dinitro-diphenyl-sulphone-*bis*-lysine derivatives and data of Schroeder *et al.* (15) provide some evidence that the peptides in partial hydrolysates of gelatin and collagen may contain the sequence: glycine-pro-hypro-glycine frequently. The nature of the effect of the hypro residues on the organization of collagen and the type of bond which the hypro forms have been studied on modified bovine collagens. A preliminary account of this work is given in the following note

1. Gustavson, K. H. *Svensk Kem. Tidskr.* **65** (1953) 70.
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The Presence of Interchain Links Between Hydroxy and Keto-Imide Groups in Collagen

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The function of the hydroxyproline (=hypro) residue, the imino acid unique for collagen, has come to the forefront by the observation that the hypro content is directly related to the organization and reactivity of collagens [1]. Its possible participation in interchain links is obvious. An attempt of characterization of such cross-links has been made, primarily by the study of the effect of inactivation of the hydroxy groups of collagen on its properties and behaviour to certain reactants. The results of the investigation of collagen with its amino groups completely and its hydroxy groups to the maximum extent acetylated indicate that a part of the hydroxy groups forms a strong bond with the oxygen of the keto-imide groups (hydrogen bond). A preliminary account of the main findings forming the basis for this conclusion will be given in this note.

every third amino acid residue of collagen is generally considered to be a pyrrolidine residue. This proportion is an integral part in recent models of collagen commencing with Astbury's classical model [9], and up to the helical structure principle of Pauling and Corey [10], although they have recently suggested occasional units of one residue of prolines of four residues [11].

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The Presence of Interchain Links Between Hydroxy and Keto-Imide Groups in Collagen

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The function of the hydroxyproline (=hypro) residue, the imino acid unique for collagen, has come to the forefront by the observation that the hypro content is directly related to the organization and reactivity of collagens [1]. Its possible participation in interchain links is obvious. An attempt of characterization of such cross-links has been made, primarily by the study of the effect of inactivation of the hydroxy groups of collagen on its properties and behaviour to certain reactants. The results of the investigation of collagen with its amino groups completely and its hydroxy groups to the maximum extent acetylated indicate that a part of the hydroxy groups forms a strong bond with the oxygen of the keto-imide groups (hydrogen bond). A preliminary account of the main findings forming the basis for this conclusion will be given in this note.

Both bovine hide powder and calfskin were used. The N-acetylation and the exhaustive acetylation (N- and O-acetylation) of the collagen were carried out according to Green *et al.*[2]. Their analytical procedures were also used. The N-acetylated collagen contained the ε-amino groups completely inactivated (0.39 mmole acetyl per g collagen). The exhaustively acetylated collagen had in addition 80 % of its hydroxy groups blocked, containing 1.78 mmole acetyl per g collagen. The O-acetylation was 1.39 mmole/g collagen. The aliphatic amino acids and tyrosine account for 0.60 mmole OH/g collagen. Assuming that these residues are preferentially acetylated, at least 0.79 mmole/g collagen of hypro should have been acetylated. Hence, out of the 1.07 mmole hypro present in the original collagen, three hydroxy groups out of four have at least reacted. The total N content of the acetylated collagen was 16.5 % and the acid binding capacity 0.68 mmole HCl per g protein, while the corresponding values for the intact collagen were 18.0 and 0.90.

The stability of collagen, measured by its shrinkage temperature, T_s, was not changed by N-acetylation. The N- and O-acetylated collagen commenced to shrink at 40—44° C, while the intact collagen showed T_s of 64—66° C. Other properties, such as the degree of swelling, were profoundly changed by the exhaustive acetylation also. These findings indicate that by acetylation of hydroxy groups *interchain* crosslinks are broken. It is also of interest to note that the T_s obtained for the O-acetylated collagen coincides with that of the skins of most cold-water fishes (cod[1]), suggesting that the hydroxy group resisting acetylation forms a stronger bond than the rest of the hydroxy groups, and that this particular group is mainly responsible for the stabilization of the teleost collagen.

Indications of the nature of the other partner in the interchain bond were obtained by investigating the linking of polyphenols such as condensed tannins (Mimosa) by collagen and O-acetylated bovine collagen. It has been proved conclusively that the main reaction in the irreversible fixation of these tannins by collagen involves hydrogen bonding of the polyphenols on the keto-imide group (the carbonyl oxygen), since collagen and hydrated modified polyamides with the —CO—NH— link as the predominant reactive group show the same trend of reaction towards the condensed type of vegetable tannins [3]. By the N-acetylation, the fixation of mimosa tannin is slightly decreased, due to the elimination of the small fraction of tannin fixed by the amino groups, by their acetylation. The exhaustively acetylated collagen possesses markedly *greater* binding capacity for the mimosa tannins than intact collagen, the increase being of the order of 50—75 %. Thus, the intact collagen fixed 60 % mimosa tannins, on the basis of collagen, while the O-acetylated collagen fixed 95—105 %. A number of other vegetable tannins showed all increased binding, percentages increase of 15—40 having been recorded according to the degree of affinity of the individual tannins for the —CO—NH-link. In view of these data, amplified by our present knowledge of tanning reactions, the rupture of links between the hydroxy groups and the keto-imide group is indicated to occur in the O-acetylation.

In gelatin, the main part of these interchain hydrogen bonds on the —CO—NH— links apparently have been severed since its binding capacity for mimosa tannins reaches values of an order of 140 % tannin on the protein, figures [5] comparable with those by the polyamide [4] and considerably higher than those found for the O-acetylated collagen. These data support the present thesis that by acetylation of the hydroxy group of collagen the hydrogen bond which it forms with the carbonyl oxygen of the —CO—NH-link is split, consequently increasing the number of potential sites for hydrogen bonding of the polyphenolic tannin on the collagen.

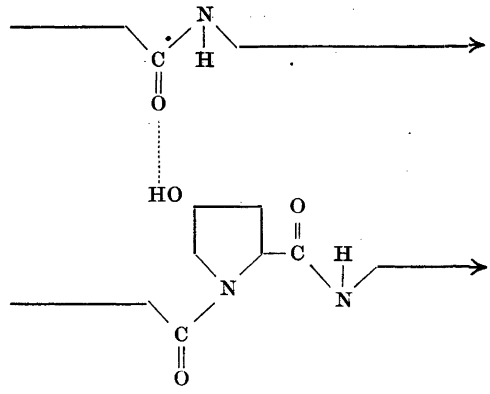
Additional support of this view is given by data forming the first experimental indication of the participation of the hydroxy group of collagen in tanning processes, more particularly for the binding of non-ionic chromium complexes. The fixation of tanning agents by the hydroxy groups of polyvinyl-alcohols has been demonstrated by Elöd and Schachowskoy [6].

The sulphito-chromium sulphates, obtained by adding 2—2.5 moles of Na_2SO_3 per each mole Cr_2O_3 to a solution of the 67 % acid chromium sulphate, described elsewhere[7], contain preponderantly non-ionic complexes and small amounts of negatively charged chromium complexes, as proved by electrophoresis and ion exchange. They are prone to aggregate. These chromium complexes are practically inert to the modified polyamide [7]. However, they have been found extremely useful as specific

agents for the evaluation of the coordination faculty of collagen [4], since their fixation by collagen is mainly governed by the number of free sites available on the protein for hydrogen bonding. By the heat-denaturation of collagen, for instance, the non-ionic chrome fixation is increased by 50—75 %, indicating the rupture of cross-links in collagen and the formation of additional sites for the binding of these complexes [4]. Since the polyamide does not bind the sulphite-complexes[7], and steric hindrance due to the molecular size is improbable from comparison with other agents of greater molecular weight which are fixed by the polyamide [3], it appears that some other groups than the keto-imide link must be instrumental for the fixation of the nonionic sulphito-sulphato-chromium complexes.

Collagen (hide powder) treated for 24 h with the sulphito complexes at a concentration of 20 g Cr_2O_3 per liter fixed 21.8 % Cr_2O_3 of its own weight. The O-acetylated collagen showed very much lower fixation, or 15.2 % Cr_2O_3. It should be noted that the cationic, and gradually even the anionic protein groups, are involved in the reaction. However, the main fixation has been indicated to be located on non-ionic protein groups. The hydroxy group is indicated to form the principal site of binding, in view of the marked decrease of the chrome fixation resulting from its acetylation and inactivation. This finding, with due consideration of numerous other facts which cannot be enumerated in this note, supports the main thesis of this paper that the hydroxy group of collagen, probably mainly that of hypro, is able to form a strong interchain link with the —CO—NH-group of the type.

The rupture of such a bond in the various pretreatments mentioned and by heat denaturation would satisfactorily explain both the increased fixation of vegetable tannins (by the freed —CO—NH—bond) and of the non-ionic complex chromium compounds (by the freed OH—bond) as well as the effects of the blocking of the hydroxy group, *i. e.*, the impaired hydroxy-coordination of the chromium complexes, but increased binding of the keto-imide-attached vegetable tannins and the large T_s decrease ($\triangle T_s$: —22° C) found. In view of the marked reactivity of native collagen for the agents mentioned, it is probable that only a few of the total number of the hydroxy and keto-imide groups are able to form the type of crosslink suggested.



That the hypro residue mainly supplies the hydroxy group is made probable by the following facts. The content of aliphatic hydroxyamino acids is somewhat higher in teleost collagen than in the bovine type [8]. However, the T_s values follow the trend of the hypro content, being lowered with decreasing content of this imino acid [1]. If the aliphatic hydroxy group should form the main interchain link, the T_s values should show the reverse trend. The evidence for the crosslinking being *intermolecular* has already been given.

Professor R. S. Bear, Department of Biology, M. I. T., Cambridge, Mass., has kindly informed the author that the type of bond suggested is compatible with the steric conditions in the present model of the collagen helix [9].

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Phosphorus-containing Amino Acids and Peptides from Acid Hydrolysates of Casein and Pepsin

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The importance of the phosphorus-containing amino acids and peptides has been further shown by findings on the function of the phosphoproteins in living cells [1,2] and by experiments with di-isopropyl fluorophosphate [3]. This paper contains results obtained with two phosphoproteins, casein and pepsin.

Experimental and results. For preparation of phosphopeptides technical casein was used. Otherwise casein prepared according to Hammarsten (Grave, Stockholm) or pepsin twice crystallized from ethanol (Worthington Biochem. Sales Co., Freehold, N. J.) was employed. Techniques outlined in earlier papers *cf.* [2] have been applied.

When casein hydrolysed at 100° C for various periods was fractionated on two successive Dowex 50 columns, different proportions of the total phosphorus could be recovered in the phosphoserine peak:

5 hrs	10 hrs	20 hrs
14.8 %	16.4 %	21.4 %

For the preparation of phosphopeptides large amounts of casein were hydrolysed for 20 hours and fractionated through several columns. The two final steps were elution from a Dowex 50 column with 0.01 *N* hydrochloric acid, and elution from a Dowex 1 or 2 column generally with 0.5 *N* formic acid. The separation was in both cases traced with an automatic conductivity recorder [4]. The results are summarized in Table 1. All fractions gave one spot on paper chromatograms coloured with ninhydrin or phosphate reagent. End-group determinations were performed as described by Sanger and Thompson [5]. Some peptides gave no DNP-amino acid after hydrolysis. It may be that the amino group is blocked in some way, although it is possible to prepare the DNP-derivative of free phosphoserine. Phosphoserylglutamic acid from phosphopeptone was described as long ago as 1933 [6]. It is well established that the acid hydrolysis used gives a higher degree of degradation and a simpler mixture than enzymatic digestion [7].

Pepsin was analysed by the same technique. Fig. 1 shows a conductivity curve of the hydrolysate run through a Dowex 50 column. The high peak could be shown to

Table 1.

Dowex 50 column: Effluent volume / Resin bed volume	Final chromatographic system (resin and formic acid concentration)	Amino acids after hydrolysis (2 *N* HCl, 120°, 20 hrs)	N-terminal amino acid
0.95	Dowex 1, 0.25 *N*	Glu, Ser, Val	Glu
		Glu, Ser	Glu
		Val, Ser, Glu	—
1.25	Dowex 2, 0.5 *N*	Ser, Glu	—
		Ser	
1.7	Dowex 2, 0.5 *N*	Ser, Ala	—
		Ala, Ser	Ala
1.9	Dowex 2, 0.5 *N*	Val, Ser	Val
		Thr	
2.5	Dowex 2, 0.5 *N*	Leu, Ser	Leu
		Ileu, Ser	
2.9	Dowex 2, 0.5 *N*	Leu, Ileu, Ser	Leu or Ileu

Abbreviations after Brand and Edsall. The peptides are listed in the same order as they emerge from the Dowex 1 or 2 column.

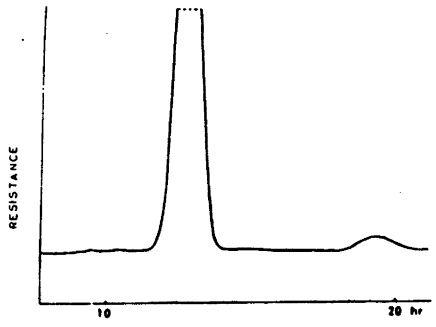

Fig. 1. A conductivity curve from the hydrolysate of 4 g pepsin run through a Dowex 50 column. Elutrient: 0.01 N hydrochloric acid. Column dimensions: 1.03 × 39.5 cm. Flow rate: 6 ml/hr.

be phosphoserine by paper chromatography before and after hydrolysis, and by X-ray diffraction diagram (Fig. 2). It contained 14.8 % of the phosphorus. The lower peak contained after hydrolysis the amino acids serine, threonine and glutamic acid.

There is one phosphorus atom per molecule of pepsin, and Perlmann has shown by experiments with different phosphatases that the phosphorus is probably bound to two different amino acids [8]. The isolation of phosphoserine shows that the phosphorus is bound to serine as in all typical phosphoproteins. A more detailed report of these experiments will be given elsewhere.

This investigation was sponsored by grants from the *Medical Faculty, Uppsala* (for the casein project), and from the *Therèse and Johan Andersson Foundation, Stockholm* (for the pepsin project).

After this investigation was complete the author's attention was drawn to a note reporting the isolation of phosphoserine and threonylphosphoserylglutamic acid from pepsin [9].

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Fig. 2. X-ray powder diagram of phosphoserine from pepsin. Flat film, with a distance of 10.0 cm between specimen and film.

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Viscosities of Dilute High Polymer Solutions

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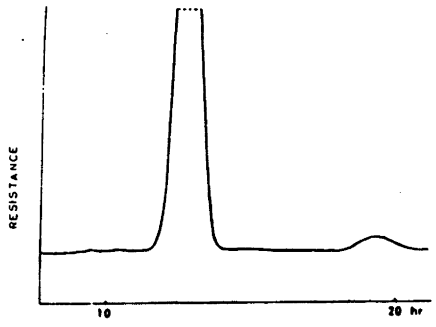
During the years 1950—51 an apparatus for the very precise determination of relative viscosity [1] was built at this Institute. In the first measurements on high polymer solutions an effect was observed, in which the plot of η_{sp}/c *vs.* c was linear only down to the concentration 0.0001 g/ml, whereas at still lower concentrations the curve tended to bend downwards. A probable explanation was that the concentration, determined by weighing before the viscosity measurement, had changed in the viscometer due to adsorption of solute on its walls. The results of measurements of adsorption by Cutler and Kimball [2] indicated that this explanation is valid. It is of course very difficult to obtain a fairly good estimate of the magnitude of the adsorption effect at these very low concentrations.

It later seemed that the 'bending down' might partly be due to the fact that at these low concentrations the molecules are free from each other, which gives a lower hydrodynamic interaction, so that the viscosity diminishes more than corresponds to the decreasing concentration.

During the past year some measurements on polystyrene in toluene have been done in an attempt to verify this. In these experiments part of each solution was used in the viscometer, and after a measurement its concentration was checked turbi-



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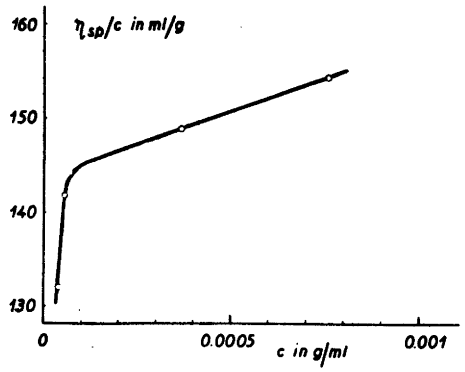

Fig. 1. Viscosity of polystyrene in toluene. $\eta_{sp}/c = f(c)$. $M \sim 500\,000$.

dimetrically against that of the remainder of the solution. It was evident that the bending down of the curve could not be explained as due to concentration change only. The adsorption was only about 3 % at the lowest concentration, whereas a linear relationship had demanded 14 %. Also upon critical examination the shape of the curve indicated that an adsorption of the size in question is unacceptable, as the 'bending down' would have a considerably flatter progress then.

Very recently some other measurements of viscosity of very dilute polymer solutions have been published, where the results have been quite different from those described here. For polystyrene in toluene, Streeter and Boyer [3] have found that the curve in question bends upwards and passes through a maximum. Possibly the different results are due to the different manipulation of the solution before the measurement, so that the higher viscosity from the measurements of these authors depends on the *very long* shakening of the solution during which a certain expansion of the molecules is possible. On the other hand the results they obtained upon concentrating the solutions indicate that it is not probable that the bending down of the curve found by the present author is due to some molecular breakdown.

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A New Sarsasapogenin Glucoside from *Narthecium ossifragum* (L.) Huds.

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The occurrence of a saponin fraction from *Narthecium ossifragum* was reported in a recent communication [1]. The saponin was obtained by concentration of a diluted methanolic extract, which previously had been extracted with petroleum ether. Further work has led to the following observations. During evaporation under reduced pressure the main portion of the saponin precipitated in the form of minute white scales, which were filtered off. The mother liquor was successively extracted with ether, and small quantities of chloroform, which removed α-methoxy-$\Delta^{\alpha,\beta}$-butenolide [2]. By continued extraction with more chloroform a sticky, greenish extract was obtained, which was evaporated to dryness and dissolved in ethanol. On careful addition of water a second crop of saponin could be obtained.

After repeated recrystallisation from hot diluted ethanol the saponin formed glistening, white platelets, a small quantity of which was further purified by chromatography on wet silica gel [3]. The purified saponin, when heated appreciably above 200° C, gradually darkened and finally melted to a dark brown syrup. The highest m.p. obtained lay in the neighbourhood of 285°. The saponin had a hemolytic index of about 110 000.

The purified saponin was submitted to mild hydrolysis and samples were withdrawn from the hydrolysis mixture at intervals. Paper chromatography of the samples showed that the hydrolysis led to an almost immediate liberation of arabinose, more slowly followed by galactose. After complete hydrolysis also xylose and glucose could be spotted on the chromatograms.

The crude saponin, as could be shown by paper chromatography [4], yielded on hydrolysis one single sapogenin, which after repeated recrystallisation from methanol melted at 197—99°. $[\alpha]_D^{20} -72°$. (Found: C 75.0; H 10.70. Calc. for $C_{27}H_{44}O_3$ +

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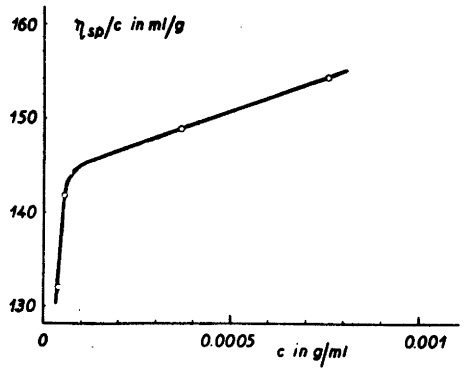

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CH_3OH: C 75.0; H 10.78. Found, after drying: C 77.2; H 10.64. Calc. for $C_{27}H_{44}O_3$: C 77.8; H 10.65.)

The acetylated sapogenin, after recrystallisation from methanol, melted at 128 −30°. $[\alpha]_D^{20}$ −67°. (Found: C 73.2; H 10.21. Calc. for $C_{29}H_{46}O_4 + CH_3OH$: C 73.4; H 10.27. When dried by melting in vacuum, the acetate had m.p. 142−44°. (Found: C 76.2; H 10.19. Calc. for $C_{29}H_{46}O_4$: C 75.9; H 10.11.)

The chemical and physical properties of the substance are close to those of sarsasapogenin. The melting point of the sapogenin showed no depression on admixture with an authentic sample of sarsasapogenin. The identity of the two substances was further confirmed by the infrared spectra.

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1. Stabursvik, A. *Acta Chem. Scand.* **7** (1953) 1220.
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3. Stoll, A., Angliker, E., Barfuss, F., Kussmaul, W. and Renz, J. *Helv. Chim. Acta* **34** (1951) 1460.
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The Carotenoids of *Shepherdia canadensis* (L.) Nutt.

ARNULV STABURSVIK*

Institutt for Organisk Kjemi, Norges Tekniske Högskole, Trondheim, Norway

Through the courtesy of Mr. Olav Gjærevoll, Museum of the Royal Norwegian Society of Sciences, Trondheim, a small sample of the fruits of *Shepherdia canadensis* has been obtained. The fruits were collected by Mr. Gjærevoll in Alaska, where they are called "soap berries". They are eaten by the natives, after being whipped with sugar to a fluffy mass.

An aqueous extract of the fruits indeed gave an extremely rich and stable foam on shaking. The fruits had a moderately bitter taste. Work on isolation of the saponin, and, if possible, of the bitter principle will be attempted.

The fruits were intensely red in colour, due to substances of carotenoid nature, and it was considered worth while first to examine these substances. The carotenoids of other members of the *Elaeagnaceae* have already been subjected to such investigation. From the fruits of the sea buckthorn, (*Hippophaë rhamnoides* L.) zeaxanthin (Karrer and Wehrli [1]) and physaliene (Winterstein and Ehrenberg [2]) have been isolated. More recently cryptoxanthin, lycopene, β-carotene and γ-carotene have been added to the list by Bielig [3]. In *Elaeagnus longipes* Gray, lycoxanthin, lycopene and γ-carotene have been found by Geiger-Vifian and Müller [4].

By chromatographic separation of the carotenoids of *Shepherdia canadensis* the fractions shown in Table 1 were obtained.

Table 1.

Zone No.	Colour	Absorption maxima, mμ, solvent petroleum ether		
1	Colourless	365	346	330
2	Diffuse khaki	475	447	425
3	Pale yellow	425	400	379
4	Khaki	477	448	
5	Pale yellow	425	399	378
6	*Yellow-orange*	*485*	*455*	*432*
7	Yellow	466	437	414
8	Yellow-orange	485	456	432
9	*Orange*	*496*	*462*	*434*
10	*Red*	*504*	*473*	*444*
11	*Dark red*	*503*	*473*	*445*
12	Salmon	485	452	
13	Pale yellow	425	399	378
14	Pale salmon	472	443	420
15	Yellow	448	425	400
16	Mauve	490	455	430
17	Salmon	Single max. at 450		
18	Yellow	450	421	400
19	Yellow	450	422	400

The zones are numbered in order of increasing absorbtive power. The zones 1−11 were chromatographed on calcium hydroxide, the other zones on calcium carbonate.

The fractions 6 and 9−11 (printed in italics), contained the main bulk of the carotenoids. The fractions 12−19 contained very small amounts of pigments.

Fraction 11 gave after several recrystallisations from benzene-methanol dark, bluish

* Research fellow of *Norges Almenvitenskapelige Forskningsråd.*

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red crystals which melted sharply at 137°corr. After saponification the substance melted at 162°corr. The absorption maxima corresponded closely to the values for lycoxanthin, whose m.p. is 164°, according to Zechmeister and Cholnoky [5]. These authors have synthesised lycoxanthin acetate. Its m.p., 137°, is the same as that of the substance isolated from *Shepherdia*. Since esters with higher fatty acids would be expected to have lower melting points, the identity of the isolated substance with lycoxanthin acetate seems very probable.

The neighbouring zone, fraction 10, was chromatographically identical with lycopene from tomatoes. Fraction 9 was not obtained in a crystalline form. After repeated chromatographic separation it seemed to contain no traces of other pigments and gave the absorption bands of γ-carotene [6]. However, in contrast to γ-carotene, the substance had two fairly pronounced peaks at 353 and 369 mμ.

No definite conclusions can be reached with respect to the identity of the other carotenoids. The fractions 1 and 4 displayed absorption maxima very similar to those of phytofluene [7], and α-carotene [8], respectively.

The pale yellow zones 3 and 5 showed the absorption bands of η-carotene [9] or ζ-carotene [10]. The orange zones 6 and 8 both displayed the spectrum of β-carotene.

The fractions 12—14 were epiphasic, but on saponification they turned hypophasic, consequently the pigments were xanthophyll esters. The absorption spectra of fractions 18 and 19 corresponded closely to chrysanthemaxanthin [11] and flavoxanthin [12]. The latter, when dissolved in ether, gave a weak blue colour with hydrochloric acid.

No attempt was made to correlate the zones 2, 7, and 15—17 with known caroteoids.

Experimental. Ripe fruits of *Shepherdia canadensis* were collected at Beaver Creek, White Mountains, Alaska, during the summer of 1953. Sugar was added as a preservative, the mixture heated until boiling, and stored in a 1 lb. glass jar until used.

The fruits were washed with water, treated with acetone to extract the carotenoids, and the carotenoids transferred to petroleum ether (b. r. 40—70°) by addition of water. The petroleum ether extract was washed with water and dried over anhydrous sodium sulphate. All operations were carried out in an atmosphere of pure nitrogen. After evaporation to dryness at 30°, the carotenoids were redissolved in petroleum ether and chromatographed on a column (2 × 20 cm) of precipitated calcium carbonate (Riedel-de Haën). The column was developed with petroleum ether containing a trace of acetone until zone 11 had just left the column. Each of the zones 12—19 were cut out separately and their absorption spectra measured in petroleum ether solution with a Beckmann DU spectrophotometer.

The eluate from the column was collected in two fractions; the first fraction contained the zones 1—8, and the second fraction the zones 9—11. The latter was rechromatographed on a column of calcium hydroxide (Riedel-de Haën) and developed with petroleum ether, containing just enough acetone to cause a slow downward movement of the pigment bands. Each zone was rechromatographed separately until homogeneity, and the eluted substances dissolved in 1 ml benzene. Upon careful addition of methanol the pigments from the zones 10 and 11 crystallised.

Also the first fraction of the eluate, containing the zones 1—8, was chromatographed on calcium hydroxide, and the absorption spectra recorded.

I wish to express my thanks to Professor N. A. Sörensen for his kind interest in the work.

1. Karrer, P. and Wehrli, H. *Helv. Chim. Acta* **13** (1930) 1104.
2. Winterstein, A. und Ehrenberg, U. *Hoppe-Seyler's Z. physiol. Chem.* **207** (1932) 25.
3. Bielig, H. J. *Ber.* **77B** (1944) 748; *Chem. Abstracts* **44** (1950) 617h.
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The Constitution of Phenolphthalein Oxime

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Phenolphthalein oxime is shown to be a derivative of 2,3,1-benzoxazine and not one of phthalimidine as hitherto assumed.

The oxime undergoes rearrangement and hydrolysis to *p*-hydroxybenzoyl benzoic acid and *p*-aminophenol not only in acid solution (as known) but also in alkaline solution.

N-methyl hydroxylamine reacts with phenolphthalein in alkaline solution similarly to hydroxylamine proper while O-methyl hydroxylamine does not react at all.

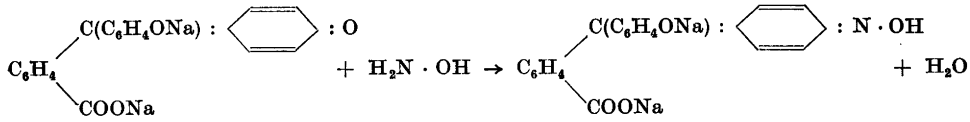
It is suggested that phenolphthalein hydrazide contains a six-membered heteroring instead of the hitherto supposed five-membered ring.

The similarity of the rearrangement of phenolphthalein oxime and that of triphenylmethyl hydroxylamine is pointed out.

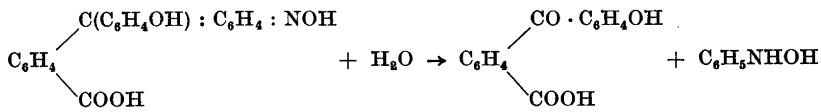
Friedländer [1], in 1893, described a reaction between phenolphthalein and hydroxylamine in alkaline solution:

$$C_{20}H_{14}O_4 + H_2NOH \rightarrow C_{20}H_{15}O_4N + H_2O$$

in which a yellow compound, named phenolphthalein oxime, was formed. It was at first supposed to be a quinone oxime:



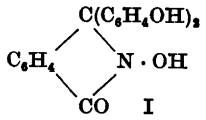
The free oxime was insoluble in water, soluble in alkali and in dilute sulfuric acid. On boiling, the yellow solution in sulfuric acid gradually lost its colour and from the solution an almost quantitative yield of *p*-hydroxybenzoyl benzoic acid and *p*-aminophenol, 1 mole of each from 1 mole of the oxime, could be obtained. Friedländer interpreted this hydrolysis in the following way:



The primarily formed, then unknown, phenylhydroxylamine was supposed to rearrange into *p*-aminophenol.

Later [2], however, he observed that tetrabromo phenolphthalein oxime — with bromine in the four *ortho*-positions to the phenolic hydroxyl groups — under similar conditions gave a dibromo *p*-aminophenol with bromine still in *ortho* positions to the phenolic hydroxyl group and so he had to abandon the quinone oxime hypothesis.

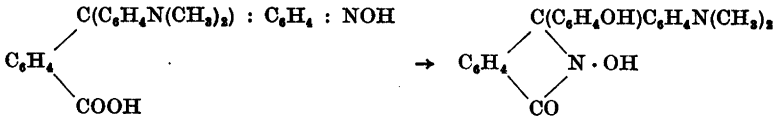
Several investigators [3] have discussed the constitution of phenolphthalein oxime and the conclusion generally accepted seems to be that the compound has the following structure:



I

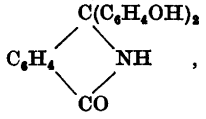
It must be mentioned, however, that Thiel and Diehl [3b] do not feel entirely satisfied: »Es wird nach dem hier Gesagten sich als nötig erweisen, für das Oxim in jedem der besprochenen Zustände eine besondere Konstitutionsformel aufzustellen, eine Aufgabe, die angesichts der hier herrschenden, sicher sehr komplizierten Verhältnisse wohl nicht ganz leicht wird.» Their doubt is mainly caused by the different colour of the substance at pH 10 (orange) and pH 13 (pure yellow) and the fact that 3′,3″-dinitro and higher nitrated phenolphthaleins do not form oximes.

Buu-Hoï [4], in 1940, suggests for the oxime of phenol-dimethylaniline-phthalein the quinone oxime formula for the primarily formed product which by recrystallization from alcohol is rearranged into the more stable heterocyclic form:



There are, however, several facts which are incompatible with the accepted formula I.

1. Phenolphthalein imide, formed from phenolphthalein and ammonia, has undoubtedly the constitution



but it is insoluble in dilute acids. It seems, therefore, quite improbable that a compound with the constitution I should be more basic than the imide.

2. Phenolphthalein oxime, when treated in alkaline solution with dimethyl sulfate, yields a yellow trimethyl ether which in boiling alcoholic potassium hydroxide splits off one methyl group [3b]. As methoxyl amine is stable to hydrolysis by alkali it appears unlikely that the $>NOCH_3$ group should be saponifiable.

Furthermore, the dimethyl ether obtained by acidifying the alkaline solution is yellow and readily soluble in ethyl ether. On short standing, however,

the ethereal solution deposits colourless crystals which are only slightly soluble in ether. When redissolved in alkali the yellow modification of the dimethyl ether again is formed when the solution is made acid. Obviously two forms of phenolphthalein oxime dimethyl ether exist and formula I does not account for this possibility.

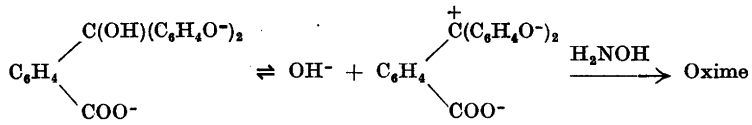
3. Phenolphthalein, in alkaline solution, does not react at all with methoxylamine, H_2NOCH_3. A solution has been kept for hours on a boiling water bath without any reaction occurring.

Against the quinone oxime formulation, besides the fact already mentioned, tells the reaction to be described later in this paper between phenolphthalein and N-methyl hydroxylamine which leads to quite similar products.

Thus the necessity of another formula for phenolphthalein oxime is evident.

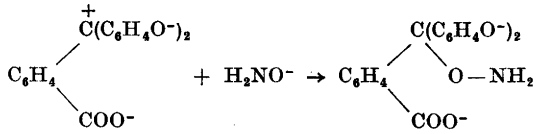
As methoxylamine does not react with phenolphthalein it must be assumed that the hydroxyl group is involved in the condensation reaction. In neutral or only slightly alkaline solutions, *e. g.* phenolphthalein in an alcoholic solution of free hydroxylamine, no reaction takes place.

When an aqueous alkaline solution of phenolphthalein is brought to the "fading equilibrium" at room temperature, hydroxylamine added, and the solution placed in a spectrophotometer the absorption maximum of the red solution (at 552 mμ) at first can be seen to decrease rapidly and after a while the decrease is far slower. The obvious explanation is that the coloured divalent phenolphthalein anion reacts fairly rapidly with hydroxylamine and soon the reverse of the fading reaction determines the rate of reaction:

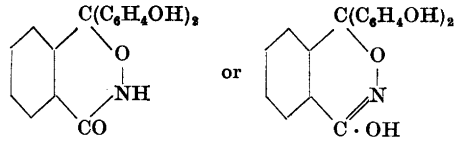


At higher temperatures hydroxylamine apparently also reacts with the faded ion.

Since the reaction proceeds most rapidly in strongly alkaline solution it seems reasonable to assume that it is the hydroxylamine anion, H_2NO^-, that reacts with the phenolphthalein anion, the reaction being then quite similar to the fading reaction with hydroxyl ions:



and the constitution of the product obtained on acidification consequently is supposed to be



II III

It is here assumed that the red phenolphthalein anion has the constitution of an ampho-ion. Usually a quinone-phenolate structure is ascribed to this ion; but it seems to me that the ampho-ion structure gives a better picture as it brings out the perfect symmetry of the two phenolic groups. Of course some resonance structure results from the ionization process and the formulation is only a suitable way of illustrating the essential properties. Moreover, in a recent paper Davies and Jones [5] have shown that the infrared spectrum of the red phenolphthalein salts contains no "quinone frequencies".

The heteroring system assumed here to exist in the phenolphthalein oxime molecule — 2,3,1-benzoxazine — apparently has never been assigned to any existing compound so it cannot be inferred from analogous compounds whether or not such a substance might possess base properties. It appears, however, reasonable. The fact that the trimethyl ether has one saponifiable methyl group tends to show that the ether is derived from structure III, the grouping $-C(OCH_3)=N-O-$ being a modified hydroximic acid ester. The relatively high acidity of the oxime — it can be dissolved in potassium carbonate solution — is explained in the same way. The different colour of the solutions at different degrees of alkalinity depends on the different ionization — whether one has to do with a monovalent or a trivalent anion. The reason why the polynitro phthaleins do not form oximes is that the nitrophenolates do not effect ionization at the central carbon atom: their colour is the usual colour of nitrophenols in alkaline solutions.

The easy conversion of the yellow dimethyl ether to the colourless one is explained as a rearrangement of the dimethyl ether of III into that of II.

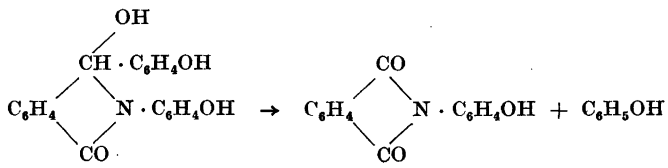
For further investigation of the reaction between phenolphthalein and hydroxylamine the latter was replaced by N-methyl hydroxylamine. To an alkaline solution of phenolphthalein was added the equivalent amount of N-methyl hydroxylamine and the mixture was kept at 60—70°. After 5—10 minutes the violet-red colour had disappeared and was replaced by a blood-red colour, and on acidification a yellow compound was precipitated. It contained nitrogen, but it has not been possible to obtain a pure product because a suitable solvent for recrystallization could not be found. — On longer standing of the reaction mixture at 60—70° the red colour faded considerably. After an hour the solution was made acid with acetic acid and an almost colourless compound slowly crystallized out. Obviously a rearrangement had taken place. The product obtained could be identified as the *p*-methylaminophenol salt of *p*-hydroxybenzoyl-*o*-benzoic acid.

In the alkaline solution thus were found products entirely analogous to the products of acid hydrolysis of the oxime proper. This fact of course invited to an examination of the oxime in alkaline solution, and it could be shown that a similar hydrolysis could be effected, although considerably slower. The oxime was dissolved in a hot aqueous solution of potassium carbonate and kept at 90° for 12 hours in a well stoppered flask. Addition of acetic acid caused a slow crystallization of a light yellow substance. (The oxime is precipitated instantaneously on acidification, and none was found; the oxime was completely transformed.) The product was, as expected, the *p*-aminophenol salt of hydroxybenzoyl benzoic acid.

These reactions throw light upon an observation made by Friedländer and others in connection with the preparation of phenolphthalein oxime: When

an excess of hydroxylamine is used the oxime is accompanied by a small amount of a byproduct which could be identified as N-(*p*-hydroxyphenyl) phthalimide.

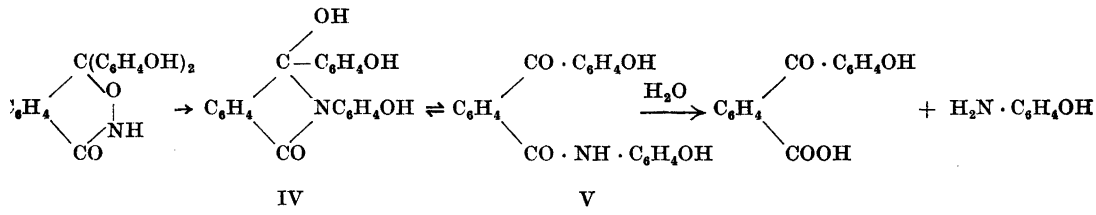
Orndorff [3f] assumed the formation of this compound to be due to the splitting off of a molecule of phenol from a rearranged form of the oxime, the reaction in some way being catalyzed by hydroxylamine:



It is, however, difficult to see any reason for such a reaction, and it has been possible to show that a second molecule of hydroxylamine is involved in the reaction and *p*-aminophenol is formed. When phenolphthalein (10 g) in alkaline solution was heated to 90° for one and a half hour with two equivalents of hydroxylamine 1.3 g of the phthalimide derivative and 0.4 g of *p*-aminophenol could be isolated besides 8 g of the oxime.

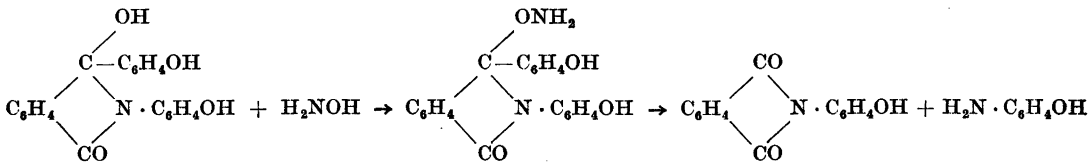
REARRANGEMENT OF PHENOLPHTHALEIN OXIME

The formation of *p*-hydroxybenzoyl benzoic acid and *p*-aminophenol from the oxime obviously implies a rearrangement of the oxime prior to the hydrolysis:



IV V

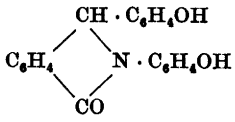
The same rearrangement also precedes the reaction with a second molecule of hydroxylamine:



It is necessary to assume that this reaction takes place before the rearrangement product IV is hydrolyzed because *p*-hydroxybenzoyl benzoic acid does

not react with hydroxylamine in alkaline solution: After two hours at 90° the acid could be recovered almost quantitatively.

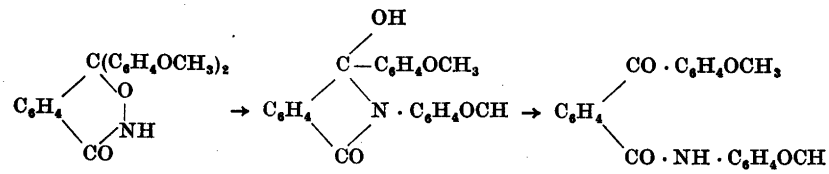
The oxime dissolved in a solution of sulfuric acid in alcohol is reduced by zinc to the compound [3a,e,f].



This reaction also must occur *after* rearrangement, but *before* hydrolysis.

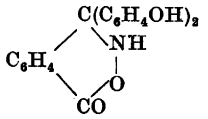
The intermediate compound IV (or V) can, as shown by Orndorff, [3f] be obtained by acetylating the oxime with acetic anhydride with subsequent hydrolysis of the triacetate.

While the trimethyl ether of the oxime can be boiled for any length of time with acetic anhydride without rearrangement the dimethyl ether when boiled in acetic anhydride for one or two minutes is rearranged, but not acetylated.



The product obtained in this way is insoluble in alkali.

The rearrangement of phenolphthalein oxime is somewhat similar to the Beckmann rearrangement: an organic radical migrating from carbon to nitrogen. It also recalls the transformation of triphenylmethyl hydroxylamine into benzophenone anil discovered by Stieglitz [6]. As triphenylmethyl hydroxylamine is supposed to be a N-derivative one might consider the possibility of phenolphthalein oxime having the structure



Against this hypothesis the following objections can be made: (1) The non-reactivity of methoxylamine; (2) the observation made by Stieglitz that triphenylmethyl N-methylhydroxylamine only with great difficulty and with extremely low yields can be rearranged while by phenolphthalein N-methyl oxime the rearrangement proceeds even more readily than by the simple oxime, and (3) the saponification of the trimethyl ether.

In fact, the question arises if not the trityl hydroxylamine hitherto regarded as a N-derivative really is an O-derivative. In an excess of trityl chloride hydroxylamine forms a di-trityl hydroxylamine and it seems, on sterical grounds, very unlikely that a N-ditrityl hydroxylamine can be formed since ammonia is unable to form a ditritylamine. Furthermore, benzaldehyde

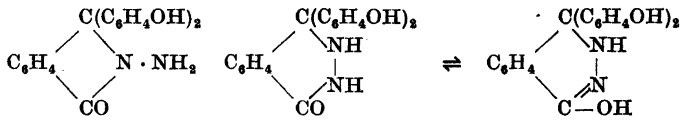
when heated with trityl hydroxylamine gives an oxime which has been assigned the formula $(C_6H_5)_3C \cdot \underset{\ddot{O}}{N}{:}CH \cdot C_6H_5$, but just as well might be O-trityl benzaldoxime. In fact, the same compound is obtained from benzaldoxime sodium on treatment with trityl chloride.

If, on the other hand, trityl hydroxylamine is an O-derivative the Stieglitz transformation taking place in the presence of phosphorus pentachloride in an anhydrous solvent becomes quite analogous to the rearrangements found in the phenolphthalein oxime group:

$$(C_6H_5)_3C \cdot O \cdot NH_2 \rightarrow (C_6H_5)_2C(OH) \cdot NHC_6H_5 \rightarrow (C_6H_5)_2C{:}NC_6H_5 + H_2O$$

PHENOLPHTHALEIN HYDRAZIDE

Hydrazine reacts with an alkaline solution of phenolphthalein with formation of a hydrazide [7]. In the literature the constitution of this substance is given as VI



VI VII VIII

It seems, however, more reasonable to assume the formation of a sixmembered ring VII or VIII. The compound is insoluble in dilute strong acids, so a free amino group is improbable. It seems to exist in two forms: The compound obtained by acidification of the alkaline solution in which it is formed is readily dissolved in warm acetic acid, but when the solution is brought to boiling it crystallizes out and is now sparingly soluble in acetic acid. It can be brought back to the soluble form again by dissolving it in alkali and acidifying the solution.

The two modifications probably correspond to the structures VII and VIII. They have the same melting point, but long before melting the crystals turn red, indicating some inner rearrangement of unknown nature.

EXPERIMENTAL

Phenolphthalein oxime

The oxime was prepared according to the procedure of Orndorff and Yang [3f].

Crystalline salts with sulfuric, hydrochloric, and perchloric acids are readily prepared by dissolving the oxime in warm (60—70°) dilute acids and cooling. The hydrochloride is sparingly soluble even in hot water and is best obtained by the addition of a little hydrochloric acid to a solution of the sulfate or perchlorate. They are all yellow. The oxime is recovered unchanged by addition of sodium acetate to aqueous solutions of the salts.

The only solvent dissolving considerable amounts of the oxime seems to be pyridine (or alkylpyridines). Addition of alcohol to such a solution precipitates the oxime in a very pure state, m.p. 216°.

Rearrangement of the oxime in alkaline solution. 15 g of the oxime are dissolved in a boiling solution of 5 g of potassium carbonate in 100 ml of water. The flask is then stoppered tightly in order to avoid oxidation from the atmosphere and is kept at 90° overnight. The still hot solution is acidified with acetic acid (10 ml in 50 ml of water). No precipitate is formed immediately, indicating that no unchanged oxime remains, but on cooling a light yellow crystalline substance separates. After half an hour the compound is filtered off and washed with cold water. Yield 14 g, m.p. after recrystallization from alcohol 200–01°. When recrystallized from water the substance is brownish-grey due to oxidation. (Found: C 68.2; H 4.90; N 4.08; 4.09. Calc. for $C_{20}H_{17}O_5N$: C 68.3; H 4.84; N 3.99).

From the reaction mixture strong acids precipitate directly *p*-hydroxybenzoyl benzoic acid and from the filtrate potassium carbonate precipitates *p*-aminophenol. The product formed in the process thus is the aminophenol salt of hydroxybenzoyl benzoic acid.

Phenolphthalein oxime ethers

The trimethyl ether was prepared and saponified according to Orndorff and Yang. The yellow dimethyl ether has m.p. 176–78° [3b]. It dissolves readily in ether, but after a few minutes the yellow colour of the solution has faded and colourless crystals appear. The compound is now only slightly soluble in ether and melts at 156–57°. (Found: N 3.91. Calc. for $C_{22}H_{19}O_4N$: N 3.88).

Rearrangement. 5 g of the dimethyl ether in 10 ml of acetic anhydride was heated to boiling for 1–2 minutes and poured into water. As the acetic anhydride became hydrated a crystalline compound separated. The colourless compound after recrystallization from alcohol had m.p. 171–72°. (Found: C 73.1; H 5.41; N 3.90. Calc. for $C_{22}H_{19}O_4N$: C 73.2; H 5.31; N 3.88).

The product is insoluble in alkali and is supposed to be *p*-anisoyl-*o*-benzoic *p*-anisidide or its cyclic isomer.

When treated similarly with diethyl sulfate phenolphthalein oxime yields a triethyl ether which can be saponified by alcoholic potassium hydroxide to a diethyl ether, a yellow compound, m.p. 157–58°. This ether retains its yellow colour when crystallizing from ethyl ether. (Found: N 3.62. Calc. for $C_{24}H_{23}O_4N$: N 3.59).

After treatment with acetic anhydride in the same manner as the dimethyl ether a snow-white compound is obtained, m.p. 201–02°, when recrystallized from alcohol. (Found: C 73.8; H 6.00; N 3.60. Calc. for $C_{24}H_{23}O_4N$: C 74.0; H 5.96; N 3.59).

The substance is insoluble in alkali and quite similar to the methyl compound described above.

Phenolphthalein and N-methyl hydroxylamine.

20 g of phenolphthalein and 7 g of N-methyl hydroxylammonium chloride are dissolved in a solution of 20 g potassium hydroxide in 80 ml of water. The solution is kept in a stoppered flask in a water bath at 65–70°.

In about 10 minutes the violet-red colour of phenolphthalein had disappeared and was replaced by a blood-red colour. A sample was taken out, diluted and acidified with acetic acid. A yellow substance separated, very soluble in alcohol, methanol, acetic acid and acetone, insoluble in non-polar solvents. No solvent suitable for recrystallization was found. Evaporation of alcohol from a solution left a glassy brick-red mass. The nitrogen content was too low, apparently due to an admixture of unreacted phenolphthalein.

The main portion was kept at the temperature mentioned for an hour when dilute acetic acid (20 + 80 ml) was added. The solution was now light yellow and no precipitate appeared at once. On standing for half an hour with occasional scratching and stirring 19 g of light greyish-yellow crystals were filtered off and washed with dilute alcohol and water. It was dried in an exsiccator protected against light. When exposed to sunlight the crystals become salmon-coloured.

The dry substance melts with decomposition at 125–27°. (Found: N 3.64; 3.65. Calc. for $C_{22}H_{21}O_5N$: N 3.69).

When the reaction mixture is made acid with strong acids *p*-hydroxybenzoylbenzoic acid is precipitated. *p*-Methylaminophenol was not isolated, but the filtrate when made alkaline rapidly darkened on exposure to oxygen, indicating the presence of this compound.

Phenolphthalein and excess of hydroxylamine

A solution of 10 g of phenolphthalein, 12 g of potassium hydroxide, and 5 g of hydroxylammonium chloride in 50 ml of water was kept at 90° for an hour and a half and then acidified with acetic acid. After cooling the precipitate was filtered off, washed with a little water and immediately treated with a solution of 20 ml conc. sulfuric acid in 150 ml water. The oxime dissolved, while *p*-hydroxyphenyl phthalimide could be filtered off. The oxime was set free with sodium acetate. The first mentioned filtrate was neutralized with potassium carbonate and *p*-aminophenol separated. Yields: 8.0 g of oxime; 1.3 g of imide, m.p. (from alcohol) 292–93°; 0.4 g of aminophenol, m.p. 185°.

To 1.3 g of the imide corresponds 0.7 g of aminophenol, but this compound is considerably more soluble in water than is the imide.

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Note on β-Heparin

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The statement made by Marbet and Winterstein that β-heparin is a chemical entity has been confirmed by chemical and physicochemical analyses. The rate of acid hydrolysis and the optical rotation of the compound indicate that there is a resemblance between the internal structure of β-heparin and of chondroitin sulfuric acid. With respect to the anticoagulant activity, it is concluded that the mechanism of action of β-heparin differs in some respects from that of heparin and of heparin monosulfuric acid. The anticoagulant strength is found to be greatly dependent upon the nature of the coagulation system used for its assay.

In 1951 Marbet and Winterstein [1,2] reported the isolation of a new mucopolysaccharide from the lungs of some species of animals; it was named β-heparin. This β-heparin showed a resemblance to chondroitin sulfuric acid. It contained galactosamine and was levorotatory. Unlike chondroitin sulfuric acid, however, it exerted a relatively strong anticoagulant activity. Heparin and heparin monosulfuric acid, the only previously known natural anticoagulants of this type, contain glucosamine.

The present paper describes the results of an investigation carried out in order to establish the chemical entity of β-heparin.

Some evidence is also presented suggesting that the mode of the anticoagulant action of β-heparin may differ from that of the usual heparin.

Two samples of β-heparin, one from sheep lungs and one from ox lungs — which through the courtesy of Drs. Winterstein and Marbet, Hoffman-La Roche, Basel, had been given to professor Jorpes — were kindly put at my disposal for analysis. I was thereby enabled to confirm all the chemical findings of Marbet and Winterstein and to add some additional information in favor of the supposition that β-heparin is a chemical entity. With respect to the anticoagulant action of this form of chondroitin sulfuric acid, it was possible to show that the biological activity is greatly dependent upon the coagulation system in which it is measured.

EXPERIMENTAL

1. *Analyses.* We determined the moisture, ash, sulfur and acetyl contents and the optical rotation of the sheep β-heparin and confirmed the data reported by Marbet and Winterstein. For the acetyl determination, the method of Lemieux and Purves [3] was

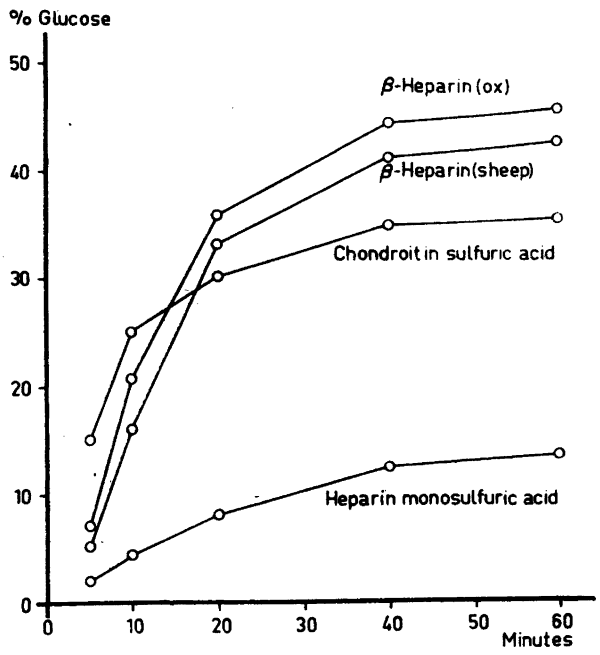

Fig. 1.

adopted instead of the methods of Viditz [4] and Kunz [5] used by the Swiss workers. The first-mentioned procedure has been found to be the most reliable for heparin [6]. The analysis of the single amino sugars was performed according to the ion exchange method developed by Gardell [7] in this laboratory. Heparin monosulfuric acid (prepared from ox lung with the method of Jorpes and Gardell [8]) was also analyzed as a reference substance. In accordance with the previous findings, heparin monosulfuric acid gave only D-glucosamine, although a rather low value, 27.5 %, was obtained as compared to the theoretical figure (42.8 %), possibly due to partial decomposition of the amino sugar during the preliminary hydrolysis. On the other hand, the β-heparin preparation was found to contain

Table 1. Anticoagulant activity of β-heparin, chondroitin sulfuric acid and heparin monosulfuric acid.

The figures represent the activity in international heparin units per mg water free substance. Samples 1 and 2 were analyzed with the thrombin method on three different days and sample 3 on two days.

	Studer and Winterstein [14]	Ox blood *In vitro*	British Pharmacopoeia 1953	U. S. P. XIV 1950	*In vivo*
1. β-Heparin (sheep)	50.4, 49.9; 56.3, 51.6; 49.1	3.4 4.2	0	<2	3 (cat) 3—4 (dog)
2. β-Heparin (ox)	24.6, 25.7; 24.3, 24.3;	8.3	3—4	<3	6—8 (cat) 8 (dog)
3. Heparin monosulfuric acid	7.7, 7.3;	4	<3	6	
4. Chondroitin sulfuric acid		0		0	

D-galactosamine as the only amino sugar component, the quantity found being 28.1 %. It may therefore be concluded that the anticoagulant activity of the preparation is not due to contamination with heparin.

The homogeneity of this preparation was then examined by electrophoresis on a slab of hyflo super-cel [9]. Electrophoresis was performed twice at pH 4.7 (0.1 *M* acetate buffer) for 8 and 12 hours, respectively. In both runs only one peak was observed.

The chemical and physical analyses described above seem to support the statement of Marbet and Winterstein that β-heparin is a chemical entity *per se*.

2. *Acid hydrolysis.* Several polysaccharides, such as heparin, heparin monosulfuric acid and hyaluronic acid, can be differentiated by comparing the rate of hydrolysis in 7.5 % (by volume) sulfuric acid at 100° C [10]. The β-heparin preparations (both from sheep and ox) were therefore hydrolyzed under the aforementioned conditions. The result was compared with those for chondroitin sulfuric acid, prepared from ox cartilage with the method of Strandberg [11] and for heparin monosulfuric acid. As can be seen from Fig. 1, the rate of hydrolysis of β-heparin closely resembled that of chondroitin sulfuric acid, suggesting a similarity in the internal structure of these two compounds.

3. *Anticoagulant activity.* Marbet and Winterstein measured the anticoagulant activity of β-heparin from ox lungs by the thrombin method and reported appreciably high values, about 36 international heparin units per mg. Although Blombäck *et al.*[12] recommend this method for heparin assay, it seemed of interest to ascertain whether the other assay methods would provide any different results. The anticoagulant activity of the β-heparin preparations, the chondroitin sulfuric acid and the heparin monosulfuric acid was therefore determined *in vitro*, using the four methods described in the paper of Blombäck *et al.* *. An *in vivo* determination was also carried out on a cat and a dog, the coagulation time being determined according to the technique of Bergqvist [13]. The results of these experiments are summarized in Table 1.

As can be seen in Table 1, the anticoagulant activity of the β-heparin preparation was remarkably low except with the thrombin method. The activity of heparin monosulfuric acid was fairly constant, irrespective of the method applied. The chondroitin sulfuric acid was inactive. It can be concluded from these findings that the mechanism of action of β-heparin differs in some respects from that of heparin and of heparin monosulfuric acid.

* The author is greatly indebted to Drs. Margareta and Birger Blombäck for the assays of the anticoagulant activity.

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Die Reaktion von Chinonmonochlorimid mit Lignin

I. Spezifität der Reaktion auf *p*-Oxybenzylalkoholgruppen und deren Bestimmung in verschiedenen Ligninpräparaten

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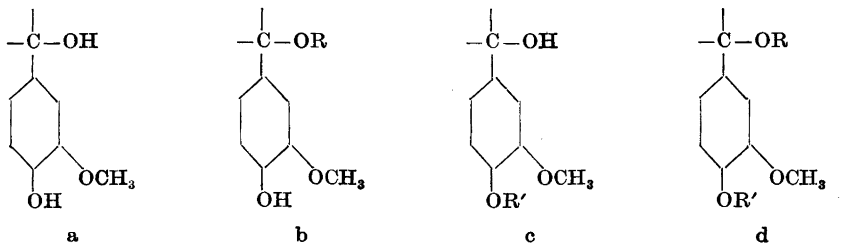
Unsere derzeitige Kenntnis von der Struktur der Ligninbausteine und deren Verbindung untereinander gründet sich zu einem grossen Teil auf Analogien im Verhalten ligninähnlicher Modellsubstanzen und verschiedener Ligninpräparate. Mit Hilfe dieser vergleichenden Methodik konnte bereits die Anwesenheit gewisser Strukturelemente sehr wahrscheinlich gemacht werden, jedoch stehen strenge Beweise noch aus. In einer vor kurzem erschienenen Übersicht über neuere Ergebnisse auf dem Gebiet der Lignin-Strukturforschung weisen daher Adler und Lindgren [1] abschliessend auf die Bedeutung des direkten Nachweises bestimmter Gruppen, vor allem der vielfach postulierten Benzylalkohol- und Benzyläthergruppen, im nativen Lignin hin.

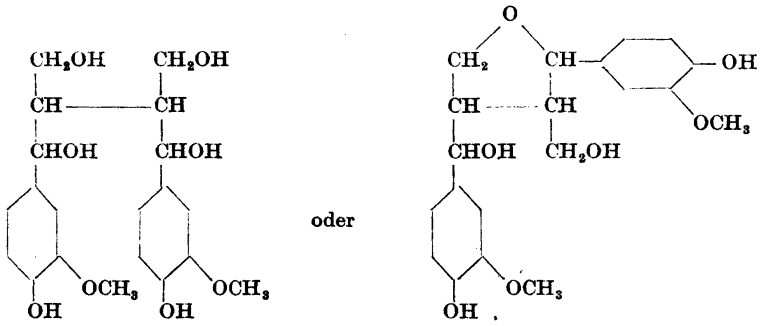
Adler und Gierer [2] haben bereits einige Versuche in dieser Richtung kurz mitgeteilt. Es wurde gezeigt, dass Brauns' »native lignin» aus Fichtenholz mittels methanolischem Chlorwasserstoff schon bei Zimmertemperatur methyliert wird, und dass diese Reaktion der Hauptsache nach auf einer Verätherung von Benzylalkoholgruppen (Typus a und c) oder einer Umätherung von Benzyläthergruppen (Typus b und d) beruhen muss. Fernerhin wurde nach Umsetzung von »native lignin» mit Bromwasserstoff und darauffolgender Behandlung mit Natriumbicarbonat das Auftreten von Chinonmethidgruppen spektrophotometrisch nachgewiesen. Diese ebenfalls unter milden Bedingungen verlaufende Reaktion weist auf das Vorhandensein von phenolischen Benzylalkohol-oder Benzyläthergruppen (Typus a und b) hin.



In der vorliegenden Arbeit wird nun versucht, unter Einhaltung möglichst milder Reaktionsbedingungen (Zimmertemperatur, 0,01 *N* NaOH) freie phenolische Benzylalkoholgruppen (Typus a) durch eine Kupplungsreaktion spezifisch nachzuweisen und zu bestimmen.

Dieser Typ von reaktionsfähigen Gruppen im Ligninmolekül wurde von Lindgren [3] und Mikawa [4] als sogenannte »Gruppen X» für die rasch verlaufende Anfangsphase der Sulfitierung bei neutralem pH verantwortlich gemacht. Setzt man ihre Anwesenheit im Lignin voraus, so müssen sie auch an anderen Ligninreaktionen, wie Alkylierung mit alkoholischer Salzsäure [2], Reaktion mit Thioglykolsäure [5], Umsetzung mit Schwefelwasserstoff [6], beteiligt sein.

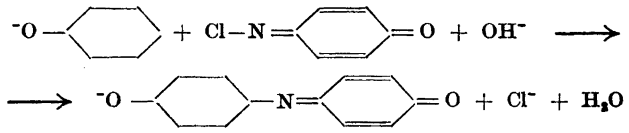
Freudenberg und Mitarbeiter [7] fanden vor kurzem als Zwischenprodukt der enzymatischen Dehydrierung des Coniferylalkohols Dehydrodiconiferylalkohol, d,l-Pinoresinol und ein drittes Dimeres, dem sie vorläufig die Struktur



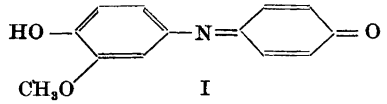
zuschreiben. Es ist denkbar, dass die *p*-Oxybenzylalkoholgruppierungen in solchen oder ähnlichen Zwischenstufen beim weiteren biologischen Aufbau des Ligninmoleküls im verholzenden Gewebe teilweise intakt bleiben.

DIE KUPPLUNGSREAKTION UND IHRE SPEZIFITÄT

Chinonmonochlorimid reagiert in schwach alkalischer Lösung mit Phenolen unter Bildung tiefblauer Farbstoffe, sogenannter Indophenole [8]:



Es wurde nun gefunden, dass Brauns' »native lignin» sowie das in entharztem Fichtenholzmehl enthaltene Lignin ebenfalls mit Chinonmonochlorimid unter Bildung eines Indophenols reagieren. Dieses konnte in kristallisierter Form isoliert und als Chinon-mono-[3-methoxy-4-oxy-anil] (I) identifiziert werden, worüber in einer folgenden Mitteilung näher berichtet werden soll.



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Diese Reaktion des Lignins ist offenbar auf die Tatsache zurückzuführen, dass nicht nur einfache Phenole (z. B. Phenol, Guajakol) sondern auch in *p*-Stellung substituierte Phenole mit Chinonmonochlorimid reagieren, und zwar nur dann, wenn der Substituent unter den Kupplungsbedingungen abgespalten wird [9].

Eine Reihe ligninchemisch interessanter Modellsubstanzen wurde daher auf ihr Verhalten gegenüber Chinonmonochlorimid in schwach alkalischer Lösung untersucht, wobei folgende Gesetzmässigkeiten festgestellt wurden:

1) Eine freie phenolische Hydroxylgruppe ist für das Zustandekommen der Reaktion erforderlich.

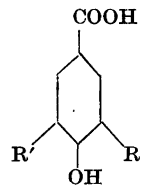
2) Die Kupplung in *p*-Stellung ist gegenüber der Kupplung in *o*-Stellung sehr stark bevorzugt, sodass die letztgenannte praktisch vernachlässigt werden kann.

3) Bei besetzter *p*-Stellung tritt Kupplung nur dann ein, wenn der betreffende Substituent bei der Kupplung abgespalten wird.

In Tabelle 1 sind die positiv reagierenden *p*-substituierten Phenole, aufgeteilt in 5 Verbindungsklassen, angeführt.

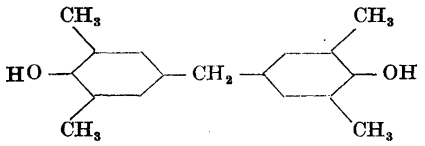
Tabelle 1.

Positiv reagierende p-substituierte Phenole:

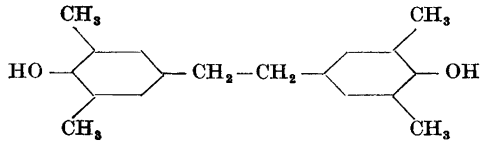


A) *p-Oxybenzoesäuren:*
1) *p*-Oxybenzoesäure (R = R' = H) *
2) Vanillinsäure (R = OCH_3, R' = H)
3) Syringasäure (R = R' = OCH_3)

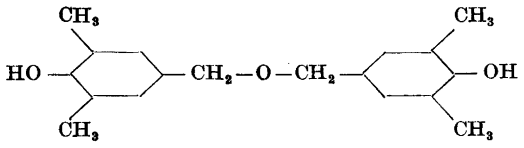
B) *p,p'-Dioxydiphenylmethane:*
4,4'-Dioxy- 3,5,3',5'-tetramethyl-diphenylmethan:



C) *p,p'-Dioxydiphenyläthane:*
4,4'-Dioxy- 3,5,3',5'-tetramethyl-diphenyläthan:

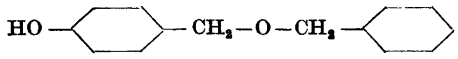


D) *p-Oxydibenzyläther:*
1) 4,4'-Dioxy- 3,5,3',5'-tetramethyl-dibenzyläther:



* Sehr schwache Farbreaktion.

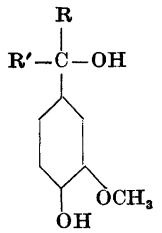
2) *p*-Oxydibenzyläther:



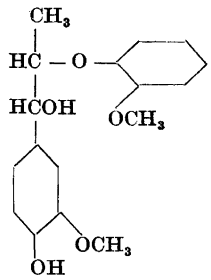
E) *p-Oxybenzylalkohole:*

a) *Guajacylcarbinole:*

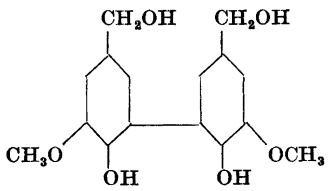
1) Vanillylalkohol (R = R′ = H)
2) Apocynol (R = CH_3, R′ = H)
3) Äthylguajacylcarbinol (R = C_2H_5, R′ = H)
4) Dimethylguajacylcarbinol (R = R′ = CH_3)
5) Guajacylglycerin (R = CH_2OH–$CHOH$–, R′ = H)



6) 1-[4-Oxy-3-methoxy-phenyl]-1-oxy-2-[2-methoxy-phenoxy]-propan *

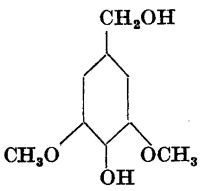


7) Dehydrodivanillylalkohol *

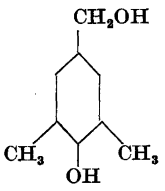


b) *Andere p-Oxybenzylalkohole:*

1) Syringylalkohol

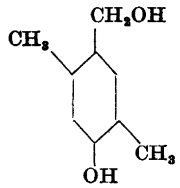


2) 2,6-Dimethyl-4-methylol-phenol



* Die Darstellung dieser beiden Verbindungen, die mir, ebenso wie mehrere andere in Tab. 1 aufgeführte Substanzen, freundlicherweise von Herrn Prof. E. Adler überlassen wurden, ist noch nicht veröffentlicht.

3) 2,5-Dimethyl-4-methylol-phenol



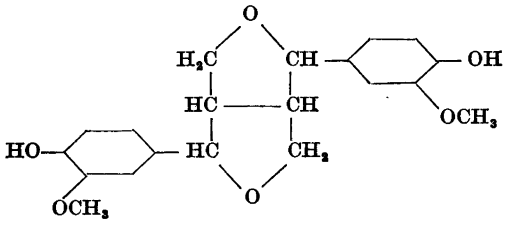
4) α-ständiges Carbonyl oder eine zum Phenolkern konjugierte C—C Doppelbindung im p-Substituenten verhindern die Kupplung.

Eine Ausnahme bilden jedoch die Vinylenhomologen zu Typus A und Typus E (Tabelle 1), z. B. Ferulasäure und Coniferylalkohol, welche eine intensive Farbreaktion geben. Der bei diesen Kupplungsreaktionen gebildete Indophenolfarbstoff ist jedoch instabil; die blaue Farbe verschwindet bereits nach wenigen Sekunden.

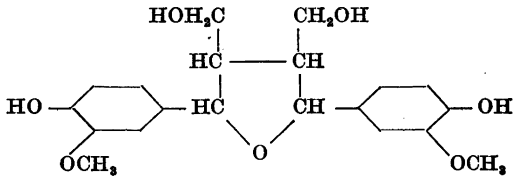
5) Verätherung der alkoholischen Hydroxylgruppe in Typus E mit einem aliphatischen Alkohol hebt die Kupplungsfähigkeit auf.

So kuppeln z. B. Vanillylalkoholmethyläther und das als Ligninstrukturtyp besonders interessante Pinoresinol (II) nicht mit dem Reagenz. Guajacylcarbinole des Typus E (Tabelle 1) verlieren ihre Kupplungsfähigkeit durch Behandlung mit methanolischer Salzsäure vollkommen, was auf Verätherung der alkoholischen Hydroxylgruppe unter Bildung von p-Oxybenzylalkyläthern vom Typus b (siehe S. 1319) beruht [10a].

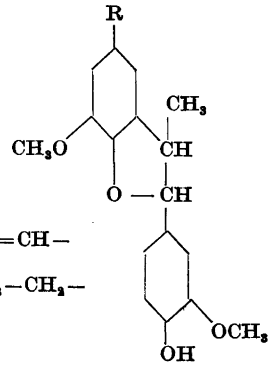
In diesem Zusammenhang sei auf den eigentümlichen Unterschied im Verhalten der eben erwähnten p-Oxybenzylalkyläther und der beiden untersuchten p-Oxydibenzyläther (Typus D, Tabelle 1) gegenüber dem Reagenz hingewiesen. Während die ersteren keine Kupplungsreaktion geben, reagieren die letztgenannten glatt mit dem Reagenz. Dies könnte darauf hindeuten, dass die Ätherbindung in p-Oxydibenzyläthern unter den



II



III



IV R = $CH_3-CH=CH-$

V R = $CH_3-CH_2-CH_2-$

Kupplungsbedingungen aufgespalten wird, worauf die Spaltprodukte im Sinne der *p*-Oxybenzylalkohole (Typus E) reagieren.

Der Dibenzyläther Olivil (III) gibt dagegen die Kupplungsreaktion nicht; sein Hydrofuranring scheint demnach wie der des Pinoresinols (II) ungespalten zu bleiben. Ebenso geben die cyclischen Benzylaryläther Dehydrodiisoeugenol (IV) und Dihydro-dehydrodiisoeugenol (V) keine Kupplungsreaktion.

Die Gesetzmässigkeiten 1—5 gleichen denen, die Ziegler und Mitarbeiter [11] bei der Kupplung von Benzoldiazoniumchlorid bzw. von Nitrobenzoldiazoniumchloriden mit *p*-substituierten Phenolen fanden.* Ziegler und Gartler [9] beschrieben auch die Kupplung von Chinonmonochlorimid mit *p*-Oxybenzylalkoholen (Typus E) und *p,p'*-Dioxydiphenylmethanen (Typus B), nicht aber die mit den Typen A, C und D (Tabelle 1).

Die Abspaltung des *p*-Substituenten bei der Kupplungsreaktion wurde bisher nur bei vier Guajacylcarbinolen (E) näher untersucht. In allen vier Fällen konnte nach der Kupplungsreaktion der erwartete Aldehyd bzw. das erwartete Keton aus dem Reaktionsgemisch abdestilliert und als Dinitrophenylhydrazon durch Schmelzpunkt und Mischschmelzpunkt identifiziert werden.

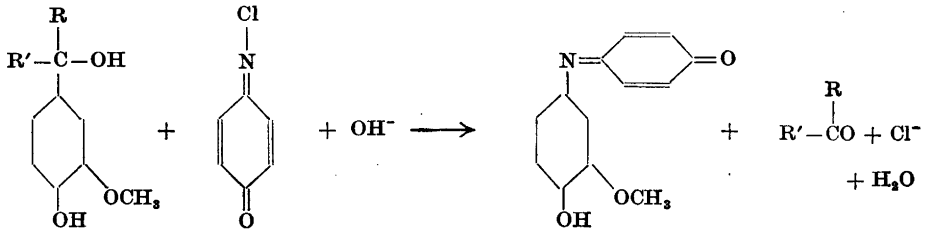
So entstand aus

Vanillylalkohol..... Formaldehyd,
Methyl-guajacyl-carbinol ..... Acetaldehyd,
Äthyl-guajacyl-carbinol ..... Propionaldehyd,

und aus

Dimethyl-guajacyl-carbinol .... Aceton.

Die Kupplung dieser vier Guajacylkarbinole mit Chinonmonochlorimid kann deshalb folgendermassen formuliert werden:



Das hierbei entstehende Indophenol ist, wie in einer folgenden Mitteilung gezeigt werden soll, identisch sowohl mit dem aus Guajakol, wie dem aus Lignin erhaltenen.

Bei der Untersuchung der Frage, welche von den in Tabelle 1 angeführten Strukturen für die Kupplungsreaktion des Lignins verantwortlich ist, scheidet Strukturtypus A ohne weiteres aus, da Fichtenholzlignin keine Carboxylgruppen enthält [10b]. B, C und D können, wie unten gezeigt wird, ebenfalls ausge-

* Die Reaktion von *p*-Nitrobenzoldiazoniumchlorid mit verschiedenen Modellsubstanzen und Ligninpräparaten wird derzeit hier untersucht.

schlossen werden. Als einzige Ursache für das Zustandekommen der Kupplungsreaktion kann daher die Benzylalkoholstruktur (E) im Lignin angesehen werden.

Diese zunächst aus Modellversuchen hergeleitete Annahme konnte direkt am Lignin bestätigt werden. Verschiedene Lignin-und Fichtenholzpräparate, in denen entweder die phenolischen Hydroxylgruppen oder die α-ständigen Hydroxylgruppen oder beide Arten von Hydroxylgruppen blockiert waren, wurden auf ihre Kupplungsfähigkeit mit dem Reagenz geprüft und kolorimetrisch miteinander verglichen. In Tabelle 2 sind die unter vergleichbaren Bedingungen (siehe Versuchsteil) gemessenen Extinktionswerte und die prozentuelle Extinktion, bezogen auf den »native lignin»-Wert, eingetragen.

Tabelle 2.

Präparat	Extinktion (siehe Versuchsteil)	% des »native lignin»-Wertes
»Native lignin» nach Brauns [a]	0,428	100
»Native lignin» mit Pyridin und Essigsäureanhydrid bei Zimmertemperatur acetyliert [b]	0,035	8,2
»Native lignin» bei Zimmertemperatur sulfitiert [b]	0,052	11,9
»Native lignin» mit 0.5 %iger methanolischer HCl bei Zimmertemperatur methyliert [b]	0,043	10,0
»Native lignin» regeneriert aus methyliertem »native lignin [b]	0,220	51,5
»Native lignin» mit $NaBH_4$ bei Zimmertemperatur reduziert [b]	0,417	97,5
Alkohollignin extrahiert aus entharztem Fichtenholzmehl mit siedender 3 %iger äthanolischer HCl	0,027	6,4
Dioxan-Lignin extrahiert aus entharztem Fichtenholzmehl mit 0.35 % iger HCl in 85 %igem Dioxan	0,040	9,4
Holzmehl entharzt [a]	0,088	20,6
Holzmehl entharzt und mit Pyridin-Essigsäureanhydrid bei Zimmertemperatur acetyliert [b]	0,005	1,2
Holzmehl entharzt und mit Diazomethan bei Zimmertemperatur methyliert [b]	0,007	1,6

[a] Von sämtlichen Ligninpräparaten wurde die gleiche Gewichtsmenge, von den Holzmehlpräparaten das 3,6—fache hiervon verwendet (siehe Versuchsteil).
[b] Siehe Versuchsteil.

Wie aus Tabelle 2 hervorgeht, kann die Kupplungsfähigkeit des Lignins mit dem Reagenz durch Methylierung, Sulfitierung oder Acetylierung weitgehend (bis auf ca. 1/10) herabgesetzt werden.

Das Verhalten des mit 0.5 % iger methanolischer Salzsäure bei Zimmertemperatur methylierten »native lignin» steht im Einklang mit der bereits erwähnten Tatsache, dass benzylalkoholische Hydroxyle durch alkoholische Salzsäure sehr glatt veräthert werden [10a]. Gruppen vom Typus a, welche die Kupplungsreaktion geben müssen, werden dabei also in Gruppen vom Typus b (siehe S. 1319) umgewandelt, denen, wie oben gezeigt, die Kupplungsfähigkeit abgeht.

Dies bestätigt die von Adler und Gierer [2] ausgesprochene Auffassung, dass die Methylierung mit methanolischer Salzsäure bei Zimmertemperatur

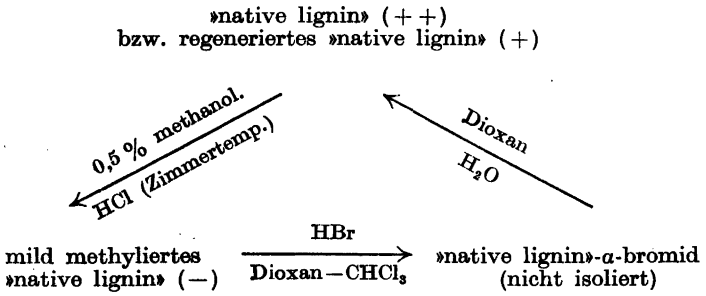
u. a. in einer Verätherung von *p*-Oxybenzylalkoholgruppen besteht. In analoger Weise steht die Aufhebung der Kupplungsfähigkeit durch äusserst milde Sulfitierung (siehe Tab. 2 und Versuchsteil) in Übereinstimmung mit Lindgrens Ansicht [12], dass die leichtest sulfitierbaren Gruppen des Lignins *p*-Oxybenzylalkoholgruppen — neben *p*-Oxybenzyläthergruppen — sind.

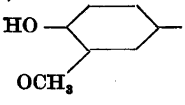
Alkohollignin, welches bekanntlich durch Kochen von Holz mit äthanolischer Salzsäure gewonnen wird, gibt erwartungsgemäss aus denselben Gründen keine nennenswerte Kupplungsreaktion. Die schon von Holmberg [13] vertretene Ansicht, dass die Äthoxygruppen im Äthanollignin durch Verätherung von benzylalkoholischen resp. durch Umätherung von Benzyläthergruppen entstehen, erhält hierdurch eine weitere Stütze.

Das Ausbleiben der Kupplungsreaktion bei diesen Ligninpräparaten vor allem bei dem unter milden Bedingungen methylierten »native lignin» kann als wichtiges Argument für die Spezifität der Reaktion auf freie *p*-Oxybenzylalkoholgruppen angesehen werden, denn dadurch lassen sich die Strukturtypen B, C und D (Tabelle 1) für das Lignin ausschliessen. Behandelt man nämlich derartige Modellsubstanzen unter den gleichen milden Bedingungen (Zimmertemperatur, 24 Stunden) mit 0.5%iger methanolischer Salzsäure, so bleibt die Kupplungsfähigkeit in vollem Umfang erhalten, während sie bei fünf untersuchten Guajacylcarbinolen des Strukturtypus E (Tabelle 1) — wie beim »native lignin» — durch Ätherbildung fast vollständig aufgehoben wird.

Ein weiterer Beweis für die Spezifität der Kupplungsreaktion auf freie *p*-Oxybenzylalkoholgruppen gelang durch Rückführung des in Tabelle 2 angeführten mild methylierten »native lignin»[2] in sogenanntes »regeneriertes native lignin», wobei die Kupplungsfähigkeit bis zu rund 50 % wieder hergestellt werden konnte. Fig. 1 zeigt schematisch den »Reaktionszyklus»:

Fig. 1.



Mit Natriumborhydrid reduziertes »native lignin»[2] gab die Farbreaktion in ungefähr gleicher Intensität wie das Ausgangsmaterial. Da Natriumborhydrid alle Ketogruppen zu Alkoholgruppen reduziert, kann man daraus schliessen, dass »native lignin» keine nennenswerten Mengen von Guajacongruppen  enthält.

»Native lignin» stellt bekanntlich nur einen verhältnismässig kleinen Teil der Gesamtligninmenge des Holzes dar (8—10 %) [14a]. Um zu untersuchen, wie sich die Hauptmenge des Lignins im Holz gegenüber dem Reagenz verhält, wurde die Kupplungsreaktion auch mit feingemahlenem Holzmehl durchgeführt. Tabelle 2 zeigt, dass die Farbintensität bei der Kupplung der 3,6-fachen Gewichtsmenge Holzmehl (gleiche Ligninmengen) nur rund 20 % von der beim »native lignin» beobachteten ausmacht. Da die Kupplungsreaktion mit Holzmehl in Suspension ausgeführt wurde, dürfte dieser Wert als ein Minimum anzusehen sein. Immerhin scheint das extrahierbare »native lignin» einen relativ höheren Gehalt nicht nur an phenolischen Hydroxylgruppen [15] sondern auch an freien *p*-Oxybenzylalkoholgruppen zu besitzen als die Hauptmenge des Lignins.

Das nach der Methode von Stumpf und Freudenberg [16] dargestellte Dioxan-Lignin gab eine noch schwächere Farbreaktion. Trotz der verhältnismässig milden Extraktionsbedingungen dürfte dieses Ligninpräparat während der Isolierung zumindest an den besonders säureempfindlichen *p*-Oxybenzylalkoholgruppen chemisch verändert worden sein.

BESTIMMUNG DER *p*-OXYBENZYLALKOHOLGRUPPEN IN »NATIVE LIGNIN»

Die Intensität der Farbreaktion des »native lignin» wurde kolorimetrisch bestimmt und mit der des Guajakols, bzw. verschiedener Guajacylcarbinole verglichen. Hierbei ergaben Guajakol und die in Tabelle 3 angeführten Guajacylcarbinole Extinktionswerte zwischen 0,301 und 0,348. Bei der Kupplung der 10-fachen äquivalenten Menge »native lignin» (mittleres M. G. der Phenylpropaneinheiten ca. 210) unter den gleichen Bedingungen (siehe Versuchsteil) wurde ein Extinktionswert von 0,428 erhalten. Auf etwa jede 7.—8. Phenylpropaneinheit des »native lignin» kommt demnach eine freie *p*-Oxybenzylalkoholgruppe.

Tabelle 3

Substanz (0,047 mg Guajakol bzw. äquivalente Menge Guajacylcarbinol)	Extinktion (Versuchsbedingungen s. Exper. Teil)	% Phenylpropaneinheiten in »native lignin» mit *p*-Oxybenzylalkoholstruktur (ber. aus nebenstehenden Extinktionswerten)
Guajakol	0,341	12,3
Vanillylalkoho	0,308	13,6
Methyl-guajacyl-carbinol	0,301	13,9
Äthyl-guajacyl-carbinol	0,320	13,1
Dimethyl-guajacyl-carbinol	0,315	13,3
Guajacylglycerin	0,348	12,0
»Native lignin»	0,428	—

EXPERIMENTELLER TEIL

Herstellung der »native lignin»-Derivate

»*Native lignin*» nach Brauns [17]. Reinigung siehe Lit.[14b]. OCH_3 — 14,67 %.

»*Native lignin*» *mit Diazomethan methyliert* nach Brauns [17]. OCH_3 — 18,26 %.

»*Native lignin*» *mit Pyridin und Essigsäureanhydrid acetyliert* nach Brauns [18].

»*Native lignin*» *mit Natriumborhydrid reduziert* [3]: 1 g »native lignin» wurde in 80 ml Alkohol und 20 ml Wasser gelöst und zur rotbraunen Lösung 0,5 g Natriumborhydrid in kleinen Anteilen zugesetzt. Die Lösung hellte sich hierbei etwas auf und trübte sich gegen Schluss der Natriumborhydrid-Zugabe. Die Trübung verschwand durch Zusatz von weiteren 20 ml Wasser.

Nach Stehen über Nacht wurde das pH der Lösung durch Zugabe einiger Tropfen konzentrierter Salzsäure von 10,5 auf ca. 1,5 gebracht und die Lösung im Vakuum zur Trockne eingeengt (Max. Temp. 35° C).

Der Rückstand wurde in 50 ml Wasser suspendiert, durch eine Sinternutsche filtriert und mit Wasser bis zur Cl^--Freiheit gewaschen. Nach Trocknen im Vakuumexsikkator und Umfällen aus Dioxan-Äther [14b] wurden 0,8 g eines fast weissen Produktes erhalten, das keine Phloroglucin-Reaktion mehr gab und keine Carbonylgruppen mehr enthielt (Titration der bei der Umsetzung mit salzsaurem Hydroxylamin freiwerdenden Salzsäure).

»*Native lignin*» *mit methanolischer Salzsäure methyliert* [3]: 0,5 g »native lignin» wurden in 10 ml absolutem Methanol gelöst und 2,5 ml einer 2,5 %igen methanolischen Salzsäure zugesetzt. Nach 24-stündigem Aufbewahren bei Zimmertemperatur wurde mit verdünnter Natronlauge vorsichtig neutralisiert und die Lösung im Vakuum zur Trockne eingeengt. Der dunkelbraune Rückstand wurde mit 5 ml einer Mischung von absolutem Methanol und absolutem Aceton (1 : 1) ausgezogen, vom Ungelösten abfiltriert und die Lösung tropfenweise unter lebhaftem Rühren in 80 ml Wasser eingetragen. Nach Stehen über Nacht wurde abfiltriert, mit Wasser gut gewaschen und im Vakuumexsikkator getrocknet. Reinigung durch Umfällen aus Dioxan-Äther [14b]. Helles crèmefarbenes Pulver. Ausbeute 0,24 g. OCH_3 — 20,5 %.

»*Regeneriertes native lignin*»: 40 mg des bei Zimmertemperatur mit 0,5 %iger methanolischer Salzsäure methylierten »native lignin» wurden in 2 ml absolutem Dioxan gelöst und 2 ml mit HBr gesättigtes Chloroform langsam unter Schütteln zugesetzt. Ein Teil der Substanz fiel hierbei aus und ballte sich zu Klümpchen zusammen. Nach 5 Minuten wurde im Vakuum zur Trockne eingeengt, der braungelbe Rückstand in 2 ml Dioxan und 2 ml Wasser aufgenommen und erneut im Vakuum bei Zimmertemperatur eingedunstet. Der Rückstand löste sich nur teilweise in 100 ml Äthanol. Mit 2 ml der filtrierten alkoholischen Lösung wurde direkt die kolorimetrische Bestimmung (s. unten) durchgeführt.

Sulfitiertes »*native lignin*»: 40 mg »native lignin» wurden in 2 ml absolutem Dioxan gelöst, 2 ml 10 %ige Natriumbisulfitlösung zugesetzt und die Lösung 2 Stunden bei Zimmertemperatur aufbewahrt. Einengen im Vakuum. Der hellgelbe Rückstand wurde 3 mal mit je 2 ml absolutem Dioxan ausgezogen und das Ungelöste mit etwas absolutem Dioxan nachgewaschen. Der beim Einengen der vereinigten Dioxanextrakte erhaltene Rückstand wurde in 100 ml Äthanol gelöst und mit 2 ml dieser Lösung die kolorimetrische Bestimmung (s. unten) durchgeführt.

Herstellung der Holzmehlpräparate

Holzmehl: Feingemahlenes Fichtenholzmehl wurde gesiebt und zur Entharzung 72 Stunden mit Alkohol-Benzol (1 : 1) extrahiert. Trocknen im Vakuum bei 40° über P_2O_5.

Holzmehl mit Diazomethan methyliert: Feingemahlenes entharztes Fichtenholzmehl wurde in einer Lösung von Diazomethan in Äther suspendiert und 2 Wochen bei Zimmertemperatur aufbewahrt. Die Diazomethanlösung wurde in dieser Zeit 3 mal erneuert. Hierauf wurde abfiltriert, zunächst mit Methanol und dann mit Wasser gewaschen und im Vakuum bei 40° über P_2O_5 getrocknet. OCH_3 — 16,3 %.

Holzmehl mit Essigsäureanhydrid und Pyridin acetyliert: 2 g feingemahlenes entharztes Holzmehl wurden in einer Mischung von 5 ml Essigsäureanhydrid und 2 ml Pyridin suspendiert und 24 Stunden bei Zimmertemperatur geschüttelt. Hierauf wurde scharf

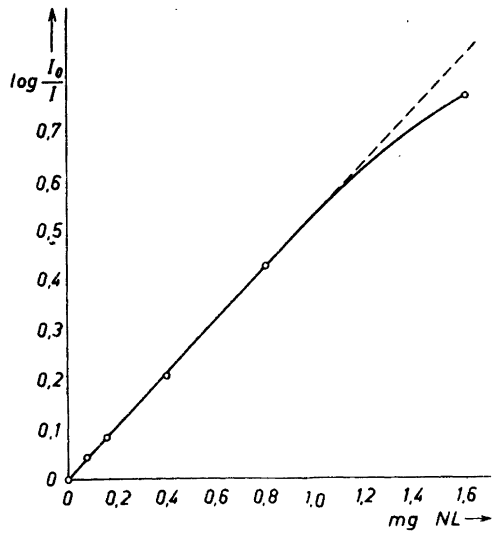


Fig. 2. Kupplungsreaktion von »native lignin» mit Chinonmonochlorimid (2 ml äthanolische »native lignin»-Lösung 0,08, 0,16, 0,4, 0,8 oder 1,6 mg enthaltend). Reaktionsbedingungen siehe unten.

abgesaugt, mit Wasser einigemale gut nachgewaschen und im Vakuumexsikkator über NaOH und P_2O_5 getrocknet.

Chinonmonochlorimid: nach Willstätter und Mayer [19]. Reinigung durch 2-malige Vakuumsublimation (10 mm Hg, 60°), Schmp. 86°.

Kupplung mit Modellsubstanzen

Je 0,1 Millimol Modellsubstanz wurde in 10 ml Alkohol gelöst. 1 ml der Lösung wurde mit 1 ml einer 1 mmol. Chinonmonochlorimidlösung und 1 ml 0,04 *N* Natriumhydroxyd versetzt. Nach einer Stunde wurde mit einer Blindprobe (ohne Modellsubstanz) verglichen. Phenole mit freier *p*-Stellung sowie *p*-substituierte Phenole der Strukturen A—E (Tabelle 1) geben eine intensive Farbreaktion (grünblau bis blau), während alle anderen untersuchten Modellsubstanzen gar nicht oder nur äusserst schwach reagieren (gelb bis gelbgrün).

Kolorimetrische Bestimmungen

Die kolorimetrische Bestimmung der *p*-Oxybenzylalkoholgruppen in »native lignin» bzw. dessen Derivaten wurde unter verschiedenen Bedingungen (Reagenzkonzentration, Alkalikonzentration und Gesamtvolumen) durchgeführt. Hierbei ergab sich, dass der gefundene relative Gehalt der einzelnen »native lignin»-Präparate an *p*-Oxybenzylalkoholgruppen, bezogen auf »native lignin», (siehe Tabelle 2, Spalte 2) weitgehend unabhängig von den gewählten Konzentrationsverhältnissen ist. Das Lambert-Beer'sche Gesetz gilt über einen weiten Konzentrationsbereich, sofern mindestens die gleiche Gewichtsmenge Chinonmonochlorimid wie »native lignin»-Präparat zur Reaktion kommt (siehe Fig. 2).

Bei der Ermittlung der in den Tabellen 2 und 3 angeführten Extinktionswerte wurde wie folgt verfahren:

Einwagen: 1,18 mg Guajakol bzw. äquivalente Menge der in Tabelle 3 angeführten Guajacylcarbinole
20,00 mg »native lignin» bezw. Ligninpräparat (siehe Tabelle 2)
2,88 mg Holzmehl bzw. Holzmehlpräparat (siehe Tabelle 2)

Die Einwagen der Modellsubstanzen, des »native lignin» und der Ligninpräparate wurden in je 50 ml Äthanol gelöst und zu je 2 ml der Lösungen 0,2 ml alkoholische Chinonmonochlorimidlösung (1 mg enthaltend), sowie 1 ml 0,04 *N* NaOH zugesetzt. Nach einstündigem Stehen bei Zimmertemperatur wurden 2 ml der Lösung mit Alkohol auf 25 ml verdünnt und die Extinktion bei 640 mμ gegen eine entsprechende Blindprobe gemessen.

Die Einwagen der Holzmehlpräparate wurden in 2 ml Alkohol suspendiert und nach Zugabe von Reagenz und NaOH eine Stunde lang bei Zimmertemperatur geschüttelt. Nach Filtration der Lösungen wurde analog wie bei den Modellsubstanzen und Ligninpräparaten verfahren.

Einwirkung 0,5 %iger methanolischer Salzsäure auf Modellsubstanzen der Typen B, C, D und E (Tabelle 1)

Je 0,01 Millimol Modellsubstanz wurde in 1 ml 0,5 %iger methanolischer Salzsäure gelöst und die Lösung bei Zimmertemperatur 24 Stunden aufbewahrt. Hierauf wurde zu jeder Probe 1 ml einer 1 mmol. Chinonmonochlorimidlösung und 1 ml 0,2 *N* NaOH zugesetzt und nach einer Stunde mit einer entsprechenden Blindprobe (1 ml Methanol anstatt 1 ml 0,5 %iger methanolischer Salzsäure) verglichen.

Modellsubstanzen der Typen B, C und D zeigten nach dieser sehr milden Behandlung mit methanolischer Salzsäure unverminderte Kupplungsfähigkeit (gleiche Farbintensität wie bei der Blindprobe), während die untersuchten Guajacylcarbinole des Typus E — ebenso wie »native lignin» — ihr Kupplungsvermögen zum grössten Teil einbüssten.

Nachweis der aus Guajacylcarbinolen bei der Kupplungsreaktion abgespaltenen Carbonylverbindungen

Je 100 mg Guajacylcarbinol wurden unter leichtem Erwärmen in 10 ml Wasser gelöst und 100 mg Chinonmonochlorimid, gelöst in 2 ml Alkohol, zugegeben. Nach Zusatz von 1 ml 1 *N* NaOH wurde unter N_2-Strom in eine Vorlage von 15 ml 0,3 %iger 2,4-Dinitrophenylhydrazin-Lösung (in 2 *N* HCl) destilliert. Schon beim Übergehen der ersten Tropfen begann das entsprechende Dinitrophenylhydrazon auszufallen. Es wurde weiterdestilliert bis sich kein Niederschlag mehr bildete. Dieser wurde abfiltriert, mit Wasser gut gewaschen, getrocknet und gewogen. Tabelle 4 zeigt die Ausbeuten, Schmelzpunkte und Mischschmelzpunkte der erhaltenen Dinitrophenylhydrazone nach Umkristallisieren aus Alkohol.

Tabelle 4.

Guajacylcarbinol	Abgespaltene Carbonylverbindung	Dinitrophenylhydrazon		
		Ausbeute % d. Th.	Schmp.	Misch-Schmp.
Vanillylalkohol	Formaldehyd	11,2	160—162°	160 —165°
Methylguajacylcarbinol	Acetaldehyd	65,5	164—165	162,5—165°
Äthylguajacylcarbinol	Propionaldehyd	48,5	148—150°	147,5—150°
Dimethylguajacylcarbinol	Aceton	46,0	124—125°	124 —125°

SUMMARY

Quinone monochloroimide reacts with lignin in weakly alcaline solution to yield a blue dyestuff.

This coupling reaction was investigated in more detail using about 80 model compounds chemically related to lignin and was found specific to the *p*-hydr-

oxybenzylalcohol structure in lignin. The specificity was further shown by the fact that the reaction is not given by "native lignin" derivatives and wood powders, the phenolic and/or the benzylalcoholic groups of which were blocked by etherification or esterification.

With the aid of a simple colorimetric method the amounts of free *p*-hydroxybenzylalcohol groups in different lignin preparations were determined. In Brauns' "native lignin" from spruce about every 7th—8th guaiacylpropane unit was found to have a *p*-hydroxybenzylalcohol structure.

The coupling reaction allows a more detailed study of the initial phase of common lignin reactions such as methylation with methanolic hydrochloric acid, sulphonation, or reaction with thioglycolic acid, and represents a criterion for the chemical intactness of lignin preparations isolated in various ways.

Herrn Professor E. Adler, Chalmers Tekniska Högskola, Göteborg, danke ich für sein reges Interesse an dieser Arbeit.

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Stemphylone, a Root-Killing Substance from *Stemphylium radicinum*

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In testing some plant pathogenic fungi for antibiotic and germ-inhibiting effects substrates from *Stemphylium radicinum* were found to inhibit the germination of *i.a.* seeds of *Lepidium sativum*.

The active substance can be extracted with chloroform and recrystallized from ethanol or acetone. The substance is slightly soluble in water, but soluble in many organic solvents, concentrated sulphuric acid, and hydrochloric acid. It is optically active.

With dinitrophenylhydrazine an orange dinitrophenylhydrazone is obtained. As reactions for aldehydes are negative, the substance is consequently supposed to be a ketone. It has been called stemphylone.

The formula $C_{12}H_{12}O_5$ is proposed as composition of the substance.

A solution of stemphylone in concentrated H_2SO_4 has an intensive blue fluorescence in ultraviolet light.

The maxima of the UV absorption spectrum are reached at 340 mμ and 271 mμ.

Neergaard [1] has found production of crystals in some *Stemphylium* cultures on agar substrates. These crystals have been proved to consist of stemphylone.

Stemphylone is slightly inhibitory to *Escherichia coli* and *Streptococcus aureus*.

By the technique used an effect has been observed at concentrations down to 5×10^{-5} on germinating seeds of *Lepidium sativum*.

At concentrations permitting germination but inhibiting complete development, no visible changes are seen on the stem and the seed-leaves, whereas the root has changed somewhat. It is much shorter than normal, and is brown to brownish black coloured while the root-hairs are missing.

EXPERIMENTAL

All melting points are uncorrected micro melting points. The microanalyses are made by Mr. P. Hansen.

Production of stemphylone. A 2 % solution of malt extract (A/S Alfred Benzon) was poured into glass-bottles in layers of 3 cm, sterilized, and inocula-

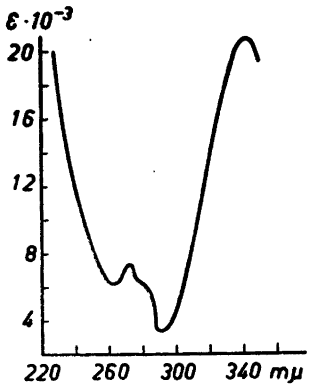

Fig. 1. UV spectrum o stemphylone in ethanol..

ted with spores from *Stemphylium radicinum* (M., Dr. & E.) Neerg. 1939 „Carrot IV" [2], which had previously been cultivated on slices of raw carrot. The bottles were left at a temperature of 22°, and the production of stemphylone was followed by a fluorescence reaction. One drop of the substrate was added to 5 ml of concentrated H_2SO_4, and the fluorescence compared with a standard solution.

After four weeks the mycelium was filtered off, and the filtrate extracted with 1/5 vol. of chloroform. The chloroform layer was dried with Na_2SO_4, added charcoal, and filtered. After evaporation the residue was a brownish crystal mass.

Yield 1 g per litre of substrate. Recrystallized from ethanol and then from acetone. Almost colourless needles. M.p. 220° with destruction. (Found: C 61.3; H 5.3. Calc. for $C_{12}H_{12}O_5$ (236.2): C 61.3; H 5.1.)

Optical activity; With a solution of 126 mg of stemphylone in 10 ml of a mixture of ethanol and chloroform (1 vol. + 2 vol.) $[\alpha]_D^{22} = -125°$ was found.

Fluorescence. A solution of 0.06 mg of stemphylone in 100 ml of concentrated H_2SO_4, and a solution of 0.05 mg of quinine in 100 ml of dilute H_2SO_4 had equal intensity of fluorescence when compared in a simple photoelectric fluorophotometer.

UV spectrum. The absorption spectrum was measured in a solution of ethanol in a Beckmann model DU quartz spectrophotometer (Fig. 1).

Dinitrophenylhydrazone. After recrystallization from pyridine orange needles were obtained. M.p. 245° (dest.). An alcoholic solution is coloured scarlet by NaOH. (Bamberger's test for nitrophenylhydrazones). The sample used for analysis was dried at 165° for 1 h. (Found: C 52.2; H 3.9; N 13.4. Calc. for $C_{18}H_{16}O_8N_4$ (416.3); C 52.0; H 3.9; N 13.5.)

Isolation of crystals from an old agar culture; A few milligrams of crystals were collected with a needle and recrystallized from ethanol. M.p. 220°. The isolated compound was in all respects identical with stemphylone.

Determination of the effect of stemphylone on germinating seeds of Lepidium sativum. Filter paper 9 cm in diameter was placed on the bottom of a Petri dish, and 3 ml of an aqueous solution of stemphylone were poured

on it. About 50 seeds were placed on the moist filter paper and left in the thermostat at a temperature of 22°. At concentrations down to 10^{-3} there was no germination. Growth-inhibition could be observed at concentrations between 10^{-3} and 5×10^{-5}.

SUMMARY

A ketonic compound, stemphylone, was isolated from substrates of *Stemphylium radicinum*. Production, purification, and analysis of the compound are described.

The most interesting property of stemphylone is the toxic effect on germinating seeds, where especially the root development is inhibited.

My thanks are due to P. Neergaard, Dr. agron., for having drawn my attention to the production of crystals in the agar cultures of *Stemphylium* species, and for the fungus cultures used.

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*iso*Thiocyanates X. Synthesis and Characterization of *cis*-Crotyl *iso*Thiocyanate

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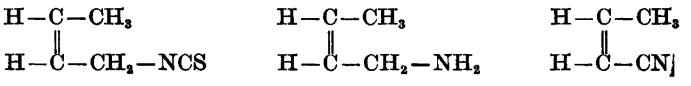
In the first paper of this series [1] the synthesis and characterization of 3-butenyl, α-methallyl, β-methallyl and *trans*-crotyl *iso*thiocyanate were reported. The preparation of the heretofore unknown *cis*-isomer of the latter (I), which was desired in current investigations, has now been effected and an account of the results are given in the present communication.

Nearly all methods used in the preparation of *iso*thiocyanates require the corresponding primary amines as starting materials. *cis*-Crotylamine (II), which is needed in the present case, appears to be a hitherto unknown substance, although the *trans*-isomer is mentioned repeatedly in the literature.



I II III

In view of our previously described successful reduction of allyl cyanide to 3-butenylamine with lithium aluminium hydride [1], it was a logical extension to apply the same reagent to *cis*-crotonitrile (III). The latter has become easily accessible as a result of the extensive studies of Bruylants and his coworkers [2-4] on the isomerization of allyl cyanide. Quite unexpectedly, the reduction of *cis*-crotonitrile (III) did not take the desired course. Numerous experiments, conducted under widely varied and carefully controlled conditions, yielded *n*-butylamine as the only seizable reaction product, indicating simultaneous reduction of the nitrile-group and the conjugated double bond. Although reduction of a conjugated carbon-carbon double bond with lithium aluminium hydride has been noticed occassionally (*cf.* Ref.[5]) its occurrence in the present type of compounds seems rather unprecedented. Hatch and Nesbitt [6] reduced *iso*crotonic acid to *cis*-crotyl alcohol without saturation of the double bond, a fact which renders the present observation even more surprising.

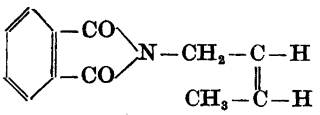
Consequently, a different approach to the synthesis of *cis*-crotylamine was sought. We formerly obtained the *trans*-isomer from *trans*-crotyl bromide by Gabriel-synthesis and subsequent hydrazinolysis [1]. A similar sequence of reactions has been effected now with *cis*-crotyl chloride as the starting material.

Table 1.

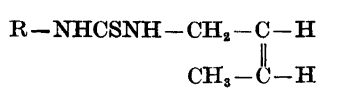
Amine	Formula	Composition	M. p., °C.	Analyses Nitrogen Calcd.	Nitrogen Found	Sulphur Calcd.	Sulphur Found
Ammonia	Va	$C_5H_{10}N_2S$[a]	88.5	21.53	21.54	24.63	24.51
Aniline	Vb	$C_{11}H_{14}N_2S$[b]	87	13.58	13.36	15.54	15.63
p-Toluidine	Vc	$C_{12}H_{16}N_2S$[b]	61	12.73	12.50	14.55	14.76
α-Naphthylamine	Vd	$C_{15}H_{16}N_2S$[b]	120.5	10.93	10.98	12.50	12.46

[a] Recrystallized from water. [b] Recrystallized from aqueous ethanol.

The latter was obtained from 3-chloro-2-buten-1-ol (kindly furnished by Dr. L. F. Hatch); the carbinol was dehydrochlorinated and catalytically reduced to *cis*-crotyl alcohol. This was finally converted into *cis*-crotyl chloride, all steps being conducted as described by Hatch and Nesbitt [6]. *cis*-Crotyl chloride reacted smoothly with potassium phthalimide in dimethylformamide solution to N-*cis*-crotylphthalimide (IV), the reaction proceeding without stereomutation as evident from the nature of the further reaction products as well as from the sharp melting point.



IV



V

a. R = H
b. R = C_6H_5
c. R = *p*-$CH_3C_6H_4$
d. R = *α*-$C_{10}H_7$

A preliminary attempt to utilize the mild hydrazinolysis procedure of Schumann and Boissonnas [7] with phenylhydrazine proved fruitless, the starting material being recovered in good yield. The desired cleavage of (IV) was then effected by the ordinary Ing and Manske procedure [8], after it had been demonstrated that (IV) did not stereomutate under the influence of the acid employed in the reaction. The crude *cis*-crotylamine hydrochloride was in turn submitted to reaction with thiocarbonyl chloride and alkali according to the modified Dyson procedure [9] which we previously had found useful for the synthesis of numerous *iso*thiocyanates. This method is more satisfactory and convenient than the Andreasch-Kaluza procedure formerly used for the synthesis of *trans*-crotyl *iso*thiocyanate [1]. The new *cis-iso*thiocyanate was obtained as a distillable, colourless liquid with a strong mustard smell. Owing to its supposed instability the main part of the product was immediately submitted to reactions with ammonia, aniline, *p*-toluidine and *α*-naphthylamine to give the highly crystalline thiourea-derivatives (V, a—d), for which data are presented in Table 1. From this it will be seen that three of the four derivatives have melting points below those of the corresponding *trans*-thioureas [1], as would be expected, whereas *cis*-crotylthiourea (Va) melts 28° higher than the *trans*-isomeride.

The allocation of *cis*-configuration to the higher-melting crotylthiourea was further strengthened by studies of the infra-red spectra. In Fig. 1 are schematically presented the spectra of various *cis*- and *trans*-crotylthioureas. Apart from the intensive band at *ca.* 6.50 μ, attributable to the C = S-stretching

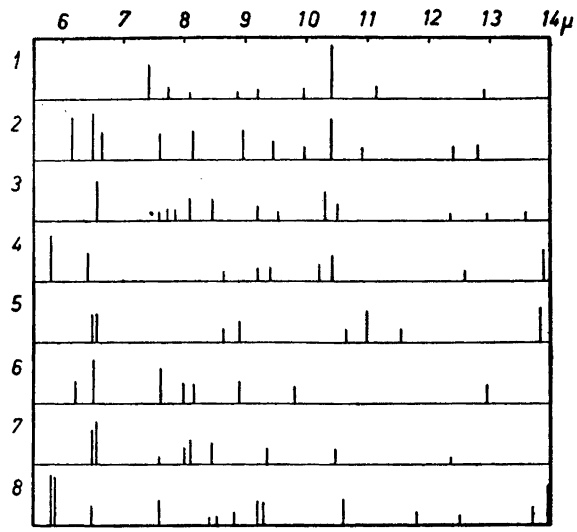


Fig. 1. Schematic presentation of infra-red absorption spectra. 1. trans-*Crotyl* iso*thiocyanate (in addition to the lines presented above, this substance exhibited a very strong absorption band at 4.60 μ, attributable to the N = C = S-group); 2.* trans-*Crotylthiourea; 3.* trans-*Crotylphenylthiourea; 4. N-*trans-*Crotylphthalimide; 5.* cis-*Crotylamine hydrochloride; 6.* cis-*Crotylthiourea; 7.* cis-*Crotylphenylthiourea; 8. N-*cis-*Crotylphthalimide.*

vibration, the most striking feature of the spectra is the characteristic line at about 10.3—10.4 μ in the *trans*-derivatives, which can be assigned to the out-of-plane deformation vibrations of the two hydrogen atoms attached to the *trans*-double bond, as has been frequently noticed in various structurally related compounds (*cf. e.g.* Ref.[10]). Throughout the present work this *trans*-absorption line proved of much diagnostic value because its absence, in lack of a correspondingly characteristic *cis*-absorption band, indicated *cis*-configuration of the compound under investigation. Thus the spectroscopic results are considered to be a proof of the correct assignment of structure, despite the anomalous melting point data. Another representative can therefore be added to the very limited group of crystalline, authentic *cis*-compounds melting higher than their corresponding *trans*-isomerides.

Further confirmation of the stereochemical relationship was incidentally obtained when a small sample of the original *cis*-crotyl *iso*thiocyanate, which had been kept for two weeks at 0°, was found to yield essentially pure *trans*-crotylthiourea upon reaction with ammonia in the usual way. Infra-red inspection of the specimen clearly demonstrated it to be the *trans*-isomer (*cf.* Fig. 1), formed by spontanous rearrangement at 0° of the original product, which had consequently consisted of the labile *cis-iso*thiocyanate. No further studies were made of the rate with which the stereomutation takes place.

Paper chromatography of *cis*- and *trans*-crotylthiourea, performed according to our standard method [11], gave undiscernible spots, a fact of no serious consequence however for the systematic search for naturally occurring

*iso*thiocyanates, because neither of the crotyl derivatives have so far been encountered in nature.

With all the standard thioureas derived from unsaturated five-carbon *iso*thiocyanates on hand *, a few comments can be made regarding some similar products of dubious structure listed in the literature. Luchmann [12] treated γ-chlorobutylamine with potassium hydroxide at elevated temperature and obtained an unsaturated amine, forming a phenylthiourea with m.p. 94—7° upon treatment with phenyl *iso*thiocyanate. Tentatively, the structure (VI) was suggested for the product, but Galand [13] later assumed Luchmann's product to be an impure *trans*-crotylthiourea (VII). The first possibility can now be definitely excluded, because authentic 3-butenylphenylthiourea has the m.p. 124—126° [1]. Inspection of the melting points in Table 1 seems to support Galand's interpretation. The same author [13] further reported the reduction of allyl cyanide to an unsaturated amine, characterized by its phenylthiourea-derivative of m.p. 88—91°. The latter was claimed to be authentic (VI), an assignment which has now proved clearly untenable.

$CH_2=CH-CH_2-CH_2-NHCSNH-C_6H_5$ VI

$C_6H_5-NHCSNH-CH_2-C(H)=C(H)-CH_3$ VII

The formation of *cis*-crotylamine by the reduction of allyl cyanide, as performed by Galand, seems to be a very remote possibility. Hence, the reported m.p. of the phenylthiourea-derivative is not reconcilable with any of the authentic samples, a fact which lends support to the belief that Galand's derivative represents an impure preparation. In 1907 Stein [14] reported the isolation from Indian rape seeds of an unsaturated five-carbon *iso*thiocyanate, which was characterized by its phenylthiourea-derivative with m.p. 53°. No derivative with nearly as low a m.p. has been encountered during our studies of the isomeric *iso*thiocyanates; no explanation can be offered for the observation of Stein. Again, an unsaturated five-carbon *iso*thiocyanate, isolated by Schimmel & Co.[15] from *Brassica juncea* Hook et Thoms, was characterized as its thiourea-derivative with m.p. 69—70°, depressed on admixture with authentic *trans*-crotylthiourea. From the series of authentic derivatives prepared in this laboratory we conclude that the essential oil above does not represent any pure unsaturated five-carbon *iso*thiocyanate. Finally, comparison with the authentic samples on hand indicates that the alleged synthetic crotyl *iso*thiocyanate of Searle [16], for which a thiourea-derivative with m.p. 91.8—92.5° is reported, hardly represents any unsaturated five-carbon *iso*thiocyanate.

EXPERIMENTAL **

cis-*Crotylphthalimide* (IV). A suspension of 23.2 g of potassium phthalimide in 95 ml of dimethylformamide was placed in a three-necked flask, provided with thermometer, dropping funnel, reflux condenser and a mercury-sealed stirrer. 10.7 g of *cis*-crotyl chloride [6] were added dropwise under vigorous stirring and the suspension kept for 3 hours

* That is, excepting isomers containing an α,β-double bond. No attempts have been made to synthetize such compounds which would most likely be very unstable as derivatives of vinylamine.

** All melting points recorded are determined with a calibrated thermometer in capillary tubes in an oil bath.

at 80°. After stirring for another 3 hours at room temperature 475 ml of water were added and the mixture extracted with one 140-ml and two 50-ml portions of chloroform. After drying the chloroform layer, the solvent was removed *in vacuo* and the residual oil brought to crystallization on cooling and scratching. After one recrystallization from aqueous ethanol, 19.0 g (80 %) of essentially pure *cis*-crotylphthalimide remained. Two additional recrystallizations from dilute ethanol furnished a specimen for analysis. M. p. 65°. (Found: C 71.86; H 5.46; N 6.92. Calc. for $C_{12}H_{11}O_2N$ (201.2): C 71.62; H 5.51, N 6.96).

The corresponding *trans*-derivative was previously found [1] to melt at 75—76°. Infra-red data for both isomers are presented in Fig. 1.

cis-*Crotylamine hydrochloride*. After a preliminary experiment had shown that *cis*-crotylphthalimide could be recovered unchanged from the acid treatment employed in Ing and Manske's hydrazinolysis procedure, the above imide was deacylated as formerly described for the *trans*-derivative [1]. From 14 g of *cis*-crotylphthalimide there was obtained 7.1 g (95 %) of crude *cis*-crotylamine hydrochloride, which was used in the following step without further purification. A sample for analysis was recrystallized twice from 2-butanone and once from anhydrous ethanol and ether. The hydrochloride separated in flat prisms with m. p. 138°. Its infra-red spectrum is reproduced in Fig. 1. (Found: N 13.09; Cl 32.93. Calc. for $C_4H_{10}NCl$ (107.6): N 13.02; Cl 32.95).

cis-*Crotyl* iso*thiocyanate* (I). A solution of 5.82 g (54 mmoles) of *cis*-crotylamine hydrochloride in 54 ml of 1 *N* NaOH was slowly added to a cooled solution of 6.23 g (54 mmoles) of thiocarbonyl chloride in 70 ml of chloroform. After addition of 92 ml of 1 *N* NaOH the solution was shaken for one hour at room temperature. A copious brown precipitate was removed by filtration through a sintered glass plate and thoroughly washed with chloroform. The filtrate was separated and the aqueous layer extracted with three 10-ml portions of fresh chloroform. The organic phase was dried over magnesium sulphate and the solvent removed by distillation through a small column. The residue was distilled *in vacuo* and the *iso*thiocyanate (2.47 g) was collected as a vesicant colourless liquid, b. p. 71—73° at 11 mm. Owing to the rather small amount of substance on hand, no attempts were made to purify the sample further for analysis; it was immediately transformed into its thiourea-derivatives for characterization.

The new *iso*thiocyanate should be used shortly after its preparation. Otherwise stereomutation may occur as noticed from the infra-red spectrum (*cf.* Fig. 1) of a small specimen which had been kept for two weeks at 0°. During this period it was quantitatively converted into the stereoisomeric *trans*-crotyl *iso*thiocyanate.

cis-*Crotyl-thioureas* (V, a—d). On reaction with ammonia, aniline, *p*-toluidine and *a*-naphthylamine in the usual way, the freshly prepared *cis*-crotyl *iso*thiocyanate above was rapidly transformed into the respective thioureas (V, a—d) for which analyses and melting points are presented in Table 1. The infra-red spectra of the four derivatives were determined; some of the results, supplemented with the spectra of the corresponding *trans*-isomers are recorded in Fig. 1.

Upon paper chromatography in chloroform solution as previously described [11], *cis*-crotylthiourea (Va) gave a spot with an R_{Ph}-value of 0.76, while the spot of the corresponding *trans*-derivative had an R_{Ph}-value of 0.77.

Infra-red absorption spectra. The infra-red absorption spectra were determined in Nujol mulls with one exception, *viz.* that of *trans*-crotyl *iso*thiocyanate. This substance was applied directly to the rock salt plates as a thin film. The measurements were conducted on a Beckman IR-2 instrument.

SUMMARY

A synthesis of *cis*-crotyl *iso*thiocyanate, proceeding from *cis*-crotyl chloride *via cis*-crotylphthalimide and *cis*-crotylamine, is described. The stereochemical authenticity of the new *iso*thiocyanate has been secured through studies of the infra-red absorption spectra. Spontaneous stereomutation of the *cis-iso*thiocyanate has been observed, even at 0°.

The *iso*thiocyanate has been characterized by its transformation into thioureas upon reaction with aniline, *p*-toluidine, α-naphthylamine and ammonia. The derivative with the latter represents a new member of the rather limited group of authentic *cis*-compounds, melting higher than their corresponding *trans*-isomers.

The chemical nature of various *iso*thiocyanates and derivatives, reported in the literature and of questionable structures, are discussed in the light of our own results.

We are much indebted to Dr. L. F. Hatch, the University of Texas, Austin, U.S.A., for his generous gift of a sample of 3-chloro-2-buten-1-ol, which facilitated the preparation of *cis*-crotyl chloride considerably.

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Paper Chromatography of Vitamin K_1 and Related Compounds with some Observations on Products of Ultra-violet Irradiation

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Although a number of methods for the determination of vitamin K_1 and other naphthoquinones have been described [1] each has certain limitations. In searching for a simple, sensitive, and specific method, we have directed our attention to filter-paper chromatography, since the naphthoquinones have a characteristic fluorescence on filter-paper [2]. In this report the R_F values of a series of compounds related to vitamin K_1 are given in three different solvent systems along with the fluorescent colors observed under ultra-violet light. At the same time some observations of the effect of ultra-violet irradiation on these compounds are presented.

EXPERIMENTAL

Chromatography. Ascending technique and Whatman No. 1 filter-paper were used throughout. The filter-paper was soaked in 5 % (v/v) solution of Dow Corning Silicone No. 1107 in *cyclo*hexane [3] or chloroform and allowed to dry in air. Unless otherwise noted, 10 μg of the naphthoquinones in ethanol solution were applied to the paper with a 1 μl Carlsberg pipette; the dimer of menadione was in acetone solution. Vitamin K_1 was used as a control substance and run on all chromatograms. These were developed overnight (16—20 hrs) at room temperature under black cloaks to prevent photolysis.

Materials. Vitamin K_1 and vitamin K_1-oxide were products of Merck & Co., Inc. Menadione and phthiocol were products of Hoffmann-La Roche & Co., Inc. The dimer of menadione was prepared by irradiation of menadione [4]. The other naphthoquinones, recently synthesized and examined biologically [5] were kindly donated by Hoffmann-La Roche & Co.

Solvents. Preliminary observations suggested that the substances were more stable in acidified solvents, as chromatography of a single compound in a non-acidified alcohol-water system often resulted in the development of two spots. Of thirty solvent systems examined, only three proved useful, and these are listed in Tables 1 and 2.

Detection of the substances. The developed chromatograms were dried at room temperature, held before a Philips No. 57202 E 170 ultra-violet lamp (3 665 Å) and the fluorescent colors noted. After an exposure of 45—60 sec. to the ultra-violet light, the color

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of the fluorescence was again noted and the spot outlined. The fluorescent color brought out by spraying with a solution of 2 *N* KOH in ethanol was also recorded. In quantitative measurements the outlined spot was traced on millimeter paper and the area obtained by direct counting.

Irradiation. The compounds and the vitamin K_1 control were applied to the coated filter-paper, allowed to dry, and then held before the ultra-violet lamp (3 665 Å) for 2—3 minutes. Identical quantities of the same substances which were not irradiated, were placed on the same paper. It was chromatographed and dried in the manner described and then examined under the lamp for fluorescence.

RESULTS

The R_F values presented in Tables 1 and 2 were obtained from chromatograms run on the same batch of silicone-coated paper and in fresh solvents. The latter was especially important, for as the solvents aged, the R_F values diminished, although their relative values remained the same.

The R_F values of the various naphthoquinones in those solvent systems that seemed most useful are listed in Table 1 together with a notation of their fluorescent colors. It may be seen that as the length of the side-chain increased, the R_F values decreased. However, compounds containing 25 and 30 carbons (X and XI respectively) were not significantly different. Vitamin K_1 and its oxide had the same R_F values.

"Before activation" denotes the fluorescent colors emitted by the chromatographed substance when initially held before the ultra-violet lamp. This fluorescence was clearly evident after the substance was chromatographed and less obvious when the sample was first applied to the paper. Exposure to the ultra-violet lamp for 45—60 seconds induced a permanent change in the fluorescent color of most compounds and an increase in the area of fluorescence. The fluorescent colors are tabulated under "After activation". Spraying with alcoholic KOH after activation produced yet other colors, and these are listed in the last column. The fluorescence after activation was stable for at least four months.

Quantitative chromatographic measurements of vitamin K_1 were carried out in the ethyl alcohol and *iso*propyl alcohol systems. The fluorescence after activation (*i.e.*, the green fluorescence) was clearly visible in concentrations as low as 0.5 μg, while the fluorescence before activation (*i.e.*, the red fluorescence) was not apparent to the unaided eye until 5 μg were present. The color change produced by activating quantities greater than 10 μg was restricted to the periphery of the spot, the central portion retaining its red fluorescence. Spraying KOH on quantities less than about 2.5 μg obliterated the fluorescence.

In ethyl alcohol quantities greater than 5 μg developed as elliptical spots; in *iso*propyl alcohol spots were elliptical at concentrations above 40 μg. In both solvents 80 μg or more of the vitamin developed as long ellipses which began at the point of application and swept forward.

Between 1.5 and 50 μg, the log of the concentration was linearly related to the area of the spot. In concentrations of 0.5 μg, the area was not related to the concentration of the vitamin, but to the unaided eye the intensity of fluorescence appeared to be related to concentration.

Table 1. R_F values and fluorescence of 2-methyl-1,4-naphthoquinone and its derivatives.

No. of compound	Formula of group in 3-position or designation of compound	Solvent system			Color of fluorescence		
		Ethyl-alcohol-acetic acidwater (75 : 25 : 225)	*Iso*propyl alcohol-acetic acidwater (600 : 25 : 375)	*n*-Propyl alcohol-acetic acidwater (600 : 25 : 375)	Before activation	After activation	After activation and spraying with KOH
I	—H (menadione)	0.71	0.82	0.89	red	blue	green
II	(dimer of menadione)	0.73	0.88	0.97	red	blue	green
III	—OH (phthiocol)	0.63	0.72	0.80	red	red	(cherry red in visible light)
IV	$-CH_2-CH=C(CH_3)-CH_2-CH_2-CH=C(CH_3)-CH_3$	0.55	0.66	0.73	red	green	orange
V	$-CH_2-CH=C(CH_3)-[CH_2-CH_2-CH=C(CH_3)]_2-CH_3$	0.49	0.58	0.68	red	green	orange
VI	$-CH_2-CH=C(CH_3)-[CH_2-CH_2-CH_2-CH(CH_3)]_2-CH_3$	0.42	0.46	0.54	red	green	orange
VII	$-CH_2-CH=C(CH_3)-[CH_2-CH_2-CH=C(CH_3)]_3-CH_3$	0.31	0.40	0.47	red	green	orange
VIII	$-CH_2-CH=C(CH_3)-(CH_2)_3-CH(CH_3)-(CH_2)_3-CH(CH_3)-(CH_2)_3-CH(CH_3)-CH_3$ (vitamin K_1)	0.19	0.28	0.36	red	green	orange
IX	(the 2,3-oxide of vitamin K_1)	0.19	0.27	0.38	red	green	orange
X	$-CH_2-CH=C(CH_3)-(CH_2-CH_2-CH_2-CH(CH_3))_4-CH_3$	0.12	0.16	0.26	red	green	orange
XI	$-CH_2-CH=C(CH_3)-CH_2-(CH_2-CH=C(CH_3)-CH_2)_4-CH_2-CH=C(CH_3)-CH_3$ (vitamin K_2)	0.14	0.18	0.30	red	green	orange

Table 2. R_F values and fluorescence of irradiated 2-methyl-1,4-naphthoquinone and its derivatives.

No. of compound	Formula of group in 3-position or designation	Solvent system: Ethyl alcohol-acetic acidwater (750 : 25 : 225)	Solvent system: *Iso*propyl alcohol-acetic acidwater (600 : 25 : 375)	Solvent system: *n*-Propyl alcohol-acetic acidwater (600 : 25 : 375)	Color of fluorescence: Before activation	Color of fluorescence: After activation	Color of fluorescence: After activation and spraying with KOH
I	—H (menadione)	0.00 0.73	0.00 0.80	0.00 0.89	blue red	blue blue	blue green
II	(dimer of menadione)	0.00 0.74	0.00 0.89	0.00 0.93	blue red	blue blue	blue green
III	—OH (phthiocol)	0.63	0.74	0.81	red	red	(cherry red in visible light)
IV	$-CH_2-CH=C(CH_3)-CH_2-CH_2-CH=C(CH_3)-CH_3$	0.00 0.51 0.72 0.81	0.00 0.63 0.81 0.85	0.00 0.73 0.82 0.89	blue none none green	blue green green green	blue orange none orange
V	$-CH_2-CH=C(CH_3)-[CH_2-CH_2-CH=C(CH_3)]_2-CH_3$	0.00 0.49 0.68 0.77	0.00 0.57 0.78 0.85	0.00 0.66 0.80 0.91	blue none none green	blue green green green	blue orange none orange
VI	$-CH_2-CH=C(CH_3)-[CH_2-CH_2-CH_2-CH(CH_3)]_2-CH_3$	0.00 0.43 0.62 0.72	0.00 0.49 0.73 0.87	0.00 0.51 0.78 0.89	blue none none green	blue green green green	blue orange none orange
VII	$-CH_2-CH=C(CH_3)-(CH_2-CH_2-CH=C)_3-CH_3$	0.00 0.34 0.62 0.77	0.00 0.41 0.71 0.86	0.00 0.44 0.79 0.89	blue none none green	blue green green green	blue orange none orange
VIII	$-CH_2-CH=C(CH_3)-(CH_2)_3-CH(CH_3)-(CH_2)_3-CH(CH_3)-(CH_2)_3-CH(CH_3)-CH_3$ (vitamin K_1)	0.00 0.18 0.49 0.74	0.00 0.28 0.54 0.85	0.00 0.33 0.62 0.87	blue none none green	blue green green green	blue orange none orange
IX	(the 2,3-oxide of vitamin K_1)	0.00 0.19 0.49 0.75	0.00 0.17 0.47 0.83	0.00 0.28 0.53 0.87	blue none none green	blue green green green	blue orange none orange
X	$-CH_2-CH=C(CH_3)-(CH_2-CH_2-CH_2-CH)_4-CH_3$	0.00 0.13 0.39 0.76	0.00 0.17 0.47 0.83	0.00 0.28 0.53 0.87	blue none none green	blue green green green	blue orange none orange
IX	$-CH_2-CH=C(CH_3)-CH_2-(CH_2-CH=C(CH_3)-CH_2)_4-CH_2-CH=C(CH_3)-CH_3$ (vitamin K_2)	0.00 0.12 0.28 0.72	0.00 0.22 0.38 0.84	0.00 0.31 0.49 0.88	blue none none green	blue green green green	blue orange none orange

Table 2 shows the R_F values of the same compounds that had been chromatographed after irradiation. Phthiocol showed one fluorescent spot, menadione and its dimer two, and the other compounds four. With the exception of phthiocol, all irradiated compounds showed one stationary spot ($R_F = 0.00$) of blue fluorescence that was not altered by the KOH spray. One of the components of each irradiated substance had an R_F value corresponding to that of its non-irradiated counterpart (*cf.* Tables 1 and 2): thus, the R_F value of non-irradiated vitamin K_1 in ethyl alcohol, *iso*propyl alcohol, and *n*-propyl alcohol were 0.19, 0.28 and 0.36, respectively; the corresponding R_F values of one component of the irradiated vitamin were 0.18, 0.28, and 0.33. In addition to the stationary spot and to the spot with the same R_F value as the non-irradiated substance, every irradiated compound with long alkyl side chains (Nos. IV—XI) showed two other spots. One fluoresced only after activation, a fluorescence that was eliminated by spraying with KOH. The fourth and most mobile component fluoresced green without prolonged activation. The R_F values of the third and fourth spots were inversely related to the length of the side-chain on the parent compound.

DISCUSSION

The results presented in Table 1 show that reversed phase paper chromatography is applicable to the separation and identification of naphthoquinones. Their R_F values were inversely related to the length of the side-chain in the 3-position up to a 30 carbon grouping. The sensitivity of the method, which was 0.5 μg for qualitative analysis and 1.5 μg for quantitative analysis, could probably be increased by instrumental measurements of fluorescence. In view of the relative roundness of the spots, the *iso*propyl alcohol system is probably the most useful, especially for quantitative studies.

Of the three different fluorescent colors exhibited by some of the compounds, the fluorescence observed "after activation" appeared to be the most intense. It was visible to the unaided eye when concentrations as low as 0.5 μg of the fluorescing compound were present. This fluorescence was in all likelihood due to a photochemical product of the original naphthoquinone. The original compound was probably represented by the fluorescence "before activation" which required the presence of about 5 μg of the compound for visibility. The fluorescence after the KOH spray was intermediate in intensity.

It is known that photo-activation of naphthoquinones can induce dimerization [4,6] and oxidation [6], but what substances are produced under the conditions described here is unknown. It is not clear that irradiation of menadione produced its dimer, for in all observations the R_F values of the migrating spot of menadione were slightly less than the dimer. It is of further interest that the dimer itself could be further photo-activated to another compound or compounds with $R_F = 0.00$. This latter spot appeared on chromatograms of all the irradiated substances with the exception of phthiocol.

All irradiated compounds revealed a component with R_F values indistinguishable from the non-irradiated compound. These probably represent the unchanged, original substance. That these spots, in some cases, did not fluoresce

"before activation" (compounds IV—XI, Table 2) in contrast with the same non-irradiated compounds (Table 1) can probably be ascribed to the presence of quantities less than 5 μg.

In addition to the stationary spot and the one presumably representing the unchanged compound, naphthoquinones with long alkyl side chains (IV —XI), when irradiated and chromatographed, produced two other fluorescing spots. Of these, the one of smaller R_F had notable fluorescent properties: it did not fluoresce until "after activation", and the fluorescence was quenched by KOH. Both characteristics are consistent with the presence of quantities less than 2.5 μg. This spot may represent an intermediate product in the change from the original to the main fluorescing product of ultra-violet irradiation, the substance with the highest R_F value. Its fluorescent color was not quenched by KOH, which suggests a concentration greater than 2.5 μg. Irradiation may well have produced other compounds the fluorescence of which the eye could not detect or which did not fluoresce at all.

SUMMARY

Vitamin K_1 and some related compounds were chromatographed on silicone-coated filter-paper. In the three solvent systems studied, R_F values were, in general, related to the length of the side-chain in the 3-position. By their fluorescence in ultra-violet light, quantities as low as 0.5 μg were visible to the unaided eye. The area of fluorescence was linearly related to the log of the concentration from 1.5 to 50 μg. The R_F values of the fluorescent spots resulting from ultra-violet irradiation of the same compounds were also determined.

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Ternary Transition Metal Oxide Phases of the Fluorite Structure

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The cubic fluorite structure (C 1 type) occurs frequently among the following four kinds of compounds.

1) Halides, especially fluorides, of the larger divalent cations and combinations of univalent and tri-, quadri- or hexavalent cations.

2) Oxides, sulfides, selenides and tellurides of univalent ions, mostly alkalies. If the general formula is written AX_2, the atom A is here the negative rather than the positive ion — the antifluorite arrangement.

3) Oxides of large quadrivalent ions.

4) Intermetallic compounds.

The A and X atoms occupy the following positions in the space-group O_h^5—$Fm3m$:

4 A atoms in 4(*a*) 0,0,0; $\frac{1}{2},\frac{1}{2},0$;↶
8 X atoms in 8(*c*) ± ($\frac{1}{4},\frac{1}{4},\frac{1}{4}$; $\frac{1}{4},\frac{3}{4},\frac{3}{4}$;↷)

Each A atom is situated at the centre of a cube of eight X atoms located at the corners, and each X atom is tetrahedrally surrounded by four A atoms. The crystal is of an unsymmetrical valence type. From a geometrical point of view A—X contact is obtained when the radius ratio $r_A/r_X \geq \sqrt{3}-1 = 0.73$. This relation is also fullfilled or nearly so for all compounds of the C 1 type, earlier reported, with the exception of the high temperature modification of ZrO_2[1] for which the ratio r_A/r_X is 0.66, if Goldschmidt's radii values are chosen.

The only quadrivalent ions, for which the geometrical condition for the formation of a stable dioxide lattice of the C 1 type is fullfilled, are found among the rare earth metals and the actinides. The existence of oxide phases of the fluorite structure has been well established in the following cases: CeO_2[2,3], PrO_2[4,5], ThO_2[6], PaO_2[7], UO_2[2,8,9], NpO_2[10], PuO_2[11], and AmO_2[7,12]. The isomorphous high temperature modification of ZrO_2 has been reported to be stabilized at room temperature at the presence of fairly small amounts of MgO in the lattice. The saturated mixed crystal of ZrO_2 with MgO was proposed to correspond to the formula $Mg_2Zr_3O_8$[13,14] or $MgZr_3O$[14]. According to Ruff and Ebert [14] the atoms occupy the following positions in the cubic unit cell

with the lattice constant $a = 5.08$ Å: 2 Mg in 0,0,0; $\frac{1}{2},\frac{1}{2},\frac{1}{2}$, 3 Zr in $0,\frac{1}{2},\frac{1}{2}$; ↶, and 8 O in $\pm$ ($\frac{1}{4},\frac{1}{4},\frac{1}{4}$; $\frac{1}{4},\frac{3}{4},\frac{3}{4}$; ↶). This structure is most unlikely, however, partly because of some extremely small interatomic Mg-Zr distances and partly because such a configuration is not in any way consistent with a diffraction pattern of a face-centered lattice.

In the present investigation ternary oxide phases of the C 1 type (defective lattice) containing two transition metals A and B — where B = Ti or Zr — were prepared in two ways.

1) Binary A, B alloys of different compositions, sintered in zirconia crucibles in a high-frequency vacuum induction furnace, were finely powdered and oxidized with steam in the presence of hydrogen in great excess at temperatures between 600° C and 800° C.

2) Oxides of the two transition metals were mixed in different proportions and heated in evacuated and sealed silica tubes at temperatures between 700° C and 1 100° C.

Since the double-oxides of the C 1 type could not be prepared in a pure state, the composition of the phases could not be determined with any accuracy. The purest samples were obtained from method 1 (binary oxides as impurities), while only very small amounts of these oxides could be prepared according to method 2. Attempts to syntethize them from binary oxides in a vacuum furnace at temperatures between 1 200° C and 1 800° C were in vain, probably due to the instability at these high temperatures.

The phase analysis of the samples was performed by means of powder photographs taken with Cu-$K\alpha$ radiation in cameras of the Guinier type.

Black ternary oxides of the fluorite structure were found to exist with the following combinations of atoms: A = Fe, Co, Ni and B = Ti, A = V, Cr, Mo, W, Mn and B = Zr. No similar oxides were obtained in the cases of A = V, Cr, Mn, Fe, Co, Ni and B = Nb, Ta, Mo, W. The lengths of the cubic axes were measured to have the same approximate values for all the phases and to vary between $a = 5.09$ Å and $a = 5.13$ Å, which together with the phase analysis from the powder photographs indicates comparatively large homogeneity ranges. The intensities of the diffraction lines were observed to agree well with the $p|F|^2$ values calculated for a disordered distribution of the A and B atoms over the four-fold atomic position. As pointed out above the phase composition could not be determined with any certainty, but the A/B ratio seems to lie within the approximate limits $1/3 \lesssim \text{A/B} < 1$ for all the oxides reported. As the Ti and Zr atoms in all probability are ionized to the quadrivalent state he general formula can be written as $A_K^{z+}B_{4-K}^{4+}O_{8-K/2(4-z)}$, where z^+ designates the valency of the A component and has a maximum value of 4 if all metal atom positions are assumed to be occupied. As $1 \lesssim K < 2$ in the formula, some oxygen positions will be empty for $z < 4$. If $z > 4$, which may be the case for A = V, Mo and W, all oxygen positions would in all probability be occupied and some metal atom sites be empty.

For $z = 2$ and $K = 2$ in the general formula given above, the phase composition ABO_3 is obtained, meaning compounds of the ilmenite structure (E 2_2 type)[15], when A = Mn, Fe, Co, Ni and B = Ti. No analogous compounds were found to exist for A = V—Ni and B = Zr, Nb, Ta, Mo, W, although

phases related to the corundum structure (D 51 type)[16] were obtained in the cases of A = Cr and B = Mo and W [18]. The perovskite structure (E 2_1 type)[17] also has the composition ABO_3 but with one divalent and one quadrivalent metal ion rather than two trivalent ions as in the ilmenite structure. No phase of the E 2_1 type could be found in the A—B—O systems which were investigated. The oxide phases of the C 1 type reported above all seem to have the A/B ratio smaller than unity and the oxygen content greater than that corresponding to 3/2 oxygen atoms per metal atom. The latter relation is accentuated by the fairly large volume per metal atom (about 33.5 Å^3) as compared, for instance, with that of ilmenite (26.2 Å^3).

The reported ternary oxide phases of the C 1 type all have a mean lattice constant of $a = 5.11$ Å giving interatomic O—A,B (4-coordination) and O—O (6-coordination) distances of 2.21 and 2.56 respectively. As the radius ratio $r_{Ti}^{4+}/r_0^{2-} = 0.48$ is considerably smaller than that calculated for anion-cation contact in the C 1 lattice, 0.73, the Ti—O distances are greater than those observed for rutile [19], anatase [20] and brookite [21], about 1.95 Å. The Zr-O distances in baddeleyite [22] — of a slightly deformed C 1 type — vary between 1.95 and 2.65 Å. The smallest O—O distances observed for all metal oxides seem to be near 2.55 Å, and this gives an explanation to the fact that the dioxides of the fluorite structure where the radius ratio between the cations and the anions is smaller than 0.73 have approximately the same lattice constant, about 5.11 Å (corresponding to O—O distances of 2.56 Å).

SUMMARY

Ternary A—B—O phases of the C 1 type, where A and B are transition metals, have been prepared by different methods and investigated by X-ray methods. The general formula of these compounds is proposed to be $A_K^{z+}B_{4-K}^{4+}O_{8-K/2(4-z)}$, where A = Fe, Co, Ni in combination with B = Ti and A = V, Cr, Mo, W, Mn in combination with B = Zr; K varies to some extent within the approximate limits $1 \lesssim K < 2$, and z^+ designates the valency of the A component. All the oxides are black and have the same axial lengths, varying between $a = 5.09$ Å and $a = 5.13$ Å, which corresponds to mean interatomic A,B—O and O—O distances of 2.21 Å and 2.56 Å respectively. The oxides are stable at room temperature in spite of unfavourable geometrical conditions for the formation of a lattice of the C 1 type.

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Iodometric Analysis of Certain Types of Selenenyl Compounds

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A rapid and accurate method has been worked out for the iodometric analysis of aromatic selenenyl bromides, thiocyanates, alkoxides and amides.

Some observations have been made on rearrangement reactions involving selenenyl alkoxides and amides.

Selenenyl compounds, with the formulae RSeX where X is an electronegative atom or group, are derivatives of divalent electropositive selenium. In displacement reactions with nucleophilic reagents, the selenium atom acts as the electrophilic part of the molecule [1,2], the atoms or groups X being eliminated as anions. This is analogous to the behaviour of sulphenyl [3] and tellurenyl [4] compounds.

Some years ago, a method was worked out by one of us [3] for the iodometric analysis of aromatic sulphenyl thiocyanates and amides. The procedure was based on the reaction of the sulphenyl compounds with an excess of sodium thiosulphate, to form sulphenyl thiosulphates, and back-titration of the excess of sodium thiosulphate with iodine. The principle of this method has now been found to be appliccable to the iodometric analysis of the analogous selenenyl compounds, and also selenenyl alkoxides and bromides. *o*-Nitrobenzene- and 2,4-dinitrobenzeneselenenyl compounds have been studied, and the results are described below.

Selenenyl compounds react with thiosulphate ion to give the corresponding selenenyl thiosulphates [1]:

$$ArSeX + S_2O_3^{--} = ArSeS_2O_3^- + X^-$$

Here, X may be bromide, thiocyanate, or other electronegative groups the anions of which have a lower nucleophilic reactivity than thiosulphate ion towards divalent selenium. In the case of selenenyl alkoxides and amides,

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where the anions X^- have strong basic properties, an acid must be present in order to neutralize the anions and bring about a complete change:

$$ArSeOR + S_2O_3^{--} + H^+ = ArSeS_2O_3^- + ROH$$
$$ArSeNH_2 + S_2O_3^{--} + 2\ H^+ = ArSeS_2O_3^- + NH_4^+$$

The product, *o*-nitrobenzeneselenenyl thiosulphate, has earlier been isolated as the potassium salt [1] and found to be indifferent to iodine [5]. The same applies to the 2,4-dinitrobenzene analogue, as shown by the results of the present analyses.

The reactions with thiosulphate ion are of interest as a basis for iodometric analysis only if the anions X^- or their neutralization products are indifferent to iodine, so that the excess of sodium thiosulphate can be back-titrated accurately with iodine. This requirement is satisfied for bromides, thiocyanates, alkoxides and amides.

PREPARATION AND PURIFICATION OF COMPOUNDS

The melting points are in °C, and those recorded during the present work are corrected, except for the amides, where the melting points were determined on a Kofler hot bench since the compounds decompose on heating.

o-*Nitrobenzeneselenenyl bromide* was prepared from the selenenyl cyanide and bromine as described by Behaghel and Seibert [6].

2,4-*Dinitrobenzeneselenenyl bromide* was obtained by bromination of the diselenide according to Behaghel and Müller [7]. The diselenide was prepared as follows: 97 g (0.36 mole) of crude 2,4-dinitrobenzeneselenenyl cyanide [8] was suspended in 100 ml of glacial acetic acid; 15 ml (0.18 mole) of pyridine and 7 ml (0.36 mole) of water were added, and the mixture was boiled under reflux for one hour. After cooling, the diselenide was filtered off and washed with ethanol and ether. Yield, 73 g (85 %) of a very pure product.

This is an adaption of Hoggarth and Sexton's method for the preparation of disulphides [9]. The procedure gives a better yield of diselenide and a purer product than methods based on treatment of the selenenyl cyanide with alkalies or sodium acetate.

o-*Nitrobenzeneselenenyl thiocyanate* [1,10] was prepared from the bromide, dissolved in a mixture of ethyl acetate and methanol, by addition of a methanol solution of potassium thiocyanate. This method was used by one of us [1] in the first synthesis of a selenenyl thiocyanate, and has later been employed by Rheinboldt and Perrier [10] for the preparation of a large number of selenenyl thiocyanates. The product is difficult to obtain in a pure state. After four recrystallizations from carbon tetrachloride, the melting point remained practically unchanged; the purity, however, was only 99.2 %, as checked by iodometric analysis. After seven recrystallizations, this figure had increased to 99.6 %. A total number of fifteen recrystallizations were necessary to ensure a product which, according to the thiosulphate-iodine method of analysis, contained no impurities. Melting point, 113.5°; the literature value [10] is 113—114°.

2,4-*Dinitrobenzeneselenenyl thiocyanate* [10] was obtained from the bromide and potassium thiocyanate, as above. This substance had a tendency to decompose when treated with warm carbon tetrachloride for recrystallization in the usual manner, and was purified as follows: About 0.5 g was dissolved, by gentle heating, in 150 ml of carbon tetrachloride, and the filtered solution was cooled in a carbon dioxide-ethanol freezing mixture until part of the liquid had frozen. The beaker and its contents were then allowed to stand at room temperature until the solvent had melted and the selenenyl thiocyanate had changed from a plastic state to a crystalline mass (about two hours). After three such recrystallizations, the melting point was 96.2°, as compared with the literature value [10] of 94—95°. The losses during the recrystallizations were rather large.

The selenenyl *alkoxides* were prepared from the bromides and the appropriate alcohol, by heating under reflux for two hours in presence of an equivalent amount of silver acetate, which serves as a bromide acceptor. This procedure was first used by Behaghel and Müller [7], who believed that selenenyl acetates were formed. The identification of the

products, in the case of the 2,4-dinitrobenzeneselenenyl derivatives, as selenenyl alkoxides, is due to Cook and Donia [11].

o-*Nitrobenzeneselenenyl methoxide* was isolated from the filtered reaction mixture by cooling in a carbon dioxide-ethanol freezing mixture. The product was recrystallized nine times from anhydrous methanol, by cooling as above, and thus appeared as thin, yellow needles which melted at 54.2°. A substance having approximately the same melting point (52°) has been prepared, in the same way, by Behaghel and Müller [7]. Although the latter authors identified the product as o-nitrobenzeneselenenyl acetate, there is no doubt, according to the analytical results given later, that the substance is the methoxide.

o-*Nitrobenzeneselenenyl ethoxide*, isolated as described above for the methoxide, has not been prepared before. It was obtained as small, needle-shaped crystals with a yellow colour. After six recrystallizations from anhydrous ethanol, the solution each time being cooled in a carbon dioxide-ethanol freezing mixture, the substance melted at 26.2°.

The above alkoxides may be obtained from o-nitrobenzeneselenenyl thiocyanate, in the same way as from the bromide. A heating time of 30 minutes was found to be sufficient. In this case, silver thiocyanate is formed, as a side product, instead of silver bromide.

2,4-*Dinitrobenzeneselenenyl methoxide* [11] and the corresponding *ethoxide* [11] were recrystallized three times from the respective anhydrous alcohols. The compounds are readily obtained in a pure state. The melting points were 134.2° for the methoxide and 131° for the ethoxide; Cook and Donia [11] give 131—133° and 128—129°, respectively.

In the recrystallization of selenenyl alkoxides from alcohols, the alcohol corresponding to the alkoxide group contained in the substance must be used; otherwise, rearrangements will take place, as discussed later. Traces of water present may cause decompositions.

o-*Nitrobenzeneselenenyl amide* was prepared from the bromide (5.5 g) dissolved in chloroform (150 ml), by treatment with dry ammonia gas for two hours at room temperature. The ammonium bromide was removed by filtration, and the solvent distilled off *in vacuo*. The crude product was recrystallized five times, by dissolving in carbon tetrachloride, filtering, and cooling in a carbon dioxide-ethanol freezing mixture. The substance is very unstable and could not be obtained in a pure state; the recrystallized product, which melted at about 129°, was by iodometric analysis shown to contain 3.5 % of impurities. The amide probably decomposes into the imide, with liberation of ammonia, as does the corresponding sulphenyl amide [12].

2,4-*Dinitrobenzeneselenenyl amide* was prepared as described above, from 6.5 g of the bromide dissolved in 100 ml of chloroform. After filtering, the chloroform was distilled off at atmospheric pressure, until crystals began to separate out, and the solution was then allowed to cool. Yield, 4.7 g (89 %). After six recrystallizations from chloroform, the substance was analytically pure. The amide appears as orange-coloured prisms which melt at 140°. The crystals decompose slowly when stored at room temperature.

These amides have not been prepared before. The latter amide is formed also when the thiocyanate, dissolved in chloroform, is treated with ammonia gas.

ANALYTICAL PROCEDURE AND RESULTS

The o-nitrobenzene- and 2,4-dinitrobenzeneselenenyl bromides, thiocyanates, alkoxides and amides may be analyzed iodometrically as follows:

About two tenths of a millimole of the substance is weighed into a 250 ml flask, and dissolved, with gentle heating when necessary, in 4 ml of ethyl acetate. 10 ml of 96 % ethanol and 1 ml of glacial acetic acid are added, and then, with a swirling movement of the flask, 25 ml of about 0.01 *N* sodium thiosulphate. After standing for 15 minutes, 100 ml of water and 10 ml of 0.2 % starch solution are added, and the excess of sodium thiosulphate is back-titrated with standard 0.01 *N* iodine.

A blank titration is carried out, with the difference that the glacial acetic acid is added after the dilution with water, not before, and the titration is then performed immediately, in order that the acid shall not decompose the thiosulphate ion, which now is present at a larger concentration.

The difference between the numbers of milliliters of 0.01 *N* iodine consumed in the blank and the real titration gives the amount of selenenyl compound present, in hundredths of millimoles.

The time of standing, before back-titration, may be varied according to the nature of the substance analyzed and the excess of sodium thiosulphate present. The selenenyl bromides and thiocyanates react very rapidly with thiosulphate, and titration can begin immediately after the thiosulphate has been added. The selenenyl amides react slowest, and the time, 15 minutes, is sufficient for these when an excess of about 5 ml of sodium thiosulphate is present.

The thiocyanate ion is indifferent to iodine only in presence of potassium iodide [13]. The amount of iodide introduced during the titrations were found to be sufficient for this purpose.

The selenenyl thiosulphates, which occur as products, form yellowish green solutions. The end point in the titrations, when starch is used as an indicator, might therefore be thought to be observed a little late relative to the blank titrations, where the selenenyl thiosulphate is not present. The results, however, indicate that this is not the case, if care is taken in observing the end point.

The period of drainage of the pipet, on delivery of the sodium thiosulphate, should be a little longer than usual, due to the effect of the ethyl acetate-ethanol vapours.

The ethyl acetate used as a solvent had been distilled over iodine in order to remove small amounts of oxidable impurities. The 0.01 *N* sodium thiosulphate solution was prepared fresh every day by dilution from a 0.1 *N* stock solution. The 0.01 *N* iodine solution was made up with 2 % of potassium iodide, in order to depress effectively the volatility of iodine. As a result of this relatively large amount of potassium iodide, the titer value decreased by 0.4 % only in the course of nine weeks. The solution was standardized against reagent grade arsenous oxide.

The weighings were performed on a micro balance, and the back-titration volumes of iodine were read on a 10 ml micro buret.

The results for those compounds which, by this method, were judged analytically pure, are given in Tables 1 and 2. It appears that the method gives consistent and accurate results. The average deviation from the theoretical

Table 1. Iodometric analysis of o-nitrobenzeneselenenyl compounds.

Substance employed, mg	Time of standing, minutes	Iodine consumed, ml 0.009993 *N*	Substance found, mg	Deviation from theory, ‰
		Thiocyanate, ArSeSCN		
None		24.85		
52.96	5	4.37	53.03	+1.3
52.18	5	4.70	52.18	0.0
		Methoxide, $ArSeOCH_3$		
None		24.85		
47.28	15	4.47	47.28	0.0
55.22	15	1.09	55.11	−2.0
45.67	5	5.17	45.64	−0.6
		Ethoxide, $ArSeOC_2H_5$		
None		24.85		
37.94	5	9.42	37.95	+0.2
39.77	5	8.68	39.77	0.0

values, for the 16 analyses listed in the tables, is 0.7 parts per thousand, and the average absolute error is 0.04 mg.

The samples of *o*-nitrobenzeneselenenyl bromide and amide and 2,4-dinitrobenzeneselenenyl bromide, which were available for analysis, gave results which were not in agreement with the theoretical values. It is believed, on the basis of the consistency of the results, that the discrepancies are due to impurities present in the samples, and not to a failure of the analytical method.

Thus, in four separate analyses of 2,4-dinitrobenzeneselenenyl bromide, the results were 0.53, 0.35, 0.33 and 0.44 % too high. The sample used had

Table 2. Iodometric analysis of 2,4-dinitrobenzeneselenenyl compounds.

Substance employed, mg	Time of standing, minutes	Iodine consumed, ml	Substance found, mg	Deviation from theory, ‰
		Thiocyanate, ArSeSCN 0.009993 *N* iodine		
None		24.85		
58.78	5	5.52	58.75	−0.5
36.50	5	12.84	36.50	0.0
		Methoxide, $ArSeOCH_3$ 0.01002 *N* iodine		
None		26.42		
51.85	5	7.77	51.81	−0.7
55.64	5	6.39	55.64	0.0
53.96	5	7.04	53.86	−1.8
		Ethoxide, $ArSeOC_2H_5$ 0.009993 *N* iodine		
None		24.85		
55.37	5	5.84	55.30	−1.2
51.51	5	7.14	51.51	0.0
		Amide, $ArSeNH_2$ 0.01006 *N* iodine		
None		25.06		
53.87	15	4.61	53.92	+0.9
54.78	15	4.24	54.89	+2.0

been recrystallized seven times from chloroform, and melted at 117.5°, as compared with the literature value [7] of 118°. The too high values may be due to impurities of tribromide or hydroxide, which would also react with sodium thiosulphate, and give a lower equivalent weight.

A single analysis of *o*-nitrobenzeneselenenyl bromide, recrystallized once from chloroform, gave a 2 % too low result. Four separate analyses of a sample of *o*-nitrobenzeneselenenyl amide gave results which were 3.6, 3.5, 3.3 and 3.4 % too low. As mentioned earlier, this amide is unstable and could not be obtained in a pure state in spite of repeated recrystallizations.

The present method takes account of compounds only which react with sodium thiosulphate, to give products which are indifferent to iodine. Thus, impurities of diaryl selenides and diselenides, and other compounds which do not react with thiosulphate, will be disclosed by this method of analysis, whereas by ordinary analysis for total selenium content, their presence may not be noticeable.

As a result, it has been observed that many of the selenenyl compounds studied here are more unstable, and more difficult to obtain pure, than believed hitherto.

SOME REARRANGEMENT REACTIONS

In the course of the work, some observations were made on re-esterification reactions of 2,4-dinitrobenzeneselenenyl alkoxides. These compounds, which are esters of the selenenic acid, ArSeOH, react with alcohols:

$$ArSeOC_2H_5 + CH_3OH \rightleftharpoons ArSeOCH_3 + C_2H_5OH$$

Thus, the ethoxide when heated in anhydrous methanol for two hours under reflux, gave practically pure methoxide, as shown by melting point and mixed melting points. The methoxide, treated with anhydrous ethanol under similar conditions, gave a mixture of the two alkoxides. It appears that the above equilibrium may be approached from either side, and that the formation of methoxide from ethoxide takes place more readily than the reverse process. Recrystallization of higher 2,4-dinitrobenzeneselenenyl alkoxides from methanol [11] does therefore not seem advisable.

2,4-Dinitrobenzeneselenenyl amide was found to take part in similar displacements:

$$ArSeOR + NH_3 \rightleftharpoons ArSeNH_2 + ROH$$

The methoxide and ethoxide, in chloroform solutions, on treatment with ammonia gas for two hours, were transformed into the amide. The reverse reactions proceed slowly under anhydrous conditions, but the amide was converted almost completely into the ethoxide when heated in 96 % ethanol under reflux for 90 minutes.

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Hyaluronic Acid

VI. An Electron Microscope Study of Potassium Hyaluronate *

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An electron microscopical study of potassium hyaluronate shows that potassium hyaluronate molecules are filamentous with a length of several thousand Å and a width less than 30 Å. This agrees with other evidence concerning its molecular size.

The object of this investigation was to obtain visual evidence of the molecular configuration in potassium hyaluronate. Since electron microscopy is capable of revealing structures in colloidal range we tried to achieve our object by this technique. From the high viscosity of its dilute solutions, the streaming birefrigence, and its ability to spin into threads it was concluded that hyaluronic acid has a filamentous structure. Further evidence of this point of view has recently been furnished by one of us [1], as it was found that a graph giving corresponding values of specific viscosities and products of molecular weight and concentration was in agreement with Staudinger's empiric viscosity rule, which is valid for randomly coiled flexible long-chain molecules. In a forthcoming paper it will be stated that dilute solutions of potassium hyaluronate display elastic recoil, a property which may be attached to molecules of the above mentioned type.

From measurements of viscosity and double refraction of flow Meyer and Palmer [2], and Blix and Snellman [3] estimated mean particle lengths for different preparations of hyaluronic acid of 4 700 to 10 000 Å corresponding to molecular weights ranging from 2×10^5 to 5×10^5, the longest particles being found for umbilical cord hyaluronate. Recently we have determined the molecular weight of potassium hyaluronate of umbilical cord origin to be in the order of 5×10^5 [4].

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* Part I: *Acta Chem. Scand.* **3** (1949) 584; Part II: *Ibid.* **7** (1953) 603; Part III: *Ibid* **8** (1954) 292; Part IV: *Acta Pharmacol. Toxicol.* **10** (1954) 83; Part V: *Acta Chem. Scand.* **8** (1954) 937.

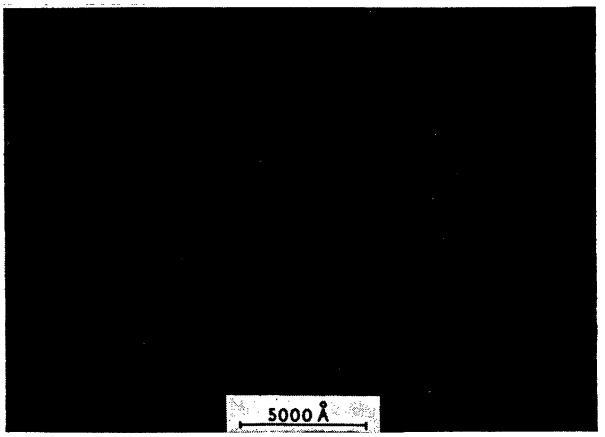

Fig. 1. Electron micrograph of potassium hyaluronate close to true focus. Explanation in text.

Assuming a length of 5 to 10 Å of the disaccharide unit, unbranched chains, and a degree of polymerisation of about 1 000, the length of this hyaluronate molecule would be 5 000 to 10 000 Å, and the transversal diameter about 5—10 Å. The present electron microscopy study substantiated this anticipation to a certain extent.

TECHNIQUE

For examination in the electron microscope, the sample must be placed on a membrane, only a few hundred Å in thickness, of a suitable material. It is normal practice to use a plastic film made from a solution of polyvinyl formal (Formvar) in ethylene dichloride placed on a supporting metal grid.

As the molecules seeked for are only about 5 to 10 Å in diameter, they would not be expected to yield sufficient contrast for detection in the electron microscope. We have therefore shadowed our specimens with palladium in a vacuum-evaporator at an angle of 1 : 3 by the technique of Wiliams and Wyckoff [5].

The easiest way to prepare a specimen from the potassium hyaluronate would be to place a droplet of a dilute solution of the material on the Formvar film and allow it to dry. During the drying proces, however, the dissolved molecules agglutinate so that the single molecules can not be detected. Other methods of preparation have been tried without success.

The Formvar film is usually slight hydrophobic; this sometimes causes the droplet to withdraw from areas, originally covered. These areas when inspected in the electron microscope do not usually display any structure but in our case on micrographs made from these parts some structural elements can be seen (Figs. 1 and 2). The pictures seem to agree well with those expected for threadlike molecules.

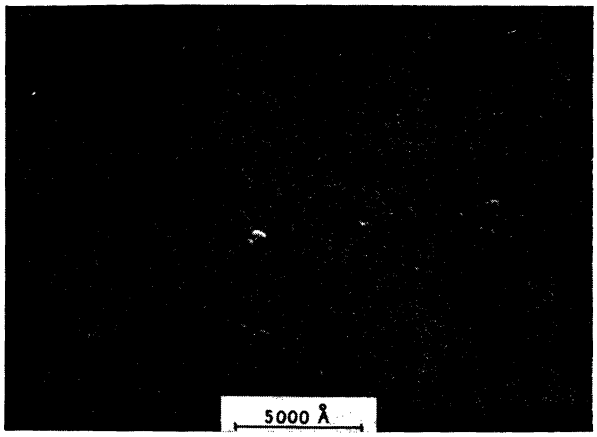

Fig. 2. Electron micrograph of potassium hyaluronate slightly underfocussed. Explanation in text.

This led us to the conclusion that some of the molecules, coming into close contact with the Formvar membrane, adhered so strongly to it, that they would be left on the membrane if the solvent was removed.

This assumption was confirmed by several experiments in which droplets of the solution were allowed to stay on the Formvar membrane for a short time only (60 sec.), and then removed completely. If allowed to stay for a considerable longer time, the membrane is rendered hydrophilic preventing a complete removal of the solvent.

The density of molecules on the membrane is a function of the concentration of the solution and the time the drop is left on the membrane, but in all cases the same filamentous structures were observed in the pictures.

By this method of preparation it is even possible to use solutions containing some amount of soluble salts because of the small amount of solvent and consequently of salt left on the membrane.

The electron micrographs shown here were taken of a highly purified, protein-free preparation from umbilical cords, the chemical composition of which corresponds closely to the theoretical values. The solutions were not dialysed. They contained about 0.01 % of the hyaluronate.

The electron microscope used in this study was an RCA type EMU 2B, capable of giving a resolution of about 20 Å. A focussing series of pictures were taken at an original magnification of 13 000, and the best focussed ones were later optically enlarged.

As seen from the micrographs * the molecules of potassium hyaluronate seem to form a dense network of anastomosing filaments, the individual fibers (molecules, it is supposed) having a relative smooth form and being fairly

* Unfortunately it was not to be avoided that many details disappeared in the reproduction.

constant in width all over their extent. Besides, all the threads are alike, a fact which really suggests that individual molecules, indeed, are observed on these micrographs. The observed patterns do not arise from the supporting film, as a micrograph of the latter taken under the same conditions as that of hyaluronate solutions, the only difference being the absence of hyaluronate, does not display structures.

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Fractional Precipitation of Serum Proteins by Heavy Metal Ions

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Salts of heavy metals are known to be powerful protein precipitating agents. A large number of investigations on the interaction of metals with proteins have been published, and the fractional precipitation of human serum by addition of zink and barium salts has been reported by Cohn *et al.* [1,2]. The experiments to be described here offer a different approach to a similar problem. A fractional precipitation of equine and bovine serum by means of ions of heavy metals was attempted.

METHODS

Serum was obtained fresh from spontaneously coagulated equine or bovine blood. Solutions of the following salts were used as precipitants: $PbCl_2$ (saturated solution), $CdCl_2$ (0.1 *M*) and $CuCl_2$ (1 *M*). The serum samples were acidified with 1 *N* HCl to a pH where no precipitate appeared when the precipitating solution was added. The solution of the precipitating salt was then added in excess and the pH was increased stepwise by dropwise addition of 1 *N* NaOH with constant stirring. The precipitates obtained were isolated by centrifugation, but they were not washed, because the presence of small amounts of the different serum proteins was favorable for the subsequent identification of the compounds on the electrophoresis diagram. All procedures were performed in the cold (ice bath). The precipitates were dissolved in 0.9 % NaCl by addition of a few drops of HCl. All solutions were dialysed against 0.9 % NaCl to remove heavy metal ions. The solutions were then dialysed against the phosphate buffer to be used during the electrophoresis, which was performed in our Tiselius apparatus[3]. Further details on the procedures appear from the experiments described below and from the legends to the figures.

RESULTS

The precipitates obtained by addition of $PbCl_2$ to bovine serum consisted mainly of albumin and α-globulin. The highest selectivity was obtained at pH 5.0 and the results of such experiments are presented in Fig. 1. When equine serum was precipitated with $PbCl_2$ at pH 6.7, the precipitate also contai-

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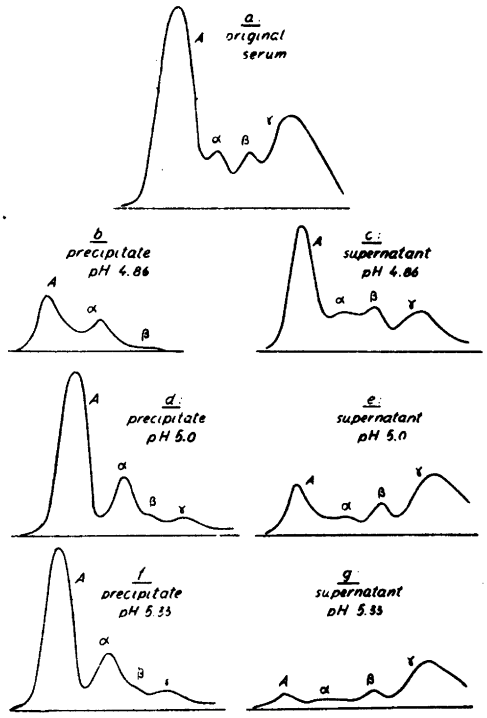

Fig. 1. Precipitation of bovine serum with lead chloride. 15 ml serum + 30 ml saturated $PbCl_2$ + 1 N HCl to pH 4.6. Precipitation by addition of 1 N NaOH. a) Original serum; b) precipitate and c) supernatant at pH 4.86; d) precipitate and e) supernatant at pH 5.00; f) precipitate and g) supernatant at pH 5.33. Electrophoresis: NaCl (μ = 0.025) — phosphate (μ = 0.075) buffer pH 7.6; 120 min., 18.0 mA. Descending patterns. Same relative concentration in all diagrams.

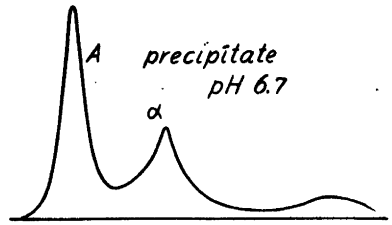

Fig. 2. Precipitate from horse serum with lead chloride. 50 ml serum + 50 ml saturated $PbCl_2$ + AcOH. Addition of 1 N NaOH to pH 6.7. Electrophoresis: NaCl (μ = 0.075) — phosphate (μ = 0.0125) buffer pH 7.6; 150 min., 18.1 mA. Ascending pattern.

ned mainly albumin and α-globulin (Fig. 2). This occurred also when the horse serum had been previously dialysed against 0.9 % NaCl (Fig. 3). When the horse serum was dialysed previously against distilled water less albumin was precipitated, and the content of α-globulin dominated the precipitate (Fig. 4). The precipitate of euglobulin formed during the dialysis against distilled water (*cf.* Svensson [10]) was negligible and no change was detected in the shape of the electrophoretic pattern.

When $CdCl_2$ was added the precipitation took an entirely different course. A precipitate obtained with bovine serum at pH 6.9 contained mainly γ-globulin. Appreciable amounts of α- and β-globulin were also precipitated and appeared as a single peak on the electrophoresis diagram. Albumin was almost totally retained in the supernatant (Fig. 5).

When the precipitation was performed with cupric ions a heavy green precipitate appeared during dialysis of the solutions against 0.9 % NaCl. The filtrates from these mixtures were blue-green and contained appreciable

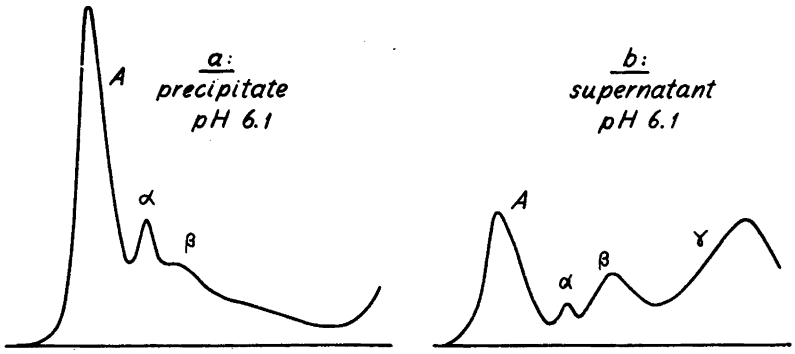

Fig. 3. Precipitate (a) and supernatant (b) from precipitation of horse serum (dialyzed against 0.9 % NaCl) with lead chloride. 20 ml dialyzed serum + 40 ml saturated $PbCl_2$ + AcOH. Addition of 0.5 N NaOH to pH 6.1. Electrophoresis: NaCl (μ = 0.025) — phosphate (μ = 0.075) buffer pH 7.6; 120 min., 18.0 mA. Concentration of precipitate relative to supernatant: 0.96. Ascending patterns.

amounts of protein. Evidently copper forms very slightly dissociable compounds with the proteins. In order to completely remove the copper the solutions were dialysed against sodium cyanide containing solutions. In this manner clear solutions were obtained, but the procedure was discontinued because we found that dialysis of normal serum against solutions containing cyanide produced a change of the electrophoretic pattern of the serum proteins. This was not reversed by a subsequent dialysis against cyanide free solutions. It follows from these experiments that the copper ion is less suitable for the fractional precipitation of serum proteins. A comparison of the results obtained at pH 4.5 with and without the addition of cyanide indicates that the copper protein compounds which precipitate during dialysis contain the bulk of the γ-globulins (Fig. 6).

It appears from these results that it is possible to achieve a fractional precipitation of serum proteins by means of ions of heavy metals. However, the specificity of the precipitations could be improved by combining the method

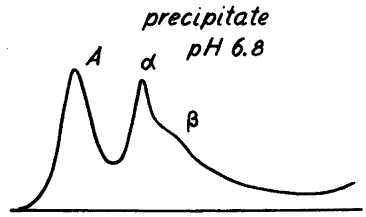

Fig. 4. Precipitate from dialyzed horse serum with lead chloride. 20 ml serum (dialyzed against distilled water) + 40 ml saturated $PbCl_2$. Addition of 0.5 N NaOH to pH 6.8. Electrophoresis: NaCl (μ = 0.025) — phosphate (μ = 0.075) buffer pH 7.6; 120 min., 18.0 mA. Ascending pattern.

here described with other methods of fractional precipitation of proteins. The following description of the use of a lead precipitation in combination with other methods may serve as an illustration of the possibilities of the method.

To 1 volume of bovine serum (electrophoresis curve in Fig. 7 a) was added 2 vol. of saturated $PbCl_2$ and enough 1 *N* HCl to prevent precipitation. After adjusting the pH to 5.0 with 1 *N* NaOH the precipitate was removed by centrifugation and dissolved in 0.9 % NaCl by addition of a small amount of 1 *N* HCl. The precipitate showed the electrophoresis diagram in Fig. 7b. A small amount of saturated ammonium sulphate was now added in order to precipitate the lead as sulphate. After centrifugation the solution was neutralized, and further amounts of $(NH_4)_2SO_4$ were added to 55 % saturation. The precipitate obtained contained the rest of the globulins, mainly α-globulin (Fig. 7c). The filtrate consisted almost entirely of albumin (Fig. 7d) and was precipitated by dilution to 40 % saturation with $(NH_4)_2SO_4$ and addition of an equal volume of 2 % tricresol in water. The final concentration of 1 % tricresol and 20 % saturation with $(NH_4)_2SO_4$ had in a previous paper [4] been found favorable for the selective precipitation of albumin. The precipitate was dissolved in a small amount of phosphate buffer and dialysed until free from cresol. The electrophoresis diagram showed an albumin preparation of high purity containing only traces of other components (Fig. 7e).

DISCUSSION

It was reported previously that protein precipitating anions, such as sulphosalicylate and tungstate, can be used in the fractional precipitation of serum proteins [5]. In a recent paper the applicability of this method in the fractionation of serum of different animal species was demonstrated [6]. The anions form dissociable compounds with the proteins, which may be selectively precipitated at appropriate pH values.

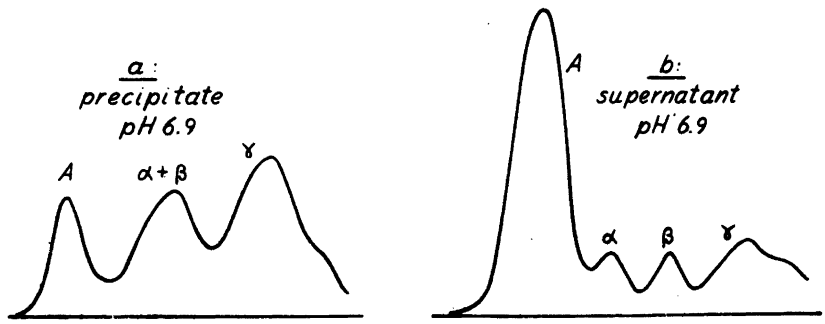

Fig. 5. Precipitate (a) and supernatant (b) from precipitation of bovine serum with cadmium chloride. 15 ml serum (same as Fig. 1a) + 15 ml 0.9 % NaCl + 7.5 ml 0.1 M $CdCl_2$ + 2.4 ml 0.25 N HCl. Addition of 1 N NaOH to pH 6.9. Electrophoresis: NaCl (μ = 0.025) — phosphate (μ = 0.075) buffer pH 7.6; 120 min., 18.0 mA. Concentration of precipitate relative to supernatant: 2.7. Descending patterns.

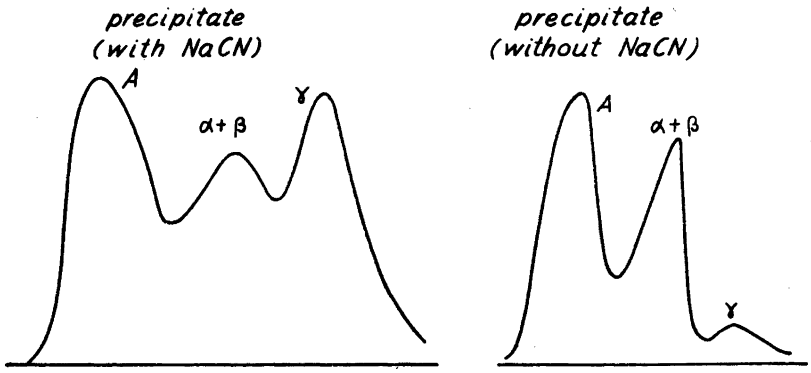

Fig. 6. Precipitate from bovine serum with copper chloride dialyzed subsequently against 0.9 % NaCl with (a) and without (b) added NaCN (0.1 %). 40 ml serum + 40 ml 0.9 % NaCl + 2 ml 1 N HCl + 2 ml 1 M $CuCl_2$. Addition of 1 N NaOH to pH 4.5. Electrophoresis: NaCl (μ = 0.075) — phosphate (μ = 0.025) buffer pH 7.5; 150 min., 18.2 mA. Descending patterns.

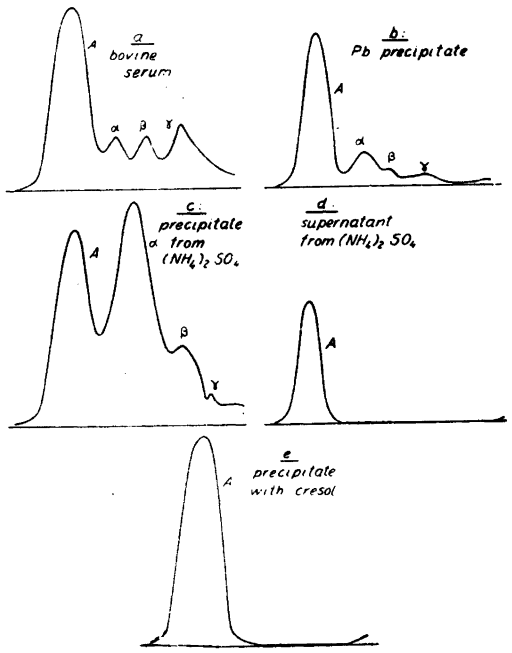

Fig. 7. Preparation of bovine serum albumin. a) Original serum; b) Lead precipitate at pH 5.0; c) Ammonium sulfate precipitate from solution of b; d) Filtrate from ammonium sulfate precipitation; e) Precipitate from d) with tricresol. Electrophoresis: NaCl (μ = 0.025) — phosphate (μ = 0.075) buffer pH 7.6; 120 min., 18.0 mA. Descending patterns. Relative concentrations: a) 1.0; b) 1.2; c) 8.1; d) 1.2; e) 3.2

These results suggested a study of the applicability of protein precipitating cations for the same purpose. The work was initiated in 1947 but had to be abandoned again until recently. In the meantime the results of Cohn's group on the fractional precipitation of proteins by means of various metal ions appeared [1,2]. Our results together with those of Cohn's group show that it is possible to perform a fractional precipitation of serum proteins by heavy metal ions.

While Cohn produced fractional precipitation in human serum with zink and barium salts, we have used bovine and equine serum and have studied lead, cadmium and copper as precipitants, because these salts are known as potent precipitants of proteins.

Our results show a considerable difference in the action of the various salts on the serum proteins. Addition of lead chloride to bovine serum caused a precipitation of albumin and of α-globulin (at pH 5.0). The precipitate obtained with cadmium (at pH 6.9) contained mainly globulins, especially γ-globulin. Copper produced a rather unspecific precipitation, but a very insoluble compound was formed with the γ-globulin, from which copper could be removed only by dialysis against solutions containing sodium cyanide. However, dialysis against sodium cyanide changed the electrophoretic pattern of normal serum, so that this method could not be recommended though it has been used by Kunkel [7].

Heavy metals have been proposed as reagents in the turbidimetric estimation of elevated concentrations of globulin (especially γ-globulin) in human sera. Thus Kunkel [7] used highly diluted serum and small concentrations of copper or zink sulphate to produce a turbid precipitate of γ-globulin. He found that Hg, Pb, Cd and U salts produced a similar effect. Under the conditions used in our experiments (bovine or equine serum in high concentrations) Pb precipitated selectively albumin and α-globulin.

Lower dilutions were used by Wunderly and Wuhrmann [8] in their cadmium test for increased globulin content in human serum. This reagent gives a positive reaction not only with γ-globulin, but also with increased α-globulin concentration. This is in accordance with the results of cadmium fractionation of bovine serum recorded in the present paper.

Doladilhe [9] reported that lead chloride precipitated mainly the euglobulins when added to dialysed horse serum. He drew this conclusion from ammonium sulphate precipitations. We have tried above to reproduce his experiments, following the precipitation by electrophoretic analysis. It will be seen that our results are not in accordance with those of Doladilhe. Typical for our results with lead precipitation was the separation of albumin and α-globulin in the precipitate. Though the composition varied to some extent with the experimental conditions (pH and salt concentration), γ-globulin did not appear in the precipitate. Doladilhe has not specified his conditions as to pH, salt concentration and concentration of lead, but it appears difficult to explain all discrepancies between our results and his solely on this basis.

A great deal of work has appeared on the combination of metals with proteins. An important result of these investigations has been the realization that apart from the free carboxylic groups also the thiol group and the various basic groups play a role in the formation of metal compounds. Probably the

marked differences in the behaviour of the cations studied here (Cd^{++}, Cu^{++}, Pb^{++}) are caused by variations in the affinities to the various metal combining groups in the proteins as well as by variations in the solubilities of the compounds formed. It appears possible to modify the solubilities during the precipitations by varying the salt concentrations, and thereby to enhance the selectivity of the fractionation procedure. The method therefore appears adaptable and has probably a wide range of applicability. The example recorded on the preparation of bovine albumin shows its usefulness in combination with other methods of protein fractionation.

SUMMARY

1. Cadmium, copper and lead chloride were used as precipitants in the fractionation of bovine and horse sera.

2. Lead showed the highest selectivity, precipitating albumin and α-globulin from bovine as well as from horse serum.

3. Cadmium precipitated mainly globulins. Copper lacked selectivity under the conditions used. With the γ-globulin fraction it formed a compound containing firmly bound copper.

4. The preparation of pure bovine serum albumin is described, using lead precipitation in combination with other methods.

This investigation was aided by a grant from *Teknisk-kemisk Fond.* Thanks are also due to Messrs. *Ferrosan* Ltd., Copenhagen, for support. Mr. Ole Tolboe took part in some of the experiments.

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pH Dependence of Choline Esterase Activity at Various Substrate and Inhibitor Concentrations

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The pH dependence of choline esterase activity has been studied in several investigations[1-6,13]. This dependence was also shown in our laboratory by the electrometric method for determinations of choline esterase activity, which seems especially applicable for determinations involving pH variations. It was also of interest to analyse the inhibitory effect of a chemically stable reversible and competitive inhibitor by means of pH variations. 1,5-bis-(*p*-N-dimethyl-N-allyl-ammonium phenyl)-pentanon-3 [7] was the inhibitor used.

EXPERIMENTAL

The following experiments were performed:

1. Measurements of the dependence of enzyme activity on pH at constant substrate concentration. The enzymes used were a) acetylcholine esterase (AChE) and b) choline esterase (ChE).
2. Measurements of activity-pS curves at different pH values. (AChE).
3. Measurements using 1,5-bis-(*p*-N-dimethyl-N-allyl-ammonium phenyl)-pentanon-3, synthesized by Tammelin [8] according to Copp [9], as the inhibitor. I_{50} was determined at different pH values. (AChE and ChE.)

For all determinations of enzyme activity the electrometric method described by Tammelin [10] was applied. The temperature in all experiments was 25° C. No determinations were run above pH 9 owing to the nature of the glass electrodes used.

Buffer. To avoid unnecessary change of ionic media at different pH values, a buffer solution principally composed as described by Britten and Robinson [11] was used. 100 ml of their stock solution plus different amounts of 0.2 *M* NaOH were made up to 400 ml with 0.2 *M* NaCl, thus maintaining the concentration of Na^+ constant.

Enzymes. For determinations on AChE cobra venom was used. A 0.005 % cobra venom preparation in the following solution was made: 0.1 *M* $MgCl_2$, 0.05 *M* KCl, 0.05 *M* NaCl. The solution was kept in a refrigerator and was stable during the course of the experiments.

For determinations on ChE blood serum was used. 15 ml of serum were diluted to 100 ml with the salt solution described above and kept in a refrigerator.

Substrates. For measurements of pH-activity curves the following substrates were used (final concentrations): acetylcholine iodide (ACh) $7.3 \cdot 10^{-3}$ *M*, acetyl-β-methylcholine iodide (MeCh) $1.8 \cdot 10^{-2}$ *M*, butyrylcholine iodide (BuCh) $6.5 \cdot 10^{-3}$ *M*. The same concentration of acetylcholine iodide was used in I_{50} determinations. It was controlled

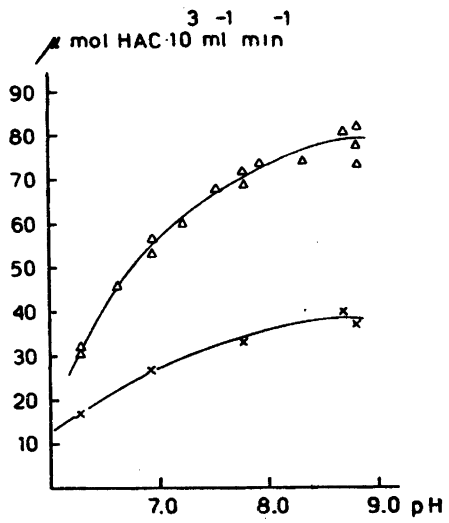

Fig. 1. pH-activity curves for AChE at constant substrate concentration. △—△ *ACh;* ×—× *MeCh.*

that the spontaneous hydrolysis of the substrates in the pH region used nowhere exceeded 3 % of the total hydrolysis [8,12].

Calibration. As the pH variations arising from evolution of acid vary at different pH values, each buffer solution was calibrated by infusion of 3 × 0.02 ml of 0.15 *M* acetic acid. An Agla syringe was used in the experiments. Serum and cobra venom gave different calibration values. The calculations used were those described by Tammelin [10].

Inhibitor. A 10^{-2} *M* solution of the inhibitor in distilled water was prepared and kept in a refrigerator. The solution was stable during the course of the experiments.

Reaction mixtures. For pH-activity and pS-activity curves the reaction mixture in the vessels had the following composition: 3 ml buffer solution, 3 ml enzyme solution and after stabilization of the E.M.F. value, 0.6 ml of substrate solution.

For I_{50} curves the enzyme solution was three times stronger and only 1 ml was used in each vessel. The vessels contained 2 ml of varying amounts of inhibitor.

The pH was determined in the final reaction mixtures.

RESULTS

pH-activity curves. a) *AChE.* The pH-activity curves for the systems AChE-acetylcholine and AChE-acetyl-β-methylcholine were measured. Fig. 1 shows the two curves. b) *ChE.* Fig. 2 shows the pH-activity curves for the systems ChE-acetylcholine and ChE-butyrylcholine.

pS-activity curves. Fig. 3 shows curves where the varying acetylcholine concentrations are plotted against the activities.

I_{50}-curves. Fig. 4 shows the curves obtained when pI_{50}-values for AChE and ChE are plotted against pH-values.

DISCUSSION

Table 1 shows that AChE splits acetylcholine and acetyl-β-methylcholine and ChE splits acetylcholine and butyrylcholine at a constant ratio. This ratio seems to be independent of the pH.

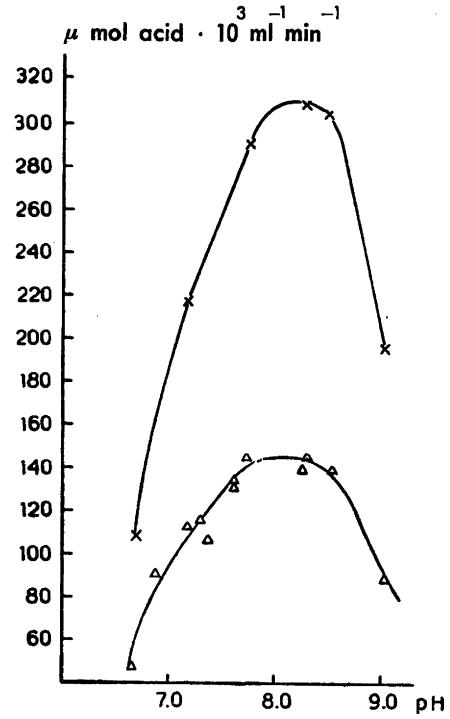

Fig. 2. pH-activity curves for ChE at constant substrate concentration. △—△ *ACh;* ×—× *BuCh.*

The curves of hydrolysis obtained with AChE seem to reach a plateau at pH 8.6. This has earlier [3,13] been shown to be part of an activity optimum reaching from pH 8.3 to pH 9. The activity of ChE reaches its optimum between pH 7.7 and 8.3 in agreement with the values found by Bergman and Wurzel [5]. Below pH 6 the activity of the enzyme decreases rapidly to zero.

The inhibitory effect of 1,5-bis-(*p*-N-dimethyl-N-allyl ammonium phenyl)-pentanon-3 decreases with decreasing pH. For both AChE and ChE the pI_{50}-pH curve starts to fall at pH 7.9.

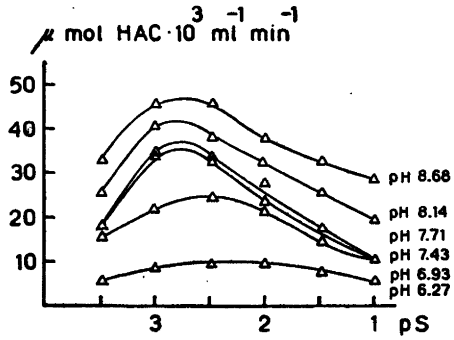

Fig. 3. pS-activity curves for AChE at different pH-values. Substrate ACh.

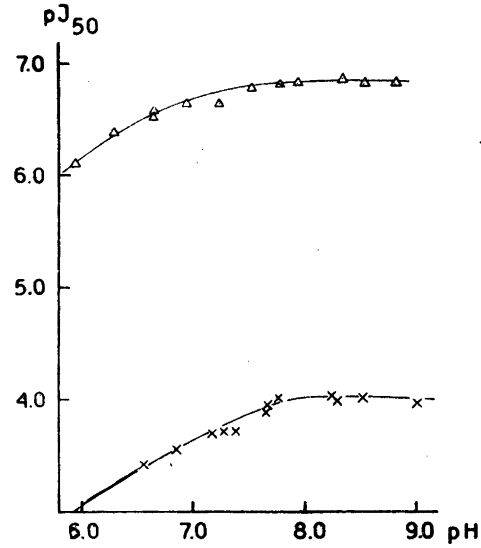

Fig. 4. pI_{50}—pH curves. △—△ AChE; ×—× ChE. Inhibitor: 1,5-(p-N-dimethyl-N-allyl-ammonium phenyl)-pentanon-3. Substrate ACh.

The pS-activity curves show, that the optimum substrate concentration increases below pH 7.43. The pH-dependence of the enzyme-substrate complex seems to be similar to the pH dependence of the enzyme-inhibitor complex. This may be reasonable, as both the substrate and the inhibitor are quaternary ammonium compounds.

From the pI_{50}-pH curves it can be seen that care must be taken in comparing I_{50} values obtained when working at a different pH. Thus the pH in Warburg

Table 1.

pH	ChE		
	μmoles acid · 10^3 ml^{-1} min^{-1}		ratio
	ACh	BuCh	ACh/BuCh
9.01	90	196	0.46
8.47	140	305	0.46
8.27	145	308	0.47
7.73	145	290	0.50
7.17	114	218	0.46
6.65	49	109	0.45
	AChE		
	μmoles acid . 10^3 ml^{-1} min^{-1}		ratio
	ACh	MeCh	ACh/MeCh
8.80	78	37	2.1
8.68	81	40	2.1
7.77	70	33	2.1
6.93	55	27	2.0
6.27	32	17	1.9

determinations is about 7.4 and in electrometric determinations usually 8.0, so that I_{50} values determined by these two methods are not exactly comparable.

Experience during this investigation shows that the electrometric method for determinations of choline esterase activity is very well suited for determinations of I_{50} values at different pH values, as in this case the relative activity values obtained by the apparatus can be used directly. As soon as it is necessary to compare true activity values, calibrations of the solutions must be performed. This is a rather time-consuming procedure, which has to be repeated for each enzyme, buffer solution and pH. If the calibrations are done with great care, the method is exact.

SUMMARY

Using an electrometric method for determinations of choline esterase activity, the activity-pH dependence for AChE and ChE has been measured. AChE activity was found to reach a plateau at pH 8.6 and was found not to decrease again before pH 9. An optimum for ChE activity was found between pH 7.7—8.3.

By the same method the inhibition-pH dependence has been measured using 1,5-bis-(*p*-N-dimethyl-N-allyl-ammonium phenyl)-pentanon-3 as an inhibitor. The inhibition was found to decrease with decreasing pH below pH 7.9. (AChE and ChE).

The pS maximum at different pH-values has been measured for AChE. It was found that the pS maximum increases with decreasing pH below pH 7.43.

The value of the electrometric method for determinations of activity-pH dependence is discussed.

My sincere thanks are due to the Director of the Research Institute of National Defence Dept. 1, Professor G. Ljunggren and to the Director of the Institute of Organic Chemistry and Biochemistry, University of Stockholm, Professor K. Myrbäck, for their kind interest in this work.

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Metabolism of Bile Acids in Rat Liver Slices and Homogenates. Bile Acids and Steroids 15

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The conjugation and 7 α-hydroxylation of desoxycholic acid to taurocholic acid [1] found to take place in the rat *in vivo* has also been demonstrated in rat liver slices [2].

These reactions have now been studied both in rat liver slices and homogenates. The techniques used and some results will be reported in this paper. For the quantitative estimation of the reaction products the paper chromatographic procedures of Sjövall [3] for free and conjugated bile acids have been used extensively.

EXPERIMENTAL

Animals. The white female rats used throughout all experiments had an average weight of 150 g. The average liver fresh weight was 5—6 g. The experiments were always started in the morning to get as uniform conditions as possible. The animals, which had free access to their normal diet up to this time, were killed by a blow on the head, the liver excised and immediately placed in ice cold buffer solution or homogenizing medium.

Labelled bile acids. The acids were prepared according to Bergström, Rottenberg and Voltz [4]. The desoxycholic acid-24-^{14}C used in the experiments reported here, was 99 % pure as measured by paper chromatography. The rest of the activity was within the limits of normal deviation from the background, with no definite spot on the paper. The stock solutions contained 1 mg per ml of the different bile acids as sodium salts in water adjusted to pH 7.5.

Buffer solution. The buffer solution was an isotonic Krebs-Ringer phosphate buffer pH = 7.4 [5].

Homogenizing medium. The homogenizing medium consisted of a 0.08 *M* Sørensen phosphate buffer pH = 7.4 and 0.12 *M* sucrose solution, the whole being approximately isotonic. (10.1 g Na_2HPO_4 + 1.4 g KH_2PO_4 + 43 g sucrose per liter.) The solution was freshly prepared every month and stored at 4—6° C.

Preparation of slices. The liver (5—6 g) was cut in 5—7 pieces, each of them placed on a cooled Petri dish and cut vertically by means of a razor blade by hand. Each section had an approximate thickness of 0.5 mm. The slices were kept under ice cold buffer solution until they were used. Average time between killing the rat and beginning of the incubation, 20 minutes.

Preparation of homogenates. The liver was first cut in slices as mentioned above but in the homogenizing medium instead of the Krebs-Ringer buffer. To the slices was then added three times the amount (v/w) of homogenizing medium at 0° C. The mixture was transferred to a homogenizer with a plastic pestle (loose fitting, width between glass and

pestle 0.8 mm) and homogenized for 30 sec. with a motor of 1/6 HP and 1 500 RPM. In order to keep the speed constant, a motor of this strength is desirable. The whole was then poured into a precooled centrifuge tube and centrifuged at 400 × g for 5 minutes. The supernatant was used and the precipitate discarded. The average time between killing the rat and finishing the homogenization was 15 minutes.

After extensive experimentations of which some data are recorded in Table 1, these conditions were found to give optimal results. Preliminary slicing of the liver before homogenizing was found to be necessary as it was impossible to use a tissue press. The necessary pressure, even during a very short time (2—5 sec), resulted in an inactive preparation. Miller and Warren [7] have made similiar observations. In accordance with Bucher *et al.*[8] it was found essential to have a loose fitting pestle but in this work addition of nicotinamide to the homogenizing medium did not influence the results.

Dry weights and N-content. 1 g liver fresh weight corresponds to 300 mg dry weight. 1 ml of the supernatant homogenate = 78 mg dry weight, (54 mg thereof from homogenizing medium). Corrected dry weight, 24 mg per ml supernatant. Nitrogen content about 3.4 mg N per ml.

Incubation and extraction of the reaction products. Slices (1—2 g) or homogenates were shaken (100 strokes per min.) with the appropriate additions in 25 ml Erlenmeyer flasks or centrifuge tubes at 37° C for 2 hours in air unless otherwise stated. A bile acid/tissue ratio of 1 : 10 000 was generally used. The reaction was stopped by addition of 5 volumes of ethanol and the mixture was acidified with a few drops of glacial acetic acid.

After 1 hour the mixture was filtered or centrifuged. The residue was washed 3 times with 2 ml of ethanol. The combined filtrates were then blown to dryness in a stream of air at 50° C. From 2 ml of homogenate (800 mg fresh weight liver) about 100 mg dry weight residue was obtained of which 75 % was sucrose or inorganic salts from the medium. About 90 % of the activity was recovered with no selective loss of any one of the reaction products.

The residue was dissolved in 1 or 2 ml of 70 % aqueous ethanol and used without further purification for paper chromatography.

Paper chromatography. We used essentially the methods developed by Sjövall [3] and added about 1/50 of the incubation residue to each strip. This gave us about 2 mg substance with an activity of 300—1 000 cpm. The strips were saturated in the chromatographic vessels overnight and run 12 hours for the butanol and 4 hours for the isopropylether/heptane chromatograms. They were dried for five min. at 80° C and cut at the upper and lower ends so that they could be put on a metal sheet for counting of radioactivity.

Table 1. Different experimental conditions for preparing rat liver homogenates.

2 ml rat liver homogenate corresponding to 800 mg fresh weight + 125 μg desoxycholic acid-24-^{14}C, pH = 7.4, 2 hrs 37°. Total conversion to tauro acids = 100 %. Average values each out of 2 similiar experiments.

No.	Variations in the preparation of the homogenate	% Conversion of desoxycholic acid to tauro acids
1	13 000 RPM, motor strength 1/80 HP	0
2	4 000 » » » »	90
3	2 500 » » » »	90
4	1 700 » » » »	75
5	1 500 » » » 1/6 HP	95
6	Same as 5, homogenizing time 5 seconds	5
7	» » 5, » » 20 »	100
8	» » 5, » » 40 »	100
9	» » 5, » » 60 »	95
10	Same as 8, glass pestle; centrifuged 5 minutes at 400 × g	95
11	» » 8, » » » 5 » » 2 000 × g	40
12	» » 8, plastic pestle; » 5 » » 400 × g	100
13	» » 8, » » » 5 » » 2 000 × g	45

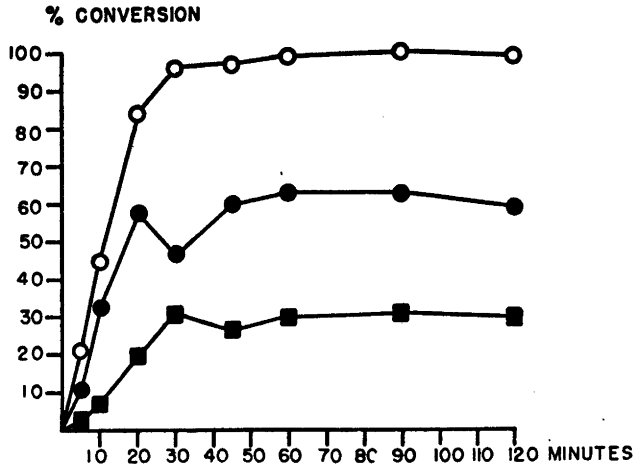

Fig. 1. Incubation of [^{14}C] *desoxycholic acid (125 μg) with 2 ml of rat liver homogenate for different times. Taurine conjugates (○), taurodesoxycholic acid (●) and taurocholic acid (■).*

The counting of the strips. We used an automatic scanner for measuring the radioactivity of the various strips with a mica-end window, lead shielded G. M. tube (Tracerlab. Inc.). Attached was a printing time counter and an automatic strip feeder. The apparatus was designed in the workshop of this institute and will be published elsewhere in due course. The strip was advanced 1 cm at each step and the same length was counted by the G. M. tube through an appropriate mask.

We normally used 200 preset counts, the time required being printed on a paper strip by means of the printing time meter. The standard error of the results from the average with a preset of 200 counts was about 12 %. The background was 35 ± 4 c/m. With higher preset counts the deviation decreases. (Preset 400 ± 10 %; preset 600c ± 8 %; preset 1 000c ± 6 %). The time for measuring a 30 cm strip with 200 preset counts was about 3 hours.

The counts obtained under these conditions (Whatman No. 1 filter paper) were about 35 % of those obtained when the same amount of labelled compound was plated on copper planchets in infinitely thin layer.

Reaction products. The identity of the formed cholic acid was verified after hydrolysis. *via* chromatography and isotope dilution, as described earlier[1,2].

RESULTS

A) *Influence of different experimental conditions on the rate of conjugation and oxidation.* Some results of preparing the homogenates under different conditions are shown in Table 1. The best method so far is No. 12 as described in detail in the experimental part. We also made some preliminary experiments on the inhibition of the reaction and found that the homogenate was totally inactivated by heat (5 min., 60° C), pressure (forcing through a tissue press) and heavy metalions (Cu, 10^{-3} *M*). Cyanide inhibited 50 % at 10^{-4} *M* concentration.

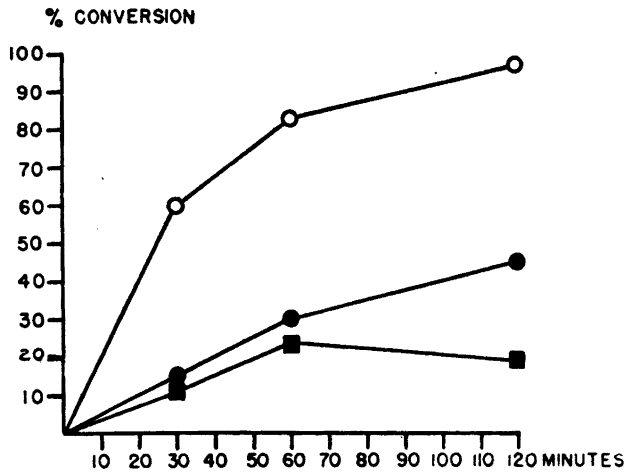

Fig. 2. Incubation of [^{14}C] *desoxycholic acid (250 μg) with rat liver slices (2 g) for different times. Taurine conjugates (○), taurodesoxycholic acid (●) and taurocholic acid (■).*

B) *The effect of the incubation time.* The influence of the incubation time is shown in Fig. 1 and 2. It is to be noticed in Fig. 1 that taurodesoxycholic acid reaches the plateau value 10 min. earlier than taurocholic acid, indicating that the reaction proceeds from free desoxycholic acid *via* taurodesoxycholic acid to taurocholic acid. This of course cannot be regarded as definitely proven by this experiment. Another observation supporting this course for the reaction is that in all our experiments we were unable to detect free cholic acid.

In a similiar experiment with rat liver slices (Fig. 2) the time for the completion of the reaction is somewhat longer. This is to be expected because of the slow diffusion of the added bile acid into the cells of the slices.

C) *Variation of the amount of desoxycholic acid.* In order to get data on the capacity of rat liver homogenates to convert desoxycholic acid into taurodesoxy and taurocholic acid increasing amounts of this bile acid were added to a fixed amount of rat liver homogenate, (2 ml homogenate corresponding to 800 mg liver fresh weight). In Fig. 3 the results of a typical experiment are shown. A practically complete conjugation of the added bile acid is obtained up to 250 μg per vessel. An inhibition is seen when 400 μg or more of the bile acid was added to the incubation mixture. This gives a minimum inhibition concentration of about 11 mg/100 ml (average for 4 experiments). Essentially the same data were obtained in 2 runs with rat liver slices. The content of free taurine present in these experiments corresponds to three times this amount [9]. Addition of free taurine did not change the results. Data on the influence of other bile acids and of the reaction products will be published in a later communication.

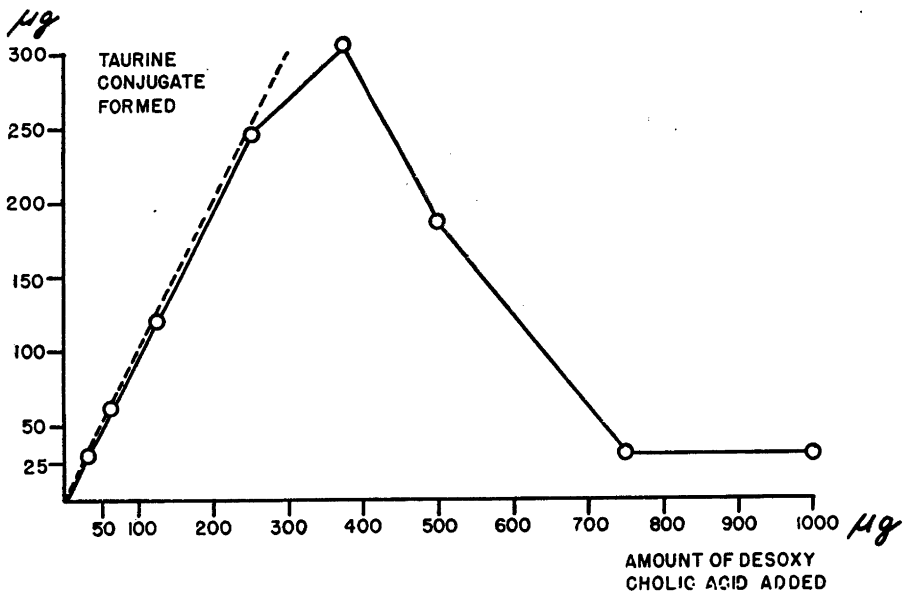

Fig. 3. Incubation of different amounts of [^{14}C] *desoxycholic acid with 2 ml of rat liver homogenate. Taurine conjugates (○). Interrupted line corresponds to total conjugation of bile acid present.*

SUMMARY

1) The experimental conditions for the conversion of desoxycholic acid-24-^{14}C into taurodesoxy and taurocholic acid by rat liver slices and homogenates have been studied.

2) The time curves of these processes and also the capacity of the rat liver to conjugate have been investigated.

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1e,2e-Dichloro-4e,5e-dibromo*cyclo*hexane, a Disordered Crystal Structure

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The crystal structure of one modification of 1e,2e-dichloro-4e, 5e-dibromo*cyclo*hexane has been determined. The space group requires the molecule to possess a center of symmetry which is incompatible with chemical evidence. The structure is therefore assumed to be disordered and Fourier projections support this assumption.

In a previous publication Hassel, Wang Lund and Lunde [1] reported the preparation of several 1,2,4,5-tetrahalogeno*cyclo*hexanes. The addition of bromine to 1,2-dichloro*cyclo*hexene(4) led to two different 1,2-dichloro-4,5-dibromo*cyclo*hexanes, one melting at 171° C, the other at 240° C. The structure of the former has been settled by X-ray [2] and electron diffraction [3] methods to be 1a,2a-dichloro-4e,5e-dibromo*cyclo*hexane [4]. The compound melting at 240° C was shown to be dimorphous with an α-modification stable below 90—95° C and a β-modification stable at higher temperature. Corresponding phases α and β have been shown to exist both for the corresponding tetrachloro- and the tetrabromocompound. Further, the crystals of the α-modifications of the three compounds have been found isomorphous, as well as those of the β-modifications [1,5]. Both structures are monoclinic with somewhat different crystallographic data.

From the fact that both modifications crystallize in the monoclinic space group $P2_1/c$ with two molecules in the unit cell, it follows that the center of the molecule has to lie in the twofold position which requires a center of symmetry. This fits well into the picture of a molecular structure with all four substituents in equatorial positions in those cases where the halogens are all of the same kind. In the case of a mixed compound of the type 1,2-dichloro-4,5-dibromo*cyclo*hexane it must however be concluded that the structure is of a disordered type in such a manner that the average symmetry corresponds to that of a center.

This kind of disorder has been found by Hendriks for *p*-chloro-bromobenzene [7], and a more recent demonstration has been given by Hassel and Hadler Vihovde [8] in the crystal structure of *trans* 1,4-chloro-bromo*cyclo*hexane.

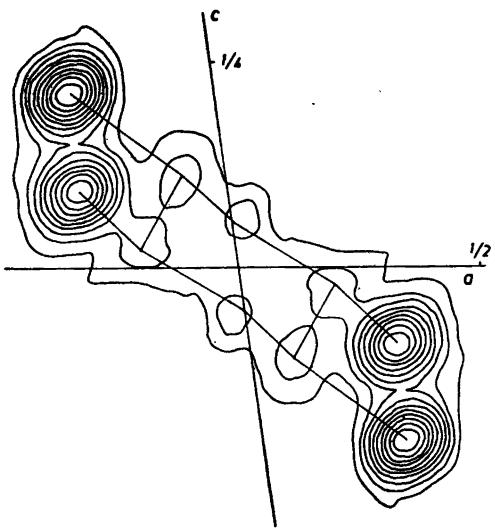

Fig. 1. Fourier projection along [010].

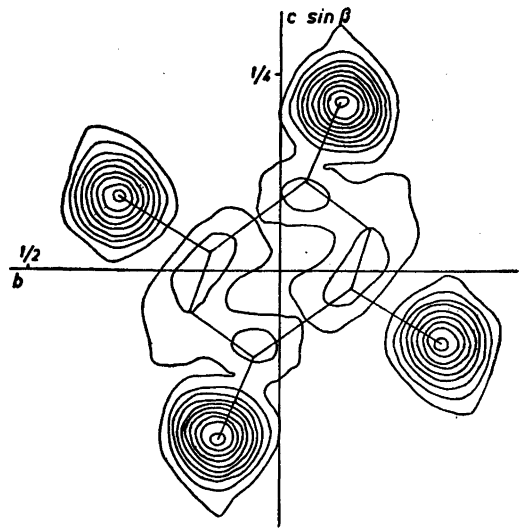

Fig. 2. Fourier projection along [100].

As rather few cases of such displacement disorder are known in structures of organic compounds it was found worth while to study in more detail one of the disordered modifications of the compound in question.

The structure of the β-modification of the tetrabromocompound has been worked out earlier by Haak [6]. The α-modifications represent however a new type of structure, therefore this was chosen for detailed study. To repeat, the crystallographic data are: $a = 6.29$ Å, $b = 6.93$ Å, $c = 10.81$ Å, $\beta = 98°$. Weissenberg equator diagrams of the zones ($h0l$) and ($0kl$) were taken with Cu—$K\alpha$ radiation using the multiple film technique. Three double sets of films were given exposure times in the ratio 20/5/1. Heavily exposed films did not give any signs of reflexions not compatible with the space group $P2_1/c$. For the estimation of intensities a standard scale was prepared using one of the stronger reflexions from the very same crystal used when taking the diagrams.

In the calculation of theoretical structure factors a common atomic scattering factor for all halogen atoms was taken to be the arithmetic mean of the f-values for chlorine and bromine, given by the Hartree and Thomas-Fermi f-curves respectively.

Trial and error analysis taking into account only the strongest reflexions soon gave a reasonable agreement between observed and calculated structure factors. Further refinement gave as result the Fourier maps reproduced in Figs. 1 and 2. In these the interval between contour lines corresponds to 3 elctrons/Å^2 starting at 3 electrons/Å^2. The halogen parameters obtained from the maps are given in Table 1. The presence of the carbon atoms gives rise to clearly separated peaks, but only approximate coordinates can be given. The x and z values listed in Table 1 are determined from the (010) projection,

Table 2. Observed and calculated structure factors.

hkl	$\lvert F_{obs}\rvert$	$\lvert F_{calc}\rvert$	*hkl*	$\lvert F_{obs}\rvert$	$\lvert F_{calc}\rvert$
100	1.9	3.5	$50\bar{8}$	0	1.0
200	26.1	26.8	$60\bar{8}$	5.7	4.3
300	21.2	20.6	$10\bar{1}0$	4.7	4.3
400	3.2	6.3	$20\bar{1}0$	6.2	6.7
500	9.5	10.9	$30\bar{1}0$	5.4	4.5
600	0	2.3	$40\bar{1}0$	0	0.5
700	0	3.2	$50\bar{1}0$	5.0	4.0
002	0	3.0	011	6.7	4.0
102	32.4	34.9	012	6.0	8.1
202	3.0	3.6	013	6.7	6.5
302	10.6	11.8	014	16.6	17.2
402	7.8	9.0	015	12.9	13.7
502	3.1	3.3	016	10.3	10.4
602	0	3.8	017	3.8	3.0
004	0	2.2	018	0	1.5
104	0	0.7	019	3.1	2.7
204	0	0.6	0110	3.5	3.1
304	0	1.2	020	11.3	9.7
404	0	0.5	021	31.5	27.1
504	0	0.3	022	7.0	6.0
604	0	0.3	023	0	1.7
006	10.0	11.0	024	3.5	4.3
106	9.0	8.6	025	6.3	6.3
206	0	2.2	026	4.1	5.7
306	8.2	10.6	027	4.3	4.5
406	0	0	028	0	1.7
506	0	3.4	029	6.7	6.2
008	2.1	2.0	031	8.2	8.0
108	8.5	10.3	032	5.8	7.2
208	6.4	6.9	033	0	0.7
308	0	2.1	034	6.4	6.2
408	5.1	6.6	035	16.1	15.3
0010	6.2	6.1	036	3.4	3.7
1010	0	0.5	037	0	0.9
$20\bar{1}0$	4.3	4.2	038	5.6	5.2
$10\bar{2}$	23.5	23.2	040	15.6	13.0
$20\bar{2}$	23.2	19.9	041	5.0	4.4
$30\bar{2}$	0	4.5	042	6.6	6.6
$40\bar{2}$	13.6	14.2	043	9.6	9.8
$50\bar{2}$	6.4	4.6	044	4.7	5.0
$60\bar{2}$	4.7	5.9	045	0	2.9
$10\bar{4}$	0	1.8	046	0	1.4
$20\bar{4}$	5.1	0.9	047	0	2.8
$30\bar{4}$	4.7	4.6	048	0	1.3
$40\bar{4}$	5.3	3.0	051	4.6	5.6
$50\bar{4}$	2.2	4.2	052	3.3	2.8
$60\bar{4}$	0	0.8	053	2.4	3.4
$10\bar{6}$	5.4	3.3	054	4.9	5.4
$20\bar{6}$	10.7	9.8	055	0	1.0
$30\bar{6}$	1.9	2.6	056	4.3	3.8
$40\bar{6}$	4.8	3.0	057	3.5	4.0
$50\bar{6}$	5.2	4.4	060	5.1	4.1
$60\bar{6}$	0	0.7	061	3.1	2.9
$10\bar{8}$	13.3	12.9	062	2.5	2.3
$20\bar{8}$	6.2	6.7	063	4.3	6.0
$30\bar{8}$	8.1	8.2	064	0	3.2
408	7.7	6.9	065	0	0.5

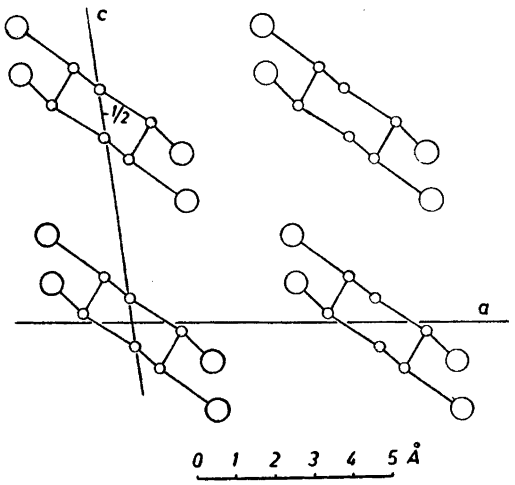

Fig. 3. Projection of the structure of a-1e,2e,4e,5e-tetrahalogeno cyclohexane on (010).

the y values from the (100) projection. In Figs. 3 and 4 are reproduced projections of the structure on (010) and (100).

In order to normalize calculated structure factors and to determine an appropriate temperature factor the ratio of observed to calculated structure factors was plotted as a function of sin ϑ. To this set of points a curve of the

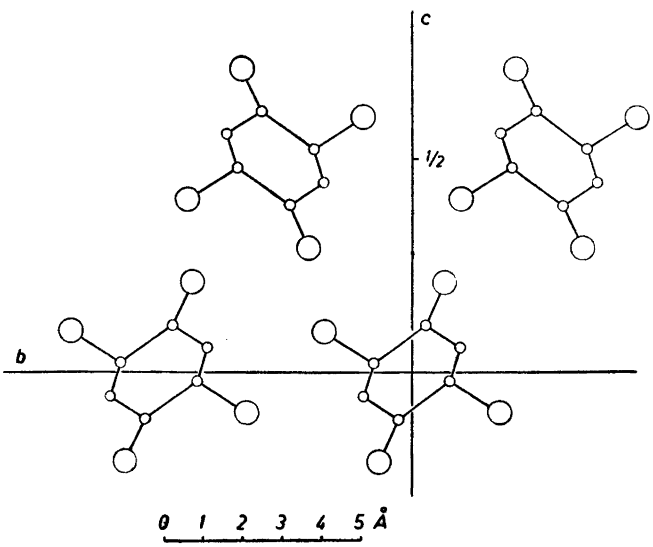

Fig. 4. Projection of the structure of a-1e,2e,4e,5e-tetrahalogeno cyclohexane on (100).

Table 1. Parameters of halogen and carbon atoms.

	x	y	z
Hal_1	−0.305	0.322	0.094
Hal_2	−0.294	−0.121	0.211
C_1	−0.195	0.138	0.021
C_2	0.088	0.050	0.111
C_3	0.003	−0.178	0.054

form $k \exp(-a \sin^2 \vartheta)$ was adjusted by inspection. For both projections the constant a was found equal to 2.16. After normalizing calculated structure factors and taking the temperature factor into account the agreement factor $\Sigma||F_{obs}|-|F_{calc}||/\Sigma|F_{obs}|$ was calculated to be 0.18 and 0.17 for the two projections (010) and (100) respectively. In Table 2 observed and calculated F-values are listed.

From the Fourier maps it will be seen that the two crystallographically nonequivalent halogen peaks are of about the same height. Rough calculations give a total number of 25 electrons associated with both peaks. Although such a number cannot be expected to be too reliable it is of interest to compare these features of the structure with the situation in the structure of 1a,2a-dichloro-4e,5e-dibromo*cyclo*hexane of m.p. 171° C. There the crystallographically nonequivalent halogen peaks 1 and 5 were found distinctly different, the maximum values being in the ratio 1/1.6, and the number of electrons associated with them were estimated to be 17 and 35. The statement made in the beginning that the present structure has to be of a disordered type in order to conform to the space group $P2_1/c$ has thus been substantiated.

In the case of the corresponding modification of 1,2,4,5-tetrachloro*cyclo*hexane intensity data have been obtained and Fourier maps worked out. The structure of that compound is analogous to that which has been presented in detail here. The isomorphous relationship between the two compounds indicated by the unit cell dimensions has thus been confirmed.

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Pyrazole Studies

VIII *. Oxidation of 4-Alkylsubstituted Pyrazol-5-ones with Tertiary Butylhydroperoxide

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The preparation of 4-alkyl-4-hydroxysubstituted pyrazol-5-ones in anhydrous alkaline-alcoholic solution containing ca. 70 % of tert. butanol with tert. butylhydroperoxide is described. The presence of water causes a decrease in the yield of hydroxy compound. When the pyrazolone is unsubstituted at N_1, only unidentified, oily products have been isolated in experiments with water containing solvents.

In neutral anhydrous solution N_1-substituted 4-alkylpyrazol-5-ones are oxidised by tert. butylhydroperoxide to the corresponding bis-pyrazolones whereas pyrazolones unsubstituted at N_1 are oxidised to unidentified oily substances.

In the preceding paper in this series was shown that 4-alkylsubstituted pyrazol-5-ones dissolved in methanol are oxidised by oxygen in the presence of an excess of triethylamine, forming a 4-alkyl-4-hydroxysubstituted pyrazol-5-one and triethylamine oxide, 1 mole of oxygen being consumed per mole of pyrazolone. The stoichiometry of this reaction was found to correspond to the following sequence of reactions (Py meaning a pyrazol-5-one molecule minus the two hydrogen atoms at C_4):

$$\mathrm{PyRH} + (\mathrm{C_2H_5})_3\mathrm{N} \rightleftharpoons \mathrm{PyR^-} + (\mathrm{C_2H_5})_3\overset{+}{\mathrm{N}}\mathrm{H} \quad (1)$$

$$\mathrm{PyR^-} + \mathrm{O_2} \rightleftharpoons \mathrm{PyROO^-} \quad (2)$$

$$2\ \mathrm{PyROO^-} + 2\ (\mathrm{C_2H_5})_3\overset{+}{\mathrm{N}}\mathrm{H} \rightleftharpoons \mathrm{PyROH}\ldots\bar{\mathrm{O}}\ldots\mathrm{HORPy} + (\mathrm{C_2H_5})_3\mathrm{NO} \quad (3)$$

$$\mathrm{PyROH}\ldots\bar{\mathrm{O}}\ldots\mathrm{HORPy} + \mathrm{H_2O} \rightarrow 2\ \mathrm{PyROH} + (\mathrm{C_2H_5})_3\mathrm{N(OH)_2} \quad (4)$$

The substance presumed as the result of reaction (2) is a hydroperoxide ion. The formation of hydroperoxides is well known from investigations of the oxidation by air of aliphatic or alicyclic unsaturated hydrocarbon systems

* VII: *Acta Chem. Scand.* 8 (1954) 1007.

(*cf. e. g.* Bolland [1]), hydrogen atoms in α-position to the double bond being attacked by an oxygen molecule, resulting in the formation of a hydroperoxide group. In the pyrazol-5-one the hydrogen at C_4 is in α-position both to the C = N double bond in the pyrazolone nucleus and to the C = O double bond outside the nucleus and is therefore strongly activated. This applies to both 4-unsubstituted and to 4-monosubstituted pyrazol-5-ones, but only in the latter case has it been possible to isolate oxidation products of definite structure after treatment of the primary reaction product with water or hydrochloric acid.

In order to get some evidence as to the possibility of alkylhydroperoxide ions to react with alkylpyrazolone ions we investigated the reaction between 4-alkylsubstituted pyrazol-5-ones and tertiary butylhydroperoxide in alcoholic solution containing sodium ethoxide. This method proved to be an excellent method for preparing 4-alkyl-4-hydroxysubstituted pyrazol-5-ones. Here, no doubt, the reaction is:

$$PyR^- + (CH_3)_3COO^- \rightarrow PyRO^- + (CH_3)_3CO^-$$

or

$$PyRH + (CH_3)_3COOH \rightarrow PyROH + (CH_3)_3COH$$

followed by:

$$PyRO^- + Na^+ \rightarrow PyRONa$$

or

$$PyROH + C_2H_5ONa \rightarrow PyRONa + C_2H_5OH$$

We are inclined to adopt the first mentioned of these possibilities as the oxidation of 4-alkylsubstituted pyrazolones in neutral ethanolic solution, at any rate in some cases, leads to the bis-pyrazolone:

$$2\ PyRH + (CH_3)_3COOH \rightarrow PyR{-}RPy + (CH_3)_3COH + H_2O.$$

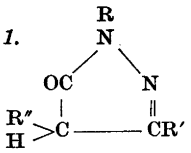
The readiness with which the pyrazolones are oxidised according to the proposed scheme is dependent on substituents present at N_1, at C_3 and at C_4, and besides on the solvent, the reactions proceeding more unambiguously with sodium ethoxide in anhydrous ethanol or in tertiary butanol than in 90 % ethanol. In the latter case pyrazolones alkyl- or arylsubstituted at N_1 (and besides substituted at C_3 and C_4) react normally, forming the 4-hydroxy-substituted pyrazolones, whereas pyrazolones unsubstituted at N_1 consume tertiary butylhydroperoxide, but the oxidation products are heavy oils which cannot be purified by distillation in vacuo (*ca.* 1 mm Hg). These oils were not further investigated; they possibly are the result of an oxidative or a hydrolytic cleavage of the pyrazole-ring.

In anhydrous neutral solution the oxidation of pyrazol-5-ones with tert. butylhydroperoxide results in the formation of bispyrazolones when the pyrazolone is substituted at N_1 whereas oils are formed when the pyrazolone is unsubstituted at N_1.

EXPERIMENTAL PART *

The pyrazolones investigated in this paper are tabulated in Table 1 which records at the same time yield, m.p. and analysis of the 4-hydroxypyrazolones formed.

* All m. p.'s uncorrected; all microanalyses by Mr. W. Egger or Mr. P. Hansen, Department of Organic Chemistry, The University, Copenhagen.

Table 1.  *Oxidation of pyrazolones with tert. butyl hydroperoxide and analysis of the 4-hydroxypyrazolones*

	R	R′	R″	Yield %	M. p. °C	Hydroxypyrazolone · Formula	Analysis	% C	% H	% N
I[2]	C_6H_5	CH_3	CH_3	44[a]	105	$C_{11}H_{12}O_2N_2$	Calc.	64.68	5.93	13.72
							Found	64.72	5.86	13.89
II[2]	C_6H_5	CH_3	C_2H_5	70	130	$C_{12}H_{14}O_2N_2$	Calc.	66.04	6.47	12.84
							Found	66.17	6.59	12.77
III[3]	C_6H_5	CH_3	*n*-C_3H_7	65	116—117	$C_{13}H_{16}O_2N_2$	Calc.	67.20	6.94	12.06
							Found	67.32	7.21	11.96
IV[3]	C_6H_5	CH_3	*n*-C_4H_9	65	95	$C_{14}H_{18}O_2N_2$	Calc.	68.27	7.37	11.38
							Found	68.34	7.18	11.50
V[4]	C_6H_5	CH_3	$C_6H_5CH_2$	95—100[b] 43[c]	145	$C_{17}H_{16}O_2N_2$	Calc.	72.85	5.75	10.00
							Found	72.81	5.64	10.23
VI[5]	C_6H_5	*Cyclo*hexano		33	140	$C_{13}H_{14}O_2N_2$	Calc.	67.81	6.13	12.17
							Found	68.08	6.03	12.26
VII[6]	CH_3	CH_3	C_2H_5	50	39	$C_7H_{12}O_2N_2$	Calc.	53.84	7.70	17.94
							Found	53.93	7.81	18.05
VIII[7]	CH_3	CH_3	$C_6H_5CH_2$	80	155—156	$C_{12}H_{14}O_2N_2$	Calc.	66.04	6.47	12.84
							Found	66.24	6.32	12.89
IX[8,9]	H	CH_3	CH_3	d	96—97	$C_5H_8O_2N_2$	Calc.	46.89	6.29	21.87
							Found	46.94	6.21	21.70
X[9,10]	H	CH_3	C_2H_5	77	128—130	$C_6H_{10}O_2N_2$	Calc.	50.67	7.09	19.71
							Found	50.20	7.19	19.70
XI[9,11]	H	CH_3	*n*-C_3H_7	65	117—118	$C_7H_{12}O_2N_2$	Calc.	53.84	7.70	17.94
							Found	53.75	7.78	17.83
XII[12]	H	CH_3	C_3H_5	65	103—104	$C_7H_{10}O_2N_2$	Calc.	54.53	6.53	18.18
							Found	54.28	6.43	17.93
XIII	H	CH_3	*n*-C_4H_9	65	101—102	$C_8H_{14}O_2N_2$	Calc.	56.47	8.29	16.47
							Found	56.63	8.24	16.43
XIV	H	CH_3	C_6H_{11}	86	140	$C_{10}H_{16}O_2N_2$	Calc.	61.20	8.22	14.28
							Found	61.36	8.32	14.36
XV[13]	H	CH_3	$C_6H_5CH_2$	60	143—144	$C_{11}H_{12}O_2N_2$	Calc.	64.68	5.93	13.72
							Found	64.73	6.03	13.70
XVI[6]	H	CH_3	C_6H_5	70	190	$C_{10}H_{10}O_2N_2$	Calc.	63.15	5.30	14.73
							Found	63.23	5.20	15.13
XVII[5]	H	*Cyclo*hexano		15	195—197	$C_7H_{10}O_2N_2$	Calc.	54.53	6.53	18.18
							Found	54.70	6.81	18.28
XVIII[14]	H	C_6H_5	$C_6H_5CH_2$	95	185—186	$C_{16}H_{14}O_2N_2$	Calc.	72.17	5.30	10.52
							Found	72.04	5.15	10.74

a) Methanolic solution
b) Anhydrous solution
c) 10 % of water present
d) Obtained only by oxidation by air, not with tert. butylhydroperoxide

All the pyrazolones were prepared by current methods and showed the m.p.'s indicated in the literature. For XIII and XIV no indications were found and we, therefore, indicate in Table 2 yield, m.p. and analyses of these substances, prepared from hydrazine hydrate and the corresponding *a*-substituted ethyl acetoacetate as indicated by Veibel *et al.*[6].

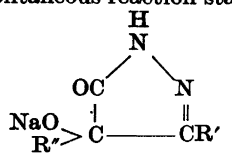
Table 2. Data for the pyrazolones XIII and XIV.

	R	R′	R″	Yield %	M.p.	Formula	Analysis	% C	% H	% N
XIII	H	CH_3	C_4H_9	95	197–98°	$C_8H_{14}ON_2$	Calc.	62.30	9.15	18.17
							Found	62.50	9.18	18.35
XIV	H	CH_3	C_6H_{11}	95	225–26°	$C_{10}H_{16}ON_2$	Calc.	66.63	8.95	15.55
							Found	66.86	9.09	15.67

1. Oxidation with tert. butylhydroperoxide in anhydrous solution in the presence of sodium ethoxide

As examples the oxidation of 3-methyl-4-ethylpyrazol-5-one (X) and of 3-phenyl-4-benzylpyrazol-5-one (XVIII) are described.

6.3 g (0.05 mole) of X, 30 ml of 2 *N* sodium ethoxide in ethanol (0.06 mole), 75 ml of tertiary butanol and 7.5 ml (0.08 mole) of tert. butylhydroperoxide, prepared as indicated by Milas and Surgenor [15], are mixed at room temperature. A spontaneous reaction starts which causes a slow increase of temperature of the mixture, 35–40° being reached after *ca.* 30 minutes and maintained there for a further hour during which the flask is shaken at intervals. A precipitate separates which after cooling is filtered off and dried over night in a vacuum desiccator. Yield 6.6 g. From the mother liquor further 0.8 g were obtained. Total yield 7.4 g = 90 %, the substance presumed to be XIX, M = 164.1.



XIX: R′ = CH_3, R″ = C_2H_5
XX: R′ = C_6H_5, R″ = $C_6H_5CH_2$

Dissolved in glacial acetic acid the substance may be titrated with perchloric acid, 0.1642 g using up 9.90 ml 0.1000 *N* perchloric acid. M calc. 164.1, found 165.7, *i.e.* purity of the substance 99 %.

The value of pK_B cannot be estimated from the titration curve, the hydroxypyrazolone ion being so strong a base that acetate ions are quantitatively liberated when the sodium salt is dissolved in glacial acetic acid so that the titration curve is practically that of the acetate ion, not that of the hydroxypyrazolone ion (*cf.* Fritz [16]). In Table 3 are recorded the potentiometer readings by titration of the sodium salts of the oxidation products of X and XIV and of acetic acid, one millimole being titrated in each case. It is seen that the figures obtained are identical within the limit of error (purity of the anhydrous sodium acetate and of the sodium salt of the oxidation product of XIV 98 %, of the oxidation product of X 99 %).

The hydroxypyrazolone itself is, as are all the other 4,4-disubstituted pyrazol-5-ones investigated, too weak a base to be titratable with perchloric acid in glacial acetic acid. It is, on the other hand, too weak an acid to be titrated with sodium hydroxide, even if its sodium salt may be isolated in the manner indicated.

The sodium salt is soluble in methanol. Excess methanolic hydrochloric acid is added, sodium chloride precipitated and filtered off. From the filtrate the solvent is removed *in vacuo* (bath temperature *ca.* 40°). The residue crystallises and is purified by recrystallisation from an ethyl acetate-petroleum ether mixture. Yield 85 % m.p. 128–130°. The substance is rather soluble in water, readily soluble in alcohols. For analysis, see Table 1.

The overall yield of the 4-ethyl-4-hydroxypyrazolone from 4-ethyl-pyrazolone is 77 %.

7.5 g (0.03 mole) of XVIII are dissolved in a mixture of 18 ml of 2 *N* sodium ethoxide (0.036 mole) and 40 ml of tert. butanol by heating. 5 ml (0.055 mole) of tert. butylhydroperoxide are added and the mixture kept for 5 minutes at 70° when a solid suddenly precipitates. The mixture is kept at 70° for a further quarter of an hour and shaken at intervals, and then left for a couple of hours at room temperature. When cold the sodium salt is filtered off with suction and dried in a stream of air. Yield quantitative.

0.2902 g of XX are dissolved in 50 ml of glacial acetic acid and titrated with 0.1 *N* perchloric acid in glacial acetic acid. M calc. 288.3, found 290. As for XIX the titration

Table 3. Titration of the sodium salts of the oxidation products of X and XIV and of acetic acid with perchloric acid in glacial acetic acid.

ml 0.1000 *N* perchloric acid in glacial acetic acid	Sodium salts of the oxidation products of X	Sodium salts of the oxidation products of XIV	Sodium acetate
	MV	MV	MV
0.0	93	92	93
1.0	102	101	102
2.0	110	110	109
3.0	117	117	116
4.0	124	124	123
5.0	132	133	131
6.0	141	141	140
7.0	151	152	150
8.0	164	165	165
9.0	188	192	190
9.5	213	231	224
10.0	333	353	355
10.5	369	376	377
11.0	381	386	387
13.0	401	402	404

curve is not that of the hydroxypyrazolone ion but that of the acetate ion. The sodium salt is readily soluble in methanol.

When the salt (8.4 g) is dissolved in water (50 ml) with slight heating another solid suddenly separates out. Evidently the sodium salt is hydrolysed by water. The solution is neutralised with acetic acid, the precipitate filtered off and dried in a vacuum desiccator. Yield 7.5 g (95 %); m.p. 185–86°. Recrystallised from aqueous acetone (50 %) the hydroxypyrazolone is obtained as fine felted needles, from water as a microcrystalline powder; m.p. unchanged 185–86°. For analysis see Table 1.

The sodium salt may also be prepared by oxidation with tert. butylhydroperoxide in ethereal solution. 0.01 mole of the pyrazolone is dissolved in 6 ml of 2 *N* sodium ethoxide. 25 ml of ether are added and a clear solution obtained by shaking. Then 2 ml (0.02 mole) of tert. butylhydroperoxide are added and the solution refluxed for 6 hours on an infra-bath. The sodium salt of the 4-hydroxypyrazolone is precipitated in a more easily filterable form than by the procedure described above. Yield 80 %.

When the sodium salt is treated with acetic anhydride a diacetate is obtained. 2 g of the sodium salt are dissolved in 10 ml of acetic anhydride by heating. The solution is kept over night at room temperature. The next day ether is added to precipitate sodium acetate which is filtered off. The filtrate is distilled *in vacuo* to remove ether and excess acetic anhydride. An oily residue is left which readily crystallises. The crystals are dissolved in ethyl acetate and precipitated with petroleum ether. Yield 1 g (40 %); m.p. 122–23°. (Found: C 68.35; H 5.59; N 7.89. Calc. for $C_{20}H_{18}O_4N_2$: C 68.57; H 5.14; N 8.00.)

2. Oxidation with tert. butylhydroperoxide in aqueous-alcoholic solution in the presence of sodium ethoxide

Here the oxidation of 1-phenyl-3-methyl-4-benzylpyrazol-5-one (V) is described as an example.

13.2 g (0.05 mole) of V, 12 ml 5 *N* aqueous potassium hydroxide (0.06 mole), 50 ml of tert. butanol and 5 ml (0.055 mole) of tert. butylhydroperoxide are mixed and kept at room temperature for 24 hours. The solution assumes a faint yellow colour after 3–4 hours No evolution of heat is observed.

After 24 hours the mixture is acidified with conc. hydrochloric acid and *ca.* 50 ml of water are added. The solution is concentrated *in vacuo* (bath temp. *ca.* 50°) to remove the organic solvent. Hereby a somewhat sticky precipitate is formed which is filtered off and dried in a vacuum desiccator. Yield 11.3 g with m.p. *ca.* 90° which dissolved in ethyl acetate and precipitated with petroleum ether yield 6 g (43 %) of the pure substance with m.p. 145°.

Recrystallised from methanol the hydroxy compound is obtained in long rhombic crystals; m.p. unchanged. For analysis see Table 1.

When methanol is used as solvent instead of tert. butanol a yield of only 30 % is obtained. In anhydrous solution the yield is 95—100 % when the procedure outlined above is followed.

3. Oxidation with tert. butylhydroperoxide in neutral anhydrous solution

5 g (0.025 mole) of II are dissolved in 50 ml of ethanol. 5 ml (0.055 mole) of tert. butylhydroperoxide are added and the mixture refluxed for one hour after which 25 ml of water are added. A precipitate is formed which after 48 hours at room temperature is filtered off and dried. Yield 4.5 g (90 %) of 4,4'-bis(1-phenyl-3-methyl-4-ethylpyrazol-5-one), m.p. 160—165° (*cf.* Veibel and Westöö [17]).

When X is oxidised in this manner no bis-pyrazolone is obtained but only heavy oils which so far have not been further investigated.

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Studies on Antimetabolites

III. Synthesis of "*Neo*histamine", the β,β-Dimethyl Analogue of Histamine *

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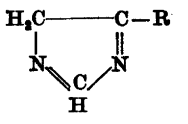
The β,β-dimethyl analogue (II) of histamine, a potential antihistaminic agent, and its N,N-dimethyl derivative (VI), have been prepared. The intermediate ethyl α-4(5)-imidazole*iso*butyrate (IV) was obtained by Bredereck and Theilig's reaction from ethyl γ-bromo-α,α-dimethylacetoacetate. The 4(5)-*tert*butylimidazole and α-4(5)-imidazole*iso*butyronitrile were analogously prepared. Reduction of α-4(5)-imidazole*iso*butyramide with lithium-aluminium hydride afforded "*neo*histamine". Similar treatment of the N,N-dimethylamide gave N,N-dimethyl-"*neo*histamine" (VI). Dimethylaminomethylation of methyl *iso*propyl ketone afforded a high yield of 4-(N,N-dimethylamino)-3,3-dimethylbutanone-2 (VII), which was brominated to (VIII). The attempted ring closure of VIII to VI was not successful.

Among the numerous compounds tested for antihistamine activity those, which are derivatives of histamine or at least contain an imidazole nucleus, deserve special attention, because of their obvious structural relationship to histamine (I) itself. In spite of this fact the number of imidazole derivatives tested is comparably low[1,2,3] and so far no potent histamine antagonist has been detected amongst them. Following up the investigations regarding the possibility of designing antimetabolites by the introduction of a suitably situated *gem*dimethyl group into the molecule of a metabolite, which proved successful in the group of plant growth substances discussed in Part I of this series[4], we have now prepared the α,α-dimethyl analogue (II) of histamine,for convenience called "*neo*histamine", and its N,N-dimethyl derivative. It is the purpose of the present paper to describe the synthetical work, the results of the physiological testing of the compounds will be discussed elsewhere.

The synthesis of 4(5)-alkylimidazoles by conventional methods requires such difficultly available intermediates as α-ketoalcohols, α-ketoaldehydes

* Part II, *Acta Chem. Scand.* 8 (1954) 1211.



I R = $CH_2CH_2NH_2$

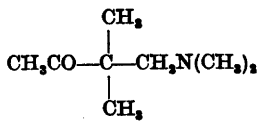
II R = $C(CH_3)_2CH_2NH_2$

III R = $C(CH_3)_3$

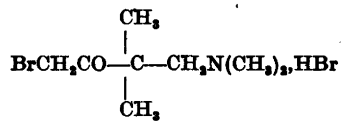
IV R = $C(CH_3)_2COOC_2H_5$

V R = $C(CH_3)_2CN$

VI R = $C(CH_3)_2CH_2N(CH_3)_2$



VII



VIII

or α-aminoketones [5]. Additional difficulties arise from the fact that in the present case the starting material should contain a *gem*dimethyl group adjoining the keto function. Several unsuccessful attempts were made to prepare "*neo*-histamine" along such lines. Recently, however, Bredereck and Theilig demonstrated [6], that α-haloketones are readily converted to imidazole derivatives when heated with formamide. This reaction opened a route to the synthesis of imidazoles carrying a quarternary side chain carbon atom at position 4(5), as it was found that the α-bromoketones required as intermediates could easily be prepared by bromination of the ketones, and directly converted to imidazole derivatives without isolation. In this way pinacolone, ethyl α,α-dimethylacetoacetate, and α,α-dimethylacetoacetonitrile were converted *via* their (not isolated) bromoderivatives into *tert*butylimidazole (III), ethyl α-4(5)-imidazole*iso*butyrate (IV), and α-4(5)-imidazole*iso*butyronitrile (V), respectively, and isolated as their hydrochlorides in 10—35 % yield.

"*Neo*histamine" was obtained from α-4(5)-imidazole*iso*butyric acid *via* the acid chloride and amide by reduction with lithium-aluminium hydride in tetrahydrofuran and isolated as the dihydrochloride. N,N-dimethyl-"*neo*-histamine" (VI) was prepared analogously *via* the N,N-dimethylamide and isolated as the dipicrate. Attempts were made to prepare this compound by ring closure from the hydrobromide of 1-bromo-4-(N,N-dimethylamino)-3,3-dimethylbutanone-2 (VIII), but no crystalline material could be isolated from the reaction mixture, which, however, gave a positive Pauly test suggesting that an imidazole derivative may have been formed. The intermediate bromo-hydrobromide (VIII) was obtained by bromination of the Mannich base (VII), 4-(N,N-dimethylamino)-3,3-dimethylbutanone-2, by the general method of Mannich and Gollasch [7]. The Mannich base (VII) was conveniently prepared by dimethylaminomethylation of methyl *iso*propyl ketone.

All the imidazole derivatives prepared gave a crimson colouration in the Pauly diazo reaction test [8].

EXPERIMENTAL *

α,α-Dimethylacetoacetonitrile. This compound has been described [9–11], but no details appear to be available for its preparation. — α-Methylacetoacetonitrile [11] (97 g) was added to a stirred solution of sodium (23 g) in anhydrous ethanol (300 ml), followed by methyl iodide (142 g). After standing overnight at room temperature the mixture was refluxed for 4 hours on the water bath. A solution of sodium (4.6 g) in anhydrous ethanol (75 ml) was added followed by methyl iodide (30 g). After refluxing for 6 hours most of the solvent was removed by distillation using a short column, the residual semi-solid mass pored into water, the oil taken up in ether, dried (Na_2SO_4) and fractionated. The yield of almost colourless oil, b. p. 162—165°, was 72 g (65 %). Lit. b. p. 163—164° [10,11]; 162°/748 mm [12].

Imidazole derivatives from α-bromoketones. General procedure. The keto compound (1.2 moles) was dissolved in carbon tetrachloride (200 ml) and brominated with stirring by slow addition of a solution of bromine (1.0 mole) in carbon tetrachloride (100 ml), the temperature being kept below 10° by external cooling. As soon as the bromine colour had disappeared the solvent and dissolved hydrogen bromide was carefully removed by distillation *in vacuo* on a water bath at maximum 30°. Formamide (400 ml) free from ammonia was added, and the mixture stirred at 160—180° for 4 hours. Excess formamide was distilled off *in vacuo* (maximum 190° in the bath), the semi-solid mass dissolved in a little water, made alkaline with solid potassium carbonate and continuously extracted with ether for 15 hours. The extract was distilled *in vacuo*, yielding faintly yellow oils, which only partly crystallised. They were dissolved in a little anhydrous ethanol, and their solutions saturated with dry hydrogen chloride. The hydrochlorides were obtained in crystalline state by careful addition of dry ether until a permanent cloudiness resulted.

4(5)-Tertbutylimidazole (III) (from pinacolone) was collected at 140—145°/7 mm. The *hydrochloride* formed colourless prisms from diethyl malonate, m. p. 168—169°. Yield 35 %. (Found: Cl 21.8; N 17.2, 17.3. $C_7H_{13}ClN_2$ requires: Cl 22.1; N 17.4.)

α-4(5)-Imidazoleisobutyronitrile (V) (from α,α-dimethylacetoacetonitrile) was collected at 140—200°/7 mm. The *hydrochloride* crystallised from ethanol-ether in prisms, m. p. 153—155°. Yield 10 %. (Found: Cl 20.4; N 24.3. $C_7H_{10}ClN_3$ requires: Cl 20.6; N 24.5.)

Ethyl α-4(5)-imidazoleisobutyrate (IV) (from ethyl α,α-dimethylacetoacetate [13]) was collected at 140—200°/7 mm. The *hydrochloride* formed prisms from ethanol-ether, m. p. 125—126°. Yield 25 %. (Found: Cl 15.8; N 12.6, 12.7; C_2H_5O 20.7. $C_9H_{15}ClN_2O_2$ requires: Cl 16.2; N 12.8; C_2H_5O 20.6.)

α-4(5)-Imidazoleisobutyric acid hydrochloride was obtained by refluxing the ethyl ester with excess 20 % hydrochloric acid and evaporation of the solvent *in vacuo* on the water bath. The residual syrup crystallised on standing for some hours. For analysis it was recrystallised from anhydrous ethanol-ether. Prisms, m. p. 183—184°. (Found: Cl 18.3; N 14.5, 14.6. $C_7H_{11}ClN_2O_2$ requires: Cl 18.6; N 14.7.) — An identical compound (mixed m. p.) was obtained by refluxing α-4(5)-imidazole*iso*butyronitrile (1.0 g) with 20 % hydrochloric acid (25 ml) for 4 hours, evaporation to dryness, dissolving the solid in anhydrous ethanol (25 ml) filtering from undissolved material, evaporation to dryness and crystallisation of the residual solid from ethanol-ether.

"Neohistamine", β,β-dimethylhistamine (II). Crude α-4(5)-imidazole*iso*butyric acid hydrochloride (from 29 g of the ethyl ester) was heated under reflux for 1/2 hour with thionylchloride (75 ml). Excess thionylchloride was evaporated, the last traces being removed *in vacuo*, and the residual oil was cautiously treated with concentrated aqueous ammonia (50 ml) with cooling in an ice bath. When the oil had dissolved, excess ammonia and water was completely removed by evaporation *in vacuo* on the water bath. The semi-solid residue was dried overnight under reduced pressure at 60° over phosphorus pentoxide. To the dry salt mixture there was added anhydrous tetrahydrofuran (300 ml) and, with stirring, powdered lithium-aluminium hydride (15 g) in small portions. After the addition the mixture was refluxed overnight on the water bath, and worked up by the general procedure of Amundsen and Nelson [14]. Water (16 ml) was carefully added with vigorous stirring, followed by 20 % aqueous sodium hydroxide (12 ml) and again water (55 ml). The organic layer was separated from the precipitated salts by filtration with

* All boiling and melting points uncorrected.

suction, and the filter cake was thoroughly washed with several portions of hot tetrahydrofuran. The combined filtrate and washings were concentrated to an oil, which was dissolved in anhydrous ethanol (50 ml) and saturated with dry hydrogen chloride. "*Neo*-histamine" dihydrochloride at once started to separate. After storing for some hours in the refrigerator, the crystals were collected and washed with ethanol. Yield 12.1 g. An additional amount was obtained by concentration of the mother liquor to a small volume. Total yield 12.9 g (46 %). The product was crystallised from methanol-ether. Prisms, m. p. 240–245° (decomp.). The compound was very soluble in water and soluble in methanol but almost insoluble in hot or cold ethanol. (Found: C 39.5; H 6.96; Cl 33.1; N 19.7. $C_7H_{15}Cl_2N_3$ requires: C 39.6; H 7.13; Cl 33.4; N 19.8.) The *dipicrate* formed long yellow needles from water, m. p. 209–211°. (Found: C 38.2; H 3.36; N 20.9. $C_{19}H_{19}N_9O_{14}$ requires: C 38.2; H 3.21; N 21.1.)

N,N-Dimethyl-"neohistamine", β,β,N,N-tetramethylhistamine (VI) was analogously prepared from ethyl α-4(5)-imidazole*iso*butyrate hydrochloride (11 g) *via* the N,N-dimethylamide (from 25 % aqueous dimethylamine). The dihydrochloride was obtained as a glass, which could not be induced to crystallise. It was dissolved in water (100 ml) and poured into a hot solution of picric acid (15 g) in water (700 ml). On cooling the *dipicrate* separated as long yellow needles. Yield 7.0 g. For analyses it was repeatedly recrystallised from water. M. p. 200–201°. (Found: C 40.2; H 3.76; N 20.0. $C_{21}H_{23}N_9O_{14}$ requires: C 40.3; H 3.71; N 20.2.) – The *dihydrochloride* was obtained by treatment of the picrate with concentrated hydrochloric acid, extraction of the picric acid with hot benzene, treatment with decolourising carbon and evaporation to dryness. A glass was obtained which slowly crystallised. Prisms from ethanol-ether. M. p. 207–210° (decomp.). (Found: C 45.0; H 7.99; Cl 29.6; N 17.4. $C_9H_{19}Cl_2N_3$ requires: C 45.0; H 7.97; Cl 29.5; N 17.5.)

4-(N,N-Dimethylamino)-3,3-dimethylbutanone-2 (VIII) Methyl *iso*propyl ketone (725 g), dimethylamine hydrochloride (240 g), 40 % aqueous formaldehyde (260 ml) and ethanol (500 ml) were mixed and heated at reflux on the water bath overnight. The mixture, which initially formed two layers, slowly became homogenous. Volatile products were distilled off on the water bath under reduced pressure, the re idual syrupy mass or crystalline cake was dissolved in water, made strongly alkaline with 6 *N* sodium hydroxide, ice being added to prevent an excessive raise in temperature, and the free base extracted with ether. The combined extracts were washed and dried (K_2CO_3). The ether was removed and the residual oil fractionated *in vacuo* using an efficient column. The Mannich base formed a colourless liquid, b. p. 54–56°/10 mm. (Found: N 9.56; neutr. equiv. 143. $C_8H_{17}NO$ requires: N 9.78; neutr. equiv. 143.2.) The amine gave a positive iodoform test. – *Hydrochloride*, colourless needles from acetone, m. p. 146–146.5°. (Found: Cl 19.9; N 7.7. $C_8H_{18}ClNO$ requires: Cl 19.7; N 8.0). *Semicarbazone* of the free amine, needles from water, m.p. 132–133°. (Found: C 54.1; H 9.87; N 27.6. $C_9H_{20}N_4O$ requires: C 54.0; H 10.0; N 28.0.) *Methiodide*, from the free amine and excess methyl iodide in ethanol, crystal powder which decomposed at about 182° without melting. (Found: I 44.5; N 5.0. $C_9H_{20}INO$ requires: I 44.4; N 4.9.)

Hydrobromide of 1-bromo-4-(N,N-dimethylamino)-3,3-dimethylbutanone-2. (VIII). The aminoketone (VII) (143 g) was dissolved in glacial acetic acid (200 ml), 66 % aqueous hydrobromic acid (100 ml) added with cooling followed by a solution of bromine (160 g) in glacial acetic acid (100 ml). The mixture was heated on a water bath until the bromine colour had disappeared. The solvent was removed *in vacuo* on the water bath, and the syrupy residue at once dissolved in acetone (400 ml). (It is important to avoid heating this mixture for an unnecessarily long time, as this causes decomposition of the bromoketone.) On cooling a crystalline product was obtained, which was isolated and thoroughly washed with acetone. Crystallisation from ethanol-ether yielded glistening prisms (192 g, 65 %) m. p. 130–132°. (Found: Br 52.2; N 4.65. $C_8H_{17}Br_2NO$ requires: Br 52.8; N 4.62.)

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Studies on the Extraction of Metal Complexes

XVIII. The Dissociation Constants and Partition Coefficient of Tropolone

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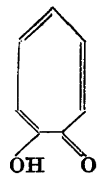
For the elaborate treatment of our metal extraction data it is always necessary to have accurate values for the dissociation constants and partition coefficients of the organic acids which are used as complex forming and extracting agents for the metal ions. Such values are very often not available and must therefore be determined. All our experiments are carried out at 25° C and at 0.1 *M* constant ionic strength (mainly sodium perchlorate) in the aqueous phase — here the organic phase was chloroform.



Tropolone

EXPERIMENTAL

Very small changes were made in the set up described earlier by the author [1]. The two main changes were: the 0.1 *M* $NaClO_4$/*4* M $NaCl/Hg_2Cl_2$, Hg reference electrode, used together with a glass electrode, was exchanged for a common saturated KCl/Hg_2Cl_2, Hg electrode, and further all chloroform used was made alcohol-free and water-saturated.

A Beckman Model DU spectrophotometer with 1 cm cells was used for the light absorption measurements. Small impurities in the distilled water changed the spectra considerably when the concentration of tropolone was small; however, these impurities were removed by filtering the water through an anion-cation exchange column.

The tropolone, $C_7H_6O_2$, was kindly prepared by Dr. Hans Fernholz, University of Heidelberg — most of it in the form of barium tropolonate. Tropolone was prepared from the Ba salt [2] by adding 0.5 *M* HCl, extracting the tropolone with $CHCl_3$, and drying the $CHCl_3$ with Na_2SO_4. The $CHCl_3$ was distilled off and the tropolone was sublimed at 50—60° C and 14 mm Hg by the cold-finger technique. Melting point: 49—50°.

DETERMINATION OF THE FIRST DISSOCIATION CONSTANT

It has been pointed out by Doering and Knox [3] and also by James and Speakman [4] that tropolone can act as a base, *i.e.* forms a tropolonium ion:

$$H_2T^+ \rightleftharpoons H^+ + HT$$

$$k_1 = \frac{[H^+] \cdot [HT]}{[H_2T^+]} \approx K_1 \qquad (1)$$

Doering and Knox [3] found a marked dependence of the light absorption of acid tropolone solutions on pH at 420—470 mμ. Unexplainably enough, we did not obtain such large changes of light absorption with pH in this wave-length region * (a 0.03 *M* tropolone — 1 *M* $HClO_4$, or 1 *M* HCl, solution is almost colorless). Minor changes at 350—370 mμ (Table 1) however permitted the calculation of this first dissociation constant in 3 *M* $HClO_4$ + $NaClO_4$. It may be assumed that this constant does not change considerably with the ionic strength, as is usual for univalent cationic acids.

Table 1. Light absorption of 10^{-4} M tropolone in 3 M $HClO_4$ + $NaClO_4$ solutions.

Experiment No.	$[H^+]$ *M*	$[Na^+]$ *M*	$[ClO_4^-]$ *M*	Absorption *A* at 355	360 mμ	365
1	3	0	3	0.258	0.158	0.103
2	2	1	3	0.290	0.200	0.145
3	1	2	3	0.360	0.280	0.220
4	0.5	2.5	3	0.430	0.360	0.290
5	0.2	2.8	3	0.500	0.455	0.380
6	0.1	2.9	3	0.520	0.485	0.418
7	0.01	2.99	3	0.565 =	0.540 =	0.460 = A_0

Both HT and H_2T^+ absorb more or less light in the 350—370 mμ range. This leads to the following equation for the total light absorption *A* at a certain wave-length:

$$A = \varepsilon_0 \cdot [HT] + \varepsilon_+ \cdot [H_2T^+] \qquad (2)$$

Using the following relations for the total concentration of tropolone, which was kept constant and equal to 10^{-4} *M*,

$$C_T = [HT] + [H_2T^+] \qquad (3)$$

$$\varepsilon_0 \cdot C_T = A_0 \qquad (4)$$

$$\varepsilon_+ \cdot C_T = A_+ \qquad (5)$$

together with equations (1) and (2) we obtain

$$k_1 = [H^+] \cdot \frac{A - A_+}{A_0 - A} \qquad (6)$$

or

$$[H^+] \cdot A_+ + k_1 \cdot A_0 - k_1 \cdot A - [H^+] \cdot A = 0 \qquad (6a)$$

* The author has measured the absorption spectra of tropolone in chloroform and in aqueous solutions of different pH-values. Apart from the measurements of acid solutions above 400 mμ, all of our results were consistent with those obtained previously by other authors [3,5-7].

The data in Table 1 can be treated in different ways. The author chose two very simple methods: In the first method A_+ was chosen so that a log $[H^+]$ *versus* log $\frac{A_0-A}{A-A_+}$ plot gave the best fit with a line of unit slope. The final A_+-values used were: for 355 mμ 0.155, for 360 mμ 0.020, and for 365 mμ 0. The method gave $pk_1 = 0.00$, 0.00 and 0.04 respectively.

The second method consists of arranging the seven equations (6a) for each wave-length in three sets (experiment 1 + 2, 3 + 4, and 5 + 6 + 7, see Table 1), forming the sum of the equations of each set, and treating the three equations so obtained as normal equations (unknowns A_+, $k_1 \cdot A_0$ and k_1). This method gave $pk_1 = -0.01$, + 0.06, and + 0.07 for 355, 360, and 365 mμ respectively.

Mean value: $pk_1 = 0.03 \pm 0.04$.

DETERMINATION OF THE SECOND DISSOCIATION CONSTANT

The thermodynamic dissociation constant of tropolone has been determined by James and Speakman [4] to be $K_2 = 10^{-7.00 \pm 0.04}$ at 20° C. Bryant, Fernelius and Douglas [8] report $10^{-6.97 \pm 0.02}$ at 30° C. Both these values were found by potentiometric titrations using glass electrodes. Doering and Knox [3] give a value of $pK_2 = 6.7$.

By potentiometric titrations at 25° C of 0.005 *M* HT + 0.005 *M* $HClO_4$ in 0.1 *M* $NaClO_4$ with 0.1 *M* carbonate-free NaOH as described earlier [1] the following values for pk_2 were obtained:

6.696
6.702 mean value: 6.706 ± 0.011
6.709
6.717

Considering the influence of the ionic strength (0.1 *M*) on $k_2 = [H^+] \cdot [T^+]/[HT]$ and the difference in temperature, there is no serious disagreement between this pk_2 value and the pK_2 values given above.

DETERMINATION OF k_2/k_d BY TWO-PHASE TITRATION

In a two-phase system as is the case for metal extraction the ligand concentration $[A^-]$ will depend not only on k_2 (and on k_1 in very acid solutions) but also on the partition coefficient of HT, $k_d = [HT]_{org}/[HT]$ *, between the two liquid phases. If k_d is rather high, the relevant constant k_2/k_d may be obtained by potentiometric, two-phase titrations as earlier shown by the author[1]. Four such titrations gave the following values for $pk_2 + \log k_d$:

8.401
8.407 mean value: 8.415 ± 0.013
8.425
8.426

log k_d for chloroform is then 1.709 ± 0.017; $k_d = 51.2 \pm 2.0$.

* The symbol *p* in ref. 1 has been replaced by k_d.

DETERMINATION OF THE PARTITION COEFFICIENT BETWEEN CHLOROFORM AND WATER

Since the partition coefficient k_d was not too high a direct determination of the partition coefficient could be made by analysing the aqueous phase spectrophotometrically at 320 mμ. In three experiments 15 ml of a 0.025 M solution of tropolone in $CHCl_3$ were shaken with 15 ml of 0.01 M $HClO_4$ + 0.09 M $NaClO_4$. The following results for k_d were calculated from the analysis data:

49.6
50.5 mean value 50.4 ± 0.8; $\log k_d = 1.702 \pm 0.007$
51.1

This agrees very well with the value obtained above from k_2 and k_2/k_d.

Beer's law proved to be valid for $CHCl_3$ solutions of tropolone at 320 mμ, and no concentration dependence of k_d was observed by the twophase titrations. Molecular-weight determinations of tropolone in organic solvents show that the compound is monomeric [5,9], and since there is a strong intramolecular hydrogen bonding in tropolone [10], it can be assumed that tropolone does not associate in the organic phase.

SUMMARY

Potentiometric and spectrophotometric determinations were used for the determination of the dissociation constants and partition coefficient of tropolone at 25° C. The following values were obtained:

$pk_1 = 0.03 \pm 0.04$ ($I = 3$ M)
$pk_2 = 6.71 \pm 0.01$ ($I = 0.1$ M)
$k_d = 50.4 \pm 0.8$ for chloroform/water ($I = 0.1$ M)

The ionic strength I in the aqueous phase was kept constant using $HClO_4$, $NaClO_4$ and NaOH.

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The Solubility of Carbon Dioxide, Oxygen, Carbon Monoxide and Nitrogen in Polar Solvents

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In recent papers it was shown that the solubility of nitrogen [1], chlorine [2], hydrogen, oxygen and carbon monoxide [3], carbon dioxide [4], helium, argon, methane and ethane [5] in non-polar solvents can be predicted with fair accuracy from the equation

$$-\log x_2 = -\log x_2^i + \log \frac{\overline{V}_2}{V_1} + 0.4343\left(1-\frac{\overline{V}_2}{V_1}\right) + \frac{\overline{V}_2}{2.303\,RT}(\delta_1-\delta_2)^2 \quad (1)$$

In this equation, x_2 denotes the solubility in mole fraction, x_2^i the "ideal" solubility, $\overline{V}_2$ the partial molal volume of the dissolved gas, V_1 the molal volume of the solvent, δ_1 and δ_2 solubility parameters of solvent and gas, respectively, R the gas constant and T absolute temperature. With regard to the derivation of equation (1) reference should be made to Hildebrand and Scott [6].

Equation (1) is derived on the assumption that the molecules are non-polar and that chemical reactions are absent. If a non-polar gas is dissolved in a polar solvent, we will expect a higher value for the solubility than is calculated from equation (1). A preliminary examination [3] for hydrogen, oxygen and carbon monoxide confirmed this expectation and showed that the difference between experimental values and values calculated from equation (1) was related to the dielectric constant (ε) for the solvent. It is the aim of the present paper to provide a more detailed knowledge of the influence of the polarity of the solvent. For this purpose we have chosen twelve solvents from weakly polar (*e.g.* toluene with $\varepsilon = 2.37$) to strongly polar (*e.g.* nitrobenzene with $\varepsilon = 34.9$) excluding, however, strongly associated solvents, *e.g.* alcohols and water. In the last section, it is shown that among the chosen solvents only acetone is somewhat associated . The other solvents (toluene, chloroform, 1,2-dibromoethane, chlorobenzene, *n*-propyl acetate, aniline, methyl acetate, pyridine, benzyl cyanide, propionitrile and nitrobenzene) are considered to be either non-associated or very little associated.

APPARATUS, MATERIALS AND PROCEDURE

Solubility determinations in the solvents mentioned above have been carried out for the two gases carbon dioxide and carbon monoxide only. The solubility of oxygen and nitrogen was taken from the literature (*cf.* Table 4). The majority of the solubility measurements were carried out in the same apparatus and using the same procedure as described in a previous paper [3]. The solubility of carbon dioxide in chlorobenzene was also determined by titration of the saturated solution, and in the solvents benzyl cyanide and nitrobenzene titration only was used. The procedure was the following: a glass cylinder with the solvent was placed in a water thermostat at 25° C. Carbon dioxide from a cylinder (99.61 % CO_2) was bubbled through until saturation. A portion of the saturated solution (corresponding to about 8 ml 0.2 *M* sodium hydroxide) was drawn off by means of a bended glass tube into a 50 ml measuring glass filled with nitrogen and containing 20.00 ml of 0.2 *M* sodium hydroxide. The amount of saturated solution was determined by weighing. The measuring glass was shaken violently, and the contents were quantitatively transferred to a 500 ml flask filled with nitrogen. After heating to about 80° C, 10.00 ml of 0.2 *M* hydrochloric acid was added and then about a ten percent' excess of barium chloride solution. After cooling to room temperature, the solution was titrated with 0.2 *M* hydrochloric acid in a nitrogen atmosphere. The results were corrected for blank values, which were less than ten percent.

The carbon dioxide used in the volumetric determinations was prepared by adding concentrated sulphuric acid to potassium bicarbonate (analytical reagent, Judex). After being passed through concentrated sulphuric acid, the carbon dioxide was collected in an all-glass gasometer with mercury as closing liquid. The apparatus is described in a previous paper [7]. The gasometer was filled and emptied until sampling showed that the gas was pure. The analyses were carried out in a gas analysis apparatus as devised by Christiansen and Wulff [8]. The carbon dioxide employed contained between 99.8 and 99.9 % CO_2. The solubility determinations were corrected for the small amount of atmospheric air, *cf.* the previous paper [4].

Carbon monoxide was prepared by adding concentrated sulphuric acid to formic acid (analytical reagent, Merck). The carbon monoxide was led through one glass spiral washing bottle containing 40 % sodium hydroxide and another containing concentrated sulphuric acid and was then collected in the mercury gasometer. The gas employed contained between 99.6 and 99.9 % CO. In this case it was superfluous to correct the solubility experiments since carbon monoxide and atmospheric air have nearly the same solubility in the solvents.

The following solvents were purified by fractionated distillation in a wire-gauze column as devised by Klit [9]. The fractions employed showed the following boiling points (760 mm) and index of refraction: *toluene* (analytical reagent, Riedel-de Haën) bp. 110.75°—110.80°, n_D(25.1°) 1.4936—1.4938, *chloroform* (analytical reagent, Merck) bp. 61.15°—61.16°, n_D(20°) 1.4460—1.4461, *chlorobenzene* bp. 131.95°—131.98°, n_D (20°) 1.3840—1.3841, *normal propyl acetate* (Judex chemicals) bp. 101.66°—101.70°, n_D (20°) 1.3846, *methyl acetate* bp. 56.72°—56.85°, n_D (20°) 1.3618, *pyridine* bp. 115.51°—115.55°, n_D (20°) 1.5100—1.5101, *acetone* (analytical reagent, Merck) bp. 56.12°—56.14°, n_D (20°) 1.3588—1.3589 and *propionitrile* (Rubber Industries and Sherman Chemicals) bp. 97.31°—97.35°, n_D (20°) 1.3664—1.3665.

Benzyl cyanide and aniline (puriss Merck) was purified by fractional distillation in vacuum. The fractions used had the following constants: *benzyl cyanide* bp. 233.49°—233.55°, n_D (20°) 1.5233 and *aniline* bp. 184.32°—184.34°, n_D (20°) 1.5864. *Nitrobenzene* was first purified by fractional freezing and then by fractional distillation in vacuum; the fractions employed showed bp. 211.94°—211.95°, n_D (25°) 1.5524—1.5527 and the melting point 5.75° C.

1,2-dibromoethane (Merck) was purified by fractional freezing. The samples employed showed mp. 9.5°—9.8° C, n_D (20°) 1.5390 and the density 2.179 at 20.6° C.

Pertinent data for vapour pressures and densities used in the calculations of the solubility experiments were taken from international Critical Tables.

Table 1. Solubility of carbon dioxide and carbon monoxide. Experimental values expressed in Bunsen absorption coefficient (α) at 25.0° C.

Solvent	Carbon dioxide	Carbon monoxide
Toluene	2.22 2.20	0.172 0.170
Chloroform	3.59 3.58	
1,2-Dibromoethane	2.00 2.00	0.0771 0.0785
Chlorobenzene	2.16, 2.17 * 2.18 2.14 ** 2.16 **	
n-Propyl acetate	4.84 4.80	0.226 0.228
Aniline	1.22 1.18	0.0465 0.0462, 0.0465 *
Methyl acetate	6.47 6.39 6.42	
Pyridine	3.38 3.34	0.107 0.107
Benzyl cyanide	2.07 ** 2.03 **	0.0692, 0.0687 * 0.0698, 0.0698 *
Acetone	6.48 6.45 6.43 6.34 6.49	
Propionitrile	5.39 5.38	0.199 0.199
Nitrobenzene	2.23 ** 2.17 **	0.0819, 0.0823 * 0.0808, 0.0805 * 0.0814, 0.0808 *

* indicates that the equilibrium has been established at different pressures.
** results obtained by titration.

EXPERIMENTAL RESULTS

The results of the solubility determinations for carbon dioxide and carbon monoxide are given in Table 1.

In the calculations of the experimental values for the solubility of carbon dioxide in acetone and methyl acetate, it was necessary to introduce a correction for dissolved gas in the minor amount of solvent which was placed in the gas burette.

It appears from Table 2, which includes solubilities found by other investigators, that the results for *carbon dioxide* in 1,2-dibromoethane, aniline, pyridine and nitrobenzene are a confirmation of results obtained by Just [10] and Kunerth [11]. Our results in toluene, chlorobenzene, methyl acetate, acetone and chloroform were, respectively, about 5, 5, 8, 12 and 15 % higher than the values given by Just.

Our results for the solubility of *carbon monoxide* in aniline and nitrobenzene are about 5 % lower than stated by Just.

The degassing of benzyl cyanide and nitrobenzene was done very carefully since these two solvents have very high boiling points, 234° C and 211° C, respectively. In some experiments the degassing was carried out by evacuation with repeated freezing and melting the solvents; it turned out that experiments in which we did not freeze the solvent resulted in the same experimental value for the solubility.

The solubilities are recalculated into mole fraction in Tables 3 and 4.

CORRELATION WITH THEORY

Hildebrand and Scott [6,p.168] have developed the following expression for the heat of mixing, ΔH^M, for one mole mixture of two polar components

$$\Delta H^M = (x_1 V_1 + x_2 V_2)\,[(\delta_1 - \delta_2)^2 + (\omega_1 - \omega_2)^2]\,\Phi_1 \Phi_2 \qquad (2)$$

Φ_1 and Φ_2 is the volume fraction of solvent and solute, respectively. In equation (2) the δ's no longer have their usual meaning as the square roots of the cohesive energy densities, but must be calculated from

Table 2. Solubility of carbon dioxide and carbon monoxide expressed in Bunsen absorption coefficient at 25° C. Comparison with older measurements.

Solvent	Carbon dioxide			Carbon monoxide			
	Just [10]	Kunerth [11]	Present investigation	Just [10]	Skirrow [12]	Horiuti [13]	Present investigation
Toluene	2.11		2.21	0.166	0.167		0.171
Chloroform	3.14	3.15	3.58	0.179	0.189		
1,2-Dibromoethane	1.98	1.96	2.00				
Chlorobenzene	2.08		2.17				
Aniline	1.21	1.19	1.20	0.0491	0.0486		0.0464
Methyl acetate	5.95		6.43				
Pyridine	3.35	3.28	3.36				
Acetone	5.77	5.85	6.44	0.204	0.218	0.236	
Nitrobenzene	2.25		2.20	0.0858	0.0852		0.0813

$$\frac{\Delta E^v}{V} = \delta^2 + \omega^2 \tag{3}$$

Equation (3) expresses for each of the components the overall energy of vaporization as a sum of the energy (δ^2) arising from the dispersion forces and the energy (ω^2) arising from the dipole forces. The values of ω^2 are calculated from equation (10) in the next section. Since we are concerned in this paper with gases without permanent dipoles, equation (2) can be written

$$\Delta H^M = (x_1 V_1 + x_2 V_2)\ [(\delta_1 - \delta_2)^2 + \omega_1^2]\ \Phi_1 \Phi_2 \tag{4}$$

Using this equation and the approximations $\Phi_1 = 1$, $x_2 = n_2/n_1$ and $\Phi_2 = x_2 \bar{V}_2/V_1$, we find that the solubility can be calculated from equation (1) by adding $\bar{V}_2 \omega_1^2/2.303\ RT$. In a previous paper [4] it was shown, that the equation

$$-\log x_2 = -\log x_2^i + \frac{\bar{V}_2}{2.303\ RT}(\delta_1 - \delta_2)^2$$

accounts for the experiments somewhat better than equation (1). Consequently we may enquire whether the equation

$$-\log x_2 = \underbrace{-\log x_2^i + \frac{\bar{V}_2}{2.303\ RT}(\delta_1 - \delta_2)^2}_{A} + \underbrace{\frac{\bar{V}_2}{2.303\ RT}\omega_1^2}_{B} \tag{5}$$

is able to account for the experiments. If we use $\delta_1 = \delta'''$, *i.e.* a solubility parameter corrected for dipole attraction (*cf.* next section), we get the A-values given in column 7 in Table 3. The difference (Δ''') between A and the negative logarithm of the experimental value of the solubility is given in column 10. According to equation (5) Δ''' should be equal to B, which is given in the last column of the table. For pyridine and benzyl cyanide we get $B = \Delta'''$, but there are considerable divergences for the other solvents. A corresponding calculation for the solubility of carbon monoxide in the polar solvents gave the same qualitative result. The explanation of the failure of equation (5) may probably be found in the diverse assumptions made in its derivation. The solubility in the "basic" solvents aniline and pyridine will be discussed below.

It will be shown in the following that it is possible to account empirically for the measurements.

Values of A in equation (5) have been calculated (in Table 3) using $\delta_1 = \delta'$ and $\delta_1 = \delta''$, respectively. The differences between A-values and experimental values of $-\log x_2$ are given in column 8 and 9. On the whole these differences (Δ' and Δ'') increase in going from the less polar to the more polar solvents. Fig. 1 shows the Δ''-values as a function of the dipole moments (μ) of the solvents. The μ-values employed are given in Table 8. The points for toluene, chloroform, chlorobenzene, propyl acetate, methyl acetate, benzyl cyanide, propionitrile and nitrobenzene are grouped about a straight line through the zeropoint in the coordinate system.

Table 3. Solubility of carbon dioxide in polar solvents at 25° C. Comparison of experimental and calculated values.

$x_2^i = 0.0230$, $\bar{V}_2 = 47.7$ ml, $\delta_2 = 5.1$.

Solvent	V_1	Exp. value of $x_2 \cdot 10^4$	$-\log x_2$	A in eq. (5) calc. with δ'	δ''	δ'''	Δ'	Δ''	Δ'''	B in eq. (5)
$C_6H_5CH_3$	106.8	105	1.98	2.14	2.20	2.20	0.16	0.22	0.22	0.00
$CHCl_3$	80.7	128	1.89	2.18	2.24	2.17	0.29	0.35	0.28	0.13
1,2-$C_2H_4Br_2$	86.6	77.2	2.11	2.62	2.66	2.58	0.51	0.55	0.47	0.10
C_6H_5Cl	102.2	98.6	2.01	2.33	2.35	2.29	0.32	0.34	0.28	0.12
$CH_3COOC_3H_7$	115.6	245	1.61	2.02	2.09	2.03	0.41	0.48	0.42	0.14
$C_6H_5NH_2$	91.5	49.1	2.31	2.94	3.09	2.98	(0.63)	(0.78)	(0.67)	0.19
CH_3COOCH_3	79.9	226	1.65	2.17	2.32	2.20	0.52	0.67	0.55	0.28
C_5H_5N	80.8	121	1.92	2.60	2.74	2.46	(0.68)	(0.82)	(0.54)	0.55
$C_6H_5CH_2CN$	115.7	105	1.98	2.80	2.96	2.62	0.82	0.98	0.64	0.65
CH_3COCH_3	74.0	211	1.68	2.25	2.36	1.83	0.57	0.68	0.15	1.33
C_2H_5CN	70.9	169	1.77	2.70	2.86	2.02	0.93	1.09	0.25	1.44
$C_6H_5NO_2$	102.7	101	2.00	2.90	3.09	2.35	0.90	1.09	0.35	1.78

The values for *aniline* and *pyridine* are placed above the line showing that the solubilities in these solvents are higher than those corresponding to the dipole moments of the solvents. This higher solubility can be caused by chemical forces between carbon dioxide and the "basic" solvents. This effect is not found for carbon monoxide, and not for nitrogen and oxygen in pyridine.

Ditte [15] has shown the formation of a crystalline compound by dissolving carbon dioxide in dry aniline at about 10° C and a pressure of about 50 atm. On the other hand, if carbon dioxide at atmospheric pressure is led into dry aniline at room temperature no carbamate is formed (Faurholt and Ballund Jensen [16]); if some of the saturated solution is added to sodium hydroxide solution, all the carbon dioxide is instantaneously precipitated as carbonate on addition of an excess of barium chloride solution.

The point (10) for *acetone* is placed below the line in accordance with the assumption that acetone is somewhat associated (*cf.* the last section). If we use the molecular weight 75 for acetone — corresponding to a content of about 30 % double molecules — we get the point 10 A. This assumption, which influences only the value of x_2, is also able to account for the solubility of carbon monoxide, oxygen and nitrogen in acetone.

A comparison between the experimental and calculated values for the solubilities of oxygen, carbon monoxide and nitrogen is given in Table 4. The calculation is carried out using equation (1) with $\delta_1 = \delta''$. The differences, Δ'', between calculated and experimental values of $-\log x_2$ are (Fig. 1) plotted against the dipole moments of the solvents, and we again find an approximate proportionality. The slope of the lines is greatest for carbon dioxide, almost equal for nitrogen and carbon monoxide and smallest for oxygen. This result is possibly related to the electronic polarization, which for carbon dioxide is 6.6, carbon monoxide 4.9, nitrogen 4.4 and oxygen 4.0 ml, *cf.* Landolt Börn-

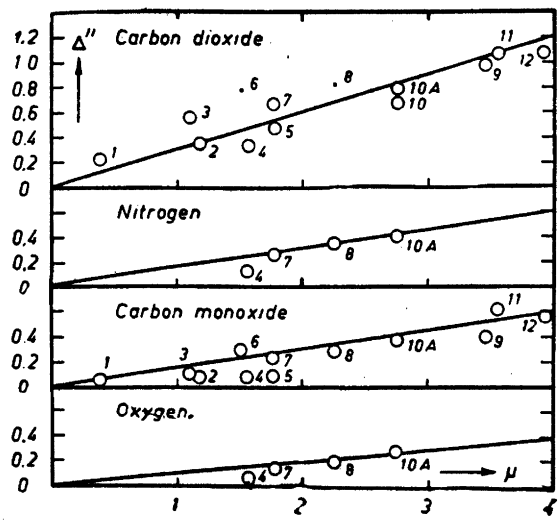

Fig. 1. Δ'' (see the text) plotted against the dipole moments of the solvents. 1. toluene, 2. chloroform, 3. 1,2-dibromoethane, 4. chlorobenzene, 5. n-propyl acetate, 6. aniline, 7. methyl acetate, 8. pyridine, 9. benzyl cyanide, 10. acetone, 11. propionitrile and 12. nitrobenzene. 10 A assuming a mean molecular weight of acetone equal 75.

Table 4. Solubility of carbon monoxide, nitrogen and oxygen in polar solvents at 25° C. Comparison of experimental and calculated values.

	x_2^i	$\bar{V}_2$ ml	δ_2
oxygen	$17.6\ 10^{-4}$	46	5.7
carbon monoxide	$15.5\ 10^{-4}$	52	5.8
nitrogen	$16.0\ 10^{-4}$	53	5.2

Solvent	Oxygen $-\log x_2$ exp.	eq.(1)	Δ''	Carbon monoxide $-\log x_2$ exp.	eq.(1)	Δ''	Nitrogen $-\log x_2$ exp.	eq.(1)	Δ''
$C_6H_5CH_3$				3.09	3.14	0.05			
$CHCl_3$				3.17[c]	3.23	0.06			
$1,2\text{-}C_2H_4Br_2$				3.52	3.61	0.09			
C_6H_5Cl	3.10[a]	3.16	0.06	3.20[a]	3.28	0.08	3.36[a]	3.47	0.11
$CH_3COOC_3H_7$				2.93	3.02	0.09			
$C_6H_5NH_2$				3.72	4.01	0.29			
CH_3COOCH_3	3.05[a]	3.19	0.14	3.07[a]	3.30	0.23	3.23[a]	3.49	0.26
C_5H_5N	3.34[b]	3.54	0.20	3.41	3.69	0.28	3.60[b]	3.94	0.34
$C_6H_5CH_2CN$				3.44	3.83	0.39			
CH_3COCH_3	2.95[a]*	3.24	0.29	2.99[a]*	3.36	0.37	3.15[a]*	3.55	0.40
C_2H_5CN				3.20	3.82	0.62			
$C_6H_5NO_2$				3.43	3.99	0.56			

a) Horiuti [13], b) Guerry [14], c) Skirrow [12].
* assuming that acetone has the mean molecular weight 75.

stein [17]. It should be noted that the solubility of carbon dioxide in 1,2-dibromoethane is found to be unexpectedly high, and that the four gases have shown smaller solubilities in chlorobenzene than is expected from Fig. 1.

CALCULATION OF THE SOLUBILITY PARAMETERS OF THE SOLVENTS

The solubility parameters in question, calculated in different ways, have been summarized in Table 5. δ' and δ'' is calculated from

$$\delta = \sqrt{\frac{\Delta H - RT}{V}} \tag{6}$$

where ΔH is the heat of vaporization at 25° C, R the gas constant, $T = 298.1°$ C and V the molal volume of the solvent at 25° C. In the calculation of δ' is used ΔH-values from

$$\Delta H^{298} = -2950 + 23.7\ T_b + 0.020\ T_b^2 \tag{7}$$

where T_b is the boiling point of the solvent, *cf.* Hildebrand and Scott[6,p.427]. In the calculation of δ'', ΔH-values from the last column in Table 7 are used; these values will be discussed below. It is seen that for each solvent, δ' is less than δ''. δ''' is finally calculated from

$$\delta''' = \sqrt{\frac{\Delta H - \omega_1^2 - RT}{V}} \tag{8}$$

where ω_1^2 is the dipole contribution to the energy of vaporization. In this equation we again have used ΔH-values from the last column in Table 7. In the calculation of ω_1^2 (cal/mole) the following equations given by Böttcher [18] are used:

$$\omega_1^2 = \frac{4\pi}{3}\frac{d}{M}N^2\frac{\varepsilon - 1}{2\varepsilon + n_D^2}\frac{n_D^2 + 2}{3}\mu^2 \cdot 2.39 \cdot 10^{-8} \tag{9}$$

$$\omega_1^2 = RT\,\frac{(\varepsilon - 1)(\varepsilon - n_D^2)}{\varepsilon(n_D^2 + 2)} \tag{10}$$

where d is density, M molecular weight, N Avogadros number, ε dielectric constant, n_D refraction index and μ dipole moment for the solvent. For the values for ε and μ used, see Table 8. For non-associated solvents these two equations give nearly the same value for ω_1^2. The results are given in Table 5, where it is seen that equation (9) gives slightly higher or lower values than equation (10). This can be considered as a criterion that the polar liquids in question — with the exception of acetone — are not considerably associated; for associated liquids, equation (9) results in distinctly lower values than equation (10). For acetone we thus calculate ω_1^2 about 22 % less from equation (9) than from (10). Since the two sets of values for ω_1^2 give nearly the same result for δ''' we have only given δ''' calculated by means of equation (10) in Table 5.

Table 5. Solubility parameters (δ) at 25° C.

Solvent	ω_1^2 cal/ml eq. (9)	ω_1^2 cal/ml eq. (10)	δ' eq. (6) a)	δ'' eq. (6) b)	δ''' eq. (8)
Toluene	0.13	0.11	8.9	9.1	9.1
Chloroform	3.4	3.7	9.05	9.25	9.0
1,2-Dibromoethane	2.7	2.9	10.4	10.5	10.3
Chlorobenzene	3.8	3.6	9.55	9.6	9.4
n-Propyl acetate	4.0	4.0	8.4	8.7	8.45
Aniline	5.4	5.3	11.2	11.55	11.3
Methyl acetate	8.6	7.9	9.0	9.5	9.1
Pyridine	17.1	15.6	10.35	10.7	9.95
Benzyl cyanide	21.0	18.5	10.85	11.25 c)	10.4 c)
Acetone	29.6	37.9	9.3	9.65	7.45
Propionitrile	54.9	50.8	10.6	11.0 d)	8.4 d)
Nitrobenzene	36.6	41.3	11.1	11.55	9.6

a) using ΔH from equation (7)
b) » » » last column in Table (7)
c) calculated from the estimated value $\Delta H = 15.2$ kcal/mole at 25° C.
d) » » » » » $\Delta H = 8.59$ » » 25° C.

As described above, we need values of the heat of vaporization for the solvents at 25° C. For toluene, chloroform and pyridine only calorimetric values at 25° C are found in the literature (*cf.* Table 7) while for most of the solvents values were given at the boiling point.

Several of the calorimetric values at the boiling points — summarized in Table 6 — show considerable disagreement. To check these values, the heat of vaporization is calculated from Clapeyrons formula, partly from values of the pressure dependence of the boiling points (column 3), partly from the temperature dependence of the vapour pressures (column 4). Deviations from ideal gas behavior were calculated from Berthelot's equation

$$P.V = RT\left[1 + \frac{9}{128}\frac{PT_c}{P_cT}\left(1—6\,\frac{T_c^2}{T^2}\right)\right] \qquad (11)$$

where P_c and T_c is critical pressure and temperature, respectively. These corrections amounted to 2.7—3.7 %.

The heat of vaporization were also calculated from Hildebrand's empirical equation [6,p.427]

$$\Delta H^b = 17.0\ T_b + 0.009\ T_b^2 \qquad (12)$$

where T_b is the boiling point in absolute temperature. This equation, which is only valid for non-polar liquids, gives too low values for polar liquids. The heats of vaporization for the liquids in question — with the exception of nitrobenzene — are only between 2 and 10 % higher than calculated from equation (12). We thus see that the boiling points include to a high degree the effect of the dipole attraction. No vapour pressure data for nitrobenzene allowing a calculation of the heat of vaporization at the boiling point (210.8° C)

was found in the literature. The calorimetric value, 7.94 kcal/mole, is considered to be much too low, since it is 2.4 kcal lower than that calculated from equation (12). The most satisfactory heats of vaporization are given in the last column of the table. For benzyl cyanide we found no data allowing a calculation of ΔH at the boiling point.

These heats of vaporization have been recalculated to 25° C (see Table 7) by means of Kirchhoff's law

$$\Delta H^{298} = \Delta H^{b} + \int_{298}^{T_b} \Delta c_p dT \qquad (13)$$

Table 6. Heats of vaporization (ΔH) at the boiling point, kcal/mole.

Solvent	Calorimetric values	Calc. by us from $\frac{dt^{27}}{dp}$	Calc. by us from vapour pressures	Calc. by us from eq(12)	Chosen value
Toluene	7.70[1] 7.83[2] 7.99[3] 7.96[4]	8.00		7.85	8.00
Chloroform	6.97[5] 7.02[4] 7.09[6] 7.08[7]	7.01		6.69	7.01
1,2-Dibromoethane	8.69[4] 8.23[8] 8.51[4]	8.65		8.35	8.69
Chlorobenzene	8.73[4] 8.54[9]	8.46	8.55[10]	8.36	8.54
n-Propyl acetate	7.89[1] 8.20[4] 8.21[11] 8.15[9] 8.49[12]	8.20		7.63	8.20
Aniline	9.71[13] 10.20[14] 10.60[15] 10.60[16]	10.43	10.54[17]	9.66	10.4
Methyl acetate	6.96[1] 7.26[4] 7.18[12] 7.28[11]	7.36	7.29[10]	6.59	7.28
Pyridine	8.49[4] 8.48[18]	8.93	8.41[19] 8.54[20]	7.97	8.48
Acetone	7.09[4] 7.27[21] 7.27[22]	7.12	7.09[23] 7.14[24]	6.57	7.14
Propionitrile		8.05	7.79[25] 7.84[26]	7.53	7.84
Nitrobenzene	7.94[13]	12.35		10.33	12.3

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where Δc_p is the difference between the molal heat capacity of the liquid and the vapour. Considering Δc_p to be independent of the temperature as a first approximation we get from equations (7), (12) and (13)

$$\Delta c_p \sim 10 + 0.011\ T_b \qquad (14)$$

Even if equation (14) can give Δc_p with an accuracy of 10—20 % only, the error in the calculated value of ΔH at 25° C is estimated to be few percent. The agreement with the heats of vaporization calculated from vapour pressures (column 4) is satisfactory.

Heats of vaporization at 25° C have also been calculated from equation (7) (see Table 7) , and in the last column of this table we have chosen such values of the heat of vaporization, which we believe are most reliable. These values — with the exception of the value for nitrobenzene — are estimated to be reliable to within 2—3 %. The calorimetric values for toluene and pyridine are in accordance with the values in column 3 and 4, while the calorimetric value for chloroform is estimated to be too high.

Table 7. Heats of vaporization (ΔH) at 25° C, kcal/mole.

Solvent	Calorimetric values	Calculated by us from eq(13)	Calculated by us from vapour pressures	Calculated by us from eq(7)	Chosen value
Toluene	9.07[1]	9.21	9.12[4]	9.09	9.12
Chloroform	7.97 (20°)[2]	7.50	7.40[5] 7.48[6] 7.10[7]	7.21	7.48
1,2-Dibromoethane		10.30	10.1[5]	9.92	10.1
Chlorobenzene		10.08	9.99[8]	9.93	10.0
n-Propyl acetate		9.27	9.33[9]	8.74	9.33
Aniline		12.8	12.79[10] 12.84[11]	12.08	12.8
Methyl acetate		7.72	7.81[12]	7.05	7.81
Pyridine	9.71 (20°)[3]	9.76	9.84[13]	9.28	9.84
Acetone		7.56	7.50[14] 7.39[15] 7.75[16] 7.45[6]	7.02	7.5
Nitrobenzene		15.1	14.3[17]	13.20	14.3

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7 and 17 are cited from Timmermans (*cf.* Table 6, Ref.[21]).

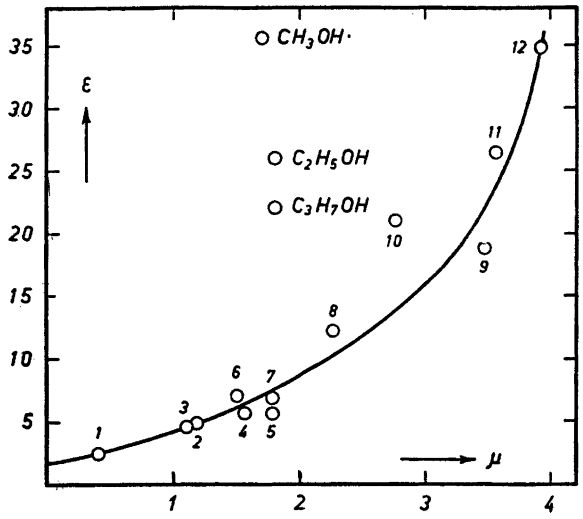

Fig. 2. The dielectric constants (ε) of polar liquids plotted against the dipole moments (μ). 1. toluene, 2. chloroform, 3. 1,2-dibromoethane, 4. chlorobenzene, 5. n-propyl acetate, 6. aniline, 7. methyl acetate, 8. pyridine, 9. benzyl cyanide, 10. acetone, 11. propionitrile, and 12. nitrobenzene.

The values of dielectric constants and dipole moments for the solvents used in this work are given in Table 8 columns 2 and 5, respectively. To get reliable values for the dielectric constants we plotted values given in the literature against the temperature; the temperature coefficients are given in the table. $d\varepsilon/dt$ as a whole increases with increasing values of ε. The values of μ in column 5 for eleven of the solvents were obtained from measurements in benzene; the value for 1,2-dibromoethane originates from a measurement in gas phase. Moreover we have reproduced the lowest and highest stated value of μ for each of the solvents, values which include measurements both in gas phase and in non-polar solvents.

ON ASSOCIATION IN THE SOLVENTS

In this section we wish to estimate the extent of association of the solvents.

Pauling [19] has given the following criterion for association in liquids. If the dielectric constants are plotted against the dipole moments, for non-associated liquids the points lie close to a simple curve, while the points for associated liquids lie above the curve. The points for the present solvents are shown in Fig. 2 together with points for the associated solvents methanol, ethanol and propanol. Since the point for acetone is placed distinctly over the curve, this solvent is considered to be associated. We get a confirmation of this estimate by regarding Yamamura's [20] freezing point measurements for mixtures of acetone and benzene.

Yamamura's measurements have been compared (Fig. 3) with the freezing points for solutions of carbon tetrachloride, carbon disulphide, *cyclo*hexane and nitrobenzene in benzene (data from *Int. Crit. Tables*). The experimental freezing point lowering is plotted against the mole percent of the solute. The curve

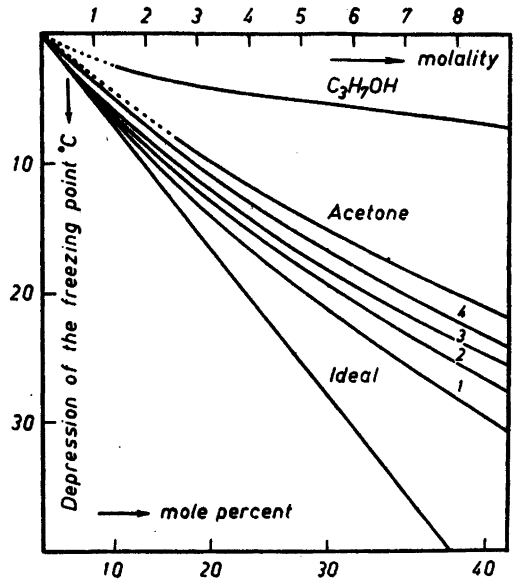

Fig. 3. Freezing point depression of benzene solutions. Solutes: 1. carbon tetrachloride, 2. carbon disulphide, 3. cyclohexane and 4. nitrobenzene.

marked "ideal" is drawn using the cryoscopic constant for benzene (5.1°). It is seen from equation (1) that the departure from ideality for molecules with molal volumes not too different from that of benzene is given by

$$\frac{\bar{V}_2}{2.303\ RT}\ (\delta_1 - \delta_2)^2$$

Using solubility parameters from Hildebrand and Scott [6, appendix 1] we calculate the following values of this term:

carbon tetrachloride	0.026
carbon disulphide	0.028
*cyclo*hexane	0.080

These numbers are in accordance with the experimental fact that the deviation from ideality is smallest for carbon tetrachloride and largest for *cyclo*hexane. Using $\delta_1 = 9.25$, for acetone we get the value 0.011, *i.e.* the curve for acetone is expected to lie near the curve for carbon tetrachloride. The much higher position cannot be explained from the polarity of acetone, *cf.* the position of the curve for the more polar nitrobenzene. An explanation would be to assume association in acetone. The curve for propanol dissolved in benzene is shown for comparison.

Finally some older criterions for association in liquids shall be mentioned. Walden [21] has investigated the connection between the surface tension (σ) and internal pressure (K) for many liquids at their boiling point. The

internal pressure was calculated from the heat of vaporization. Both the values of σ and K give information on the forces of attraction in the liquids. Walden put forward the following empirical rules:

1) The ratio between the surface tension at the boiling point and the critical pressure (p_k) for "normal" liquids is nearly constant ($\sigma/p_k \sim 0.45$, lying between 0.42 and 0.47)

2) The ratio between the internal pressure at the boiling point and the critical pressure for "normal" liquids is nearly constant ($K/p_k \sim 34$, lying between 32 and 35).

For "associated" liquids he found σ/p_k less than 0.42 and K/p_k greater than 35. Such ratios have been reproduced for several of the solvents in Table 8, and using Walden's rules, it is seen that only acetone behaves abnormally. It should be emphasized that the departure from ideal behavior decreases with increasing temperature and that Walden's rules only give information on possible association at the boiling point of the liquid.

Eötwös [22] has given the following relation between surface tension, molal volume (V) and temperature (t):

$$\sigma \cdot V^{2/3} = A - k_E \cdot t$$

Table 8. Some constants for the twelve polar solvents.

Solvent	ε 25° C	$\frac{d\varepsilon}{dt}$	μ-limits [12]	μ [12]	Eötwös constant k_E [13]	Values at the boiling point for σ/p_k	K/p_k
Toluene	2.37[1]	0.005	0.34—0.39	0.39	2.2	0.44	33
Chloroform	4.77[1]	0.018	1.15—1.18	1.18	2.1		
1,2-Dibromoethane	4.70[2]	0.008	0.79—1.10	1.10	2.2		
Chlorobenzene	5.61[3]	0.016	1.55—1.70	1.57	2.2	0.45	34
n-Propyl acetate	5.6[4]	(0.02)	1.78—1.96	1.78	2.3	0.47[a]	34[a]
Aniline	6.86[5]	0.023	1.48—1.75	1.51	2.1	0.45	34
Methyl acetate	6.68[6]	0.030	1.74—1.78	1.78	2.2		
Pyridine	12.2[7]	0.029	2.10—2.26	2.26	2.3		
Benzyl cyanide	18.8[8]	0.064	3.47—3.56	3.47	2.2		
Acetone	20.9[9]	0.10	2.71—2.85	2.76	1.9	0.39[b]	41[b]
Propionitrile	26.3[10]	0.13	3.56—4.03	3.56	1.6	0.43	
Nitrobenzene	34.9[11]	0.18	3.93—4.30	3.93	2.2		

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a) $p_k = 32.9$ atm.
b) $p_k = 47$ atm.

where A is a constant depending on the liquid in question. k_E (Eötwös' constant) was found to be about 2.12 for many liquids. Values of k_E for the twelve solvents have been summarized in Table 8. It is seen that k_E for propyl acetate and pyridine is somewhat higher than 2.12, while k_E for acetone and propionitrile is lower than 2.12. Later on it has been proved that k_E even for 'normal' liquids is not a constant, but depends on the molecular weight of the liquid, *cf.* Glasstone [23]. Among the twelve solvents just acetone and propionitrile have the lowest molecular weights, and therefore we are not able to draw any conclusion on association in these two solvents. Neither did an examination of the temperature dependence of k_E enlighten the question: values of k_E in many temperature intervals between 0° and 100° C were calculated for the twelve solvents. In no case was an essential increase of k_E with increasing temperature found (*cf.* Glasstone [23]). For acetone, aniline and pyridine we found an increase of about 10 %, for propyl acetate a decrease of about 16 %, while there was no clear variation in k_E for the other solvents.

SUMMARY

1. The solubility of *carbon dioxide* expressed as the Bunsen absorption coefficient is determined at 25° C in the following solvents: toluene 2.21, chloroform 3.58, 1,2-dibromoethane 2.00, chlorobenzene 2.17, normal propyl acetate 4.82, aniline 1.20, methyl acetate 6.43, pyridine 3.36, benzyl cyanide 2.05, acetone 6.44, propionitrile 5.39 and nitrobenzene 2.20.

For *carbon monoxide* the following solubilities were determined at 25° C: toluene 0.171, 1,2-dibromoethane 0.778, normal propyl acetate 0.227, aniline 0.0464, pyridine 0.107, benzyl cyanide 0.0694, propionitrile 0.199 and nitrobenzene 0.0813.

These solubilities are compared with previous measurements in Table 2.

2. If the solubility of *carbon dioxide* is calculated from

$$-\log x_2 = -\log x_2^i + \frac{\bar{V}_2}{2.303\,RT}(\delta_1-\delta_2)^2$$

we get values less than the experimental ones. The difference between the calculated and experimental values of $-\log x_2$ is shown to be approximately proportional to the dipole moment of the solvent, *cf.* Fig. 1.

The solubilities of *oxygen*, *carbon monoxide* and *nitrogen* are calculated from equation (1), and the same dependence of the dipole moments of the solvents was found to be valid (*cf.* Fig. 1). The different slopes of the lines in this figure are held to depend on the electronic polarization of the gases.

3. A comparison between calorimetric values of the heat of vaporization for the solvents and values calculated from the temperature dependence of the vapour pressure is given in Table 6 and 7.

4. Different criteria for association in liquids are discussed. Acetone is the only one among the twelve solvents which is assumed to be associated (*cf.* Figs. 2 and 3). It is shown that the solubility of the four gases in acetone can be explained by assuming a mean molecular weight of acetone equal to 75.

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The Use of Redox Indicators in Acidimetric Titrations

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It has been shown that weak bases ($pk_B < 10$) can be titrated acidimetrically using indicators consisting of the ceric-cerous redox system plus nitro-ferroin or ferroin. The execution of the titrations requires some skill, but they can be carried out with reasonable accuracy. Of weak bases, acetate ion, succinate ion, and pyridine have been titrated.

In acidimetric or alkalimetric titrations an acid-base system, comprising a weak acid and its corresponding weak base where these are of different colours, is generally used as indicator; with the exchange of protons a structural change takes place. Instead of a simple acid-base system like this, more complicated indicators are sometimes used. As early as 1850 Schwartz [1] added ferric chloride to gallic acid, and used as an indicator a solution of the resulting precipitate in hydrochloric acid. Schwarz recommended this in preference to litmus, which together with other vegetable extracts was generally used at the time in titrations of bases and acids.

A survey of different types of acid-base indicators has been given by Charlot and Bézier [2]. They mention precipitation indicators with a relatively small transition interval at pH ~ 11. These are applicable to the titration of weak acids, the pk_S's of which are about 8 to 9.

The purpose of the present paper is to show that in principle it is possible to use redox indicators in the titration of weak bases, the pk_B's of which are below 10. Redox indicators are normally applied to redox titrations. However, it has previously been shown that in special cases redox indicators can also be used in precipitation titrations (see *e.g.*, Rancke-Madsen [3]), subject to an abrupt change in the concentration of the reduced or oxidized component of a redox system taking place at or sufficiently close to the equivalence point. The acidimetric titrations mentioned below are based on the same principle, with the modification however that a redox system dependent on pH is added besides the redox indicator.

In the present work a ceric-cerous mixture or in some cases ceric salt alone has been used. According to Bowles and Partridge [4] the neutralization of an

acid solution of a ceric salt will take the following course: After the neutralization of a strong acid a colloidal solution will be produced, and the precipitation of the ceric hydroxide can be observed at pH ~ 2.6—2.8. Visible precipitation of cerous hydroxide will occur only at pH ~7.1.

The redox potential in a ceric-cerous solution is given by

$$E = E_o + 0.06 \log \frac{[Ce^{++++}]}{[Ce^{+++}]}$$

At pH values between *ca.* 2 and 7, $[Ce^{++++}]$, and not $[Ce^{+++}]$, of the above equation will be dependent on pH.

E_o is stated [2] to be 1.60 volts in nitric acid medium; generally, E_o is highly dependent on the medium, due to the formation of ceric complexes.

A 0.1 *M* ceric hydrogen sulphate solution, $Ce(HSO_4)_4$, 0.05 *M* in sulphuric acid, was used at first as the ceric solution. However, owing to hydrolysis this solution was rather unstable, and it was considered inadvisable to acidify the solution further: since the amount of ceric solution added could not be controlled to great accuracy, the acid concentration of the titrant would have varied. Therefore in place of this solution a 0.1 *M* solution of ammonium hexanitratocerate, $(NH_4)_2Ce(NO_3)_6$, in ethanol was used. This solution is sufficiently stable for a couple of days, but not appreciably longer. During the experimental work a fresh solution (0.28 g $(NH_4)_2Ce(NO_3)_6$ in 5 ml 96 % ethanol) was prepared every day.

The cerous salt solution was a 0.1 *M* aqueous solution of cerous nitrate, $Ce(NO_3)_3,6H_2O$.

DETERMINATION OF THE pH-TRANSITION OF VARIOUS INDICATOR SYSTEMS

The indicator systems hereafter denoted by $Ce^{+4} + Ce^{+3}$ consisted of 1 drop of a solution of a redox indicator + 0.1 ml ceric salt solution + 0.1 ml cerous salt solution. The indicator systems denoted by Ce^{+4} did not contain any cerous salt solution.

The indicator systems were added to 30 ml water (as described below under a) and b)) or to a dilute solution of sodium hydroxide (c), d), and e)), and titration was carried out until transition of colour was obtained with different 0.1 *N* acids. The titrations require some skill and should in all cases be carried out very slowly and with vigorous shaking, particularly shortly before the transition. The transitions are not reversible. The pH values were measured electrometrically.

a) Redox indicator: 0.025 *M* nitro-ferroin. Colour transition from red (becoming gradually weaker) until the disappearance of the last shade of red.

Indicator system	titrator	determinations of pH					mean value of pH
$Ce^{+4} + Ce^{+3}$	HNO_3	1.78;	1.82;	1.78;	1.81;	1.80	1.80
Ce^{+4}	»	1.92;	1.92;	1.93;	1.95;	1.90	1.92
$Ce^{+4} + Ce^{+3}$	$HClO_4$	1.54;	1.52;	1.54;	1.53;	1.53	1.53
Ce^{+4}	»	1.68;	1.73;	1.70;	1.73;	1.72	1.71
Ce^{+4}	H_2SO_4	1.92;	1.86;	1.88;	1.90;	1.83	1.88

b) Redox indicator: 0.025 *M* ferroin. Colour transition as under a).

Indicator system	titrator	determinations of pH					mean value of pH
$Ce^{+4} + Ce^{+3}$	HNO_3	2.62;	2.60;	2.54			2.59
Ce^{+4}	»	2.70;	2.76;	2.73;	2.76;	2.77	2.74
$Ce^{+4} + Ce^{+3}$	$HClO_4$	2.32;	2.30;	2.36;	2.34;	2.36	2.34
Ce^{+4}	»	2.54;	2.60;	2.55;	2.61;	2.54	2.57
Ce^{+4}	H_2SO_4	2.53;	2.47;	2.46;	2.47;	2.46	2.48

When sulphuric acid is used as titrator, a precipitate is produced, which makes it difficult to observe the transition.

c) Redox indicator: *o*-tolidine, 1 % ethanol solution. Colour transition from yellow (the ceric colour) to green. The transition to a faint green is difficult to observe, and so the determinations are more uncertain than those described under a) and b).

Indicator system	titrator	determinations of pH					mean value of pH
$Ce^{+4} + Ce^{+3}$	$HClO_4$	4.83;	5.02;	4.66;	4.80;	5.00	4.86
Ce^{+4}	»	5.00;	4.84;	5.12;	4.94;	5.00	4.98

d) Redox indicator: *o*-dianisidine, 0.2 % solution in 2 *M* acetic acid. Colour transition (to a faint red) is very difficult to observe (*cf.* c)).

e) Redox indicator: 0.3 *M* potassium iodide + 3 ml 1 % starch solution. Colour transition from colourless to blue.

Indicator system	titrator	determinations of pH					mean value of pH
$Ce^{+4} + Ce^{+3}$	$HClO_4$	3.04;	3.08;	3.07;	3.13;	3.10	3.08
Ce^{+4}	»	3.26;	3.28;	3.25;	3.22;	3.23	3.25

For the second colour transition of starch-iodine (from blue to colourless) non-reproducible pH values of between 2.4 and 2.9 were found.

f) Redox systems other than ceric-cerous are likely to be applicable as indicators. Thus, the indicator system thallic-thallous + potassium iodide, producing reversible and reproducible transitions, seems to be one possibility. The applicability of this and related indicator systems is being studied more closely at present.

THE USE OF THE REDOX SYSTEM CERIC-CEROUS + NITRO-FERROIN OR FERROIN IN CONNECTION WITH THE TITRATION OF WEAK BASES

As the colour transition for a given indicator system occurs within a very small interval of the pH scale, it is possible in principle to carry out direct titrations of suitably weak bases to the pH values corresponding to the equivalence point. However, although the pH-values at colour transitions are certainly reproducible when the same ceric solution is used, with a different ceric solution slightly different pH values are usually found. Since also other conditions (ionic strength, final volume, *etc.*) should be kept fairly constant, this procedure was rejected.

Instead, titrations were attempted in which the weak acid was titrated past the equivalence point to colour transition of one of the indicator systems mentioned above, and then the overtitration corrected by means of a blank experiment. The blank experiments were carried out on pure water, so that the end volume in the titration of the weak base was practically identical with that in the titration of water. The same indicator system was used in both titrations, and these were carried out as uniformly as possible and to the same colour transition.

CALCULATION

The concentrations mentioned in the following all refer to the final volume of the solution. Let the stoicheiometric concentration of the weak base to be titrated be C_B, and a strong acid be added in excess at a total concentration C_t. The actual concentrations of the weak base and its corresponding weak acid may be written [B] and [S], respectively. The base and acid dissociation constants for the base and its corresponding acid are k_B and k_S, respectively.

The hydrogen ion concentration in the final solution must then be as stated in equation (1), for from the strong acid a quantity of hydrogen ions corresponding to (C_B —[B]) are used for the neutralization of the weak base:

$$[H_3O^+] = C_t - (C_B - [B]) + [OH^-] \tag{1}$$

The last term, which originates from the self-dissociation of water, can be neglected under the circumstances in question, *i.e.* $[OH^-]$ is considered negligible, and rearranging (1) we obtain:

$$[B] = [H_3O^+] - C_t + C_B \tag{2}$$

Further

$$C_B = [B] + [S] \tag{3}$$

which together with (2) gives:

$$[S] = C_t - [H_3O^+] \tag{4}$$

If (2) and (4) are inserted in the usual expression for k_S, we get:

$$k_S = \frac{[H_3O^+]\,[B]}{[S]} = \frac{[H_3O^+]([H_3O^+] - C_t + C_B)}{C_t - [H_3O^+]} \tag{5}$$

In the blank experiments, a strong acid is added to pure water; if the stoicheiometric concentration of the strong acid is C_o in the final volume, then we get:

$$[H_3O^+] = C_o \tag{6}$$

since hydrogen ions originating from the self-dissociation of water can also be neglected here.

If $[H_3O^+]$ is the same in the two solutions, (6) can be inserted in (5), and after rearranging, we get:

$$C_t - C_o = \frac{C_o}{k_S + C_o} \cdot C_B = \frac{[H_3O^+]}{k_S + [H_3O^+]} \cdot C_B \tag{7}$$

It follows that the equation

$$C_t - C_o = C_B \tag{8}$$

is valid with an error the absolute magnitude of which is:

$$C_B - \frac{[H_3O^+]}{k_S + [H_3O^+]} \cdot C_B = \frac{k_S}{k_S + [H_3O^+]} \cdot C_B, \text{ which is } < \alpha \cdot C_B$$

if $k_S < \frac{\alpha}{1-\alpha} \cdot [H_3O^+]$, which for small values of α is approximately equal to $\alpha \cdot [H_3O^+]$.

Thus equation (8) is valid with a relative error $< \alpha$ on C_B, if

$$k_S < \alpha \cdot [H_3O^+] \tag{9}$$

or

$$pk_S > pH - \log \alpha \tag{10}$$

or

$$pk_B < 14 - pH + \log \alpha \tag{11}$$

If, for instance, an indicator system with a colour transition at pH = 2 is used, and if < 1 % error is required, then (8) may be used for the calculation of the quantity of base, assuming that $pk_B < 10$.

Table 1. Titration of 15.00 ml 0.0998 N CH_3COONa with 0.0928 N H_2SO_4. Indicator system: Ce^{+4} + nitro-ferroin. Uncertainty on the mean value B: μ_B = 0.02 ml. Uncertainty on single determination of A: μ_A = 0.05 ml. Uncertainty on single determination of A—B: μ_{A-B} = 0.06 ml. Uncertainty on single analysis: μ = 0.4 %. Uncertainty on mean value of determinations μ_m = 0.2 %.

Blank test No.	ml H_2SO_4	Deviation (ml)
1	7.47	+ 0.04
2	7.37	− 0.06
3	7.39	− 0.04
4	7.47	+ 0.04
5	7.45	+ 0.02
Mean value: B = 7.43 ml		

Analysis No.	A: ml H_2SO_4	A—B (ml)	% of the theoretical
1	23.51	16.08	99.7
2	23.55	16.12	100.0
3	23.61	16.18	100.3
4	23.50	16.07	99.6
5	23.64	16.21	100.5
6	23.51	16.08	99.7
7	23.49	16.06	99.6
8	23.51	16.08	99.7
9	23.56	16.13	100.0
10	23.47	16.04	99.5
Mean value: A_m = 23.54		Mean value:	99.9

Table 2. Titration of 15.00 ml 0.1 M CH_3COONa. Indicator: n.f. = nitro-ferroin; f. = ferroin. B = mean value of 5 blank experiments. μ_B = uncertainty on the mean value B. A_m = mean value of 10 single determinations A, where A = ml titrator used for direct titration of the sample. μ_A = uncertainty on A. μ_{A-B} = uncertainty on A−B. R = quantity of acetate found (mean value). μ = uncertainty on single analysis. μ_m = uncertainty on mean value of determinations.

CH_3COONa, M =	0.0998	0.0998	0.1002	0.1009	0.1009	0.1009
Indicator	n.f.	f.	n.f.	n.f.	n.f.	f.
Redox system	Ce^{+4}	Ce^{+4}	Ce^{+4}	Ce^{+4}	$Ce^{+4} + Ce^{+3}$	Ce^{+4}
Titrator	H_2SO_4	H_2SO_4	HNO_3	$HClO_4$	$HClO_4$	$HClO_4$
» , M =	0.0928	0.0928	0.1022	0.0966	0.0966	0.0966
B (ml)	7.43	1.38	7.66	6.76	8.16	0.79
μ_B »	0.02	0.04	0.02	0.04	0.06	0.03
A_m »	23.54	17.50	22.36	22.46	23.86	16.45
μ_A »	0.05	0.06	0.04	0.09	0.09	0.08
μ_{A-B} »	0.06	0.07	0.05	0.10	0.11	0.08
R (%)	99.9	100.0	100.0	100.2	100.2	100.0
μ »	0.4	0.4	0.3	0.7	0.7	0.5
μ_m »	0.2	0.3	0.2	0.3	0.5	0.3

TITRATION OF WEAK BASES

The ceric-cerous + nitro-ferroin or ferroin redox system has been tried as an indicator system in connection with the titration of the following weak bases:

acetate ion	$pk_B = 9.2$
succinate ion	$pk_B = 9.8$
pyridine	$pk_B = 8.9$

a) Acetate ion. A 0.1 *M* solution of sodium acetate was prepared by weighing and diluting to a known volume. $CH_3COONa,3H_2O$ *p.a.*, the purity of which had been controlled, was used. To 15.00 ml of this solution 1 drop of redox indicator + 0.1 ml ceric solution were added, and in some cases 0.1 ml cerous solution also. Then titration was carried out with 0.1 *N* strong acid.

As a blank test 30 ml distilled water, to which was added the same quantity of the indicator system, was titrated. The blank endpoint was determined

Table 3. Titration of 15.006 ml 0.0993 M pyridine. Indicator: n.f. = nitro-ferroin; f. = ferroin. Redox system: Ce^{+4}. Titrator: 0.1021 M H_2SO_4. B = mean value of 5 blank experiments. μ_B = uncertainty on the mean value B. A_m = mean value of 10 single determinations A, where A = ml titrator used for direct titration of the sample. μ_A = uncertainty on A. μ_{A-B} = uncertainty on A−B. R = quantity of pyridine found (mean value). μ = uncertainty on single analysis. μ_m = uncertainty on mean value of determinations.

Indicator	n.f.	f.
B (ml)	5.89	0.81
μ_B »	0.04	0.02
A_m »	20.45	15.34
μ_A »	0.09	0.06
μ_{A-B} »	0.09	0.06
R (%)	99.8	99.6
μ »	0.6	0.4
μ_m »	0.3	0.2

as a mean value of 5 determinations, and it was used in all subsequent titrations of acetate ion in the same series of experiments.

An example of a series of experiments is given in Table 1. In Table 2 this series and similar ones are given with the calculated uncertainties. The titrations of acetate ion with perchloric acid using ferroin were particularly difficult and were sometimes unsuccessful; it turned out to be profitable here not to add the indicator system until about three-quarters of the titration had been carried through.

It will be seen from the tables that there was good agreement between the titrations and the theoretical data. The uncertainty of a single determination is in all cases below 1 %, and with a suitable choice of titrator below 0.5 %. Sulphuric acid seems to be the most suitable titrator.

b) Pyridine. Similar series of experiments were carried out with the weak base pyridine. In these experiments a product was used which had been purified several times by distillation and stored over solid potassium hydroxide. Two series are given in Table 3. It will be noted that the results are in reasonable agreement with those described under a); the uncertainty is a little larger, however, and the results a little low, which is probably due to the volatility of pyridine. In this case, as in that of the acetate ion, sulphuric acid seems to be the best titrator.

c) Other weak bases. Succinate ions were titrated, giving results comparable with those described under a). The titration of aniline failed, however — probably owing to traces of impurities in the aniline. Attempt were also made at the titration of formate ions, although *a priori* this base should be considered a little too weak for titration ($pk_B = 10.2$). Thus, a complete series of experiments gave results of a mean value only 95.4 % of the theoretical.

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A Study of the Infrared Spectra of Some Stereoisomeric Diphenylpolyenes

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In spite of the rapidly increasing literature on infrared spectroscopy, relatively few papers have appeared covering stereoisomeric forms of conjugated double bond systems; and these refer mostly to conjugated diene types [1-4]. In the series of α,ω-diphenyl polyenes, of the general formula,

$$C_6H_5 \cdot (CH = CH)_n \cdot C_6H_5$$

the two steric forms of diphenylethylene [5] ($n = 1$) and the three stereoisomeric diphenylbutadienes [6] ($n = 2$) have been known for some time and characterized by their ultraviolet spectra [7].

Recently, the present authors have isolated, out of the six theoretically possible stereoisomeric diphenylhexatrienes ($n = 3$), three *cis* forms, in the chromatographically homogeneous and ultraviolet-spectroscopically pure state [8]. Furthermore, three *cis* isomers of diphenyloctatetraene ($n = 4$; total number of spatial forms, 10) have been obtained in collaboration with J. H. Pinckard [9]. Some skeleton models belonging to the sets, $n = 1$ to 4, whose configurations have been assigned with reasonable reliability are shown in Figs. 1 to 4.

Our infrared curves, taken in the region 2—15 μ, are given in Figs. 7—10 while Fig. 11 represents in a comparative manner, all bands between 5—15 μ referred to in the following discussion.

In the stereoisomeric sets studied the *cis* double bonds can be subdivided into sterically "hindered" and "unhindered" types. In this series, when a "terminal" *trans* double bond, *i.e.* a double bond adjacent to the phenyl group, assumes *cis* configuration, then the overlapping of two hydrogen-atoms creates a spatial conflict.[19] In the case of stilbene (Fig. 5) these are two ring-hydrogens, while in all higher homologs a ring hydrogen and an aliphatically bound H-atom would overlap (Fig. 6). Such "hindered" *cis* double bonds are contained in our models, Fig. 1 (bottom); Fig 2 (middle and bottom); Fig. 3, C; Fig. 4, C, D, and E.

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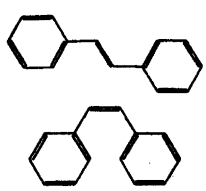

Fig. 1. Skeleton models of trans-stilbene (top) and cis-stilbene (bottom).

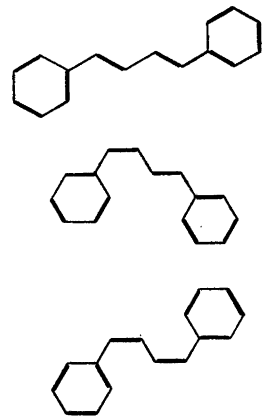

Fig. 2. Skeleton models of all-trans-diphenylbutadiene (top), cis-trans (middle), and di-cis-diphenylbutadiene (bottom).

No attempt has been made in the present paper to give a complete assignment of all bands observed in the region, 2—15 μ; however, we intend to point out and discuss from the viewpoint of *cis-trans* isomerism those wave length regions in which the curves of the individual steric forms display some characteristic features.

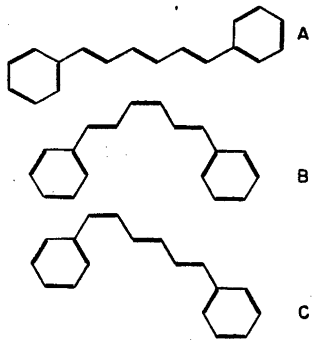

Fig. 3. Skeleton models of all-trans-diphenylhexatriene (A), Δ^3-cis-(B), and Δ^1-cis-diphenylhexatriene (C).

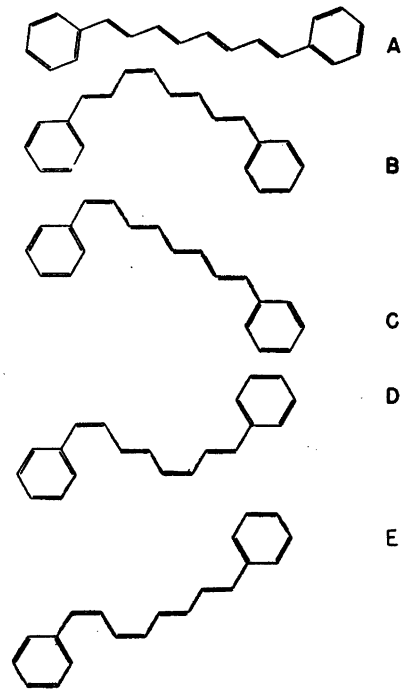

Fig. 4. Skeleton models of all-trans-diphenyloctatetraene (A), Δ^3-cis-(B), Δ^1-cis-(C), $\Delta^{1,6}$-di-cis-(D), and $\Delta^{1,3}$-di-cis-diphenyloctatetraene (E).

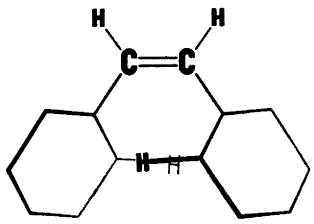

Fig. 5. Overlapping of two ortho ring-hydrogens in c i s-stilbene (according to G.N. Lewis et al.).

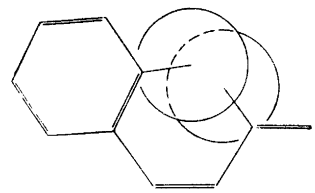

Fig. 6. Steric conflict between two hydrogen atoms in terminal-c i s diphenylpolyenes [19].

AROMATIC BANDS

Since all compounds mentioned below contain monosubstituted benzene rings, a number of bands, characteristic for such aromatic systems, appear in the curves. In most instances they are not markedly influenced by the steric configuration of the aliphatic chain and should be eliminated from discussions of spatial phenomena. This will be done in the following paragraphs.

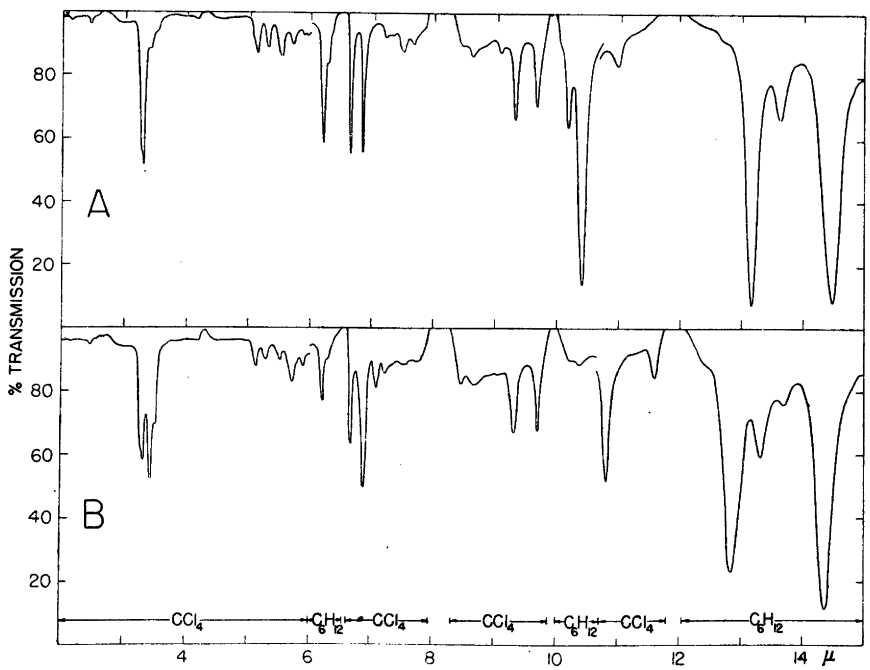

Fig. 7. Infrared curves of t r a n s- (A) and c i s-stilbene (B) in the region 2—15 μ. 1.0 % solutions in 1.0 mm cells The curves are composed of recordings in carbon tetrachloride and cyclohexane, using the sections where the transparency of the solvents was satisfactory, as indicated below curve B.

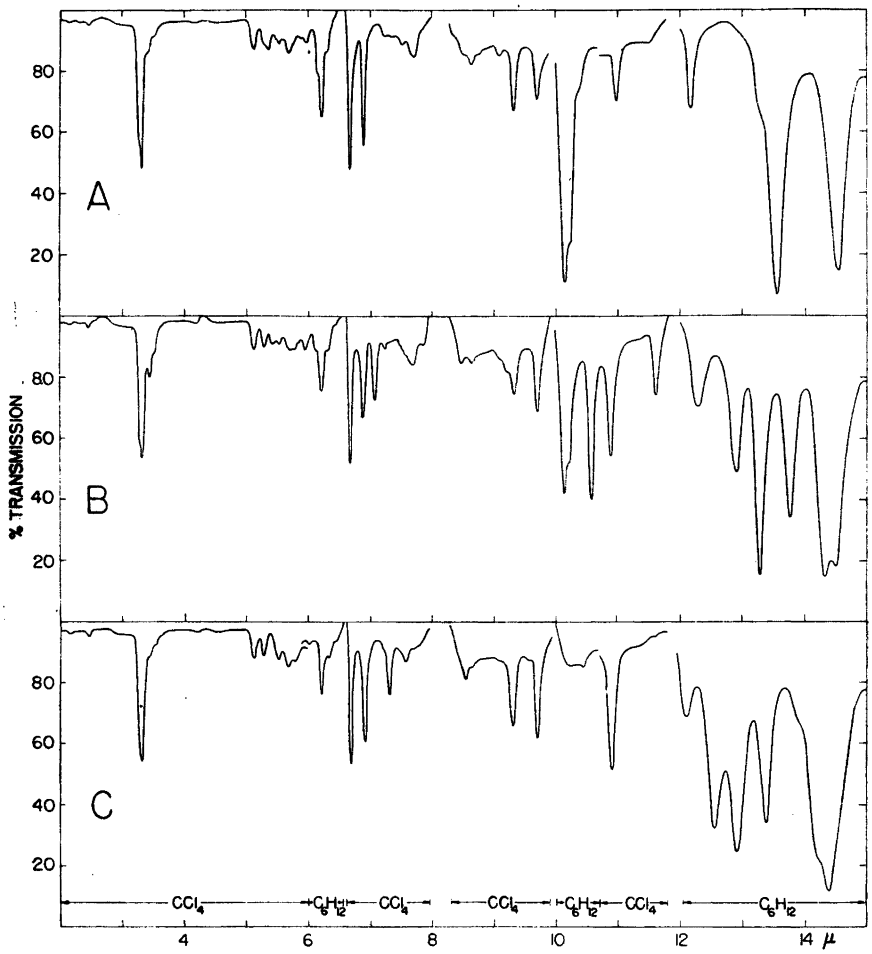

Fig. 8. Infrared curves of all-t r a n s- (A), mono-c i s- (B), and di-c i s-diphenylbutadiene (C); cf. also Legend to Fig. 7.

In the general region of CH stretching frequencies [10] there occurs a medium intensity band located at ~3.30 μ; it undergoes splitting when some *cis* configurations are assumed but this band did not give us a sufficient basis for stereochemical conclusions.

In the region, 5—6 μ several bands are observed that are characteristic for monosubstituted benzene derivatives such as toluene or diphenyl (Fig. 11; m, n), for example. According to Ingold [11] they represent combination tones and overtones. The number of bands appearing in this region is subject to variations. Probably, this phenomenon does have some stereochemical significance which, however, cannot be interpreted at the present time.

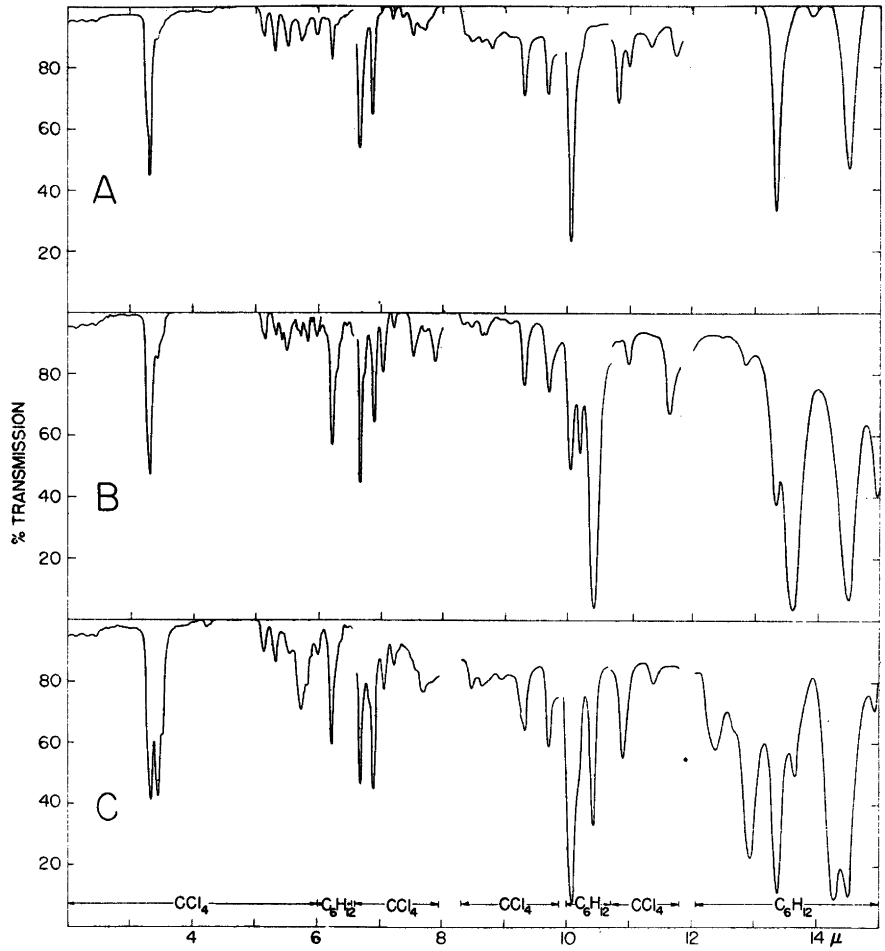

Fig. 9. Infrared curves of all-t r a n s- (A), Δ^3-c i s- (B), and Δ^1-c i s-diphenylhexatriene (C). The cyclohexane part of the spectrum of all-t r a n s-diphenylhexatriene was recorded in a saturated solution (~ 0.3 %); cf. also Legend to Fig. 7.

As is well known, the aromatic ring exhibits a CC stretching band at ~ 6.25 μ which obscures the longer wave length C = C stretching band in an aliphatic conjugated system. The shorter wave length C = C conjugated stretching band (~ 6.0 μ) is possibly present in some (but not all) curves of our stereoisomers in accordance with some data given by Rasmussen and Brattain [4].

According to Rasmussen [12] all other CC stretching frequencies of the aromatic ring are spread over a rather wide range, *i.e.* from 6 μ to 10 μ. A section of the same range is also covered by the CH bending frequencies or hydrogen in-plane vibrations in the vicinity of 7 μ. There we find two modera-

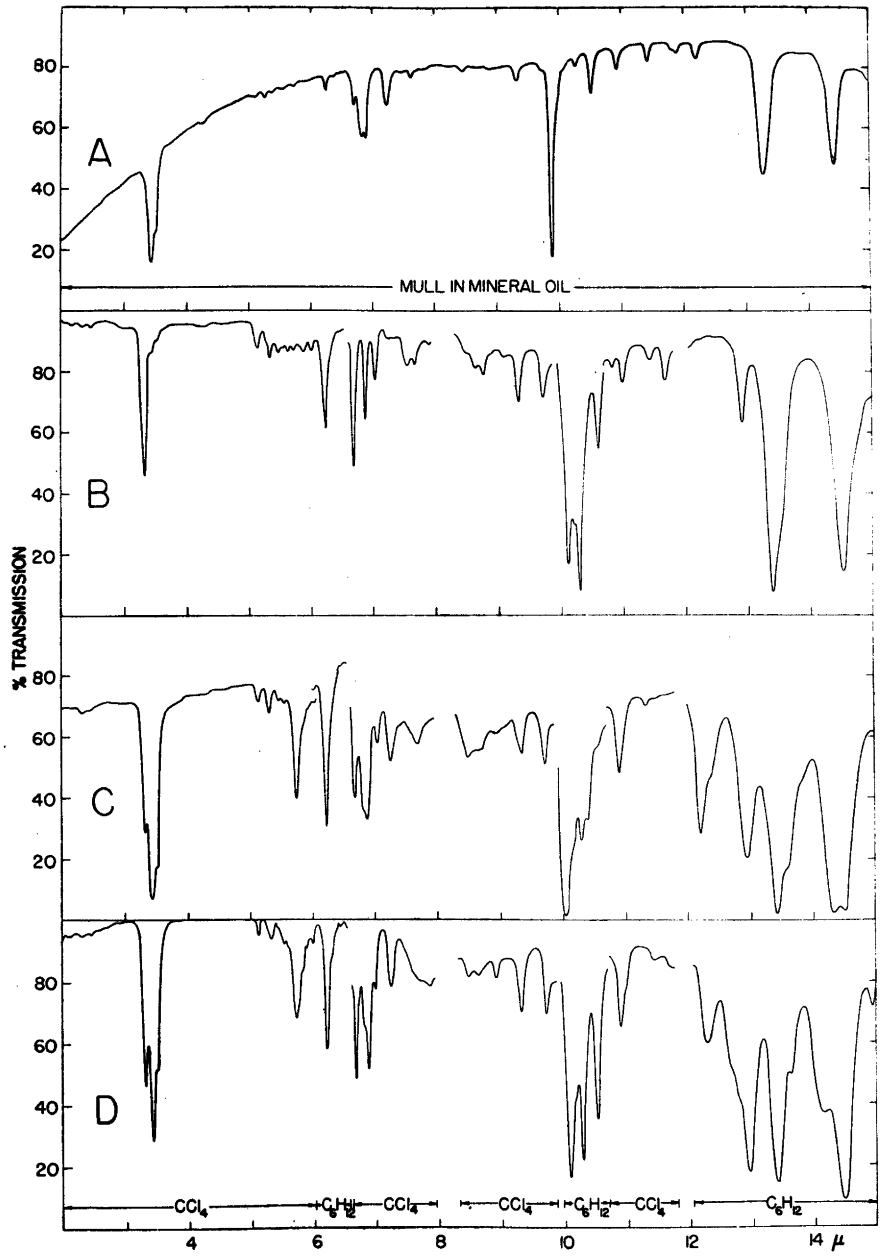

Fig. 10. Infrared curves of all-t r a n s-diphenyloctatetraene (A), Δ^{3}-c i s- (B), Δ^{1}-c i s- (C), and $\Delta^{1,3}$- or $\Delta^{1,5}$-di-c i s-diphenyloctatetraene. Curve A was taken on a mull; cf also Legend to Fig. 7.

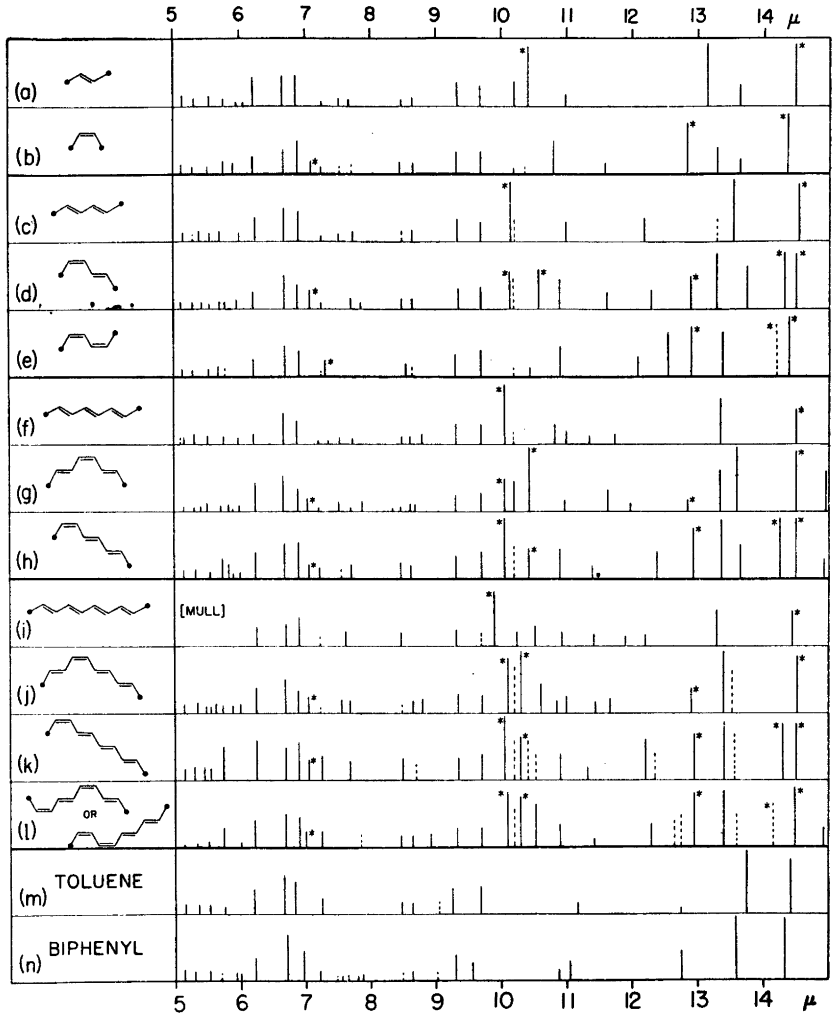

Fig. 11. General chart of the infrared bands observed between 5 μ and 15 μ represented as vertical lines at the band maxima; the heights designate relative intensities. The dashed lines denote unresolved bands which appear as shoulders on adjacent bands. The intensities and to a lesser degree even the positions of such bands are uncertain. Asterisk indicates a stereochemically significant band. The left column represents the stereochemical configuration of the aliphatic chain, with the terminal black dots indicating phenyl groups.

tely intense bands at remarkably constant wave lengths, *viz.* at 6.67 ± 0.02 μ and 6.88 ± 0.02 μ. A weaker band is located at 7.23 ± 0.02 μ whose constancy allows an easy differentiation from the stereochemically significant 7.0—7.1 μ band to be discussed below.

Between 7.5 μ and 9 μ there are several minor bands that evidently are sensitive to spatial rearrangements; they cannot be interpreted at the present time.

Two sharp, moderate bands characteristic for the monosubstituted benzene ring appear in all our curves at 9.30 μ and 9.70 μ and do not seem to be influenced either by the length or by the configuration of the aliphatic conjugated system.

As is well known, the 10—15 μ region is associated with the hydrogen out-of-plane rocking bands, intermixed with lower intensity carbon-carbon deformation bands, that arise from both rings and the aliphatic chain.

In this region we find, throughout all stereoisomeric sets studied, a moderate to weak band at the sharply constant wave length, 10.20 μ. It appears mostly as a shoulder on the adjacent strong intensity band(s) arising from the out-of-plane rocking of hydrogen atoms attached to a *trans* double bond. Because of its constancy the band can be identified easily and does not cause difficulties in this stereochemically important region, 10.0 — 10.6 μ ("*trans*-band region"). Neither is a moderate to weak band at 11 μ disturbing, although, as shown in the curves, it is evidently influenced by *trans* → *cis* rearrangements which usually shift it towards shorter wave lengths and may even split it into two distinct bands.

As demonstrated by the four all-*trans* curves (Fig. 11; a, c, f, i), the number of the bands between 11.0 μ and 12.5 μ (including that at $\sim$ 11.0 μ) is characteristic for the number n of the aliphatic double bonds: one band for $n = 1$, two for $n = 2$, *etc.* Possibly, these bands are associated with the carbon-carbon deformation frequencies of the aliphatic chain. This would be in agreement with the different appearance of the individual *cis* curves in this region. However, we are unable to offer a more precise explanation at present.

The strong bands at $\sim$ 13.5 μ and 14.5 μ are characteristic for the monosubstituted aromatic ring and represent two modes of hydrogen out-of-plane rocking in the ring [11,13]. The location of the former band is dependent on the nature of the substituent but no pertinent rules can yet be formulated. It is also possible that in case of certain *cis* configuration types this band would split into two, since the two rings would then be unequally substituted as a consequence of asymmetry of the aliphatic chain. The higher wave length band (14.5 μ) is influenced by a *trans* → *cis* rearrangement of a terminal aliphatic double bond, causing a more or less extensive splitting of the band. The latter feature will be useful in the following stereochemical discussion.

STEREOCHEMICALLY SIGNIFICANT BANDS

(*cf.* also Table 1)

Although the CH stretching bands ($\sim$ 3.30 μ) and the CC stretching bands (between 6 μ and 10 μ), as well as the CC deformation bands (10—15 μ) could be *a priori* influenced by steric changes, as mentioned above, no practical use could be made of these modes of vibrations so far. Hence, only the bands caused by the in-plane and out-of-plane vibration of hydrogen atoms attached to double bonded carbon will be considered here. The following four types of bands will have to be discussed.

1. In the CH bending region we find that the curve of each *cis* isomer studied contains a band absent from all-*trans* curves. This weak to moderate

Table 1. Stereochemically significant infrared bands in some c i s-t r a n s isomeric diphenylpolyene sets, listed in microns, at their absorption maxima. The figures in parentheses represent units of 10 % absorption [e. g. (6) stands for 60 % absorption or 40 % transmittance].

Compound	Configuration	In-plane bending vibration of H atoms attached to *cis* double bonded carbons	Out-of-plane rocking of H atoms attached to a *trans* double bonded carbon, in conjugation with:		Out-of-pl. rocking of H atoms attached to *cis* double bonded carbons	Out-of-plane rocking of a pair of equivalent H atoms in the rings:	
			any C = C bond	a *cis* C = C bond		hindered	unhindered
Stilbene	*trans*	—	10.42(9)	—	—		14.48(9)
	cis	7.09(2)	—	—	12.84(8)	14.37(9)	
Diphenyl-butadiene	*trans*	—	10.15(9)	—	—		14.54(9)
	mono-*cis*	7.07(3)	10.14(6)	10.58(6)	12.90(5)	14.33(8)	14.50(8)
	di-*cis*	7.31(2)	—	—	12.91(7)	14.20(8)	14.39(9)
Diphenyl-hexatriene	*trans*	—	10.06(8)	—	—		14.50(5)
	Δ^3-*cis*	7.03(2)	10.06(5)	10.42(10)	12.85(2)	—	14.50(9)
	Δ^1-*cis*	7.05(2)	10.06(9)	10.42(5)	12.93(8)	14.26(9)	14.50(9)
Diphenyl-octa-tetraene	*trans*	—	9.90(9)	—	—		14.44(5)
	Δ^3-*cis*	7.04(2)	10.11(8)	10.30(9)	12.91(4)	—	14.52(9)
	Δ^1-*cis*	7.05(3)	10.05(10)	10.30(7)	12.95(7)	14.30(9)	14.51(9)
	$\Delta^{1,3}$-di-*cis* or $\Delta^{1,5}$-di-*cis*	7.00(2)	10.10(8)	10.30(8)	12.95(8)	14.15(7)	14.49(9)

band that was assigned earlier to vibrations of a hydrogen atom attached to a *cis* double bonded carbon [14] is located at roughly the same wave length, 7.00—7.09 μ, in all but one of our *cis* compounds, the exception being di-*cis*-diphenylbutadiene in whose spectrum the band has been shifted to 7.31 μ. Possibly, this could be explained by the "all-hindered-*cis*" feature of this isomer, unique in the diphenylpolyene series.

2. Of the three out-of-plane rocking bands referred to in the present discussion the shortest wave length one appears at 10.0—10.6 μ. This strong band was assigned earlier to the out-of-plane rocking frequencies of hydrogen atoms attached to *trans* carbon-carbon double bonds [15]; a splitting of this band has been observed in the presence of a conjugated *trans-cis* diene group [1,2]. Since the papers mentioned refer to shorter systems and hence do not mention several features needed in our discussion, the following, more detailed treatment of the 10.0—10.6 μ region seems to be justified.

According to Table 1, only the stereoisomers having at least one double bond in the *trans* configuration possess any strong absorption band(s) in this region. Since neither *cis*-stilbene nor di-*cis*-diphenylbutadiene belongs to this type, no pertinent band appears in their spectra except perhaps for traces. The presence of such trace-bands could possibly be caused by a slight *cis* → *trans* isomerization during recording.

In the region under discussion all isomers containing both a *trans* and a *cis* double bond(s) display a doublet in contrast to the all-*trans* forms. We will tentatively formulate this as follows: *a trans* C = C bond, either isolated or in conjugation with any other bond, gives rise to a vibrational frequency of a hydrogen bonded to one of the carbon atoms. We name this band the "lower λ *trans* band". On the other hand, conjugation of a *cis* to a *trans* double bond brings about a new vibrational frequency of a hydrogen atom (linked to a *trans* C = C carbon) resulting in a "higher λ *trans* band".

Although the laws governing intensity relationships in infrared spectra are not yet well understood, our formulation seems to be in agreement with the observed relative intensities of the two *trans* bands defined above, if it is assumed that the intensities are additive. While the central *cis* double bond of diphenylhexatriene is conjugated with two *trans* double bonds, only one such possibility exists for a terminal *cis* double bond. A similar statement is valid for higher diphenylpolyenes. Consequently, the "higher λ *trans* band" should appear with lower intensity in the spectrum of a terminal mono-*cis* form than in that of non-terminal mono-*cis* forms (*cf.* the inversion of the intensity ratios of the two bands demonstrated in Fig. 11, g and h as well as j and k).

The wave length position of these two peaks is not constant from set to set. When proceeding from a to l in Fig. 11, we note that with lengthening the conjugated system the "lower λ *trans* band" migrates towards higher frequencies; it reaches approximate constancy and perhaps it is even shifted again in the opposite direction when systems containing 3—4 conjugated double bonds are reached. Analogous is the behavior of the "higher λ *trans* band" whose shift is even more extensive. Consequently, the two peaks lie more closely together in the spectra of the higher diphenylpolyenes studied than in the lower ones. Possibly, the two bands may coincide or even exchange places in still higher systems.

The tendency to shift towards higher frequencies just mentioned is also in agreement with resonance considerations. Increasing conjugation enhances the rigidity of the chain and hence its resistance to out-of-plane vibrations, which, in terms of light absorption, means a shift to higher frequencies.

While the wave length position of the "lower λ *trans* band" is constant within simpler sets, the position given in Table 1 for the all-*trans*- and Δ^1-*cis*-diphenyloctatetraenes, *viz.* 9.90 μ and 10.05 μ respectively, are markedly lower than the corresponding data listed for the two other *cis* forms. The 9.90 μ value is explained by the circumstance that in this instance the spectrum of a solid phase (mull) had to be recorded; indeed, the curve of a saturated solution (< 0.1 %) of the all-*trans* compound shows a weak but distinct band at 10.10 μ. Similar differences between liquid and solid phase infrared spectra were observed earlier [16]. For the shift to the 10.05 μ position small amounts of the all-*trans* isomer were probably responsible, formed by a slight *cis* → *trans* rearrangement as demonstrated by the appearance of a number of small all-*trans* crystals during recording of the exceptionally labile Δ^1-*cis* isomer.

3. According to several authors [17,18,14] the band arising from the out-of-plane rocking of hydrogen atoms attached to a *cis* carbon-carbon double bond appears over a wide range, *i.e.* between 12.0 μ and 14.5 μ. In this region

all our curves contain a variety of strong to moderate bands that makes the identification of the true "*cis*-band" difficult. We believe, however, that the band at 12.84—12.95 μ, present in each *cis* compound but in none of our *trans*-forms, is this "*cis*-band". Such assignment is also in agreement with an observation made by the authors on central mono-*cis*-β-carotene whose curve shows a sharp band at 12.84 μ, absent from the all-*trans*-β-carotene curve.

Some of the other bands appearing in this region may possibly be explained in terms of conjugation, in analogy to the splitting of the "*trans*-band" mentioned in Section 2. For another explanation of the richness of bands in this region, *cf.* the discussion of the $\sim$ 13.5 μ band in the Chapter, "Aromatic Bands".

4. As mentioned above, the presence of a terminal *cis* double bond results in a splitting of the 14.5 μ aromatic band, yielding a new one at a somewhat shorter wave length. If it is assumed that the latter band is caused by the out-of-plane rocking of ring-hydrogens in *o*-position to the side-chain, this splitting becomes explainable on the basis of the spatial conflict between an aliphatic hydrogen and an *ortho* ring-hydrogen (Fig. 6). The resistance to the out-of-plane rocking of such a ring-hydrogen would require energy absorption on a higher level, *i.e.* at a lower wave length, as shown by all sterically hindered *cis* compounds studied. The new band would then become a part of a doublet since the 14.5 μ band would not be expected to disappear; it represents the normal ("unhindered") vibrations of *ortho* hydrogen atoms.

Another possible interpretation of the doublet could be formulated as follows: Upon the introduction of one terminal *cis* bond the rings will no longer be substituted equally, considering their different distances from the *cis* bond mentioned. This situation would then give rise to two (instead of one) vibrational frequencies. Such an explanation seems, however, to be in contradiction to the lack of a doublet in the Δ^3-*cis*-diphenyloctatetraene spectrum (Fig. 9, j). The writers are in favor of the first explanation of the doublet given above.

EXPERIMENTAL

The preparation and purification of the substances used are described in References 1—4. All samples were chromatographically and U.V.-spectroscopically pure, and solutions of *cis* forms were re-checked chromatographically for possible *cis* → *trans* isomerization after each recording. This check gave satisfactory results except for *cis*-II-diphenyloctatetraene that underwent considerable rearrangement, possibly caused by infrared illumination; as mentioned, tiny crystals of the all-*trans* compound were visible in the cell after recording. However, rapid operations (including recording) have probably reduced the extent of this rearrangement to less than 10 %.

The solvents used were, "Eastman Spectro Grade". All but two substances were recorded in 1.0 % carbon tetrachloride and *cyclo*hexane solutions using a 1.0 mm sodium chloride cell. Some of the minor isomers were filled into a micro cell (1.0 mm). A saturated ($\sim$ 0.3 %) *cyclo*hexane solution of all-*trans*-diphenylhexatriene was used for recording that part of the spectrum which had to be taken in this solvent. For the still less soluble all-*trans*-diphenyloctatetraene a Mineral Oil (No. 7) mull was used.

The solutions were prepared and filled into the cells in dim light, and exposure to short wave length light was avoided during the recordings.

In all experiments a Perkin-Elmer self-recording infrared spectrophotometer, Model 21 was used. The curves given in Figs. 7—10 represent combinations of the regions taken in carbon tetrachloride (2—6; 6.6—7.95; 8.30—9.90; 10.70—11.80 μ) and in *cyclo*hexane (6—6.55; 10.00—10.70; 12.05—15.00 μ).

SUMMARY

Infrared spectra of several *cis-trans* isomeric diphenylpolyenes, $C_6H_5 \cdot (CH = CH)_n \cdot C_6H_5$, are given for the sets, $n = 1$ to 4, in the region 2 to 15 μ. The observed bands, so far as they can be interpreted at present, have been divided into two groups. The first one includes the bands caused by the presence of the aromatic ring, and the second group is characteristic for certain spatial configurations. Two "*cis*-absorption" regions are pointed out resulting from in-plane and out-of-plane vibrations of hydrogen atoms. As a rule, *trans* absorption bands are shown to be influenced by conjugation to a *cis* double bond, giving a doublet. An aromatic hydrogen rocking band was found to split in the presence of a terminal, *i.e.* sterically hindered *cis* double bond.

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Studies on the Chemistry of Lichens

VII *. Structure of Porphyrilic Acid **

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Porphyrilic acid, $C_{16}H_{10}O_7$, has been isolated from *Haematomma coccineum* (Dicks.). It contains two phenolic hydroxyl groups, one ether oxygen, one C-methyl group, one carboxyl group and one lactone group. The dimethyl ether has been oxidized by permanganate to a tetracarboxylic acid, which easily forms a yellow dianhydride. Accordingly, the carboxyl group is in *ortho*-position to the methyl group and the lactone group is of the phthalide type. On demethylation and decarboxylation the tetracarboxylic acid ultimately forms 1,7-dihydroxydibenzofuran (I) which has been synthetised. These results are in harmony with two alternative structures, V and VI, for porphyrilic acid.

Zopf [1], in his classical investigations on lichens, studied the grey, leprous species *Haematomma porphyrium* (Pers.), collected from rocks on the peninsula of Kullen, South Sweden. Among other substances, *e.g.* atranorin, he isolated a crystalline, colourless acid, which melted with decomposition at about 298° after darkening from 270°. A decomposition product, porphyrilin, crystallised as characteristic prisms from the melt. In ethanolic solution, porphyrilic acid gave an intense indigo-blue colour with ferric chloride. Crystals of porphyrilic acid gave a green colour with a solution of bleaching powder.

From the yellowish-green species *H. coccineum* (Dicks.), growing together with *H. porphyrium*, Zopf [2] isolated the same porphyrilic acid, atranorin and *l*-usnic acid.

H. coccineum var. *abortivum* (Hepp) was investigated by Hesse [3], who gave an incomplete description of a substance called coccinic acid (m.p. 262—264°), similar to porphyrilic acid. Zopf gave no analyses at all for porphyrilic acid, but in a single analysis of coccinic acid Hesse found: C 58.6; H 3.6 %.

A preliminary investigation of *H. coccineum* (Dicks.), carried out by H. Erdtman several years ago, indicated that the high melting, sparingly soluble

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substance isolated from this lichen by extraction with acetone in a Soxhlet-apparatus probably belonged to a new type of non-hydrolysable lichen acids.

The substance was very similar to Zopf's porphyrilic acid in properties and colour reactions, and this name was adopted for it. *H. coccineum* also contained atranorin and *l*-usnic acid, the latter substance in remarkably large amounts, up to 20 % of the dry weight.

For constitutional studies it was necessary to collect large amounts of lichen material. *H. coccineum* is a more or less pulverous, crustose lichen and is rather common on stone blocks and rocks in Sweden. Sometimes it covers large areas but it often grows together with other lichens, *e.g. H. porphyrium* and *Crocynia*-species, and it is difficult to collect large quantities of quite pure lichen. Our material was collected mainly in the vicinity of Stockholm and in Blekinge, South Sweden, and the greatest possible care was taken to avoid contamination by other species.

The acid was isolated by treating the crystallisate from the acetone extract with boiling benzene, whereby an undissolved residue of crude porphyrilic acid amounting to 2—6 % of the lichen material was obtained. It was further purified *via* its *cyclo*hexylamine salt and after crystallisation from ethanol was obtained as fine, colourless needles, m.p. 280—283°. (decomp.). After drying *in vacuo* at 180°, porphyrilic acid gave analytical values agreeing with the formula $C_{16}H_{10}O_7$. Preparations dried at lower temperatures retained water of crystallisation (1—2 molecules) and traces of ethanol and the analytical values were not quite satisfactory. The *cyclo*hexylamine salt had the composition $C_{16}H_{10}O_7$, $C_6H_{11}NH_2$, indicating that porphyrilic acid is a monobasic acid.

Porphyrilic acid is very sparingly soluble in most solvents but may be crystallised from ethanol or dioxan. The solutions in organic solvents and especially in alkali exhibited a bluish-white fluorescence in ultra-violet light. As found by Zopf [1] the alcoholic solution gave an intense indigo-blue colour with ferric chloride, indicating the occurrence of a phenolic hydroxyl in the *ortho*-position to a carbonyl function, probably a free or esterified carboxyl group [4].

The acid was recovered unchanged after boiling with 1 *N* sodium hydroxide solution as well as after treatment with cold concentrated sulphuric acid and hence is not a depside or a depsidone.

The substance did not contain any methoxyl groups and exhibited no keto-function. Kuhn-Roth determination gave evidence for the presence of one C-methyl group.

With diazomethane, the acid yielded methyl O-dimethyl porphyrilate, $C_{15}H_7O_3(OCH_3)_2COOCH_3$, m.p. 270—272°, hydrolysed by alkali to O-dimethyl-porphyrilic acid, $C_{15}H_7O_3(OCH_3)_2COOH$, also obtained with dimethyl sulphate and alkali. These derivatives were very sparingly soluble in organic solvents.

Hydrolytic titration of the dimethyl ether revealed the presence of a lactone group in addition to the free carboxyl group.

With acetic anhydride and pyridine porphyrilic acid afforded a diacetate, $C_{15}H_7O_3(OCOCH_3)_2COOH$, m.p. 262—265° (decomp.), transformed into a methyl ester, $C_{15}H_7O_3(OCOCH_3)_2COOCH_3$, by diazomethane. Acetylation with acetic anhydride alone yielded another acetate, m.p. 197—199°, which analysed approximately for $C_{15}H_7O_3(OCOCH_3)_2COOCOCH_3$. It gave acetanilide by treatment with aniline in the cold and was obviously a mixed anhydride.

When heated to the melting point, porphyrilic acid lost one molecule of carbon dioxide, yielding a characteristic decomposition product, porphyrilin, $C_{15}H_{10}O_5$, m.p. about 295—300° with sublimation, which like porphyrilic acid exhibited the blue ferric chloride reaction, the green colour with bleaching powder and the strong fluorescence in ultra-violet light. Porphyrilin contained one C-methyl group. On prolonged treatment with diazomethane it furnished a dimethyl ether, $C_{15}H_8O_3(OCH_3)_2$, and acetylation afforded a diacetate, $C_{15}H_8O_3(OCOCH_3)_2$. Hydrolytic titration showed the presence of a lactone group.

In carbonate buffer, O-dimethyl-porphyrilic acid could be oxidized with potassium permanganate to a tetracarboxylic acid (A), $C_{12}H_2O(OCH_3)_2(COOH)_4$, characterized by its tetramethylester. On heating, the acid (A) lost two molecules of water, yielding a yellow dianhydride (B), $C_{12}H_2O(OCH_3)_2(C_2O_3)_2$, m.p. about 330—335° with sublimation.

These results show that porphyrilic acid contains two phenolic hydroxyl groups, one carboxyl group in *ortho*-position to a C-methyl group and one lactone group of the phthalide type. With the exception of the carboxyl group, porphyrilin contains the same functional groups as porphyrilic acid and is obviously formed by a simple decarboxylation. One oxygen atom is unaccounted for in both compounds and is probably present as an ether bridge. The empirical formula for porphyrilic acid, $C_{16}H_{10}O_7$, may therefore be resolved into $(CH_3)(COOH)(OH)_2$ $(—CH_2—O—CO—)C_{12}H_2O$. The $C_{12}H_2O$-residue, due to its composition, appeared to contain a dibenzofuran structure, porphyrilic acid being a hexasubstituted dibenzofuran.

When boiled with hydrobromic acid, the anhydride (B) underwent demethylation and partial decarboxylation to a dihydroxy-dicarboxylic acid (C), $C_{12}H_4O(OH)_2(COOH)_2$, characterized as the tetramethylderivative, $C_{12}H_4O(OCH_3)_2(COOCH_3)_2$.

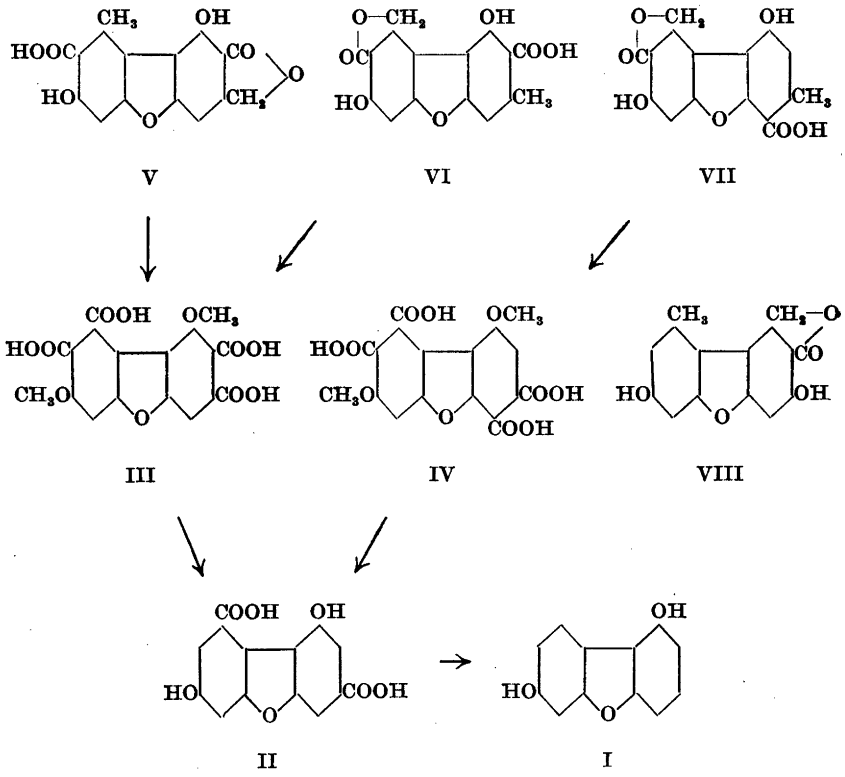
The acid (C) was further decarboxylated by heating with quinoline and a copper chromite catalyst, whereby a phenol (D), $C_{12}H_6O(OH)_2$, m.p. 162.5—164°, was obtained in 70 % yield. It yielded a diacetate, $C_{12}H_6O(OCOCH_3)_2$, m.p. 141—142°. If the above deduction is correct this phenol should be a dihydroxy-dibenzofuran.

In view of the dominating role of orcinol in lichen chemistry it seemed to be most probable that the phenol (D) was related to the dihydroxy-dibenzofuranes derived from a dehydro-diresorcinol. The symmetrical 3,7-dihydroxy- and 1,9-dihydroxy-dibenzofuranes are known [5,6], having m.p. 241° and 215° respectively. The unsymmetrical 1,7-dihydroxy-dibenzofuran, (I), m.p. 163.5—164.5°, has now been prepared. A mixed Ullmann coupling of 2-iodo-resorcinol dimethyl ether and excess of 4-iodo-resorcinol dimethyl ether yielded 2,4,2′,4′-tetramethoxy-biphenyl, m.p. 92—93° (lit.[7] m.p. 93°), 2,6,2′,6′-tetramethoxy-biphenyl, m.p. 174—175° (lit.[8] m.p. 175—176°) and the unknown unsymmetrical isomeride, 2,6,2′,4′-tetramethoxy-biphenyl, m.p. 106—107°. When boiled with hydrobromic acid, the last compound underwent simultaneous demethylation and ring closure to 1,7-dihydroxy-dibenzofuran (I) which was found to be identical with substance (D) by mixed melting point determinations carried out with the free phenols and with the acetates.

Acid (C), from which the phenol (D) (I) is obtained, must have structure (II), as it is decarboxylated only with difficulty, a property characteristic of aromatic *meta*-hydroxy-carboxylic acids. The absence of carboxyl groups in *ortho*-position to a hydroxyl group is further supported by the reddish-brown ferric chloride reaction of this acid.

For the tetracarboxylic acid (A) two structures, (III) and (IV), are probable alternatives. Both should give dianhydrides on heating and loose two molecules of carbon dioxide on demethylation with hydrobromic acid.

From the formulae (III) and (IV) several structures for porphyrilic acid may be derived. An acceptable alternative, however, must explain the smooth decarboxylation of porphyrilic acid to porphyrilin and also the blue ferric reaction of both these substances. These requirements are fulfilled by structures (V), (VI) and (VII), all of which embody the structure of orsellinic acid. In structures (V) and (VI), the free carboxyl group occupies an *ortho*-position but in structure (VII) a *para*-position to a free hydroxyl group. Compounds of the former type are known to form borate complexes, which may be detected by paper chromatographic methods [9]. The R_F values of porphyrilic acid on borate- and phosphate-impregnated paper of pH 8.7 were 0.03 and 0.15 respectively, a clear indication of the formation of a borate complex, and hence structure (VII) may be eliminated. Consequently, porphyrilic acid must be



represented by either of formulae (V) and (VI) and the structure of the acid (A) is (III).

A decision between the alternatives (V) and (VI) requires partial oxidation of O-dimethyl-porphyrilic acid to a tricarboxylic acid, decarboxylation of this acid to the dihydroxy-methyldibenzofuran and synthesis of this compound. The degradations have been accomplished and the synthetic work is in progress.

An isomer of porphyrilin is known among the lichen acids: Strepsilin, m.p. 324°, which has been isolated by Zopf[10] from *Cladonia strepsilis* Wain. According to Shibata [11] it is a derivative of 3,7-dihydroxy-dibenzofuran (VIII). The two substances show great similarities in properties and colour reactions.

EXPERIMENTAL *

Isolation of porphyrilic acid. Extraction of the lichen material with acetone for 2—3 days in a Soxhlet apparatus yielded an acetone extract, worked up as here described for a single run on air-dry *H. coccineum* (160 g, corresponding to 120 g dry, ash-free material). The acetone-solution (2 000 ml) deposited a greyish crystallisate A (7.4 g) which was collected. Evaporation of the filtrate to dryness and extraction of the residue with warm ethanol (250 ml) yielded a yellow crystallisate B (15.6 g), insoluble in ethanol. The mother liquor on evaporation yielded an oil (28 g) containing crystals. It was not further investigated.

Fraction B was dissolved by warming with a saturated solution of sodium acetate in 50 % ethanol (500 ml). The undissolved residue (0.7 g) was crystallised from chloroform (m. p. 195—196°) and identified as atranorin by hydrolysis with 100 % sulphuric acid and subsequent paper chromatography [12].

The solution on dilution with water yielded *l*-usnic acid (11.4 g), m. p. 198—199°, $[\alpha]_D^{20} = -488°$ (Chloroform, $c = 0.266$) after crystallisation from chloroform-ethanol.

Fraction A was extracted with boiling benzene, dissolved in boiling ethanol (1 500 ml) and filtered. Excess *cyclo*hexylamine (4 ml) was added, the *cyclo*hexylamine salt (6.7 g) collected after one night in the refrigerator and washed with ethanol. It was suspended in 50 % ethanol, decomposed with 2 *N* hydrochloric acid and the porphyrilic acid (4.9 g) collected and washed with water. The substance was finally crystallised from ethanol (2 500 ml), yielding fine needles (3.5 g). The analytical sample was purified *via* the *cyclo*hexylamine salt and crystallised several times from ethanol; m. p. 280—283° (decomp.) after darkening from 270°. The substance was analysed after drying to constant weight *in vacuo* at 180° (*prep. I*), at 120° (*prep. II*) and at 70° (*prep. III*). It titrates in the cold as a dibasic acid (thymolphtalein) or as a monobasic acid (bromothymol blue); the end points are not very sharp. (Found: *Prep. I* C 61.1; H 3.31; Equiv. weight 158 ± 5; 323 ± 10. $C_{16}H_{10}O_7$ requires C 61.2; H 3.21; Equiv. weight 157.1; 314.2. *Prep. II* C 57.5; H 3.76; C—CH_3 3.9; 4.0. $C_{16}H_{10}O_7$, H_2O requires C 57.8; H 3.64; C—CH_3 4.5. *Prep. III* C 56.7; H 4.06. $C_{16}H_{10}O_7$, 1.5 H_2O requires C 56.3; H 3.84.)

Colour reactions: Ferric chloride-ethanol, indigo blue; bleaching powder, green; 2,6-dichlorquinone-chlorimide (Gibbs reagent), blue; diazotised benzidine, red. Cold concentrated sulphuric acid dissolved porphyrilic acid, giving a colourless solution, from which it was recovered unchanged on addition of water. The acid is scarcely soluble in sodium bicarbonate solution, but is soluble in sodium carbonate and sodium hydroxide solutions. The solutions are colourless. Boiling with 1 *N* sodium hydroxide for one hour did not affect the acid. Alkaline permanganate was reduced rapidly in the cold, but ammoniacal silver nitrate solution only on heating.

The *cyclo*hexylamine salt was analysed after crystallisation from 50 % ethanol and drying *in vacuo* at 90°. M. p. 274—278° (decomp.). (Found: C 63.7; H 5.64; N 3.16. $C_{16}H_{10}O_7$, $C_6H_{11}NH_2$ requires C 63.9; H 5.61; N 3.38.)

* All melting points uncorrected.

Paper chromatography. The purity of porphyrilic acid and many of its derivatives and degradation products was conveniently controlled by chromatography on buffered paper with *n*-butanol-water as solvent system [9,12]. On paper (Whatman no. 1) impregnated with phosphate buffer of pH 8.7 and 11.7 the R_F values of porphyrilic acid were 0.15 and 0.02 respectively, whereas the R_F value of porphyrilic acid on paper impregnated with borate buffer of pH 8.7 was only 0.03. The spots showed a bluish-white fluorescence in ultraviolet light and could also be detected by spraying with a solution of diazotised benzidine or with Gibbs reagent.

Acetylation of porphyrilic acid. a) *Diacetoxy-porphyrilic acid.* Porphyrilic acid (0.30 g) was briefly boiled with acetic anhydride (5 ml) and pyridine (5 ml). The solution was evaporated to dryness *in vacuo*, giving a quantitative yield of the diacetate, crystallising from acetone or ethanol in needles, m. p. 262—265° (decomp.). (Found: C 60.6; H 3.58; CH_3CO 21.1, 22.1; Equiv. weight 399. $C_{20}H_{14}O_9$ requires C 60.3; H 3.54; CH_3CO 21.7; Equiv. weight 398.3.)

b) *Methyl diacetoxy-porphyrilate.* A small excess of ethereal diazomethane was added to a solution of the diacetate (250 mg) in acetone (150 ml), followed after 0.5 minutes by a few drops of acetic acid. The methyl ester crystallised from acetone in fine needles, m. p. 241—243°. (Found: C 61.5; H 3.97; OCH_3 7.60, 7.42. $C_{21}H_{16}O_9$ requires C 61.2; H 3.91; OCH_3 7.53.)

c) *Mixed anhydride of diacetoxy-porphyrilic acid and acetic acid.* Porphyrilic acid (450 mg) was boiled for 30 minutes with acetic anhydride (10 ml) and the solution evaporated to dryness *in vacuo*. The product crystallised from dry acetone in needles, m. p. 197—199°, then solidifying and decomposing gradually from 250°. (Found: C 60.2; H 3.66; CH_3CO 27.1. $C_{22}H_{16}O_{10}$ requires C 60.0; H 3.66; CH_3CO 29.3.)

d) *Reaction of the mixed anhydride with aniline.* Aniline (0.1 ml) was added to a solution of the above acetate (140 mg) in dry acetone (60 ml). After standing over-night at room-temperature, the solution was evaporated to dryness *in vacuo* and the residue treated with ether (100 ml). The ethereal solution was washed with 2 *N* hydrochloric acid, sodium carbonate solution and water and evaporated to dryness. Acetanilide (25 mg), m. p. 110—111°, was obtained and identified by direct comparison with an authentic specimen (m. p. 113—114°).

Methylation of porphyrilic acid. a) *Methyl O-dimethyl porphyrilate.* Porphyrilic acid (1 g) was suspended in acetone containing a trace of methanol and treated with an excess of ethereal diazomethane for several days, until a sample gave no colour with ferric chloride. After several crystallisations from pyridine and pyridine-ethanol the product formed fine needles, m. p. 270—272°, sparingly soluble in most organic solvents. The substance showed a very intense white fluorescence in ultraviolet light. For analysis the sample was dried *in vacuo* at 120°. (Found: C 64.1; H 4.53; OCH_3 25.8. $C_{19}H_{16}O_7$ requires C 64.0; H 4.53; OCH_3 26.1.)

b) *O-Dimethyl-porphyrilic acid.* The above ester (500 mg) in 1 *N* sodium hydroxide (20 ml) and ethanol (20 ml) was boiled under reflux for one hour, and the solution acidified with hydrochloric acid. The voluminous precipitate was collected, dried and crystallised from pyridine (50 ml), yielding small needles (400 mg). Before analysis it was recrystallised twice from pyridine and finally washed with glacial acetic acid and methanol and dried *in vacuo* at 130°. The acid gradually decomposed from about 320°. On phosphate-impregnated papers of pH 8.7 and 11.7, the acid showed R_F values 0.45 and 0.30 respectively (*n*-butanol-water). The spots were detected by means of their bluish-white fluorescence in ultra-violet light.

The pyridine solution of O-dimethyl-porphyrilic acid titrates with 0.05 *N* sodium hydroxide as a monobasic acid (bromothymol blue). After hydrolysis with excess boiling 0.05 *N* sodium hydroxide (20 minutes) and back-titration of the cold solution with 0.02 *N* hydrochloric acid, it consumed two moles of alkali (thymol blue). (Found: C 62.6, 62.9; H 4.19, 4.26; OCH_3 18.2; Equiv. weight 344; 172. $C_{18}H_{14}O_7$ requires C 63.2; H 4.12; OCH_3 18.1; Equiv. weight 342.3; 171.2.)

The same compound was obtained by methylation with dimethyl sulphate: Sodium hydroxide solution (5 ml, 40 %) was added to a suspension of porphyrilic acid (5.1 g) in water (100 ml) and methanol (50 ml), whereupon the acid passed into solution. Dimethyl sulphate (50 ml) and sodium hydroxide solution (50 ml, 40 %) were added in small portions with continuous shaking. At the end of the reaction a crystalline precipitate appeared consisting of methyl O-dimethyl porphyrilate. The reaction mixture was acidified with

hydrochloric acid, the precipitate collected and boiled for one hour with 1 *N* sodium hydroxide (150 ml) and ethanol (150 ml) to hydrolyse the ester. Acidification with hydrochloric acid yielded crude O-dimethylporphyrilic acid (5.0 g), identified by paper chromatography as described above. It was used without further purification to the oxidative degradations.

Porphyrilin. Porphyrilic acid was heated in a test tube to about 300° and the gas evolved taken up in barium hydroxide solution and identified as carbon dioxide. The decomposition product, porphyrilin, sublimed as long yellowish-white needles on the wall of the tube. Preparatively, porphyrilic acid (300—500 mg) was rapidly distilled in a test tube *in vacuo* over a free flame. Porphyrilin sublimed in 70—80 % of the theoretical yield and crystallised from methyl ethyl ketone or glacial acetic acid as white needles, m.p. 295—300° with sublimation from about 250°. It was sparingly soluble in the common solvents and gave a green colour with bleaching powder, an indigo-blue colour with ferric chloride and a blue colour with Gibbs reagent. Sodium hydroxide solution dissolved the substance giving a yellowish solution, but it was only slightly soluble in sodium carbonate solution.

On phosphate-buffered paper of pH 8.7 and 11.7, porphyrilin showed R_F values of 0.72 and 0.50 respectively. On borate-buffered paper of pH 8.7 it showed R_F = 0.69. (*n*-butanol-water.) The spots were detected in ultra-violet light (bluish-white fluorescence) and gave a redbrown colour with a solution of diazotised benzidine. Crude preparations of porphyrilin gave a second spot (R_F = 0.08, pH 11.7), having an intense yellowish fluorescence in ultra-violet light. This unknown impurity prooved very difficult to remove completely.

The analytical sample was crystallised from methyl ethyl ketone and dried *in vacuo* at 50° (*prep. I*) and at 150° (*prep. II*). The equivalent weight was determined by boiling for one hour with 0.05 *N* sodium hydroxide and back-titrating with 0.02 N hydrochloric acid (thymol blue). (Found: *prep. I.* C 62.4; H 4.40. $C_{15}H_{10}O_5$, H_2O requires C 62.5; H 4.20. *Prep. II.* C 65.9; H 3.74; equiv. weight 270 ± 10. $C_{15}H_{10}O_5$ requires C 66.7; H 3.73; equiv. weight 270.2.)

Porphyrilin dimethyl ether. Porphyrilin (300 mg) was dissolved in acetone (350 ml) containing a trace of methanol and treated with an excess of ethereal diazomethane for about two weeks until a sample gave only a faint colour with ferric chloride. The crude dimethyl ether was distilled *in vacuo* and crystallised from glacial acetic acid in needles, m.p. 267—269°. (Found: C 68.4; H 4.66; OCH_3 20.2; C—CH_3 4.4. $C_{17}H_{14}O_5$ requires C 68.5; H 4.73; OCH_3 20.8; C—CH_3 5.0.)

Porphyrilin diacetate. Porphyrilin (130 mg) was boiled briefly with acetic anhydride (3 ml) and pyridine (2 ml) and allowed to stand over night. The product (140 mg) was collected and crystallised from acetone in long needles, m.p. 235—237°. (Found: C 64.4; H 3.78; CH_3CO 24.0. $C_{19}H_{14}O_7$ requires C 64.4; H 3.98; CH_3CO 24.3.)

Permanganate oxidation of O-dimethyl porphyrilic acid. A solution of O-dimethyl porphyrilic acid (1.14 g = 3.35 mmoles) in water (200 ml) containing sodium carbonate (1.5 g) was heated on the boiling water-bath and powdered potassium permanganate (2.10 g = 13.3 mmoles) was added in small portions with stirring over 7 hours. After addition of a few drops of methanol, the manganese sludge was removed by filtration and washed with hot water. The combined filtrates were concentrated to 40 ml and acidified with conc. hydrochloric acid (5 ml). The crude acid (A) was collected, dissolved in 0.2 *N* sodium hydroxide (40 ml), the solution filtered and the acid precipitated in the cold with 2 *N* hydrochloric acid (5 ml). The compound (830 mg, yield 60 %) formed a yellowish white powder and was analysed after drying *in vacuo* at room temperature. The acid is sparingly soluble in most common solvents and possesses no sharp melting point. It easily looses water on heating, forming a yellow dianhydride (see below). (Found: OCH_3 15.3; equiv. weight 102. $C_{18}H_{12}O_{11}$ requires OCH_3 15.4; equiv. weight 101.1.)

Treatment of the acid (A) in acetone suspension with ethereal diazomethane furnished a tetramethyl ester, crystallising from acetone in prisms, m.p. 221.5—223°. (Found: C 57.5; H 4.40; OCH_3 39.8. $C_{22}H_{30}O_{11}$ requires C 57.4; H 4.38; OCH_3 40.5.)

Dianhydride of acid A. The acid (A) (980 mg) was heated *in vacuo* at 150° (loss of weight: found 8.9, calc. 9.6 %) and then sublimed *in vacuo* (1 mm) over a free flame; yield 850 mg. A small sample was resublimed (bath-temp. 270—290°, 0.05 mm) and analysed. The anhydride formed a micro-crystalline, yellow powder, m.p. 330—335° with sublimation. It was hydrolysed with boiling aqueous pyridine and the resulting solution

titrated with 0.05 *N* sodium hydroxide (bromothymol blue). (Found: C 58.5; H 2.29; OCH_3 17.0; equiv. weight 92. $C_{18}H_8O_9$ requires C 58.7; H 2.19; OCH_3 16. 9; equiv. weight 92.1.)

Demethylation and decarboxylation of acid (A). The dianhydride (B) (920 mg) of the acid (A) was heated under reflux for 3 days with hydrobromic acid (25 ml, $d = 1.5$). After cooling, the precipitate of the crude acid (C) (750 mg) was collected and crystallised from dioxan until pure, as shown by paper chromatography. The acid formed yellow needles, which gradually decomposed from about 250°. It had an R_F value of 0.55 on phosphate-buffered paper of pH 4.5 (*n*-butanol-water). The spot showed a yellowish fluorescence in ultra-violet light and gave a brown colour with diazotised benzidine. In ethanol, the acid gave a reddish brown colour with ferric chloride. It crystallised with 2 molecules of dioxan, removed completely only after drying *in vacuo* at 180°. (Loss of weight: 38.2 %. $C_{14}H_8O_7$, 2 $C_4H_8O_2$ requires 38.0 %. Found: C 57.8; H 2.74; $C_{14}H_8O_7$ requires C 58.3; H 2.80).

With ethereal diazomethane, the acid yielded a tetramethylderivative, crystallising from ethanol or acetic acid in light brownish needles, m.p. 149.5—150.5°. (Found: C 62.8; H 4.72; OCH_3 35.25. $C_{18}H_{16}O_7$ requires C 62.8; H 4.68; OCH_3 36.05).

Decarboxylation of acid (C). The acid (C) (300 mg) in quinoline (5 ml) was boiled with a copper chromite catalyst (60 mg) for 3 hours in a nitrogen atomsphere. The reaction mixture was taken up in ether, filtered and the insoluble material washed thoroughly with ether. The ethereal solution was shaken with 2 *N* sulphuric acid and water, dried with sodium sulphate and evaporated to dryness. On distillation *in vacuo* the resulting brownish oil (200 mg) yielded the crude phenol (D) (160 mg) as yellowish crystals, m.p. 150—153°. Crystallisation from benzene afforded needles, m.p. 162.5—164°, undepressed on admixture with synthetic 1,7-dihydroxy-dibenzofuran, m.p. 163.5—164.5°, described below. Colour reactions: ferric chloride, no colour: Gibbs reagent, blue; diazotised benzidine, red. The two phenols also proved chromatographically indistinguishable ($R_F = 0.14$, solvent system benzene-water) and clearly distinguished from 3,7-dihydroxy-dibenzofuran [5] (m.p. 241°, $R_F = 0.07$) and 1,9-dihydroxy-dibenzofuran [6] (m.p. 215°, $R_F = 0.75$). (Found: C 71.3; H 3.94. $C_{12}H_8O_3$ requires C 72.0; H 4.03.)

The diacetate, obtained from the crude phenol with acetic anhydride and pyridine, crystallised from methanol in needles, m.p. 141—142°, undepressed when mixed with synthetic 1,7-diacetoxy-dibenzofuran, m.p. 142—143°. (Found: C 66.7; H 4.27; $C_{16}H_{12}O_5$ requires C 67.6; H 4.26.)

2,6,2',4'-tetramethoxy-biphenyl. 4-Iodo-resorcinol dimethyl ether was prepared as described by Kauffmann and Kieser [13]. 2-Iodo-resorcinol dimethyl ether was obtained from 2-nitroresorcinol as described by Adams *et al.*[8]. 4-Iodo-resorcinol dimethyl ether (27 g), 2-iodo-resorcinol dimethyl ether (3 g) and copper bronze (90 g) was mixed thoroughly and warmed in a saltbath to 190—200°. As soon as the reaction started, the temperature in the reaction vessel rapidly rose to 260—280° and the heating was interrupted. After 10 minutes, the reaction was completed by warming to 250° for 20 minutes. The reaction mixture was extracted with acetone and the solution concentrated to an oil, which was distilled *in vacuo*. The main fraction, 10.7 g (200—220°, 1 mm), on crystallisation from methanol (40 ml) yielded a fraction A (8.2 g), m.p. 85—88°. The motherliquor on slow evaporation in the air yielded a fraction B (0.9 g), m.p. 90—100°, from which by repeated crystallisation from methanol pure 2,6,2',4'-tetramethoxy-biphenyl (0.1 g), m.p. 106—107°, was obtained. (Found: C 69.2; H 6.59; OCH_3 44.9. $C_{16}H_{18}O_4$ requires C 70.1; H 6.61; OCH_3 45.25.)

Crystallisation from methanol of fraction A gave minute amounts of 2,6,2',6'-tetramethoxy-biphenyl [8], m.p. 174—175° and 2,4,2',4'-tetramethoxy-biphenyl [7] (6 g), m.p. 92—93°.

A more convenient separation of the isomeric biphenyls was achieved by chromatography on alumina, using the method of fractional elution [14]. The column was constructed in light petroleum (b.p. 40—60°). The biphenyl-mixture (1 g, from fraction B and its mother-liquor) in benzene-petrol (100 ml; 7—93) was adsorbed on alumina (30 g). The 2,4,2',4'-tetramethoxy-biphenyl was eluted by the same solvent, and the 2,6,2',4'-tetramethoxy-biphenyl by gradually increasing the benzene-content to 30 vol. %. Yield 0.3 g, m.p. 102—104° after one crystallisation from methanol.

1,7-dihydroxy-dibenzofuran. 2,6,2',4'-tetramethoxy-biphenyl (0.47 g) and hydrobromic acid (10 ml, $d = 1.5$) were boiled under reflux in an atmosphere of carbon dioxide

for 10 hours. On cooling, the dark solution deposited greyish needles, which were collected, dried and distilled *in vacuo*, yielding a rapidly crystallising oil (0.27 g). The substance crystallised from benzene in colourless needles, m. p. 163.5—164.5°; it was also observed in a lower-melting form, m. p. 156—157°. The colour reactions were identical with those described above for the phenol (D). (Found: C 70.8; H 3.94. $C_{12}H_8O_3$ requires C 72.0; H 4.03.)

1,7-diacetoxy-dibenzofuran. This compound was obtained from the phenol with acetic anhydride and pyridine. It crystallised from methanol in needles, m. p. 142—143°. (Found: C 67.6; H 4.20. $C_{16}H_{12}O_5$ requires C 67.6; H 4.26.)

1,7-dimethoxy-dibenzofuran. Treatment of the phenol with ethereal diazomethane for two days furnished an oil which was distilled *in vacuo* and then crystallised from 50 % acetic acid. Needles, m. p. 67—68°. (Found: OCH_3 27.8. $C_{14}H_{12}O_3$ requires OCH_3 27.2.)

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Studies on Humic Acids

VI. Triquinone and Tetraquinone *

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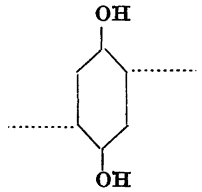
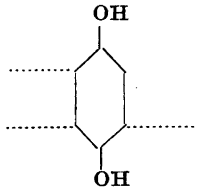
In order to gain information about the properties of phenolic polyphenyls believed to be structurally related to "phenol" or "quinone humic acids" tri- and tetrahydroquinone, their methylethers (X and XIII) and acetates as well as the corresponding tri- and tetraquinones (V and VI) have been prepared. The hydroquinones are extremely sensitive to oxidation, turning blue when exposed to air. Deep blue "quinhydrones" are obtained on partial oxidation with chromic acid. The polymerisation of the quinones in alkali has been studied and compared with that of benzoquinone. The U.V. absorption of the methylethers and acetates indicates a low degree of internuclear conjugation and especially that of the acetates exhibits similarities with the U.V. absorption of the "leucoacetates" obtained by reductive acetylation of "quinone humic acid".

Our studies on the action of weak alkali *e.g.* sodium acetate, on *p*-benzoquinone has led us to believe that the amorphous "quinone humic acids" so formed, essentially are partly oxidised polyhydroquinones containing such elements as I, II and III [1].



I



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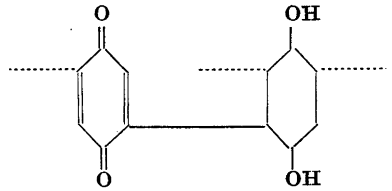
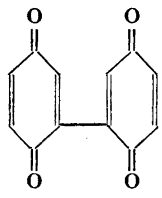


III

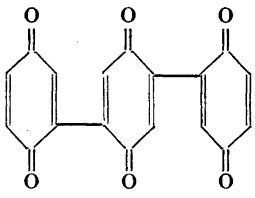
The degree of oxidation is highly dependent upon the alkalinity as shown by the amount of hydroquinone simultaneously formed in the reaction. Structure III serves only as an illustration of the theory and nothing can be said about the most probable distribution of the dissociable hydrogen atoms.

* Part V *Acta Chem. Scand.* 8 (1954) 811.

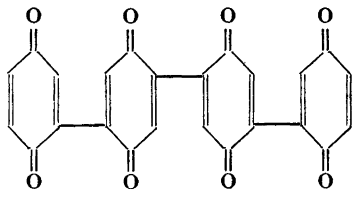
It is clear than an increased knowledge about the properties of polyhydroquinones and their corresponding quinones would be of interest in relation to the chemistry of humic acids. Diquinone (IV) and the corresponding hydroquinone are known but higher members of the series have not been described. We now record the synthesis of triquinone (V) and tetraquinone (VI) and acetates and methylethers of the corresponding hydroquinones.



IV



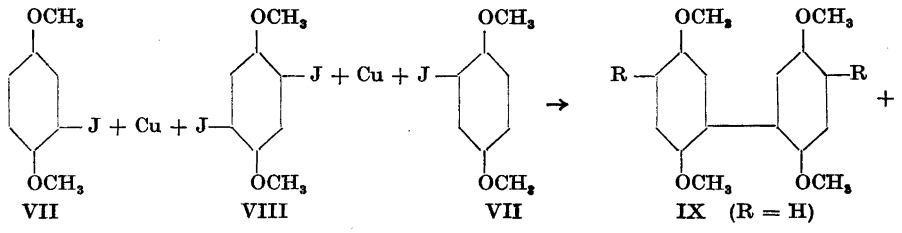
V



VI

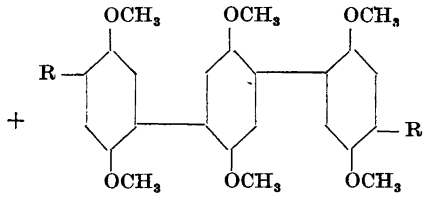
Diquinone (IV) is obtained by oxidation of the corresponding hydroquinone, 2,5,2′,5′-tetrahydroxydiphenyl, which is easily available through demethylation of 2,5,2′,5′-tetramethoxydiphenyl (IX), a product of the Ullmann coupling of iodohydroquinone dimethylether [2]. It is interesting in relation to the present research, that Barth and Schreder [3] have obtained dihydroquinone by alkali-fusion of hydroquinone. Other reaction products included a hexahydroxydiphenyl which was probably 2,4,5,2′,4′,5′-hexahydroxydiphenyl and an extremely oxidisable compound which may have been a trihydroquinone. In our previous paper we recorded the formation of dihydroquinone, albeit in a very small yield, from quinone by the action of strong sodium acetate solution.

The mixed Ullmann coupling of an excess iodohydroquinone-dimethylether (VII) and 2,5-diiodohydroquinonedimethylether (VIII) yielded a mixture containing 2,5,2′,5′-tetramethoxydiphenyl (IX) and 2,5-dimethoxy-1,4-bis-[2,5-dimethoxyphenyl]-benzene (X) from which these components could be separated by fractional distillation and crystallisation:



VII VIII VII IX (R = H)

IXa (R = Br)

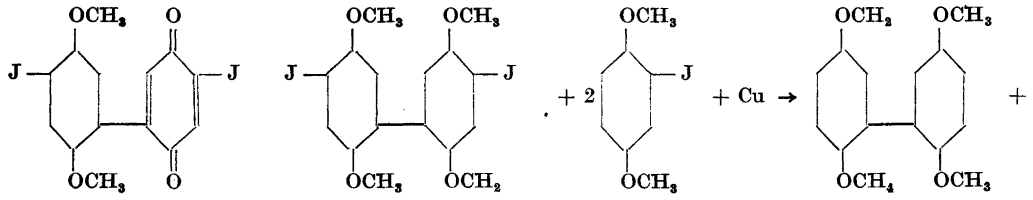


X (R = H)
Xa (R = Br)

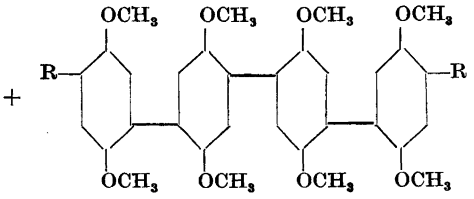
Demethylation of IX and X with pyridinium hydrochloride proceeded without complications. Trihydroquinone (X; OH instead of OCH_3) is very unstable and is easily oxidised to blue products, especially when moist. Acetylation gave the hexaacetate and oxidation with chromic acid afforded triquinone (V). With insufficient amounts of chromic acid, blueblack crystalline "quinhydrones" were obtained. By varying the amount of chromic acid, products could be obtained which may have contained 1 or 2 quinonoid nuclei. They were very similar, yielding triquinone on further oxidation and like triquinone gave trihydroquinone hexaacetate on reductive acetylation, but were not further characterised. Triquinone is a yellow substance which is very sparingly soluble in organic solvents. Only 10 mg substance were dissolved after 48 hours' continuous extraction with chloroform in a Soxhlet apparatus. Triquinone may be recrystallised from ethyl benzoate but this operation must be carried out quite rapidly to avoid extensive decomposition. Recoveries larger than 50 % were not obtained. Diquinone may be recrystallised from ethanol, but this is not advisable because the substance suffers some decomposition in ethanol solution. Triquinone is more stable to alcohol, but this is probably due to its low solubility.

During earlier studies on the action of sulphuric acid in acetic acid on quinone we were able to isolate — apart from large amounts of polymeric products and hydroquinone — a small amount of a compound $C_{18}H_{10}O_4$ which was probably an *o*-(or *p*-) diphenylbenzene derivative [4]. As it gives a diacetate and a dimethylether, it is probably a dihydroxy-*tri*phenylene dioxide. This compound could not be obtained by a similar treatment of trihydroquinone (X; OH instead of OCH_3) which was recovered unchanged. Hence trihydroquinone is probably not an intermediate product in the formation of the compound $C_{18}H_{10}O_4$.

According to Posternak *et al.*[5] oxidation of iodohydroquinone dimethylether with chromic acid gives 2,5-dimethoxy-4,4′-diiodo-diphenylquinone-2′,5′



XI XII VII IX



XIII (R = H)
XIIIa (R = Br)

(XI). This quinone was reduced to the corresponding phenol followed by methylation to 4,4′-diiodo-2,5,2′,5′-tetramethoxydiphenyl (XII). Mixed Ullmann coupling with excess iodohydroquinone dimethylether (VII) afforded a mixture of 2,5,2′,5′-tetramethoxydiphenyl (IX) and 2,5,2′,5′-tetramethoxy-4,4′-bis-[2,5-dimethoxyphenyl]-diphenyl (XIII) separated by distillation and crystallisation.

The demethylation of the quaterphenyl derivative XIII presented no difficulties but the phenol was easily oxidised in air turning blue and it was therefore acetylated to the stable octaacetate or oxidised with chromic acid to tetraquinone VI. Due to its insolubility this quinone could not be recrystallised from any organic solvent and was therefore not obtained quite pure. On reductive acetylation, however, it afforded the above octaacetate in almost quantitative yield.

In the mixed Ullmann couplings high molecular byproducts were always obtained, obviously due to coupling of the bifunctional iodides, but as the monofunctional iodide was always used in large excess the amount of such products was small.

Bromination of the di-, tri- and tetrahydroquinone methylethers IX, X and XIII in chloroform invariably yielded dibromoderivatives as the sole crystalline products and their structures are therefore certainly IXa, X a and XIII a.

The melting points of the mono—tetranuclear methylethers, acetates and quinones, and for comparative purposes, those of the parent hydrocarbons are recorded in Table 1. The melting points of the hydrocarbons and methylethers increase regularly with increasing molecular weight but the four-nuclear acetate possesses a lower melting point than the three-nuclear acetate. The former is probably dimorphous. However, no indication of dimorphism was found with the other acetates. It has not been possible to explain this abnormality but we believe that the observed low melting point is correct and that there may be some connexion with the spectrochemical peculiarities of the acetates discussed below. A similar abnormality was found for the dibrominated octamethoxyquaterphenyl (XIII a).

Table 1. Melting points of benzene-, diphenyl-, terphenyl- and quaterphenyl derivatives.

Number of nuclei	M. p. of hydrocarbon	M. p. of methylethers	M.p. of dibromomethylethers	M. p. of acetates	M. p. of quinones
1	5.5°	56°	145° *	123°	116°
2	70°	105°	155°	169°	194°
3	210°	189°	280°	244°	darkens at about 230°
4	320°	246°	261°	218° (234°)	darkens at 230—270° black at 350°

* 2,5-dibromohydroquinone dimethylether.

The U.V. absorption curves of the methylethers and the acetates have been determined since they possess a considerable theoretical interest and because the latter allow certain comparisons to be made with the U.V. absorption curves of "leucoquinonehumic acid" acetates [1].

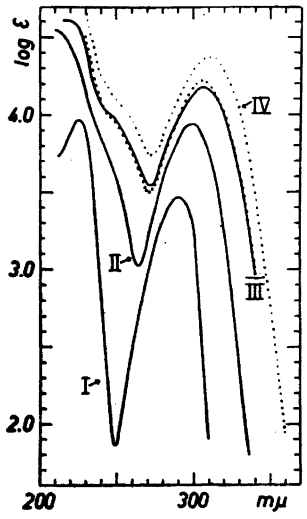


Fig. 1. Methylethers

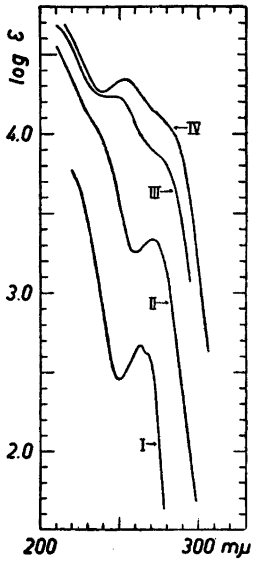


Fig. 2. Acetates

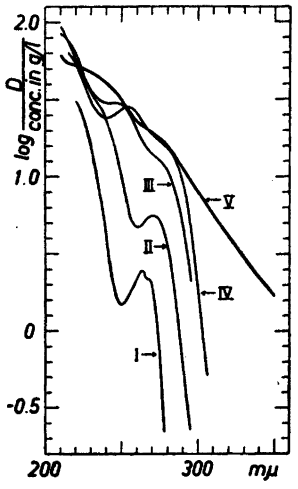


Fig. 3. Acetates on a weight by weight basis

U. V.-absorption in 95 % ethanol (————) and in dioxan (·····) for I: hydroquinone derivatives, II: dihydroquinone derivatives, III: trihydroquinone derivatives, IV: tetrahydroquinone derivatives, V: "leucoquinonehumic acid acetate."

The U.V. absorption of the methylethers (Fig. 1) exhibits regular shifts of the long-wave maximum towards red, indicating the presence of successively larger chromophores, or in other words, increasing conjugation. However, the ε-value of this maximum is, for each substance, lower than for the corresponding *p*-polyphenyl [6], indicating decreased conjugation due to the methoxyl substituents. The diphenyl K-band of the dimer, which appears as a widening of the short-wave band at about 250 mμ, also has a low intensity for the same reason. The absorption of the tri- and tetramer in the same region indicates the presence of diphenyl chromophores also in these compounds, in other words, there seems to be a certain amount of conjugation not extending over more than two nuclei. (No bands are reported for the corresponding hydrocarbons in this region [6,7].)

Fig. 2 shows the absorption curves of the acetoxy compounds. The long-wave absorption of these substances is so low and occurs at such low wavelengths (*cf.* terphenyl and quaterphenyl [6]) that there can be very little conjugation beyond the diphenyl stage. Some diphenyl conjugation seems to occur (250—260 mμ band).

In Fig. 3 the absorption of the acetoxyl compounds is compared with that of the product obtained through reductive acetylation of "sodium acetate humic acid", all curves being referred to a weight by weight basis. With increasing molecular size, the model compounds gradually approach a limit absorption curve which, if our views on the structure of the polymeric products is correct, should be similar to that of the latter at least below 300 mμ. This is indeed the case and although this product is undoubtedly more complex, it thus seems to contain a large proportion of polyphenyl elements similar to those of the model acetates.

Hydroquinone, dihydroquinone and trihydroquinone may be separated by paper partition chromatography. Employing a water saturated mixture of 9 parts of benzene and 1 part of methanol the R_F-values 0.3, 0.18 and 0.08 were obtained, bisdiazotised benzidine being used to locate the spots.

When oxidised with the theoretical amount of chromic acid, dihydroquinone affords the corresponding "quinhydrone" [3]. Similarly trihydroquinone may be oxidised to products containing one or two quinonoid nuclei. All these products possess very strong indigo blue colours and such quinhydrone-like compounds are obviously responsible for the coloration of di-, tri- and tetrahydroquinone on autoxidation in air.

In Table 2 some results of the action of alkali on various quinones are recorded. The experiments were carried out as described in part V[1] except that the amount of hydroquinone formed was estimated by oxidation of the filtrate from the humic acid and isolation of the quinone.

Table 2. Formation of humic acids and hydroquinones from various quinones by the action of alkali.

Quinone	g Quinone used	Amount of NaOH used	Time hours	Colour	ml conc. HCl used for acidification	% Humic acid	% Hydroquinone
Diquinone	0.6	15 ml 20 %	0.5	brown	12	45	1
4,4'-Dimethoxydiquinone	1.2	15 ml 20 %	1.0	green changing to brown	12	20 *	14
—„—	1.0	25 ml 8 %	1.0	green	8	20	3
Toluquinone	1.1	15 ml 20 %	0.5	green changing to brown	12	40	35

* The aqueous solution was distilled and found to contain methanol (about 45 % of theory).

The result obtained with diquinone is particularly interesting and fits in well with our theory on the polymerisation of quinone in alkali. According to this theory diquinone would be expected to yield a brown polymerisation product in which every second nucleus should be quinonoid (as in III) and no dihydroquinone should be found. The deep green solution obtained from qui-

none and toluquinone indicates a low degree of oxidation of the polymers. When shaken with air these solutions turn brown due to further oxidation. On acidification both the green and the brown solutions give amorphous humic acids which may be collected by filtration or centrifugation and washed with dilute hydrochloric acid. When they are dissolved in alkali the original colours reappear. The "leucoacetates" obtained on reductive acetylation of such quinone humic acids which are soluble in alkali with a brown colour, may be hydrolysed with alkali in the absence of air to products which on aeration are initially coloured deep green. Like diquinone tri- and tetraquinone immediately give brown solutions with alkali. In the presence of hydroquinone, however, di-, tri- and tetraquinone give green solutions with alkali, just as "diquinhydrone" and the above mentioned "quinhydrones" of triquinone. 4,4'-dimethoxydiquinone, however, like quinone gives a green colour with alkali, but as seen from Table 2 4,4'-dimethoxyquinone gives considerable amounts of the corresponding hydroquinone. A further complication consists in extensive demethylation.

EXPERIMENTAL

2,5,2',5'-Tetrahydroxydiphenyl and 2,5,2',5'-tetraacetoxydiphenyl. 2,5,2',5'-Tetramethoxydiphenyl (15 g) was heated one hour to 200—210° with freshly distilled pyridine hydrochloride (40 g). The slightly discoloured melt (a) was cooled, dissolved in water and extracted continuously with ether. The crude phenol was recrystallised from water containing a little sulphur dioxide. M. p. 240° (decomp.). — The tetraacetate was obtained from the isolated phenol or preferentially from the melt (a) by heating with acetic anhydride containing a little pyridine. The solution was poured into water and the precipitate crystallised from dilute acetic acid. Overall yield 75 %. M. p. 168—169°. (Found: CH_3COO 58.1. Calc. for $C_{12}H_6(OCOCH_3)_4$: CH_3COO 58.0.)

Diquinone (IV). The demethylation melt (a) was dissolved in water (200 ml) and the solution was poured, in portions, into a solution of sodium dichromate (40 g) in 2 *N* sulphuric acid (750 ml) with cooling and stirring. The precipitated diquinone was immediately collected and washed successively with water, alcohol and ether, dried and dissolved in hot chloroform. To the filtered solution ether was added until crystallisation occurred. On cooling pure diquinone (8.2 g or 70 % overall yield) was obtained. M. p. 192—194°. When heated with alkohol or water brown, amorphous substances were slowly formed.

2,5-Dimethoxy-2',5'-dihydroxy-4,4'-diiododiphenyl. 2,5-Dimethoxy-4,4'-diiodo-di-phenyl quinone-2',5' (XI) was dissolved in warm acetic acid and zinc dust added in portions until the solution was colourless. The reaction mixture was filtered into cold water yielding a precipitate of almost pure hydroquinone m. p. 186—190° raised to 192—193° by recrystallisation from acetic acid. (Found: OCH_3 12.9. Calc. for $C_{12}H_4J_2(OH)_2(OCH_3)_2$: OCH_3 13.1.)

2,5,2',5'-Tetramethoxy-4,4'-diiododiphenyl (XII). The above phenol was dissolved in lukewarm ethanol, an excess of dimethyl sulphate was added, followed, in portions, by the same volume of sodium hydroxide (40 g NaOH/100 ml). The tetramethylether separated almost immediately in a theoretical yield m. p. 173—177° raised to 181.5—182° by crystallisation from ethanol. (Found: OCH_3 23.6. Calc. for $C_{12}H_4J_2(OCH_3)_4$: OCH_3 23.6.)

2,5-Diiodohydroquinone dimethylether (VIII). This compound was obtained either according to the method of Robinson [8] employing an excess of iodine chloride or by the following method. Hydroquinone dimethylether (50 g) was dissolved in ethanol (200 ml) and the solution heated under reflux. In portions and alternately iodine (200 g) and precipitated mercuric oxide (75—100 g) was added. The hot solution was filtered and crude diiodohydroquinone dimethylether crystallised from the filtrate. The mother liquor was evaporated and dissolved in benzene which had previously been employed to extract the mercuric iodide precipitate. The solution was shaken with alkaline sodium iodide

solution to remove dissolved mercuric iodide and then concentrated. The diiododerivative was purified by recrystallisation from ethanol. Yield 70—80 %.

2,5-Dimethoxy-1,4-bis-[2,5-dimethoxyphenyl]-benzene (X). A mixture of monoiodohydroquinone dimethylether (19.5 g), 2,5-diiodohydroquinone dimethylether (3.5 g) and copper bronze (23 g) was heated to 180° and the temperature slowly raised. The reaction took place at 220—230° and the mixture thickened. The temperature was increased to 260—265° to complete the reaction, the whole operation requiring about 20—30 minutes. The crushed reaction mixture was mixed with cotton wool and extracted with chloroform in a Soxhlet apparatus for two hours. The brownish, chloroform-soluble product from four runs (43 g) was fractionately distilled at a pressure of 0.5 mm and the following fractions collected: 1. B. p. <185° (27 g); 2. B. p. 185—275° (13 g); 3. Residue (3 g). Fraction 1 was redistilled and an oily portion, b. p. <105° discarded. The next fraction, b. p. 105—185° (24 g) yielded 2,5,2′,5′-tetramethoxydiphenyl (18 g) on treatment with ethanol. Fraction 2 was also redistilled when a mixture (a) of the diphenyl- and terphenyl derivatives (4 g) was recovered at a temperature below 200°. The fraction 200—290° (b. 9 g) was recrystallised twice from acetic anhydride (10—20 ml) m. p. 166—183 raised to 169—186°, and once from methylethylketone yielding the pure terphenyl derivative, m. p. 188—189°. The mother liquors were evaporated and the residues mixed with fraction a and refractionated as described above. Total yield of the terphenyl derivative 4.5 g. Small crystals, sparingly soluble in ethanol and ether but soluble in chloroform. (Found: C 69.8; H 6.4; OCH_3 45.4; Mol. wt. (camphor) 410. Calc. for $C_{18}H_8(OCH_3)_6$: C 70.2; H 6.4; OCH_3 45.6; Mol. wt. 410.)

2,5-Dihydroxy-1,4-bis-[2,5-dihydroxyphenyl]-benzene and its hexaacetate. 2,5-Dimethoxy-1,4-bis-(2,5-dimethoxyphenyl)-benzene (0.5 g) was added to freshly distilled pyridine hydrochloride (4 g) and the mixture heated one hour to 200—210° in an atmosphere of carbon dioxide. The cooled melt (a) was treated with water containing sulphur dioxide (50 ml) when the phenol was obtained as a fine suspension. It is very sparingly soluble in ether and is best collected by centrifugation. The compound is relatively soluble in ethanol and is so labile towards oxygen that no attempts were made to obtain it in a pure state. When moist it is immediately coloured deep blue. In the dry state it is somewhat more stable, but is soon coloured blue superficially. The m. p. appears to be between 280—290°. — The hexaacetate is obtained by dissolving the pyridine-hydrochloride melt (a) in hot acetic anhydride (20 ml containing a little pyridine) and pouring the solution into water. Yield 91 % calculated on the methoxy compound. It is crystallised from strong acetic acid or ethyl-glycol. The compound is soluble in chloroform and sparingly soluble in ethanol and ether. M. p. 243—244°. (Found: C 61.8; H 4.6; CH_3COO 60.1; OCH_3 0. Calc. for $C_{18}H_8(OCOCH_3)_6$: C 62.3; H 4.5; CH_3COO 61.3.)

Triquinone (V). The pyridine hydrochloride melt (a) was dissolved in water (25 ml) and the suspension poured, in portions, into a solution (40 ml) of chromic acid solution made from 5 g sodium dichromate and a mixture of 1 part of concentrated and 3 parts of 2 *N* sulphuric acid. The temperature must not be allowed to rise. At each addition a transient dark coloration was observed changing into yellow. The precipitated deep yellow triquinone was immediately filtered, (double filter papers), and washed with water, alcohol and ether. Yield 90 % calculated on the methoxy compound. Triquinone may be recrystallised from ethyl benzoate by pouring the substance into the hot solvent, immediate filtration and cooling. The mother liquor is always coloured strongly brown. Reductive acetylation with zinc, acetic acid, acetic anhydride and a trace of pyridine gave the hexaacetate of the corresponding hydroquinone in quantitative yield (mix. m. p.). Triquinone has no melting point but turns dark at about 230°. (Found: C 65.8; H 2.6. Calc. for $C_{18}H_8O_6$: C 67.6; H 2.5.) The sample contained some ash which partly accounts for the low carbon content. Due to the extremely low solubility of triquinone a complete purification is difficult to achieve.

2,5,2′,5′-Tetramethoxy-4,4′-bis-[2,5-dimethoxyphenyl]-diphenyl. (XIII). A mixture of iodohydroquinonedimethylether (19 g), 4,4′-diiodo-2,5,2′,5′-tetramethoxydiphenyl (3.9 g) and copper bronze (23 g) was heated in the usual way and then continuously extracted 24 hours with chloroform. The product (18.1 g) was distilled at low pressure (0.4 mm) and a fraction collected boiling below 190° (12.5 g) which on crystallisation from alcohol yielded 2,5,2′,5′-tetramethoxydiphenyl (11 g) followed by another boiling between 190 and 300° (3.8 g). The latter was powdered and extracted with boiling acetone (75 ml). The undissolved residue (2.8 g m. p. 241—244°) was recrystallised from nitrobenzene

(25 ml), m. p. 245—246°. It may also be recrystallised from dibutylphthalate. It is very sparingly soluble in most organic solvents but somewhat soluble in chloroform. (Found: C 70.7; H 6.3; OCH_3 45.2. Calc. for $C_{24}H_{10}(OCH_3)_8$: C 70.3; H 6.3; OCH_3 45.5.)

2,5,2',5'-Tetrahydroxy-4,4'-bis-[2.5-dihydroxyphenyl]diphenyl and its octaacetate. Very finely divided 2,5,2',5'-tetramethoxy-4,4'-bis-[2,5-dimethoxyphenyl]-diphenyl (0.35 g) was introduced into a flask and pyridinium hydrochloride (5 g) distilled directly into it. Carbon dioxide was passed into the flask which was heated for one—two hours to 205—215°. After cooling, the slightly discoloured melt was either dissolved in water containing sulphur dioxide, when a colourless very fine suspension of the hydroquinone was obtained, or was directly acetylated by heating with acetic anhydride (20 ml) containing a drop of pyridine. The hydroquinone could be collected by centrifugation but because of its instability no attempts were made to characterise it. It is rapidly coloured blue even in the dry state. The octaacetate was obtained in an almost quantitative yield and was recrystallised from hot acetic acid or ethyl glycol. The melting point was found to be 215—218.5°.

In a few cases, especially, when the octaacetate had been distilled in a high vacuum and the melt induced to crystallise by moistening with methanol, a m. p. of 212—213° was observed followed by solidification and renewed melting at 233—234°. (Found: C 62.1; H 4.6; CH_3COO 60.8; OCH_3 0. Calc. for $C_{24}H_{10}(OCOCH_3)_8$: C 62.3; H 4.6; CH_3COO 61.2.)

Tetraquinone (VI). This compound was prepared from demethylated 2,5,2',5'-tetramethoxy-4,4'-bis-[2,5-dimethoxyphenyl]-diphenyl by oxidation with chromic acid in exactly the same way as triquinone. Yellowish crystal powder which turns brown from about 230° and is black at 350°. It is extraordinarily insoluble in all solvents and could not be recrystallised. (Found: C 64.6; H 2.3. Calc. for $C_{24}H_{10}O_8$: C 67.6; H 2.4.)

The preparation on ignition yielded some ash containing chromium. On reductive acetylation an almost quantitative yield of the corresponding octaacetate was obtained.

Bromination of (a) 2,5,2',5'-tetramethoxydiphenyl, (b) 2,5-dimethoxy-1,4-bis-[2,5-dimethoxyphenyl]-benzene and (c) 2,5,2',5'-tetramethoxy-4,4'-bis-[2,5-dimethoxyphenyl]-diphenyl. The phenol ethers were dissolved in chloroform and an excess (in case c only a slight excess) of bromine in chloroform added. After 30 minutes the solutions were shaken with a dilute sodium carbonate solution, dried and evaporated. The bromoderivatives were recrystallised (a) from ethanol, (b) from acetic anhydride and (c) from nitrobenzene.

From (a) slender needles (probably IX a) were obtained, m. p. 154—155°. (Found: OCH_3 28.2. Calc. for $C_{12}H_4(OCH_3)_4Br_2$: OCH_3 28.6.) From (b) small compact crystals (probably X a), m. p. 278—280° were obtained. (Found: OCH_3 32.2. Calc. for $C_{18}H_6(OCH_3)_6Br_2$: OCH_3 32.7.) From (c) a fine crystal powder (probably XIII a) was obtained, m. p. 259—261°. (Found: OCH_3 35.2. Calc. for $C_{24}H_8(OCH_3)_8Br_2$: OCH_3 35.2.)

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Die polarographische Strom-Spannungskurve von Cadmiumcyanidlösungen. Ein Beispiel von Stufenaufteilungen durch ungepufferte Komplexbildung

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Die Aufteilung oder Verzerrung einer polarographischen Stufe in einer für analytische Zwecke wenig geeigneten Grundlösung ist bereits aus den ersten Jahren der Polarographie bekannt. Ganz allgemein ist eine solche Verzerrung immer dann zu erwarten, wenn ein am Depolarisator oder am Reaktionsprodukt stark gebundener Reaktionsteilnehmer in der Grundlösung bei geringer Konzentration ungepuffert vorliegt. In diesem Falle wird der Umsatz des Depolarisators von einer starken Anreicherung oder Konzentrationsabnahme des nicht gepufferten Reaktionsteilnehmers im Elektrodenraum begleitet sein, die eine entsprechende Potentialverschiebung bedingt.

Diese Ursache der Stufenverzerrung scheint nicht gleich erkannt zu sein. So wurde von Pines [1] angenommen, die Aufteilung der Zinkstufe in Anwesenheit von kleinen Mengen Kaliumcyanid sei dadurch bedingt, dass gleichzeitig verschiedene Komplexe zwischen Zink-Ionen und Cyanid-Ionen vorliegen, die verschiedene Halbwellenpotentiale haben und nur langsam ineinander umgelagert werden.

Die Bedeutung der Wasserstoffionenpufferung für eine Stufenverzerrung oder Stufentrennung wurde 1940 von Müller [2] erkannt und an Hand der Redoxstufen von Hydrochinon-Chinon demonstriert. Sie ist neuerdings mehr eingehend von Rüetchi und Trümpler [3] bei Reduktion von organischen Depolarisatoren behandelt worden.

Die Stufenverzerrung durch kleine Konzentrationen eines ungepufferten * Komplexbildners der mit dem Depolarisator reagiert, ist von Ringbom und Eriksson [4] berücksichtigt worden. Diese Autoren scheinen indessen stillschweigend anzunehmen, dass die Gültigkeit der Ilcovic'schen Diffusionsgleich-

* Unter einer gut gepufferten Lösung versteht man gewöhnlich eine Lösung, in der sich die Wasserstoffionenkonzentration bei grosser Veränderung des potentiellen Säuregrades wenig ändert. In der vorliegenden Arbeit wird das Wort auch für solche Lösungen verwendet, bei denen die Konzentration eines anderen Reaktionsteilnehmers in entsprechender Weise vor Konzentrationsänderungen geschützt ist.

ung ohne Rücksicht auf die Beweglichkeit der einzelnen Reaktionsteilnehmer auch dann für die Berechnung des Elektrodenpotentiales als Funktion der Stromstärke angenommen werden darf, wenn der Depolarisator in der Diffusionsschicht mit einem anderen Körper reagiert. Die Ableitung dieser Gleichung hat sich aber nur mit der Diffusion eines einzelnen Körpers beschäftigt. Es ist deshalb angebracht, die Anwendung sowohl dieser Gleichung als auch die darauf aufgebauten Gesetze, auf solche Fälle zu beschränken, bei denen ihre Gültigkeit auch wirklich erwiesen ist. Die Gleichungen von Ringbom und Eriksson [4], die einer Ermittelung von Massenwirkungskonstanten aus gemessenen Potentialen bei vorgegebenen Stromstärken einer polarographischen Strom-Spannungskurve dienen sollen, sind in Wirklichkeit nur dann streng gültig, wenn die Diffusionskonstante des freien Komplexbildners und diejenigen der einzelnen Komplexe die gleichen sind wie die Diffusionskonstante des freien Depolarisators in der vorliegenden Grundlösung.

Es ist hier nicht die Stelle, auf die Bedeutung dieser Unsicherheit für die von Eriksson [5] ermittelten Massenwirkungskonstanten der Cadmiumchloridkomplexe bzw. der Cadmiumbromidkomplexe einzugehen. In diesem besonderen Falle, bei dem die Halogenidkonzentration bei fast allen Messungen die Depolarisatorkonzentration weit übersteigt, ist aber anzunehmen, dass die genannte Fehlerquelle höchstens noch ein theoretisches Interesse besitzt.

Bei der Verwendung der polarographischen Kurve für die genaue Ermittelung von Naturkonstanten ist es erforderlich, dass der Elektrodenvorgang vollständig geklärt ist, und dass der Kurvenverlauf einem reversibelen Elektrodenvorgang entspricht. Der einzige eindeutige Beweis für die Erfüllung dieser Forderungen besteht aber in einer Identität der Potentialwerte einer ruhenden Quecksilber bzw. Amalgamelektrode, bei der die errechneten Konzentrationsverhältnisse verwirklicht sind, mit den entsprechenden Potentialwerten der polarographischen Strom-Spannungskurve. So lange dieser Beweis, der an sich die Ermittelung der gesuchten Konstanten darbietet, nicht gebracht ist, sollten die polarographisch ermittelten Konstanten nur als angenäherte Werte für Überschlagsrechnungen Verwendung finden.

Die hier geäusserten Bedenken sind indessen belanglos, wenn es sich darum handelt, eine für einen polarographischen Vorgang aufgestellte Theorie zu beurteilen. In diesem Falle ist es bereits wertvoll, eine qualitative Übereinstimmung der in ungepufferten Lösungen ermittelten Kurven mit dem angenäherten theoretischen Verlauf einer entsprechenden auf reversibler Reaktion beruhenden Strom-Spannungskurve zu vergleichen. Es sollte dann auch erlaubt sein, die Rechnung durch eine nur angenähert gültige Annahme von Gleichheit der Diffusionskonstanten der einzelnen Reaktionsteilnehmer stark zu vereinfachen.

Auf diesem Hintergrunde soll hier der angenäherte theoretische Verlauf der reversiblen Strom-Spannungskurve einer cyanidhaltigen Lösung von Cadmium-Ionen besprochen werden. Drei Aufnahmen, die einem Vergleiche dienen können, sind in dieser Zeitschrift von Prytz und Østerud [6,Abb.7] veröffentlicht worden. Bei diesen Aufnahmen sind die anodischen Cyanidstufen in Bezug auf Cadmium und die kathodischen Cadmiumstufen in Bezug auf Cyanid schlecht gepuffert.

Für die anodische Cyanidstufe sind nach Kolthoff und Miller [7] folgende Reaktionsgleichgewichte verantwortlich:

$$Hg \rightleftharpoons Hg^{++} + 2e^- \quad (I)$$

und

$$Hg^{++} + 2CN^- \rightleftharpoons Hg(CN)_2 \quad (II)$$

Gleichzeitig müssen für die Cadmiumstufe zumindest die beiden Reaktionsgleichgewichte:

$$Cd^{++} + 2e^- \rightleftharpoons Cd \quad (III)$$

und

$$Cd^{++} + nCN^- \rightleftharpoons Cd(CN)_n^{(n-2)-} \quad (IV)$$

beachtet werden.

n gibt in dem Reaktionsgleichgewicht (IV) die Anzahl Cyanid-Ionen an, die mit einem Cadmium-ion ein Komplex bilden. Wenn mehrere Komplexe möglich sind, sollten sie alle hier mit einbezogen werden. Im vorliegenden Falle wird aber der Einfachheit halber nur mit einem Gleichgewichte der Form (IV) gerechnet, wobei nur die erste der bei Bildung mehrerer Komplexe erscheinende Stufenaufteilung der Cadmiumcyanidstufen in die Rechnung einbezogen wird.

Zu Vereinfachung der Rechnung sollen nunmehr die Gesamtkonzentration von Cadmium:

$$C_{Cd} = [Cd^{++}] + [Cd(CN)_n^{(n-2)-}] \quad (1)$$

und die Gesamtkonzentration von Cyanid:

$$C_{CN} = [CN^-] + n[Cd(CN)_n^{(n-2)-}] + 2[Hg(CN)_2] \quad (2)$$

definiert werden. Es sind die eckigen Klammern in gewohnter Weise für Konzentrationen eingesetzt worden. Wenn die Konzentrationen an der Elektrodenoberfläche gemeint sind, wird ein 0 bei der Konzentrationsangabe hinzugefügt.

Weiter sollen die Gesamtzahl von Cadmium-Ionen:

$$N'_{Cd} = N_{Cd} + N_{Cd(CN)_n} \quad (3)$$

und die Gesamtzahl von Cyanid-Ionen:

$$N'_{CN} = N_{CN^-} + nN_{Cd(CN)_n} + 2N_{Hg(CN)_2} \quad (4)$$

definiert werden.

In diesen Gleichungen stellen N_{Cd}, $N_{Cd(CN)_n}$ u.s.w. die Anzahl der als Indices gegebenen Ionen oder Molekel dar.

Das erste Fick'sche Gesetz gibt für die Diffusion von freien Cadmium-Ionen in Richtung der Ortskoordinate x:

$$\dot{N}_{Cd} = Dq \frac{\partial[Cd^{++}]}{\partial x} \quad (5)$$

und für die Diffusion des Cadmium-Komplexes:

$$\dot{N}_{Cd(CN)_n} = Dq \frac{\partial[Cd(CN)_n^{(n-2)-}]}{\partial x} \quad (6)$$

In diesen Gleichungen sind D die für sämtliche Reaktionsteilnehmer gleich gross angenommene Diffusionskonstante und q eine auf der Ortskoordinate x senkrecht stehende Fläche. $\dot{N}_{Cd}$ und $\dot{N}_{Cd(CN)_n}$ stellen die Molenzahlen der als Indices angegebenen Stoffe dar, die in der Zeitenheit durch die Fläche q hindurchdiffundieren.

Die Gesamtdiffusion von freien und gebundenen Cadmium-Ionen beträgt nunmehr:

$$\dot{N}'_{Cd} = \dot{N}_{Cd} + \dot{N}_{Cd(CN)_n} = Dq \frac{\partial C_{Cd}}{\partial x} \quad (7)$$

In Anlehnung an die Ableitung des zweiten Fick'schen Gesetzes für die Kugelfläche erhält man aus Gl. (7) für die Abhängigkeit der Gesamtkonzentration des Cadmiums von der Zeit:

$$\dot{C}_{Cd} = D\left(\frac{\partial^2 C_{Cd}}{\partial x^2} + \frac{2}{x}\frac{\partial C_{Cd}}{\partial x}\right) \quad (8)$$

Die Gl. (8) ist in ihrer Form mit der entsprechenden Differentialgleichung für die Diffusion eines einzelnen Stoffes identisch, der nicht mit anderen Bestandteilen der Lösung reagiert, und die als Grundgleichung bei der Ableitung der Ilcovic'schen Gleichung dient. Weiter sind auch die Anfangs- und Grenzbedingungen in der Form die gleichen wie bei der Ableitung der Ilcovic'schen Gleichung. In Anlehnung an diese aus den Lehrbüchern bekannte Ableitung kann die kathodische Stromstärke somit nicht nur als Funktion der Konzentration von Cadmium im Amalgam der Tropfenoberfläche sondern auch als Funktion von der Gesamtkonzentration der Cadmium-Ionen im Elektrodenraum angegeben werden. Mit i_d als Diffusionsstrom der kathodischen Stufe beträgt der kathodische Strom i bei gegebenem Potential:

$$i = i_d \frac{[Cd]_0}{C_{Cd}} = i_d \left(1 - \frac{C_{Cd_s}}{C_{Cd}}\right) \quad (9)$$

Das beim anodischen Stromdurchgang gebildete Quecksilbercyanid bleibt undissociiert, und es reagiert auch nicht mehr mit anderen Lösungsteilnehmern. Die durch Oxydation von Quecksilber bedingte Stromstärke i' kan deshalb in gewohnter Weise als Funktion des anodischen Diffusionsstromes i_d' und der Gesamtkonzentration von Quecksilber-Ionen an der Elektrodenoberfläche angegeben werden:

$$i' = 2i_d' \frac{C_{Hg_s}}{C_{CN}} \quad (10)$$

Die Cyanid-Ionen reagieren an der Tropfenoberfläche nur nach den Gleichgewichten (II) und (IV), und sie werden somit nicht aus der Lösung entfernt. Dies kann durch die Gleichung

$$\dot{N}'_{CN} = 0 \quad (11)$$

für die Tropfenoberfläche formuliert werden.

In Analogie zur Ableitung der Gl. (7) erhält man nunmehr für die Diffusion des gesamten Cyanides an der Tropfenoberfläche:

$$\dot{N}'_{CN} = Dq \frac{\partial C_{CN}}{\partial x} = 0 \tag{12}$$

Nach dieser Gleichung muss $\partial C_{CN}/\partial x$ gleich 0 sein, und man erhält für die Gesamtkonzentration der Cyanid-Ionen an der Tropfenoberfläche:

$$C_{CN_0} = C_{CN}$$

Für Bestimmung der Konzentrationen an der Elektrodenoberfläche liegen nun neben den Gleichungen (1), (2), (9), (10) und (13) noch folgende Gleichungen vor:

Die Massenwirkungsgleichungen der Gleichgewichte (II) und (IV):

$$[Cd^{++}] \cdot [CN^-]^n = K_{Cd}[Cd(CN)_n^{(n-2)-}] \tag{14}$$

und

$$[Hg^{++}] \cdot [CN^-]^2 = K_{Hg}[Hg(CN)_2] \tag{15}$$

Die Nernst'sche Gleichung für die Abhängigkeit des Elektrodenpotentiales von den Konzentrationen an der Elektrodenoberfläche:

$$E = E_{oHg} + \frac{RT}{2F} \ln [Hg^{++}]_0 \tag{16}$$

und

$$E = E_{1/2} + \frac{RT}{2F} \ln \frac{[Cd^{++}]_0}{[Cd]_0} \tag{17}$$

K_{Cd} und K_{Hg} stellen in den Gleichungen (14) und (15) die Massenwirkungskonstanten der Reaktionsgleichgewichte (II) und (IV) dar, während E_{oHg} für das Normalpotential des Quecksilber(II)-Ions und $E_{1/2}$ für das Halbwellenpotential des Cadmiums in indifferenter Lösung eingesetzt sind.

Aus diesen Gleichungen sind die Gesamtkonzentrationen des Elektrodenraumes von Cadmium und Quecksilber und das Potential der Elektrode E immer als Funktion der Konzentration von freien Cyanid-Ionen des Elektrodenraumes erhältlich. Die Allgemeine Lösung gibt für die Konzentration der freien Quecksilber-Ionen:

$$[Hg^{++}]_0 = \frac{a-b+\sqrt{(a-b)^2+c}}{2} \tag{18}$$

In dieser Gleichung sind:

$$a = \frac{K_{Hg}(C_{CN}-[CN^-]_0)}{2\,[CN^-]_0^2} \tag{19}$$

$$b = \frac{\frac{n}{2}\left[CN^-\right]_0^{n-2} K_{Hg}C_{Cd} + \frac{K_{Cd}}{t}}{K_{Cd} + [CN^-]_0^n} \tag{20}$$

$$c = \frac{K_{Hg}K_{Cd}\,(C_{CN} - [CN^-]_0)}{2t[CN^-]_0^2(K_{Cd} + [CN^-]_0^n)} \tag{21}$$

Die in den Gleichungen (20) und (21) vorkommende Grösse t beträgt:

$$t = \exp.\,(\frac{2F}{RT}[E_{oHg} - E_{1/2}]\,) \tag{22}$$

Mit Hilfe der Konzentration von freien Quecksilber-Ionen im Elektrodenraum erhält man nunmehr auch die übrigen Konzentrationen:

$$C_{Hg_0} = \frac{[CN^-]_0^2}{K_{Hg}} [Hg^{++}]_0 \tag{23}$$

$$[Cd]_0 = \frac{K_{Cd} C_{Cd}}{(K_{Cd} + [CN^-]_0^n)[Hg^{++}]_0 t + K_{Cd}} \tag{24}$$

Das Elektrodenpotential ist erhältlich durch Einsetzen von $[Hg^{++}]_0$ aus der Gl. (18) in die Gl. (16) und die Stromstärke durch Einsetzen der Konzentrationen aus den Gleichungen (23) und (24) in die Gleichungen (9) und (10). Die gemessene Stromstärke ist als Differenz der so erhaltenen anodischen und kathodischen Stromstärken zu errechnen.

Durch die Wahl einer Reihe vorgegebener Cyanid-Ionenkonzentrationen lassen sich somit die dazu gehörigen Spannungen und Stromstärken errechnen, sodass sich der Verlauf einer auf reversible Reaktion beruhenden Strom-Spannungs kurve auf theoretischem Wege ermitteln lässt.

Die obige Ableitung ist in der Absicht gegeben, auch in den Fällen einen Weg für die Ableitung der gesamten Strom-Spannungskurven zu zeigen, in denen sich der kathodische Strom dem anodischen überlagert. Im Falle der Depolarisationsstufe von Cadmium in Cyanidgrundlösung dürfte aber die Oxydation von Quecksilber im Spannungsbereiche der Reduktionsstufe von Cadmium so gering sein, dass sie vernachlässigt werden darf. Es soll hier auch die in diesem Falle erlaubte, für die rechnerische Ermittelung der Strom-Spannungskurve weit bequemere Ableitung gezeigt werden.

Wenn beim anodischen Kurventeil von einem Umsatz von Cadmium abgesehen werden darf, kann es auch für die Gesamtkonzentration von Cadmium in entsprechender Weise wie oben für die Gesamtkonzentration von Cyanid (vgl. Gl. (13)) gezeigt werden, dass

$$C_{Cd_0} = C_{Cd}. \tag{25}$$

Aus den Gleichungen (1), (2), (13), (14) und (25) erhält man für die Gesamtkonzentration des Quecksilbers an der Elektrodenoberfläche, die im Elektrodenraum gleich der Konzentration des Quecksilbercyanides gesetzt werden darf:

$$C_{Hg_0} = \frac{1}{2}\left(C_{CN} - [CN^-]_0 - \frac{n[CN^-]_0^n}{K_{Cd} + [CN^-]_0^n} C_{Cd}\right) \tag{26}$$

und für die Konzentration der freien Quecksilber(II)-Ionen im Elektrodenraum:

$$[Hg^{++}]_0 = \frac{K_{Hg}}{[CN^-]_0^2} C_{CN_0} \tag{27}$$

Die Stromstärke i' ist als Funktion von $[CN^-]_0$ erhältlich indem die Gesamtkonzentration von Quecksilber an der Elektrodenoberfläche aus Gl. (26) in die Gl. (10) eingesetzt wird. Die dazugehörige Spannung wird durch Einsetzen der Gl. (27) in die Gl. (16) gewonnen.

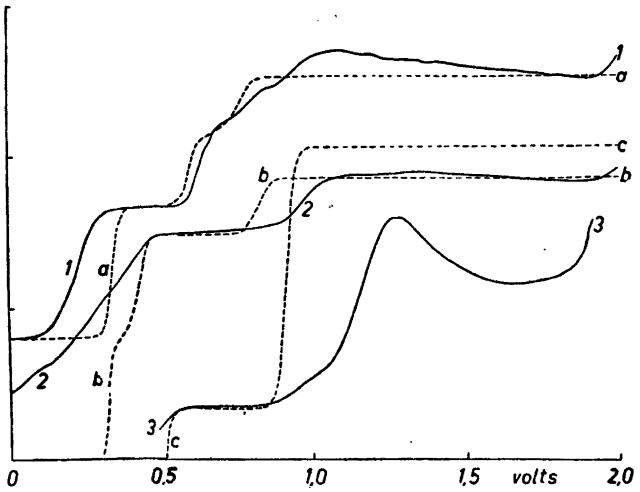

Abb. 1. Aufgenommene und errechnete Polarogramme bei 10^{-3} M Cadmium in Anwesenheit wechselnder Cyanidkonzentrationen.

Kurve 1: Von Prytz und Østerud in $2 \cdot 10^{-3}$ *M* KCN aufgenommene Strom-Spannungskurve.
Kurve 2: Von P. u. Ø. in $8 \cdot 10^{-3}$ *M* KCN aufgenommene Strom-Spannungskurve.
Kurve 3: Von P. u. Ø. in 0,04 *M* KCN aufgenommene Strom-Spannungskurve.
Kurve a: Die für die Bedingungen der Kurve 1 berechnete Strom-Spannungskurve.
Kurve b: Die für die Bedingungen der Kurve 2 errechnete Strom-Spannungskurve.
Kurve c: Die für die Bedingungen der Kurve 3 errechnete Strom-Spannungskurve.

Für die Ermittelung des kathodischen Kurventeiles kann die Konzentration von Quecksilber(II)cyanid vernachlässigt werden. In diesem Falle geben die Gleichungen (1), (2), (9), (10), (13) und (14)

$$[Cd^{++}]_0 = \frac{K_{Cd}(C_{CN} - [CN^-]_0)}{n[CN^-]_0^n} \tag{28}$$

$$[Cd]_0 = C_{Cd} - \frac{K_{Cd} + [CN^-]_0^n}{n[CN^-]_0^n}(C_{CN} - [CN^-]_0) \cdot \tag{29}$$

Die Stromstärke ist nunmehr durch Einsetzen der Gl. (29) in die Gl. (9) und die Spannung durch Einsetzen der Gleichungen (28) und (29) in die Gl. (17) erhältlich.

Auf dieser Grundlage lässt sich der Verlauf der reversiblen Strom-Spannungskurven immer dann angenähert konstruieren, wenn die notwendigen Konstanten hinreichend bekannt sind. Zum Zwecke einer solchen Aufzeichnung sind hier folgende Zahlenwerte angenommen:

Das Halbwellenpotential der Cadmiumstufe: $E_{1/2} = -0{,}59$ V gegen ges. Calomelelektrode.

Das Normalpotential der Quecksilber (II)-Ionen: $E_{oHg} = +0{,}609$ V gegen ges. Calomelelektrode.

Die Massenwirkungskonstante des Gleichgewichtes (II): $K_{Hg} = 10^{-38}$.
Die Massenwirkungskonstante des Gleichgewichtes (IV): $K_{Cd} = 1{,}4 \cdot 10^{-17}$.
Die Anzahl Cyanid-Ionen die im Cadmiumcyanidkomplex gebunden werden: $n = 4$.

Während die Komplexzerfallskonstante des Quecksilber(II)cyanides nach Literaturangabe über das Halbwellenpotential der anodischen Cyanidstufe [8] und unter Anwendung des Normalpotentiales von Quecksilber(II) errechnet wurde, sind die Daten für das Cadmiumkomplex nach den Messungen von v. Euler [9] angenommen.

Die Kurvenzüge *a*, *b* und *c* der Abb. 1 zeigen die so berechneten Strom-Spannungskurven bei Lösungen von 10^{-3} *M* Cadmium-Ionen, während die Kurvenzüge 1, 2 und 3 nach der von Prytz und Østerud [6] in ihrer Arbeit gegebene Abb. 7 abgezeichnet sind. Für die Bestimmung des Diffusionsstromes wurde die anodische Höhe der Kurve 1 deshalb verwendet, weil sie am besten abmessbar ist. Entsprechend der von den genannten Autoren verwendeten Empfindlichkeiten des Instrumentes wurden die Stromstärken der Kurve *b* in einem Masstabe gezeichnet, der 3/7 des Masstabes der Kurve *a* entsprach, während der für Kurve *c* verwendete Masstab doppelt so gross ist wie der für Kurve *a* verwendete. Bei der Berechnung der Kurve *a* wurde die Gesamtkonzentration von Cyanid gleich $2 \cdot 10^{-3}$ *M*, bei Kurve *b* gleich $8 \cdot 10^{-3}$ *M* und bei Kurve *c* gleich 0,04 *M* angenommen.

Die Kurvenzüge *a* und *b* zeigen gute Übereinstimmung mit den entsprechenden gemessenen Kurvenzügen 1 und 2. Die Potentialabweichungen dieser Kurvenpaare erzählen deshalb nichts über die Reversibilität der Reaktionen, weil die Aufnahme der Polarogramme unter Bedingungen erfolgt ist, bei denen eine beträchtliche Potentialverschiebung zu erwarten ist. Bei der hohen Depolarisatorkonzentration wird die verwendete Elektrolytbrücke zwischen der Vergleichselektrode und der polarographischen Celle ein Ohmsches Spannungsgefälle hervorrufen, dessen Grössenordnung für die Potentialabmessung von Bedeutung sein dürfte. Weiter mag die Gegenelektrode bei der am Kurvenanfang gemessenen starken anodischen Stromstärke selbst kathodisch polarisiert werden. Zu dieser Unsicherheit der Potentialabmessung der experimentellen Kurven kommt noch die Unsicherheit der konstruierten Kurven, die dadurch entstanden ist, dass die Wasserstoffionen-Pufferung bei ihrer Berechnung vernachlässigt wurde.

Die Unregelmässigkeiten am oberen Teile der Kurve 1 sind wahrscheinlich als Maximumserscheinungen zu bewerten.

Die Kurve 3 erreicht die zu erwartende Stufenhöhe überhaupt nicht. Weder für diese Tatsache noch für das ausgesprochene Minimum dieser Kurve soll hier eine Erklärung gesucht werden. Kolthoff und Lingane [9] finden eine 1 *M* Kaliumcyanidlösung als Grundlösung für die Bestimmung von Cadmium geeignet, und auch bei dieser hohen Cyanidkonzentration finden sie, dass das Abscheidungspotential nur wenig vom berechneten reversiblen Potential abweicht.

Auf diesem Hintergrund geben die von Prytz und Østerud, gezeichneten Kurven keinen Anhaltspunkt für die Annahme eines weiteren Cadmium-Cyanid-komplexes der Form $Cd(CN)_6^{4-}$.

ZUSAMMENFASSUNG

Es wird darauf hingewiesen, dass eine Verzerrung einer polarographischen Stufe immer dann zu erwarten ist, wenn sich die Konzentration eines Komplexbildners im Elektrodenraum durch die Depolarisationsreaktion merklich verändert. Als Beispiel wird die Verzerrung der Cadmiumstufe in Anwesenheit von kleinen Mengen Cyanid-Ionen herangezogen. Näherungsgleichungen für Berechnung der Strom-Spannungskurven solcher Lösungen werden für den Fall reversibler Elektrodenreaktionen ausgearbeitet.

Einige berechnete Kurvenzüge werden mit entsprechenden aus der Literatur geholten polarographischen Aufzeichnungen verglichen.

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On Metallic Phases in the Ti-P-O and Zr-P-O Systems

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METALLIC PHOSPHIDES

In a recently published paper about transition metal phosphides [1] the present author found that phosphide phases with a nonmetal content up to 50 atom-% have structures analogous to the metallic carbides and nitrides of the transition metals. The interatomic metal-metal bonds are weaker in the phosphides than in the carbides and nitrides, but the metal-phosphorus bonds seem to be strong, probably of a partial ionic nature.

TiP [1] and β-ZrP [1] are isomorphous with γ'-MoC [2] and NbN [3,4]. α-ZrP [1] exists at the approximate composition $ZrP_{0.9}$ and is of the B1 type. According to Biltz, Rink and Wiechmann [5] a titanium "subphosphide" exists at the approximate composition Ti_2P. In the present study this compound was prepared from the elements in the same way as described earlier [1], and the composition was found to correspond to the formula Ti_3P rather than Ti_2P. The Ti_3P phase is of the Fe_3P [6,7] structure, with the unit cell dimensions $a = 10.00$ Å, $c = 5.017$ Å; $c/a = 0.502$ ($V = 501.7$ Å^3). A zirconium "subphosphide" also seems to exist, but its composition and structure is unknown [8,1].

METALLIC OXIDES

Besides the earlier known TiO phase [9,10] of the NaCl structure Wang and Grant [11] have recently observed the existence of a phase (designated by δ) which is reported to be formed by a peritectoid reaction of the α—Ti and TiO phases at temperatures below 925° C.

Cubicciotti [12] and Domagala and Mc Pherson [13] did not find any traces of a ZrO phase in their investigations of the Zr—O system. They prepared the samples starting from the pure metal and the dioxide. Only the α—Zr, β—Zr and ZrO_2 phases are shown in the equilibrium diagrams published by them. This was confirmed in the present study, when the corresponding preparation method was used, *i. e.* mixtures of Zr (degassed zirconium hydride from Metal Hydrides Inc.) and ZrO_2 (Baker's Analyzed) were heated in zirconia crucibles in a high frequency vacuum induction furnace at temperatures between 1200° C and 1700° C. But if the metal was carefully oxidized with steam in the presence of hydrogen in great excess at temperatures between 600° C and 800° C a phase

of B1 type and with a cubic edge length varying between $a = 4.584$ Å and $a = 4.620$ Å was obtained [14]. The presence of hydrogen in the lattice is most unlikely because heating *in vacuo* at 1 000° C for two hours did not cause any structural change. The existence of ZrO is also confirmed by the fact that this phase could be found in many samples where Zr was oxidized *in vacuo* by means of different oxides of the metals Fe, Co, and Ni.

Because of the ease with which ZrO_2 is formed it was not possible to prepare ZrO in a pure state. The homogeneity range of ZrO seems to be comparatively large, but the limits are unknown. In contrast to ZrO it is fairly easy to prepare TiO, VO and NbO from the metals and, for instance, the oxides TiO_2, V_2O_5 and Nb_2O_5 respectively.

METALLIC PHOSPHIDE-OXIDES

In order to search for metallic ternary Ti—P—O and Zr—P—O phases, phosphide samples of different compositions were prepared by heating metal (degassed hydride from Metal Hydrides Inc.) and red phosphorus (Kahlbaum, *pro analysi*) in evacuated and sealed silica tubes at 700° C for about one week. The powdered samples were then cautiously oxidized with steam in the presence of hydrogen at various temperatures between 600° and 800° C.

The phase analysis of the specimens was performed by means of powder photographs taken with Cu-$K\alpha$ radiation in a focussing camera of the Guinier type. For measurements of the high-angle reflections additional photographs were taken with Hägg-Phragmén type focussing cameras.

Besides the binary phosphide and oxide phases the interpretation of the photographs revealed the existence of a ternary phase in each system, in both cases of a structure related to a B1 type lattice. The purest samples of these ternary compounds were obtained for the metal/phosphorus ratio equal to 3/1. Because of the difficulty of obtaining these phases in a pure state it was not possible to determine the oxygen content with any accuracy. The structure determination, however, shows the value of the metal/phosphorus ratio to be 3/1 and further makes an oxygen content of 33 atom % or slightly less very probable. The formulae are therefore written Ti_3PO_2 and Zr_3PO_2 respectively. The two phases are isomorphous and appear as black powders.

The strongest diffraction lines, which were in some cases slightly broadened, could be indexed as coming from face-centered cubic lattices. The lattice constants were found to be $a = 4.415$ Å and $a = 4.779$ Å for the Ti_3PO_2 and Zr_3PO_2 phases respectively. Besides these reflexions a series of comparatively weak lines were visible, indicating a true unit cell of hexagonal symmetry containing three metal atoms, *i.e.* one formula unit Ti_3PO_2 or Zr_3PO_2, and with the axial ratio close to that characteristic for an ideal cubic close-packing, $c/a = \sqrt{6} = 2.449$. Some "superlattice" reflections are of such an intensity that they must be due to a displacement of the metal atom positions from the rhombohedral ones — *i.e.* 0,0,0; 1/3,2/3,1/3; 2/3,1/3,2/3 — and not only to an ordering of the nonmetal atoms. Starting from the assumption that the nonmetal atoms occupy fixed positions in the lattice and that the interatomic

metal-phosphorus distances are about 0.4 Å longer than the metal-oxygen distances the most likely configuration for the phases will be obtained in the space-group D_{3d}^3—$P\bar{3}m$ with the following arrangement of the atoms:

1 Ti (Zr) in 1(*a*) 0,0,0
2 Ti (Zr) in 2(*d*) 1/3,2/3,*z*; 2/3,1/3,$\bar{z}$; with $z < 1/3$
1 P in 1(*b*) 0,0,1/2
2 O in 2(*d*) 1/2,2/3,*z*; 2/3,1/3,$\bar{z}$, with $z > 5/6$

The best agreement between the observed intensities and the calculated $p|F|^2$ values was obtained for $z_{Ti} = 0.28$ and $z_{Zr} = 0.29$. As the nonmetal atoms will be situated at the centres of metal atom octahedra the parameter values for the O atoms are assumed to be $\bar{z}_{Ti}/2$ and $\bar{z}_{Zr}/2$ for Ti_3PO_2 and Zr_3PO_2 respectively.

The intensities calculated and observed for the Ti_3PO_2 phase are compared in Table 1. The axial ratio $c/a = 2.453$ deviates to such a small extent from that characteristic of a cubical close-packed arrangement that the diffraction lines belonging to certain groups with hexagonal indices * will coincide in spite of the excellent resolution of the photographs. The cubical indices corresponding to the rhombohedral ones are given in Table 1 under the heading "B1 — indices".

Table 1. Calculated and observed intensities for the Ti_3PO_2 phase.

hkl	I_{calc}	I_{obs}	"B1-indices"	*hkl*	I_{calc}	I_{obs}	"B1-indices"
0,0,1	7	5		1,1,5	7	0	
0,0,2	2	0		2,1,0	1	0	
1,0,0	1	0		1,0,7	3		
0,0,3	3	50	1,1,1	2,0,5	12	30	3,3,1
1,0,1	42			2,1,1	14		
1,0,2	101	100	2,0,0	1,1,6	3	50	4,2,0
0,0,4	12	10		2,1,2	49		
1,0,3	21	15		0,0,8	2	0	
1,0,4	25	100	2,2,0	2,1,3	15	10	
1,1,0	68			2,0,6	13	10	
0,0,5	4	0		1,0,8	10		
1,1,1	1	0		2,1,4	14	50	4,2,2
1,1,2	1	0		3,0,0	20		
2,0,0	0	0		1,1,7	19	15	
1,0,5	23			3,0,1	0	0	
1,1,3	6	50	3,1,1	3,0,2	0	0	
2,0,1	12			0,0,9	1		
0,0,6	1	50	2,2,2	2,0,7	2		3,3,3
2,0,2	41			2,1,5	18	30	5,1,1
1,1,4	33	30		3,0,3	3		
2,0,3	13	10		1,1,8	6	0	
1,0,6	22	15		3,0,4	22	15	
2,0,4	13	15	4,0,0	1,0,9	9	0	
0,0,7	5	0		2,1,6	35	30	

* If $c/a = \sqrt{6}$ the quadratic formula for the hexagonal unit cell can be written as $\sin^2\Theta = 8C(h^2 + k^2 + hk) + Cl^2 = 9\ C\ n$ for $h + k \pm l = 3\ m$. As an example, the reflexions 0,0,9; 3,0,3; 2,1,5; 2,0,7 will coincide, all of them giving $n = 9$.

Table 2. Interatomic distances in the Ti_3PO_2 phase.

Atom	Surrounding atoms	Interatomic distance, Å	
Ti in 1(*a*)	6 Ti in 2(*d*)	2.80	2.96
	6 Ti in 1(*a*)	3.12	
	6 O	2.09	
Ti in 2(*d*)	3 Ti in 1(*a*)	2.80	3.21
	3 Ti in 1(*a*)	3.82	
	6 Ti in 2(*d*)	3.12	
	3 P	2.47	
	3 O	2.09	
P	6 Ti in 2(*d*)	2.47	
	6 P	3.12	
	6 O	3.29	
O	3 Ti in 1(*a*)	2.09	
	3 Ti in 2(*d*)	2.09	
	3 P	3.29	
	3 O	2.80	

The interatomic distances in the Ti_3PO_2 and Zr_3PO_2 phases are listed in Table 2 and Table 3, respectively.

It ought to be mentioned that the "superlattice" reflections could not be traced in the photographs for the cases when the reflections corresponding to a B1 type lattice were broad and diffuse. This may be caused by a more or less random distribution of the P and O atoms over the metalloid positions leading to mean parameter values $z_{Ti(Zr)} = 1/3$ and $\bar{z}_O = 1/6$. When these disordered samples are heated *in vacuo* for a sufficient long time at the same temperatures as that used for their preparations the lines turn sharper and the "superlattice" lines appear.

Table 3. Interatomic distances in the Zr_3PO_2 phase.

Atom	Surrounding atoms	Interatomic distance, Å	
Zr in 1(*a*)	6 Zr in 2 (*d*)	3.09	3.24
	6 Zr in 1(*a*)	3.38	
	6 O	2.29	
Zr in 2(*d*)	3 Zr in 1(*a*)	3.09	3.46
	3 Zr in 1(*a*)	3.99	
	6 Zr in 1(*d*)	3.38	
	3 P	2.61	
	3 O	2.29	
P	6 Zr in 2(*d*)	2.61	
	6 P	3.38	
	6 O	3.53	
O	3 Zr in 1(*a*)	2.29	
	3 Zr in 2(*d*)	2.29	
	3 P	3.53	
	3 O	3.09	

Table 4. Comparison between the volumes, nonmetal-metal and metal-metal distances of some metallic phases in the Ti-P-O and Zr-P-O systems.

Phase	Structure type	Axial values Å	Vol./metal atom Å³	O-metal dist. (6-coord.) Å	P-metal dist. (6-coord.) Å	Metal-met dist. (12-coord.) Å	Increase of metal-metal dist., %
α-Ti	A 3	$a = 2.952$ $c = 4.732$	17.85			2.93	—
α-Ti(O)*	L′ 3	$a = 2.961$ $c = 4.806$	18.24	2.09		2.96	0.5
TiO	B 1	$a = 4.162$ $- 4.232$	18.02— 18.95	2.08— 2.12		2.94— 2.99	0.3— 2.0
Ti_3PO_2	Ti_3PO_2	$a = 3.120$ $c = 7.652$	21.50	2.09	2.47	3.08	5.1
TiP	TiP	$a = 3.487$ $c = 11.65$	30.67		2.48	3.45 **	17.5
α-Zr	A 3	$a = 3.223$ $c = 5.127$	23.07			3.20	—
α-Zr(O)*	L′ 3	$a = 3.268$ $c = 5.244$	24.25	2.30		3.25	1.7
ZrO	B 1	$a = 4.584$ $- 4.620$	24.08— 24.65	2.29— 2.31		3.24— 3.27	1.4— 2.2
Zr_3PO_2	Ti_3PO_2	$a = 3.380$ $c = 8.279$	27.29	2.29	2.61	3.35	4.8
α-ZrP	B 1	$a = 5.270$	36.62		2.64	3.73	16.6
β-ZrP	TiP	$a = 3.677$ $c = 12.52$	36.65		2.64	3.65 **	14.2

* At the oxygen-rich phase limit (800° C).
** The metal atoms have 10-coordination.

The unit cell dimensions found in the present study for the metallic phases in the Ti—P—O and Zr—P—O systems (with the exception of the subphosphides) are listed in Table 4. The mean interatomic nonmetal-metal and the mean metal-metal distances are calculated from those data. The former ones vary very slightly for a given metal. The latter ones increase with increasing amount of phosphorus. The relative increase in relation to the intermetallic distances in the pure metals is shown in the last column of the table. These numbers may show the weakening of the metallic properties.

SUMMARY

The metallic corners of the Ti—P—O and Zr—P—O systems (nonmetal/metal ratio ≤ 1) have been investigated by means of X-ray methods.

The structures of the monophosphide phases are known earlier. Ti_3P is isomorphous with Fe_3P.

Attempts to prepare ZrO from Zr and ZrO_2 were in vain, but this phase (B1 type) could be identified in samples where Zr was oxidized by means of steam in the presence of hydrogen or with oxides of Fe, Co and Ni.

The ternary metallic phases Ti_3PO_2 and Zr_3PO_2 are isomorphous with each other and are of an interstitial structure type with the nonmetal atoms in ordered positions at the centres of metal atom octahedra. The three metal atoms in the hexagonal unit cell are displaced to a slight extent from the positions corresponding to a cubic close-packing.

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Studies on Ionic Solutions in Diethyl Ether

V. Redox Titration of Positive Triphenylmethyl Ion with Negative Triphenylmethyl Ion

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In the first three parts [1-3] of this series [1-4], it was demonstrated that a solution of lithium perchlorate in diethyl ether can be used as an ionic medium for studying ionic equilibria in ether solutions. In solutions containing small amounts of silver or halogenide ions in $LiClO_4$-ether the activity factors proved to be practically constant so that one could measure ionic concentrations directly, using emf measurements, and use concentrations instead of activities in the law of mass action. Among other things it proved possible [2,3] to calculate the solubility products of the silver halogenides and the complexity product of AgI_2^-.

We have now tried to use this ionic medium to study the reaction between triphenylmethyl perchlorate ($R^+ClO_4^-$) and lithium triphenylmethyl (Li^+R^-) *. There is much evidence for the existence of the ions, R^+ and R^-, in solutions of these substances [5], and they are likely to react forming R (triphenylmethyl radical) or R_2 (hexaphenylethane). Bent's and Keevil's experiments with cells of the type

$$Na\text{-}Hg/Na^+R^- \text{ in ether}/Na^+R^- \text{ in ether, saturated with solid } R_2/Pt$$

indicated that steady potentials can be obtained with redox solutions containing R_2 and R^-. One could expect that the redox potential during a titration of R^+ with R^- would lead to curves with at least two "shelves": one with mainly R^+ and R, one with mainly R and R^-.

When in the following we use a symbol such as R^-, this includes all sorts of complexes that R^- can form with the 2 C $LiClO_4$-diethyl ether medium; for instance molecules LiR and ether complexes.

Our experiments showed that the redox potentials were not as reproducible as those found with Ag^+ and halogenide ions. In these experiments, considerable difficulties were met with because of impurities; the various forms of

* In the following, R = $(C_6H_5)_3C$ (triphenylmethyl or trityl).

triphenylmethyl, especially R^-, are very sensitive to the decomposition products of $LiClO_4$-ether, even if the latter is stored without access to oxygen or water.

With the procedure finally adopted, we have found evidence for the formation of an intermediate step, possibly R_2^-, in addition to R (or R_2) and have also been able to calculate the order of magnitude of the various equilibrium constants involved.

MATERIALS

Preparation of $LiClO_4$ (*cf.* Gmelin [7]). In a 1 l flask, 300 g Li_2CO_3 (Kebo puriss.) was treated with 500 ml 70 % $HClO_4$ (p. a.). Most of the water was evaporated (preferably *in vacuo*) on a hot plate, the temperature of which should not exceed 150° C. The $LiClO_4$ was then recrystallized from 96 % ethanol. It was dried very carefully in an open porcelain dish at 60° C overnight, then ground in a mortar and dried for a few more hours at the same temperature. It should be well protected from dust and organic impurities to prevent decomposition during the later treatment at higher temperatures.

When the $LiClO_4$ did not smell of alcohol any more, it was recrystallized from water, sucked off on a glass filter plate, and redissolved in about 1 l water. The aqueous solution was filtered through a G 3 glass filter, and half of it was transferred to a 1 l Pyrex flask (Fig. 1 a). The flask was put into a vertical oven and the water evaporated under reduced pressure (water pump) at a temperature of 100—150° C. When almost all of the water was gone, the temperature was raised to about 200° C for one day. Next day it was kept around 230° C and then the $LiClO_4$ was melted by keeping the oven for one day at 260—270° C. The contents of the flask were kept at water pump pressure during the whole of this procedure. The temperature should never exceed 300° C.

Then an oil pump was connected and the melt kept at 1 mm Hg for at least half a day. Finally a stream of dry nitrogen was passed through the flask for a few hours, after which the melt should be allowed to cool in nitrogen. The dried salt can be kept in a closed vessel for one or two days but should preferably be used as soon as possible.

All of the *ether* used was distilled from sodium metal.

The *nitrogen* was taken from a cylinder and dried with dehydrite. The traces of oxygen were generally first removed with activated copper; always in work with Li^+R^- solutions.

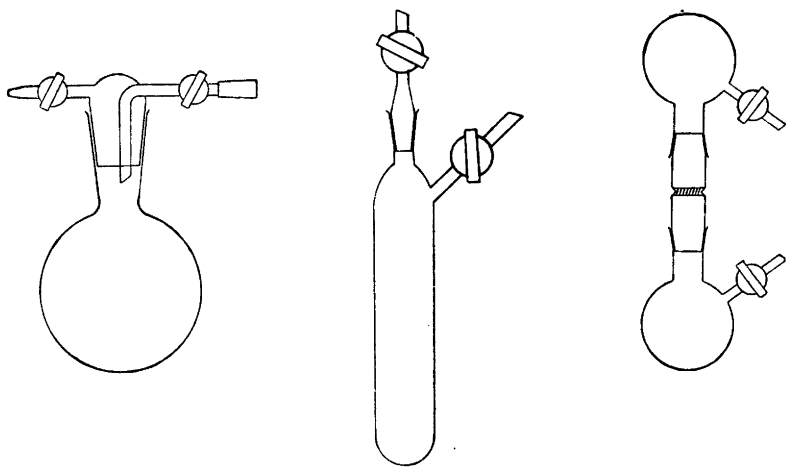

Fig. 1. a) Flask for drying $LiClO_4$ and preparing $LiClO_4$-ether solution. b) Flask for preparing and storing LiR-$LiClO_4$-ether solution. c) Apparatus for preparing and drying $LiClO_4$. Between the two flasks a G3 sintered glass filter is inserted.

Preparation and analysis of the $LiClO_4$-ether solution. When the flask was cool, it was fitted into a distillation apparatus and ether was distilled directly into the flask. The air was kept out of all vessels, if necessary by means of nitrogen, during this and all the following mixing operations. First the flask was rinsed with three portions of 50—100 ml ether. This ether was discarded after having dissolved the surface layer of the melt and all of the small drops adhering to the flask.

Then 250—300 ml of ether was distilled into the vessel and allowed to dissolve some of the $LiClO_4$ for half an hour with rather frequent but careful swirling. By then the $LiClO_4$ solution was usually close to 2 C. Half of it was transferred to another flask which was closed with a ground joint stopper with a stopcock on it to permit the release of the ether overpressure.

All joints and stopcocks should be lubricated with ether-resistant grease, prepared from starch and glycerol [3].

The other half of the solution was swirled frequently during one hour until it became very viscous, its concentration then being about 4 C. It was transferred to another vessel and the exact $LiClO_4$ concentration was determined for both solutions with the ion exchange method, described in part II [2].

The solutions should be used immediately after analysis.

The first rinsing removes most of the moisture as was shown by the following analysis. The concentration of water in the $LiClO_4$-ether solutions was determined by Johansson's [8] modification of Karl Fischer's titration method; 25 ml 2.7 C $LiClO_4$ (discarded) from a rinsing consumed 1.0 ml of an I_2 solution, 1 ml of which corresponded to 1.7 mg H_2O (0.004 % H_2O) while 50 ml 3 C $LiClO_4$-ether from the following extraction of the melt needed 0.5 ml of the same I_2 solution (0.001 % H_2O).

No Cl^- could be detected with silver nitrate after adding water to the $LiClO_4$-ether.

Preparation of LiR. LiR was prepared according to v. Grosse [9]. Lithium amalgam was first prepared by mixing 140 g Hg and 0.7 g Li in a flask which had a ground-joint stopper and a side-tube, both with stopcocks. The flask was filled with nitrogen during the mixing procedure and then evacuated by the oil pump and kept at 110° C for 5 days with occasional and very careful swirling. Finally it was cooled to room temperature and nitrogen passed through.

Triphenylmethyl chloride had been prepared according to Organic syntheses [10]; its melting point was 111—112.5° C after recrystallisation from absolute ether. Trityl chloride (4.7 g) was dissolved in 140 ml of ether, freshly distilled from sodium. This solution was pipetted (through the stopcock on the stopper) into the flask containing the lithium amalgam.

The vessel was rinsed with nitrogen, closed and shaken, and the ether overpressure released. Most of the reaction took place within half an hour but the LiR concentration increased if the vessel was left for a week and had then attained a value of 20—25 mC (16—20 % yield). The side-tube of the flask was then carefully warmed so that a polythene tube could be slipped on to it. The polythene tube was connected to the side tube of a similar flask, into which the clear LiR-ether solution could be decanted in a nitrogen atmosphere.

The remainder of the R groups may have been present either as RH or in the red crystals — probably LiR etherate — observed in the solid residue. At any rate no unreacted RCl was left; after a careful decantation and hydrolysis with alkali, tests with $AgNO_3$ showed that no chloride was present.

Analysis of the LiR-ether solution. The concentration of LiR was determined using benzyl chloride in absolute ether, as proposed by Gilman and Haubein [11] for the analysis of lithium alkyls. The previous filtration of the lithium alkyl solution recommended by these authors must be omitted in the case of lithium triphenylmethyl which decomposes on filtration through a glass filter. If the liquid has been carefully decanted it should be clear.

Example: 5 ml of LiR-ether was mixed with about 20 ml of water. The LiOH set free required 1.40 ml of 94 mC HCl for neutralization. Another 5 ml of LiR-ether was first allowed to react with benzyl chloride and then hydrolyzed. It then needed 0.60 ml of 94 mC HCl, possibly due to suspended LiOR, Li_2O, or LiOH (*cf.*[12]). *Result:* [LiR] =

$$\frac{(1.40-0.60)\cdot 94}{5} = 15.4 \text{ mC.}$$

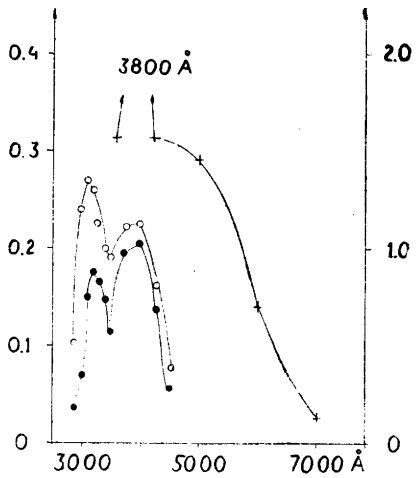

Fig. 2. Extinction curves for a) red-violet LiR-$LiClO_4$-ether solution (+, Fig. 3 c first point), bc) orange-colored solution from adding excess $LiClO_4$-ether to LiR-$LiClO_4$-ether (Fig. 3 c last point) and diluting with ether: b) protected from air (●), c) air blown through (○). The extinction scale to the right refers to a), that to the left to b) and c).

Preparation and analysis of the LiR-$LiClO_4$-ether solution. A certain amount of the solution of LiR in ether was mixed with sufficient of the stronger $LiClO_4$-ether solution to produce 50 ml solution of concentration equal to that of the weaker (about 2 C) $LiClO_4$ solution mentioned above. The vessel should permit only a small area of contact between the liquid and gas phase (Fig. 1b).

With solutions containing $LiClO_4$, we found that the benzyl chloride method for LiR gave either too high values or sometimes altogether anomalous ones. It was decided to try iodine titration instead: 5 ml LiR in $LiClO_4$-ether was pipetted into 5 ml of about 25 mC I_2 in ether. The excess of I_2 was removed with a slight excess of $Na_2S_2O_3$ and determined by back titration with an aqueous solution of I_2 using starch as indicator. Since the concentration of I_2 in ether changes rather quickly as the ether decomposes, the iodine solution should never be older than one day and a blank should be run.

Example: 5 ml of LiR-ether solution (same as in the previous example) was pipetted into 5 ml of a 23.6 mC I_2 solution in ether, the tip of the pipet being held under the surface. Reaction: $I_2 + R^- \rightarrow RI + I^-$. Excess (25 ml) of 10.2 mC $Na_2S_2O_3$ was added and titrated back with 4.08 mC I_2 in water; 21.8 ml was needed. A blank required 2.3 ml 4.08 mC I_2. *Result:*

$$[LiR] = \frac{(21.8-2.3) \cdot 4.08}{5} = 15.9 \text{ mC}.$$

The following is an example of a remarkable behaviour. The concentration of a LiR-ether solution was determined as 20.7 mC by the benzyl chloride method. It was then diluted with $LiClO_4$-ether in the ratio of 4.75 : 10 so that the final concentration of LiR could be calculated to be 14 mC. When the benzyl chloride method was applied to this diluted solution, a higher value, 16.0 mC, was found.

When the LiR-ether solution mentioned above (15.4 mC by benzylchloride, 15.9 mC by I_2 method) was diluted with an equal volume of $LiClO_4$-ether (calculated 7.7 or 7.95 mC), 8.15 mC was found with the I_2 method.

The fact that the iodine method gives slightly higher values than the benzyl chloride method might be due to some uncharged R being present which would effect the I_2 consumption ($2R + I_2 \rightarrow 2RI$) but not the consumption of acid. However, the behavior of the benzyl chloride method on dilution with $LiClO_4$-ether is unexplained.

At any rate, the I_2 method was given preference, since it was important, when a new emf titration was to be started, that the analysis for R^- in the LiR-$LiClO_4$-ether solution could be carried out quickly. In view of our analytical data we decided to subtract 10 % of the value obtained; the difference may have been due to R present, but we did not consider our data to allow an accurate correction.

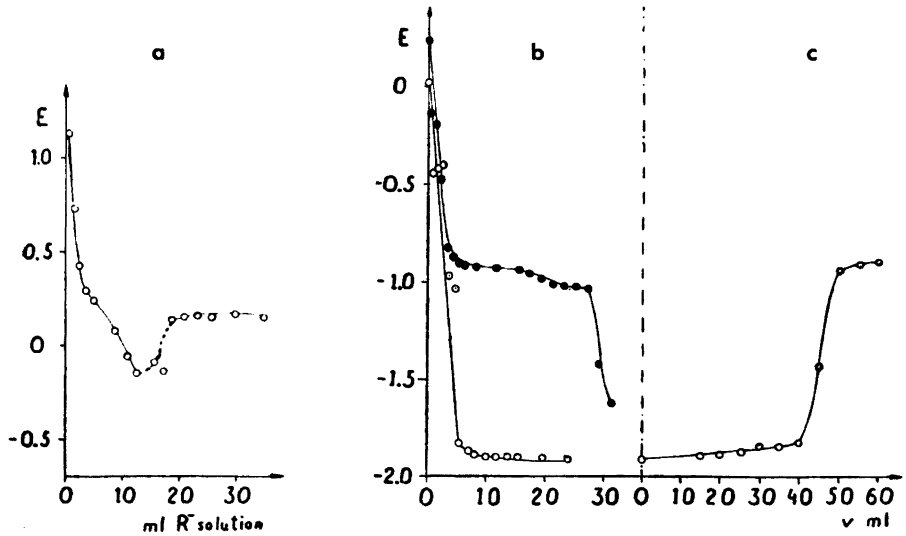

Fig. 3. Emf titration curves. a) R^- in ether was added to R^+ in 1 C $LiClO_4$-ether with 0.03 % H_2O. b) Circles ○, 7.7 mC R^- in ether was added to 10 ml 2 C $LiClO_4$ in ether less than one day old. Dots ●: 15.4 mC R^- was added to 10 ml 2 C $LiClO_4$-ether 5 days old. c) To the final solution in Fig. 3 b was added 2 C $LiClO_4$-ether one day old. — In all these experiments, old LiBr-$LiClO_4$-ether was used in the reference electrode.

The LiR-ether solution is bright red and changes rather dramatically, on mixing with $LiClO_4$-ether, to a deep red color with a violet tinge. We have made some preliminary studies on this latter solution with a Beckmann spectrophotometer (extinction curve, Fig. 2 a). Since LiR may decompose on dilution we used a Baly cell (Zeiss) with a very thin layer of the liquid, protected from air. As reference solution, $LiClO_4$-ether was used. The cells should be put into water immediately afterwards, otherwise the separable parts will become inseparable.

Preparation of $RClO_4$. Solid $RClO_4$ was prepared according to Hofman and Kirmreuther [12] and was always used within three weeks. The products obtained by Gomberg and Cone's method [13] were less pure, as seen from melting points and consumption of OH^- after hydrolysis.

1 g triphenyl carbinol was dissolved in 10 ml acetic anhydride, and the solution was cooled in an ice-salt bath to about −15 to −17° C. While the solution was still clear, 1 ml 70 % $HClO_4$ was added and the yellow precipitate filtered off, washed with acetic anhydride and dried in a nitrogen atmosphere. It was left for two days in a desiccator over P_2O_5 and should then have a melting point of 140−141° C which was not raised by recrystallization from acetic anhydride or nitrobenzene. The apparatus for filtering and drying $RClO_4$ is shown in Fig. 1 c.

When 435 mmole $RClO_4$ (as calculated from the weight) was hydrolyzed with about 50 ml water in an E-flask, 429 mmole acid (98.6 %) was found on titration with NaOH.

When the salt was dissolved in 2 C $LiClO_4$-ether, a 1.3 mC solution was obtained which kept its yellow color for a few hours. When stored the salt was always kept over P_2O_5. The color shifted slowly from deep yellow to dark brown when the salt was stored for a month or more.

The salt was decolorized on washing with ether which had been in direct contact with sodium metal and then sucked off instead of distilled. It seems likely that the cause was a small amount of NaOH in the ether.

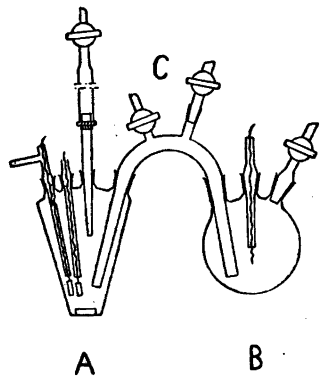

Fig. 4. A = Titration vessel with redox electrodes and stirrer. B = reference electrode vessel. C = salt bridge.

Preparation of LiBr solution. A good grade of LiBr (Kahlbaum, DABV) was dried for one day at 180° C under oil pump vacuum. Ether was distilled into the cooled flask and left in contact with the salt for a few days. The LiBr content was determined by the ion exchange method.

APPARATUS

The cell studied,

$$-\text{Ag, AgBr/0.1 C LiBr, 2 C } LiClO_4\text{/2 C } LiClO_4/R^+ + R^-\text{, 2 C } LiClO_4\text{/Pt or Au} + \quad (1)$$

was kept in a thermostated room at 20° C ± 1° C (the constancy was usually better than this). At higher temperatures, the vapor pressure of ether becomes inconveniently high.

In each titration there were two redox electrodes in the solution: a gold foil which had been ignited in a methanol flame and a palladinated Pt electrode prepared according to Hamer and Acree [14]. The Ag,AgBr electrode was prepared by Brown's method [15] and, like the Pt electrode, was dried by dipping it into absolute ethanol and then into three successive baths of absolute ether just before use.

A Radiometer of type PHM 22 was used for measuring the emf.

The glass apparatus is shown in Fig. 4; A is the titration vessel with Au and Pd-Pt electrodes, buret, magnetic stirrer enclosed in polythene, and inlet for the liquid junction. The $RClO_4$ was introduced through an inlet on one side, closed with a ground-joint stopper. B is the reference electrode vessel containing the Ag,AgBr electrode and inlet for liquid junction whilst C is the liquid junction with stopcocks for venting the nitrogen. Nitrogen was let into the apparatus through one of the electrodes which had a sidetube on top and a hole rather high up to the hollow stem.

The buret. Since ordinary stopcocks did not stop ether leakage, we tried burets from which the ether solution was let out by means of a polythene tube containing a glass bead. A considerable force was required to squeeze the tube so that liquid ran out, but after some time of use leakage occurred between additions. We then began to use a buret with a Teflon screw outlet (Johns-Manville Products) which proved to be altogether ideal for ether solutions. The upper part of the buret was joined to a tube containing a stopcock. For filling the buret, a rubber suction bulb was attached to the tube and the LiR solution was sucked up through a polythene tube drawn out to a capillary of diameter about 1 mm. This polythene tube had been slipped over the tip of the buret so that it reached the bottom of the narrow storage vessel.

When the buret was to be attached to the titration vessel, part of the polythene tube was cut off with a pair of scissors. A short part of it was left which almost reached down into the liquid, thus avoiding a too long passage of the ether solution through the gas phase. Since LiR is quickly destroyed if allowed to stand in a thin-walled polythene vessel surrounded by air, the operation should be carried out quickly and the polythene tube kept down in the nitrogen atmosphere of the storage vessels as much as possible. The ground joint of the buret should fit into that of the storage vessel.

SOURCES OF ERROR

It was important that the LiBr-ether solution should not be more than one week old when used. Otherwise the emfs were quite different from those with fresh solutions. For instance, the E at the end of the redox titration (large excess of R^-) was about —1.20 V with fresh solutions but about —2.00 V with a six weeks old solution. The electrode potential of the reference half-cell did not change; it was found that the potential difference between an Ag,AgBr electrode in an old LiBr solution and a similar electrode in a new LiBr solution was at most only a few mV, as could also be expected from the results in part I—III[1-3].

Our only explanation is that degradation products of the ether solution move through the salt bridge to the titration vessel and affect the redox potentials there.

In the first fourteen titrations carried out, we ran into difficulties which were traced back to impurities in the $LiClO_4$-ether solution. If the $LiClO_4$ melt was not dried under the oil pump vacuum, or if the first extract with ether was used, the water content of the $LiClO_4$-ether was too high.

The influence of water is demonstrated by the curve, Fig. 3a, where R^+ in 1 C $LiClO_4$-ether, containing 0.03 % water, was titrated with R^- in ether; it is quite unlike the curves obtained with less water present and using fresh solutions (Fig. 5).

The ionic strength was kept constant by always adding an equal volume of 2 C $LiClO_4$-ether. The curves in Fig. 3b show the effect of time. In curve I (○), 7.7 mC LiR was added to 10 ml of $LiClO_4$-ether, containing only 0.001 % H_2O and less than one day old. In curve II (●), 15.4 mC LiR in ether was added to 10 ml of the same 2 C $LiClO_4$-ether which was now five days old. A considerable amount of impurities had obviously formed during this time interval.

The curve in Fig. 3c was obtained when fresh $LiClO_4$-ether solution was added to the final solution from 3b, curve I. When the violet red LiR-$LiClO_4$-ether solution (extinction curve in Fig. 2a) had been decolorized, the solution at first turned light yellow, and after a few minutes the yellow color changed to orange, a color which disappeared if the solution was exposed to air. Extinction curves were taken up of this orange colored solution, after diluting 26 times with ether; the solution was protected from air in an ordinary quartz cuvette with a ground stopper, Fig. 2b. The same solution was also studied after exposure to air, extinction curve 2c. As reference solution a very weak $LiClO_4$-ether solution was used; for wavelengths less than 2 900 Å the reference solution had the larger extinction.

A maximum at 4 000 Å is present in all three curves, but only the violet red solution from Fig. 2a absorbs in the region 5 000—7 000 Å. As mentioned already a Baly cuvette was used, difficulties were met with when using ordinary cuvettes since it is necessary to dilute the solutions to avoid too large absorption, and the dilution may destroy most of the sensitive compounds.

FINAL PROCEDURE

Since the $LiClO_4$-ether solutions proved to decompose even without access to air and moisture, it was imperative to use fresh solutions and to work quickly. The elaborate technique for keeping air and moisture away that proved useful in the work with $Ag^+ - I^-$ (part III) would here have been unwieldy and had meant a great waste of chemicals; in our work with triphenylmethyl no "stock solution" could be stored even for a week. The procedures we used were compromises between the fear of contamination from the air and the urge for quick work.

Before a titration was started, the apparatus was filled with nitrogen, and the reference electrode vessel charged with 50 ml of a solution (2 C $LiClO_4$, 0.1 C LiBr.). Into the titration vessel, 5—10 ml 2 C $LiClO_4$-ether was pipetted, and the liquid junction was filled with the same solution. Then LiR in 2 C $LiClO_4$-ether was added from the buret. A few ml of LiR were always destroyed by various impurities: by traces of moisture and oxygen adhering to the electrodes, to the sintered glass-plate of the liquid junction or to the glass walls, or by impurities in the ether originally present in the titration vessel or in the ether flowing down from the liquid junction. We could not remove such impurities altogether, and it was hard to reduce the initial consumption of LiR even to its present size. When the impurities had reacted and a small excess of R^- began to appear, the solution turned red and E attained a value of about -1.2 V.

At this point a weighed amount of $RClO_4$ was carefully introduced. The color changed immediately to light yellow, and E changed to about 0.3 V. Now the main part of the titration was started: LiR was added, about 1 ml at a time until the solution after an intermediate region of dark yellow to greenish brown turned red again and E had decreased again to about -1.2 V.

The gold electrode and Pd-Pt electrode finally attained the same value; the gold electrode gave the final emf almost immediately, the Pd-Pt electrode after 5 minutes — 1 hour. In a solution containing excess LiR, the delay was rather small.

Typical E curves are shown in Fig. 5 and will be discussed below. At the second sharp drop the initial red color disappeared again after about 2 minutes; with 1 ml excess of LiR the red color remained for about 5 min, and if 1 ml more of LiR was added the color kept for almost one hour. On the other hand, red drops adhering to the buret might keep their color for up to about an hour. This indicates that R^- reacted with a component of the solution and not with a component of the gas phase especially as the contact area between liquid and gas phase rather increased slightly due to the shape of the titration vessel.

It is best to maintain a very small overpressure of nitrogen in the vessel. The solutions should be kept in the dark as much as possible.

The final reaction in which LiR is destroyed is so slow that it has been difficult to investigate it.

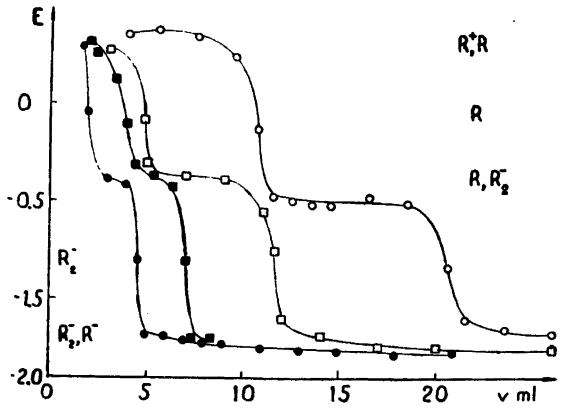

Fig. 5. Reproducible curves obtained with purified materials. Amount of $RClO_4$ added: ● *4.8,* ■ *5.3,* □ *5.4,* ○ *14.4 mg. The curves are shifted slightly sidewards, so that the total volume of the solutions was, in the order mentioned, 9.4 + v, 10.7 + v, 10.2 + v and 11 + v ml. We have assumed that the first equivalence point (R_2) was at 1.9, 3.9, 4.8, and 10.67 ml, and the second (R_2^-) at 4.5, 11.6, and 20.6 ml.*

DISCUSSION OF THE CURVE

Fig. 5 shows typical curves. They have two equivalence points (sharp drops) and three "shelves". It seems reasonable that one of the equivalence points should correspond to the prevalence of the uncharged form R (or R_2) in the solution. From the concentrations of the solutions it was quite clear that this must be the first equivalence point.

In these titrations we knew the amount of R^+ added, and also approximately the concentration of the R^- solutions. However, it was hard to determine exactly the excess of the R^- after destroying the impurities; so this excess was calculated by assuming the first sharp drop to correspond to the completion of the reaction:

$$R^+ + R^- \rightarrow 2\ R \rightleftharpoons R_2$$

The second sharp drop might be due to one of two possible causes. Either R^- reacts with the products formed when R^- previously reacted with the impurities (which may now also have reacted with R^+), or R^- reacts with R (or R_2). Table 1 shows that the amount of R^- consumed between the two equilibrium points has no relation to the amount needed for the destruction of the impurities but is almost equal to the amount of R formed at the first equilibrium point (twice the amount of R^+added).

Table 1.

Mole R^- for impurities	Mole R formed	Mole R^- used betw. eq. points	R^- betw. eq. points / R^- for impurities	R^- betw. eq. points / R formed
69.3	84.0	88.2	1.27	1.05
38.9	31.5	57.6	1.48	1.83
112.8	30.9	37.7	0.33	1.22
103.0	28.0	33.4	0.32	1.19

If the differences between the benzyl chloride and iodine analyses for R^- are ascribed to R present in the R^- solutions, the ratio will be still closer to 1. This indicates that we have a reaction:

$$R + R^- \rightarrow R_2^- \quad \text{or} \quad R_2 + 2R^- \rightarrow 2R_2^-$$

One reason why the ratio in the last column of Table 1 may come out somewhat higher than 1 is that the LiR solution is very sensitive and may be destroyed to a slight extent in the course of the titration. So, in the following calculations, we have recalculated the concentration of R^- using the amount of $R^+ClO_4^-$ weighed in, and the volume of R^- solution consumed between the two sharp drops, assuming them to correspond to the compositions R and R_2^-.

CALCULATION OF EQUILIBRIUM CONSTANTS

If we assume that R^+, R, R_2^- and R^- are the main forms of triphenylmethyl present during the titration, we may calculate from the data in Fig. 5 three "molar potentials" E_{01}, E_{02}, and E_{03}:

Symbol	$E_{01}(R^+ + e^- \rightarrow R)$,	$E_{02}(2R + e^- \rightarrow R_2^-)$,	E_{03} $(R_2^- + e^- \rightarrow 2R^-)$
●	0.32	−0.18 −0.22 −0.18	−1.38 −1.38 −1.38 −1.37
			−1.37 −1.38 −1.37 −1.37
			−1.37
■	(0.16)	−0.20 −0.20 −0.19	(−1.48) −1.40
□	0.32	−0.23 −0.22 −0.19 (−0.26)	−1.41 −1.43 −1.42 −1.41
			−1.39
○	0.30 0.36	−0.38 −0.38 −0.37	−1.36 −1.34 −1.32 −1.31
		−0.29 −0.28	−1.31

For instance, E_{01} is defined by the equation, for the emf of a cell:

Ag, AgBr/0.1 C LiBr, 2 C $LiClO_4$/2 C $LiClO_4$,/$R^+ClO_4^-$, R, 2 C $LiClO_4$/Au.

$$E = E_{01} + \frac{RT}{F} \ln \frac{[R^+]}{[R]} = E_{01} + 0.058 \log \frac{[R^+]}{[R]} \qquad (2)$$

The reference half-cell is thus always Ag, AgBr/0.1 C Br^- in 2 C $LiClO_4$.

The experimental accuracy would make it meaningless to give the figures with a higher accuracy than 0.01 V.

Values within brackets have been disregarded, usually because the points were close to an equivalence point so that the concentrations were not known with sufficient accuracy.

Disregarding one of the titrations (○) which deviates somewhat from the others, we shall use the following tentative values:

$$\begin{aligned} R^+ + e^- &\rightarrow R; \quad E_{01} = 0.32 \pm 0.05 \\ 2R + e^- &\rightarrow R_2^-; \quad E_{02} = -0.23 \pm 0.05 \\ R_2^- + e^- &\rightarrow 2R^-; \quad E_{03} = -1.40 \pm 0.03 \end{aligned} \qquad (3)$$

From these E_0 values one may calculate the equilibrium constants for a few reactions of interest

$$R^+ + R_2^- \rightleftharpoons 3R; \quad \log K = \frac{E_{01}-E_{02}}{0.058} = 9.5 \pm 1.5$$

$$R + R^- \rightleftharpoons R_2^-; \quad \log K = \frac{E_{02}-E_{03}}{2 \cdot 0.058} = 10 \pm 1$$

$$R^+ + R^- \rightleftharpoons 2R; \quad \log K = \frac{2E_{01}-E_{02}-E_{03}}{2 \cdot 0.058} = 19.5 \pm 1.5 \qquad (4)$$

$$R^+ + 3R^- \rightleftharpoons 2R_2^-; \log K = \frac{2E_{01}+E_{02}-3E_{03}}{2 \cdot 0.058} = 39.5 \pm 3$$

If, on the other hand, one assumes R_2 to predominate over R, E_{01} and E_{02} are replaced by E'_{01} and E'_{02}:

Symbol	E'_{01} ($2R^+ + 2e^- \rightarrow R_2$)	E'_{02} ($R_2 + e^- \rightarrow R_2^-$)
●	0.39	−0.32 −0.37 −0.36
■	(0.23)	−0.34 −0.35 −0.37
□	0.38	(−0.37) −0.37 −0.36 (−0.47)
○	0.43 0.36	−0.51 −0.52 −0.51 −0.44 −0.45

We shall use the following tentative values:

$$2R^+ + 2e^- \rightarrow R_2; \quad E'_{01} = 0.39 \pm 0.05$$
$$R_2 + e^- \rightarrow R_2^-; \quad E'_{02} = -0.36 \pm 0.03 \qquad (5)$$
$$R_2^- + e^- \rightarrow 2R^-; \quad E_{03} = -1.40 \pm 0.03$$

The equilibrium constants will then take the following form:

$$2R^+ + 2R_2^- = 3R_2; \quad \log K = \frac{E'_{01}-E'_{02}}{\frac{1}{2} \cdot 0.058} = 26 \pm 2$$

$$R_2 + 2R = 2R_2^-; \quad \log K = \frac{E'_{02}-E_{03}}{0.058} = 18 \pm 1$$

$$R^+ + R^- = R_2; \quad \log K = \frac{2E'_{01}-E'_{02}-E_{03}}{2 \cdot 0.058} = 22 \pm 1$$

$$R^+ + 3R^- = 2R_2^-; \quad \log K = \frac{2E'_{01}+E'_{02}-3E_{03}}{2 \cdot 0.058} = 40 \pm 1 \qquad (6)$$

Since the dissociation constant of R_2 in many solvents seems to be of the order of magnitude 10^{-4} (see Waters [16]) and the concentration of R or R_2 in the solutions is of the order 10^{-3} to 10^{-4} it is not possible to tell a priori which of the two forms will predominate in our $LiClO_4$-ether solutions. If our emf data had been more accurate, one might have drawn some conclusions from the shapes of the two first shelves; as it is, we have to leave the question open.

SUMMARY

Using 2 C $LiClO_4$-ether as ionic medium, potentiometric (redox) titrations have been made of the positive triphenylmethyl ion, R^+, with the negative triphenylmethyl ion, R^- ($R = (C_6H_5)_3C$). The emf curves show two sharp

drops which can be explained in terms of the formation of $R(R_2)$ and a complex, R_2^-. The equilibrium constants for the various rections between R^+, $R(R_2)$, R_3^- and R^- in this medium have been estimated, (4) and (6). — The work was complicated by the fact that the redox potentials were very much influenced by the decomposition products of $LiClO_4$-ether, which are formed rather rapidly but did not disturb previous work with Ag^+ and halogenide ions in the same ionic medium.

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Utilization of Ethyl Esters of α-Keto Analogues of *Iso*leucine, Leucine and Valine for Growth in Rats

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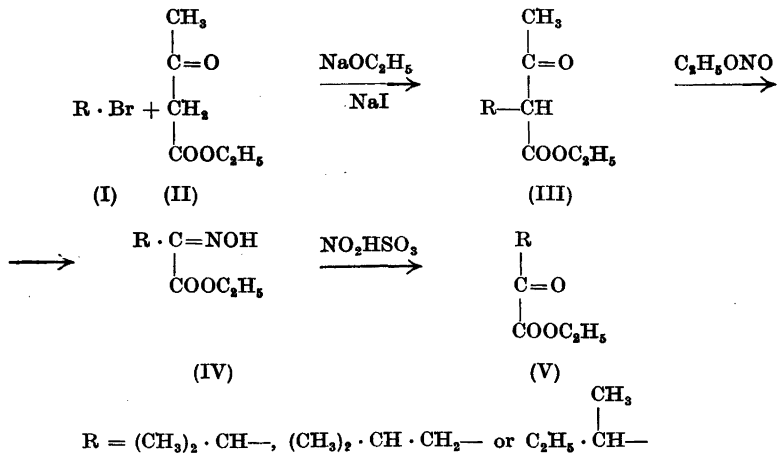
It has been established by earlier investigations that the α-keto analogues of methionine, phenylalanine, tryptophan, histidine, *iso*leucine, leucine and valine can replace these amino acids in the diet. It has also been shown [1] that the *iso*propyl ester of both L- and D-phenylalanine is able to produce growth in rats. It therefore appeared probable that amino acids could also be replaced by the esters of their α-keto analogues. In order to ascertain whether this is the case, the experiments reported in the following were made with the ethyl esters of α-keto*iso*valeric acid, α-keto*iso*caproic acid and α-keto-β-methyl-valeric acid. Strangely enough, it was found that the ethyl esters of these keto acids were poorly utilized, and that they were unable to produce growth in young rats.

EXPERIMENTAL

The ethyl esters of α-ketoisovaleric acid, α-ketoisocaproic acid and α-keto-β-methyl-valeric acid were produced as follows:



Ethyl-α-alkylacetoacetate (III) was synthetized from the corresponding alkyl bromide and ethylacetoacetate, using a method similar to that of Rupe and Courvoisier [2] for the preparation of ethyl-α-*iso*propylacetoacetate. The ethyl esters of α-oxamino acid and α-keto acid were obtained with the method described by Sen [3] for the synthesis of ethyl-α-keto*iso*valerate.

Ethyl-α-isopropylacetoacetate (III). 1 300 g of ethylacetoacetate are added to a solution of sodium alcoholate, obtained from 4.5 liters of absolute alcohol and 230 g of sodium. While stirring, the solution is heated to boiling and 340 g of NaI are added; 1 350 g of *iso*propyl bromide are then added in the course of two hours. The solution is refluxed over night. After cooling, the solution is filtered. The alcohol is distilled off *in vacuo* and 1 000 ml of water are added to the residue. The solution is then extracted three times, each time with 1 000 ml of ether, and the combined ether extracts are washed twice with 250 ml of water each time. After drying with Na_2SO_4, the ether extract is distilled *in vacuo;* the fraction distilling between 93—94.5° C at 18 mm contains the ethyl ester of *iso*propylacetoacetic acid. Yield: 49 % (850 g).

Ethyl-α-oxaminoisovalerate (IV). 425 g of ethyl-*iso*propylacetoacetate are added to a sodium alcoholate solution, prepared from 850 ml of absolute alcohol and 57 g of sodium. Ethyl nitrite, which has been dried over $CaCl_2$, is bubbled into this mixture. An ice bath is used to prevent the temperature of the solution from exceeding 40° C. The ethyl nitrite is bubbled through until the formation of heat has ceased. After the solution has stood over night at room temperature, the alcohol is removed *in vacuo*. To the residue, which consists of the sodium salt of ethyl-α-oxamino*iso*valerate, dilute sulphuric acid (1 : 3) is added with cooling until an acid reaction is obtained. Extraction with ether is then performed three times, with 100 ml each time. After drying over Na_2SO_4, the ether is removed, and the fraction is taken which distills between 128—131° C at 12 mm. Yield: 60 % (270 g).

Ethyl-α-ketoisovalerate (V). 250 g of ethyl-α-oxamino*iso*valerate are dissolved in 500 ml of 85 % formic acid. The solution is cooled to 0° C. 150 g of nitrosyl sulphate are added in small portions, at such a rate that the temperature does not exceed 5° C. When the reaction is complete, the solution is allowed to stand for one hour at room temperature, when it usually turns from greenish-blue to yellow. To this mixture are added 1 500 ml of ice water and the solution is extracted three times, with 500 ml of ether each time. The ether is removed *in vacuo* (20 mm Hg); the formic acid being removed at the same time. The residue is taken up in 500 ml of ether and extracted with 20 % Na_2CO_3, in order to remove the free α-keto*iso*valeric acid. After drying over Na_2SO_4, the ether solution is fractionated; the fraction distilling at 72° C at 16 mm contains ethyl-α-keto*iso*valerate. After redistillation, 100 g are obtained, corresponding to a yield of 40 %. (Found: C 58.0; H 8.4. Calc. for $C_7H_{12}O_3$: C 58.3; H 8.4).

Ethyl-α-ketoisocapronate and ethyl-α-keto-β-methyl-valerate are prepared in a similar way to ethyl-α-keto*iso*valerate, except that 1 500 g of *iso*butyl bromide and *sec*-butyl bromide, respectively, are used for the synthesis of ethyl*iso*butylacetoacetate (III) and ethyl-*sec*-butylacetoacetate (III). For the preparation of ethyl-α-oxamino*iso*capronate (IV) and ethyl-α-oxamino-β-methyl-valerate (IV), 460 g of ethyl*iso*butylacetoacetate and ethyl-*sec*-butylacetoacetate, respectively, are used. For the synthesis of ethyl-α-keto*iso*capronate (V) and ethyl-α-keto-β-methyl-valerate (V), 280 g of ethyl-α-oxamino*iso*capronate and ethyl-α-oxamino-β-methyl-valerate, respectively, are taken. On synthesis, the yields are as follows:

a) ethyl*iso*butylacetoacetate	43—47 %
ethyl-*sec*-butylacetoacetate	43 »
b) ethyl-α-oxamino*iso*capronate	27 »
ethyl-α-oxamino-β-methyl-valerate	56 »
c) ethyl-α-keto*iso*capronate	41 »
ethyl-α-keto-β-methyl-valerate	36 »

Ethyl-α-ketoisocapronate is redistilled *in vacuo* at 93° C at 25 mm. (Found: C 61.1; H 9.2. Calc. for $C_8H_{14}O_3$: C 60.7; H 8.9).

Ethyl-α-keto-β-methyl-valerate is redistilled *in vacuo* at 84—88° C at 21 mm. (Found: C 60.7; H 8.9. Calc. for $C_8H_{14}O_3$: C 60.7; H 8.9).

Table 1. Composition of the experimental diets.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8
	g	g	g	g	g	g	g	g
Sucrose	604.5	629.5	603.5	639.5	601.5	619.5	563.5	599.5
Sucrose-vitamin mixture (See Table 2)	150	150	150	150	150	150	150	150
Celluflour	20	20	20	20	20	20	20	20
Salt mixture *	40	40	40	40	40	40	40	40
Cod liver oil	5	5	5	5	5	5	5	5
Wheat germ oil	15	15	15	15	15	15	15	15
Inositol	1	1	1	1	1	1	1	1
Choline chloride	2	2	2	2	2	2	2	2
DL-Histidine	5	5	5	5	5	5	5	5
DL-*Iso*leucine	40	40	—	—	40	40	40	40
DL-Leucine	20	20	20	20	—	—	20	20
DL-Lysine hydrochloride	22.4	22.4	22.4	22.4	22.4	22.4	22.4	22.4
DL-Methionine	5	5	5	5	5	5	5	5
DL-Phenylalanine	20	20	20	20	20	20	20	20
DL-Threonine	10	10	10	10	10	10	10	10
DL-Tryptophan	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8
DL-Valine	—	—	30	30	30	30	30	30
Sodium bicarbonate	11.3	11.3	11.3	11.3	11.3	11.3	11.3	11.3
Ethyl-α-keto*iso*valerate	25	—	—	—	—	—	—	—
Ethyl-α-keto-β-methyl-valerate	—	—	36	—	—	—	36	—
Ethyl-α-keto*iso*capronate	—	—	—	—	18	—	—	—
	1 000	1 000	1 000	1 000	1 000	1 000	1 000	1 000

* "No. 12", *Jones* and *Foster* 1942[6].

White male rats aged from 30 to 40 days were used for the experiments. The animals were kept in individual cages and allowed to eat and drink *ad libitum*. The cages were placed in a room with a constant temperature of 26—27° C. During the two days immediately preceding the experiments, the animals were given a diet containing the same ingredients as the experimental diet, except that 18 % of an enzymatic casein hydrolysate (Aminosol Vitrum) was substituted for the amino acids. This was in order to avoid the changes in weight which otherwise occur when animals are transferred from one type of diet to another.

The diets investigated are listed in Table 1. All the diets contained the racemic form of the essential amino acids in the same concentrations as in earlier experiments [4].

Diets 1 and 2 were used to study the effect on growth of the ethyl ester of α-keto*iso*valeric acid. Diet 1 contained the ethyl ester of α-keto*iso*valeric acid instead of valine, and both valine and its ethyl ester were omitted from diet 2. The quantity of the ester amounted to 2.5 %, corresponding to 2 % of α-keto*iso*valeric acid, which had been found in earlier experiments [4] to produce satisfactory growth in rats.

Both diets 3 and 4 were devoid of *iso*leucine. Diet 3 contained 3.6 % of ethyl-α-keto-β-methyl-valerate. The concentration of the ester exceeded the theoretically calculated quantity of L-leucine (1 %) in diets 1 and 2 by 50 %. The larger quantity of the ester than that calculated was given in order to compensate for a possibly poor yield on hydrolysis and amination.

For the investigation of the ethyl ester of α-keto*iso*capronate, diets 5 and 6 were prepared. The concentration of the ester in diet 5 was 50 % greater than the calculated quantity, for the same reason as in diet 3.

Since the ethyl esters have an unpleasant smell, it was anticipated that the animals might not eat sufficient quantities. Consequently, a diet was composed which contained both ethyl-α-keto-β-methyl-valerate and 4 % of DL-*iso*leucine (diet 7). The growth produced with this diet was compared with that produced by diet 8, which contained all the essential amino acids.

The first series comprised two groups with five rats in each. One group was given diet 1 and the other diet 2. In the second series, a similar comparison was made between diets 3 and 4, and in the third series between diets 5 and 6. The results are recorded in Fig. 1.

The comparison between the effect of diets 7 and 8 was made as follows. Two groups with five rats in each were given diet 3 and diet 4, respectively, for 12 days. After this time they were given the same diet with the addition of 4 % of DL-isoleucine (diets 7 and 8). The effect of these diets on the body weight is shown in Fig. 2.

A further series of experiments was made, using groups of five rats for tests of each of the diets. The investigations covered a 10-day period. In Table 3, the means and the standard errors of the means are given both for the combined groups, each including 10 rats on the experimental diet for 10 days, and for the groups in which the experiments were continued for a longer period. On the basis of these figures, calculations can be made of the possible significant difference between the results in the different groups in the individual series.

If all the ester were converted into the respective amino acid in such a way that it could be utilized for protein synthesis, growth would be maximal. Under the experimental conditions in question, this would amount to an increase in weight of 12 ± 0.5 g per 10 days [5]. By interpolating the results of earlier experiments, an approximate figure can be given for the concentration of L-valine, L-*iso*leucine and L-leucine, respectively, that would produce the changes in weight obtained during the first 10 days, as recorded in Table 3. In these calculations, curves were drawn on the basis of the difference in the change in weight with and without 0.5 % of the corresponding DL-amino acid, *i. e.*, 0.25 % of the biologically active L form (Wretlind [5], Table IX). From these curves, a value was obtained for the quantity of L-amino acid required to produce the difference in the change in body weight obtained with the various diets, *i. e.*, those with and without the ethyl ester of the keto acid of the respective amino acids (Table 3).

RESULTS AND DISCUSSION

It is evident from Fig. 1 that when a diet lacking one of the amino acids valine, *iso*leucine or leucine was supplemented with the ethyl ester of the corresponding keto acid, it was unable to produce growth in young rats. The curves nevertheless suggest that some proportion of the relevant ethyl ester was utilized, since the loss of weight was not as marked as in the absence of this ester. This applies in particular to the ethyl ester of ketoleucine (α-keto*iso*caproic acid) and of keto*iso*leucine (α-keto-β-methyl-valeric acid). It may also be inferred from Table 3 that there is a significant difference between the loss of weight after 10 days in a comparison between the diets with and without the ethyl ester of ketovaline (α-keto*iso*valeric acid) — ($p = 0.01$), the ethyl ester of keto*iso*leucine ($p = 0.001$) and the ethyl ester of ketoleucine ($p = 0.001$). After 18 and 25 days as well, a significant difference is present between the changes in weight with and without the ethyl ester of keto*iso*leucine and ketoleucine, respectively. On the other hand, no significant difference is noted after 18 days with and without the ethyl ester of ketovaline. A possible explanation is that only two of the animals given diet 2 (without valine and its ethyl ester) survived; consequently, no reliable statistical calculations could be made. The fact that 3 out of the 5 animals on this diet died, but none of those given the ethyl ester of ketovaline, nevertheless affords

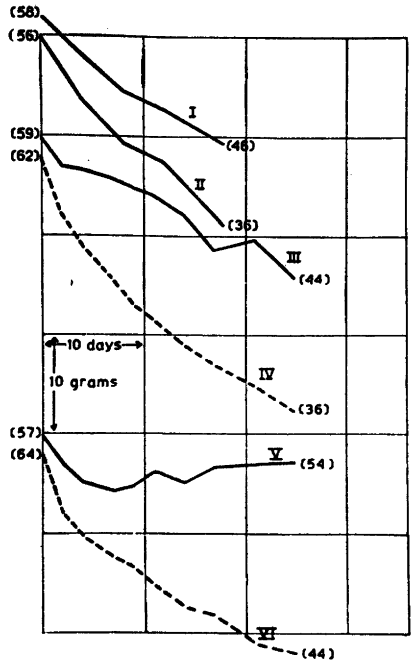


Fig. 1. Changes in weight in growing rats given diets without valine, leucine and isoleucine and diets in which these amino acids were replaced by the ethyl esters of the corresponding α-keto acids. Each curve represents the mean value for 5 rats. The figures in brackets at the beginning and end of each curve denote the mean initial and final weight, respectively.

Series 1. Curve I. Diet 1: *without valine*, with *ethyl-α-ketoisovalerate.*
Curve II. Diet 2: *without valine.*

Series 2. Curve III. Diet 3: *without isoleucine*, with *ethyl-α-keto-β-methyl-valerate.*
Curve IV. Diet 4: *without isoleucine.*

Series 3. Curve V. Diet 5: *without leucine*, with *ethyl-α-ketoisocapronate.*
Curve VI. Diet 6: *without leucine.*

additional evidence that part of the ethyl ester was converted into L-valine. Thus, it both reduced the loss of weight and prolonged the survival time.

The results obtained show that the ethyl esters of ketovaline, keto*iso*-leucine and ketoleucine cannot be converted by the organism into the respective amino acids to a sufficient extent to promote growth. It is, however, also apparent from the experiments that a small part of the esters is converted into the corresponding amino acids, since the loss of weight is less than without the relevant ester. It may be inferred from the approximative calculations incorporated in Table 3 that the reduction in the loss of weight corresponds to a content of 0.1 % of L-valine, 0.2 % of L-*iso*leucine and 0.1 % of L-leucine, respectively, in the diet. Theoretically, the concentrations of the ethyl esters in the diet correspond to 2 % of L-valine, 3 % of L-*iso*leucine and 1.5 % of L-leucine. It may therefore be concluded that only 5 to 7 % of the esters supplied is utilized.

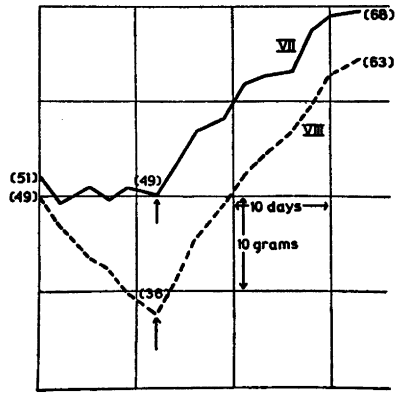


Fig. 2. The effect on growing rats of the addition of isoleucine to the diet; both diets had earlier been devoid of isoleucine, but one contained ethyl-α-keto-β-methyl-valerate. The animals represented by curve VII were given diet 3 (without isoleucine but with ethyl-α-keto-β-methyl-valerate) during the first period; after the time marked by the arrow, they were given diet 7 (diet 3 + 4 % of DL*-isoleucine). The animals represented by curve VIII received diet 4 (without isoleucine) and thereafter diet 8 (diet 4 + 4 % of* DL*-isoleucine). Each curve denotes the mean value for 5 rats. The figures in brackets are the mean initial and final weight, respectively.*

The reason for this poor utilization may be that the organism is unable to split the esters of α-keto acids. This would imply that the esterases present in the gastro-intestinal tract would only hydrolyze the keto esters to a slight extent, or not at all. As far as I am aware, no investigations have been made of this matter. Another possible explanation is that the taste and smell of the keto esters were so unpleasant that the animals refused to eat sufficient quantities of this food. It is, however, shown by the experiments recorded in Fig. 2 that this cannot have been the case. During the first period, the two groups of animals were given a diet devoid of *iso*leucine, with and without ethyl-α-keto-β- methyl-valerate, respectively. The mean initial weight was

Table 2. Sucrose-vitamin mixture.

	g
Sucrose	1.500
Aneurin hydrochloride	0.050
Riboflavin	0.100
Pyridoxine hydrochloride	0.050
Nicotinamide	0.050
Calcium *d*-pantothenate	0.250
p-Aminobenzoic acid	3.000
2-Methyl-1,4-naphthoquinone	0.020
Folic acid	0.001
Biotin	0.001

Table 3. Change in the body weight in growing rats given diets without valine, leucine and isoleucine, respectively, and diets in which these amino acids were replaced by the ethyl esters of the corresponding α-keto acids.

At the beginning of the experiments, each group comprised 10 animals. The values recorded are the means with their standard errors. The initial weight given in brackets denotes the mean for the 5 animals on which the experiments were continued more than 10 days.

Experimental series	No.	Diet	Initial weight	Change in body weight (g) after 10 days	18 days	25 days	Change in weight after 10 days, corresponding approx. to a concentration of the resp. L-amino acid of
I	1	Valine replaced by ethyl-α-keto*iso*-valerate	60 ± 2.1 (58 ± 1.9)	−8±0.8	−13±0.9*		0.1 %
	2	Without valine and the ester	54 ± 1.1 (56 ± 1.6)	−11±0.6	−19±2.7* **		
II	3	*Iso*leucine replaced by ethyl-α-keto-β-methyl-valerate	55 ± 2.0 (59 ± 2.0)	−4±1.4	−11±1.0*	−14±0.9*	0.2 %
	4	Without *iso*leucine and the ester	55 ± 2.5 (62 ± 1.9)	−13±1.1	−22±1.5*	−26±1.7*	
III	5	Leucine replaced by ethyl-α-keto*iso*-capronate	58 ± 1.5 (57 ± 2.0)	−5±0.8	−3±1.5*	−3±1.7*	0.1 %
	6	Without leucine and the ester	65 ± 3.5 (64 ± 7.2)	−13±0.9	−17±1.7*	−20±1.9*	

* After 10 days, the group comprised 5 animals only.
** 3 out of the 5 animals died between the 12th and 18th days.

about the same, *i.e.*, 51 ± 2.6 g in the group given the ethyl ester, and 49 ± 1.9 g in the group without it. After 12 days on the respective diets, the change in body weight was −2 ± 3.0 g and −13 ± 0.7 g, respectively, the difference between the two figures being significant. This implies that part of the ester of keto*iso*leucine was converted into *iso*leucine. When the two groups of animals were subsequently fed on the same diets as earlier, but with the addition of 4 % of DL-*iso*leucine, a rapid gain in weight was noted in both groups. During the 21 days from the day on which the diets were changed, the gain in weight amounted to 19 ± 3 g in the group previously on a diet with the ethyl ester of keto*iso*leucine, and 27 ± 2 g in the group on the diet without this ester. The gain in weight was thus greater in the group earlier given the diet that did not contain the ester. This difference can, however, presumably be ascribed to the fact that one group of animals was more protein-depleted than the other, and not to any difference in the nutritional value of the respective diets. It has been established by earlier experiments that growth is more rapid after protein depletion. During the first 10 days after the change to diet 8, the average gain in weight was 16 g, whereas in group XII

with diet 7, it was 12 g. The normal increase in weight with the diet in question is 12 g per 10 days [5]. This shows that the presence of the ethyl ester of keto*iso*-leucine has little effect on growth with a diet containing all the essential amino acids. Consequently, the experiments imply that the ethyl esters of the keto acids do not inhibit the normal synthesis of protein. The poor growth produced by the ethyl esters of the keto analogues of the amino acids is probably dependent on the inability of the organism to hydrolyze them into the α-keto acids.

SUMMARY

Experiments are made to ascertain the extent to which the ethyl esters of the α-keto analogues of valine, *iso*leucine and leucine are able to produce growth when the respective amino acids are lacking in the diet. It is found that these esters are unable to induce growth in young rats.

When these ethyl esters are given, a reduction is nevertheless obtained in the loss of weight occurring in the absence of the respective amino acids from the diet. This is interpreted as an indication that a small proportion (5 to 7 %) of the ethyl ester is converted into the relevant amino acid.

The explanation of the results is considered to be the inability of the organism to split these ethyl esters.

An account is given of the synthesis of ethyl-α-keto*iso*valerate, ethyl-α-keto-β-methyl-valerate and ethyl-α-*iso*capronate.

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Short Communications

A Method for the Separation of Saturated and Monounsaturated Fatty Acids through Hydroxylation

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In connection with metabolic studies [1] with oleic acid-1-^{14}C a simple method for the separation of saturated acids from oleic acid was needed to effect a sharper separation than the conventional lead salt fractionation. This separation has earlier also been made by reversed phase partition chromatography [2,3] followed by hydrogenation of the band containing palmitic and oleic acid and renewed chromatography. The oleic acid originally present is then obtained as stearic acid which is easily separated from the palmitic acid.

We have instead investigated a method involving hydroxylation of the original mixture with subsequent separation of the products formed.

Of the different hydroxylation methods tried (peracetic acid, permanganate, osmium tetroxide) we have found a procedure with performic acid in ethyl formate as described in the experimental part most suitable.

The products formed were transformed into methyl esters with diazomethane and then separated by ordinary chromatography on silicic acid. The esters of the saturated fatty acids were eluted first with pure methylene chloride (A in Table 1) after which the methyl dihydroxystearate was eluted by adding 0.75 % methanol (B) to the eluent. As evidenced in the table the hydroxylation followed by chromatography effected a separation so that a maximum of 2 % contamination is obtained when labelled palmitic or oleic acid is used. The same separation can be effected even more sharply by partition chromatography but we have found this simple method useful for routine work.

A limitation of this hydroxylation reaction is that a highly unsaturated fatty acid such as linoleic acid is not quantitatively transformed into tetrahydroxystearic acid but a number of less polar products is also

Table 1.

Expt. No.		A			B		
		mg	c/m/ mg	c/m total	mg	c/m/ mg	c/m total
Methyl palmitate (21.1 mg) + methyl oleate-1-^{14}C (21.1 mg)	1.	18.9	5	94	24.6	280	6 890
	2.	19.4	0	0	23.7	305	7 250
Methyl palmitate-1-^{14}C (21.1 mg)	3.	19.0	346	6 560	1.7	52	88
Methyl oleate-1-^{14}C	4.	1.9	59	112	23.4	320	7 490
	5.	2.0	54	108	24.6	335	8 240

21.1 mg methyl oleate corresponds to 23.4 mg methyl dihydroxystearate.

found. The products do not contaminate the saturated acids but are distributed in both the dihydroxy- and tetrahydroxy band (*cf.*[4]).

In the meantime Savary and Desnuelle [5] have published a method in which they hydroxylate with permanganate and separate the products by partition chromatography.

Experimental. All the reactions are carried out in a 15 ml round bottom flask with a 15 cm long neck (i.d. 7 mm) so that all reactions and evaporations *in vacuo* can be performed in the same vessel without losing material through spattering.

Hydroxylation of mixture of oleic and palmitic acid. 20 mg of palmitic acid and 20 mg of oleic acid-1-^{14}C in acetone solution were pipetted into the flask and evaporated to dryness. Ethyl formate (1.5 ml) and 99 % formic acid (1 ml) were added, followed by 0.12 ml of 30 % hydrogen peroxide. The solution was then left for 3 hours in a water bath at 50° and then evaporated to dryness *in vacuo*. The evaporation was repeated twice after addition of 2 ml of toluene. The residue was dissolved in a few drops of methanol and an excess of diazomethane in ether was added and the solvents again evaporated *in vacuo*.

Chromatographic separation of methyl palmitate and dihydroxystearate. Silicic acid (Baker, London) was activated by heating to 120° for 24 hours. 2 g silicic acid and 1 g of Hyflo-Supercel were mixed, stirred in methylene chloride and poured into a chromatographic tube (i. d. 20 mm) on a bed of sand. The mixture of fatty acid esters was dissolved in a few ml of methylene chloride and transferred onto the column.

The unsubstituted esters were eluted with a total of 100 ml of methylene chloride (A). The dihydroxy esters were next eluted with 100 ml of methylene chloride containing 0.75 % (v/v) methanol (B). Tetrahydroxy esters are not eluted with this solvent but require more methanol (2—3 %). The results of a typical set of reactions are shown in Table 1.

This work is part of investigations supported by *"Statens Medicinska Forskningsråd"* and *"Knut och Alice Wallenbergs Stiftelse"*.

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The Decarboxylation of Higher Fatty Acids for Tracer Work

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In connection with work on the intestinal absorption of fat in humans with carboxyl-labelled acids the necessity arose of having at hand a simple method for the decarboxylation of labelled higher fatty acids for the assay of ^{13}C in the carboxyl group.

A number of well established methods for the decarboxylation of higher straight-chain fatty acids [1-3] are described in the literature but they usually require elaborate apparatus, and we needed a method enabling us to run many decarboxylations simultaneously.

The Schmidt reaction [4-6] has been used both for the decarboxylation of short chain labelled fatty acids [3,7] as well as higher labelled fatty acids [8,9].

The present paper describes a simplified procedure for decarboxylation of higher labelled fatty acids by use of the Schmidt reaction. A simple apparatus is described in which the reaction is carried out. The samples are dissolved in benzene and introduced into the reaction vessel which contains sodium azide. Sulphuric acid is added and the carbon dioxide formed is collected in barium hydroxide.

The method has been tested using [1-^{14}C] palmitic acid and [1-^{13}C] oleic acid [10] and was found to give results in good agreement with the theoretical values.

Apparatus and Procedure. The apparatus used for the decarboxylation is shown diagrammatically in Fig. 1. At B is a three way stopcock. Materials: Sodium azide, Merck, p. a. stored in a desiccator; H_2SO_4, Merck, p. a., 97 %; Benzene, dried over KOH, distilled; 0.2 *N* $Ba(OH)_2$ stored in waxed vessels.

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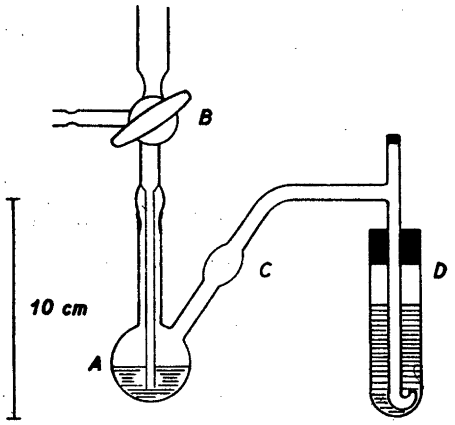

Fig. 1. Decarboxylation apparatus. For description see text.

75 mg sodium azide is introduced into the reaction vessel. The sample to be decarboxylated and containing 0.2 mmole of acid is dissolved in dry benzene and transferred to the reaction vessel with a total volume of 3 ml benzene. With a yield of about 70 % this amount gives 27 mg of barium carbonate. A continuous stream of CO_2-free nitrogen is then passed through the apparatus. Conc. sulphuric acid is used as a lubricant at both the joints. 0.5 ml of conc. sulphuric acid is let into the reaction vessel through B and the apparatus is then placed in a bath at 45° C while it is swept with nitrogen. The effluent gas is bubbled through a trap D with 0.20 *N* barium hydroxide. Wet cotton wool is used to cool the apparatus at C thus preventing benzene and SO_3 from the combustion fluid from reaching D. Total wet combustions of samples of fatty acids were carried out according to Borgström [11]. The radioactivity of ^{14}C samples was measured by a thin mica window G—M tube after plating $BaCO_3$ on aluminium planchets in infinite thickness.

Accuracy of the method. The method has hitherto been used for decarboxylation of fatty acids. The yields vary from 60 to 90 % of the theoretical.

In order to test the method a comparison of values found by combustion of [1-^{14}C] palmitic acid with values found by decarboxylation of the same fatty acid was made. The labelled palmitic acid was diluted with inactive palmitic acid so that the same value after decarboxylation as after combustion of the undiluted fatty acid should be obtained.

Values obtained from wet combustion of [1-^{14}C] palmitic acid	Values obtained from decarboxylation of diluted [1-^{14}C] palmitic acid
Specific activity	Specific activity
1 789	1 807
1 828	1 794
1 809	1 826

This work is part of an investigation supported by *The Medical Faculty of the University of Lund.*

I am greatly indebted to Professor S. Bergström for the labelled fatty acids placed at my disposal.

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On the Chemical Nature of the FMN-binding Groups in the Old Yellow Enzyme

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The observed effects of pH, anions and temperature on the kinetics of the old yellow enzyme system have suggested that the phosphoric acid ester group of FMN is attached to primary amino groups of the protein [1]. It was therefore of interest to study the effect of formaldehyde on this system. Although a great variety of reactions may take place between proteins and formaldehyde, the addition product formed instantaneously at low temperature and low concentrations of the reagent is likely to involve amino groups only [2]. We have now found that low concentrations of formaldehyde instantaneously affect both the dissociation (k_2, sec^{-1}) and reassociation (k_1, M^{-1} sec^{-1}) of the old yellow enzyme. Formaldehyde in water did not dissociate the enzyme. However, in the presence of sodium chloride it increased the rate of dissociation without previous incubation. For example, 0.12 *M* formaldehyde roughly doubled the dissociating effect (k_2) of 0.4 *M* sodium chloride above pH 7. In the neutral and acidic range, 0.12 *M* formaldehyde had no dissociating effect. The apoenzyme was affected by still lower concentrations of formaldehyde. The apoenzyme was added to solutions of FMN and formaldehyde, and k_1 determined at $t \simeq 0$. 0.02 *M* formaldehyde was found to decrease the association velocity constant (k_1) 21 % at pH 8.0, 50 % at pH 8.6, 87 % at pH 10. In the neutral and acidic range stronger formaldehyde (0.12 *M*) decreased k_1 about 25 %. Formaldehyde also decreased the FMN combining capacity of the protein in the alkaline range, but not in the neutral or acidic range. Also acetic anhydride, added to an aqueous solution of apoenzyme, and phenyl*iso*cyanate were found to inhibit k_1 and to decrease the FMN combining capacity.

The association reaction between riboflavin and apoenzyme was found to be far less sensitive to formaldehyde than was the reaction between FMN and apoenzyme. Thus, with our experimental procedure even 0.25 *M* formaldehyde at pH 10 did not inhibit this reaction.

These observations give additional evidence to the previous indications that primary amino groups are essential for the attachment of the phosphoric acid ester group of FMN to the old yellow apoenzyme.

Weber [3] has suggested that aromatic groups in the protein (tyrosine) are the more likely to act as fluorescence quenchers for FMN or FAD. Some of our kinetic data for the riboflavin–apoenzyme system could be interpreted on this basis [1]. The "old yellow" apoenzyme gives a negative nitroprusside test [4], and –SH inhibitors like $Cu^{\cdot\cdot}$, $Fe^{\cdot\cdot\cdot}$, *p*-chloromercuribenzoate or H_2O_2 do not inhibit the reaction between FMN and protein. Since unmasked –SH groups therefore are probably not present in the apoenzyme, iodine could be expected to react only with tyrosyl groups [5]. It was found that incubation of 0.3×10^{-6} *M* apoenzyme with 20×10^{-6} *M* iodine for 2 minutes at 25° and pH 7.4 completely destroyed the ability of the apoenzyme to combine both with FMN and riboflavin. The tyrosine content of the protein, measured by the Millon-Lugg test, decreased by an amount corresponding roughly to the iodine introduced into the protein. The coupling of the apoenzyme with azobenzene sulfonic acid likewise was found to decrease k_1 and the FMN combining capacity.

These observations suggest that a tyrosine residue of the apoenzyme is involved in the quenching of the fluorescence of FMN; perhaps by forming a hydrogen bond with the imino group (3) of the *iso*alloxazine nucleus [1].

The results briefly presented here are another example of the usefulness of kinetic measurements in revealing the nature of the chemical bonds between coenzymes and apoproteins [1].

A detailed report on this work will be presented in this journal.

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* Fellow Norwegian Research Council.

Kinetics of Alcohol Dehydrogenases, Studied with the Aid of a Fluorescence Recorder

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The kinetics of reactions (1), (2) and (3)

$$\text{DPNH} + \text{ADH} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{DPNH}\cdot\text{ADH} \quad (1)$$

$$\text{DPNH}\cdot\text{ADH} + \text{CH}_3\text{CHO} + \text{H}^+ \underset{k_4'}{\overset{k_4}{\rightleftharpoons}} \text{DPN}\cdot\text{ADH} + \text{C}_2\text{H}_5\text{OH} \quad (2)$$

$$\text{DPN}\cdot\text{ADH} \underset{k_5}{\overset{k_3}{\rightleftharpoons}} \text{DPN} + \text{ADH} \quad (3)$$

have previously been studied spectrophotometrically [1,2].

From the formula derived for the initial reaction velocity at $t = 0$

$$\frac{1}{e} \times \frac{da}{dt} = \frac{1}{\frac{1}{k_1 x} + \frac{1}{k_4 a}\left(1 + \frac{k_2}{k_1 x}\right) + \frac{1}{k_3}} \quad (4)$$

it is evident that all the velocity constants can be determined from initial reaction velocities if x and a can be varied within wide enough limits, and e is kept low. Since the whole system is perfectly reversible, a corresponding formula can be obtained for the reverse reaction: ADH + DPN + alcohol.

We have now found that fluorescence measurements are much more sensitive than spectrophotometric measurements, not only with flavin systems [3,4] but with DPNH systems as well. DPN does not fluoresce, but DPNH does, and this fluorescence is not quenched by the coupling of DPNH to ADH. With [DPNH] = 10^{-6} M deflections of 5 inches are observed in our apparatus with S = 0.1 and the narrowest light entrance slit (1 mm). Larger slits give proportionally higher deflections. The light absorption of this solution in 1 cm layer would be as low as $\log I_0/I = 0.006$. All the velocity constants were determined for liver ADH at pH 7.15 and 10.2; at 23.5° and 36°; and in phosphate buffers with or without 0.15 M NaCl. For yeast ADH, which is unstable at pH 10, the investigations have so far been restricted to pH 7.15. Some interesting effects have been observed.

Liver ADH: Increasing the pH from 7.15 to 10.2 at 23.5° in 0.1 M phosphate decreases k_1 ("on velocity" for DPNH) from 6.6×10^6 to 0.148×10^6; increases k_2 ("off velocity" for DPNH) from 2.7 to 5.8; scarcely changes k_5 ("on velocity" for DPN): 0.19×10^6 to 0.16×10^6; and *decreases* k_3 ("off velocity" for DPN) from 66 to 0.74. k_4 decreases from 390×10^3 to 5.6×10^6, k_4' from 9.5×10^3 to 1.6×10^3 *.

0.15 M NaCl decreases the "on velocities" (k_1 and k_5) to about half, both at pH 7.15 and pH 10.2: increases k_2 by 50% at pH 7.15 but *decreases* k_3 from 66 to 18 at pH 7.15. These effects are much less pronounced at pH 10.2. Chloride has no effect on k_4 at 23.5°, but reduced k_4' from 9.5 to 3.7×10^3. The temperature coefficient is in some cases positive, in others negative.

The *yeast ADH* differs very much from the liver ADH. Chloride decreases both k_1 and k_5, but does not interfere with the other velocity constants.

Discussion. As a result of these complicated changes it happens that chloride may stimulate or inhibit the reaction velocity in the liver ADH system, depending on the concentration of the different components. For example, if in a mixture of ADH + DPN + ethanol at 23.5° and pH 7.15 both [DPN] and [ethanol] are high, chloride increases the reaction velocity, which is then dependent mainly on k_2 that is increased by chloride. But chloride inhibits if either [DPN] or [ethanol] are low,

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* The equilibrium constant with small amounts of ADH is expressed as

$$K = \frac{[\text{CH}_3\text{CHO}]\,[\text{DPNH}]\,[\text{H}^+]}{[\text{C}_2\text{H}_5\text{OH}]\,[\text{DPN}]} = 10^{-11}$$

In the presence of ADH in excess, the equilibrium constant will be

$$K_E = \frac{k_4'}{k_4} \times [\text{H}^+]$$

because the decreasing effects of chloride on k_5, respectively k_4' then predominate. Such effects would have been hard to understand before the theory of the system was developed.

Finally, we can now calculate the influence of any of the variables on the redox potential of a solution in equilibrium. For instance, if DPN, DPNH and ADH, all in the concentration 10^{-4} *M*, were mixed with ethanol, 2×10^{-3} *M*, the system would have a potential 34 mV higher than for the same system at low [ADH]. If now 0.15 *M* NaCl were added, the potential would drop not less than 9 mV. We think such anion effects may be of physiological importance in regulating the potentials in the living tissues, where chloride-free cells are surrounded by chloride containing intercellular fluid.

The effects of chloride seem to differ considerably in various enzyme-coenzyme systems. For instance, the redox potential of the yeast ADH system is not influenced by chloride. For the oxidized form of the old yellow ferment the "off" velocity constant is increased by chloride, whereas it is decreased for the DPN · ADH complex.

The complete data will be given in a forthcoming paper in this journal.

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Studies on Antimetabolites

IV. Synthesis of the α,α-Dimethyl Analogue of (+)-Panthothenic Acid *

ÅKE JONSSON

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Following up our investigations on the possibility of designing antimetabolites by the introduction of a suitably situated *gem*dimethyl group into the molecule of an essential metabolite (outlined in Part I[1] of this series), we have now prepared the α,α-dimethyl analogue (I) of (+)-panthothenic acid (II).

The synthetical method used was essentially adopted from Kuhn and Wieland's synthesis [2] of panthothenic acid, the purification of the crude material being, however, carried out in a different way. (−)-Pantholactone was condensed with the benzylester (III) of α,α-dimethyl-β-alanine, the ester group was removed by hydrogenolysis, and the crude acid, which could not be induced to crystallise, was converted into its calcium salt, which was obtained as a microcrystalline powder. The intermediate benzylester (III) was prepared from α,α-dimethyl-β-alanine *via* the hydrochloride of α,α-dimethyl-β-alanyl-chloride, obtained from the amino acid and phosphorus pentachloride in acetyl-chloride solution. No attempt was made to improve the rather low yield of panthothenic acid analogue.

Preliminary tests with *Lactobacillus arabinosus* 17-5, using an agar cup method, showed that the panthothenic acid analogue inhibited the growth of this organism, and that this inhibition was competitively reversed by addition of (+)-panthothenic acid. The inhibition index was about 10 000. A detailed report will be published by Dr. A. Bolinder, Department of Food Chemistry, Royal Institute of Technology, Stockholm.

Experimental *. *Benzyl α,α-dimethyl-β-alaninate hydrochloride.* Ethyl β-formamino-α,α-dimethylpropionate [3] (60 g) and 20 % hydrochloric acid (150 ml) were refluxed overnight. The mixture was evaporated to dryness *in vacuo*, the dry salt extracted twice with ether, powdered, and added in small portions to acetyl chloride (400 ml) with shaking. When all had been added, finely ground phosphorus pentachloride (80 g) was added and the stoppered flask shaken overnight. Anhydrous ether (750 ml) was added and the precipitated solid was centrifuged and repeatedly washed with dry ether, mixed with benzyl alcohol (100 ml) and heated on the water bath until the evolution of hydrogen chloride ceased. The solution was filtered and diluted with much dry ether, and the precipitated ester hydrochloride filtered off and washed with ether. For purification the product was dissolved in the minimum amount of dry acetone, filtered, and reprecipitated by the addition of much ether. Lustrous plates, m.p. 94.5—96°. Yield 41 g. (Found:

* Part III. *Acta Chem. Scand.* **8** (1954) 1389.

* All melting points uncorrected.

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Following up our investigations on the possibility of designing antimetabolites by the introduction of a suitably situated *gem*dimethyl group into the molecule of an essential metabolite (outlined in Part I[1] of this series), we have now prepared the α,α-dimethyl analogue (I) of (+)-panthothenic acid (II).

The synthetical method used was essentially adopted from Kuhn and Wieland's synthesis [2] of panthothenic acid, the purification of the crude material being, however, carried out in a different way. (−)-Pantholactone was condensed with the benzylester (III) of α,α-dimethyl-β-alanine, the ester group was removed by hydrogenolysis, and the crude acid, which could not be induced to crystallise, was converted into its calcium salt, which was obtained as a microcrystalline powder. The intermediate benzylester (III) was prepared from α,α-dimethyl-β-alanine *via* the hydrochloride of α,α-dimethyl-β-alanyl-chloride, obtained from the amino acid and phosphorus pentachloride in acetyl-chloride solution. No attempt was made to improve the rather low yield of panthothenic acid analogue.

Preliminary tests with *Lactobacillus arabinosus* 17-5, using an agar cup method, showed that the panthothenic acid analogue inhibited the growth of this organism, and that this inhibition was competitively reversed by addition of (+)-panthothenic acid. The inhibition index was about 10 000. A detailed report will be published by Dr. A. Bolinder, Department of Food Chemistry, Royal Institute of Technology, Stockholm.

Experimental *. *Benzyl α,α-dimethyl-β-alaninate hydrochloride.* Ethyl β-formamino-α,α-dimethylpropionate [3] (60 g) and 20 % hydrochloric acid (150 ml) were refluxed overnight. The mixture was evaporated to dryness *in vacuo*, the dry salt extracted twice with ether, powdered, and added in small portions to acetyl chloride (400 ml) with shaking. When all had been added, finely ground phosphorus pentachloride (80 g) was added and the stoppered flask shaken overnight. Anhydrous ether (750 ml) was added and the precipitated solid was centrifuged and repeatedly washed with dry ether, mixed with benzyl alcohol (100 ml) and heated on the water bath until the evolution of hydrogen chloride ceased. The solution was filtered and diluted with much dry ether, and the precipitated ester hydrochloride filtered off and washed with ether. For purification the product was dissolved in the minimum amount of dry acetone, filtered, and reprecipitated by the addition of much ether. Lustrous plates, m.p. 94.5—96°. Yield 41 g. (Found:

* Part III. *Acta Chem. Scand.* **8** (1954) 1389.

* All melting points uncorrected.

$HOCH_2 \cdot C(CH_3)_2 \cdot CH(OH) \cdot CO \cdot NH \cdot CH_2 \cdot CR_2 \cdot COOH$

I R = CH_3 II R = H

$H_2N \cdot CH_2 \cdot C(CH_3)_2 \cdot COO \cdot CH_2 \cdot C_6H_5$

III

N 5.81; Cl 14.5. $C_{12}H_{18}ClNO_2$ requires: N 5.75; Cl 14.6).

The *picrate* was obtained by addition of a hot, saturated solution of picric acid to an aqueous solution of the ester hydrochloride. Yellow needles from aqueous ethanol, m.p. 147—148°. (Found: C 49.7; H 4.60; N 12.8. $C_{18}H_{20}N_4O_9$ requires: C 49.6; H 4.62; N 12.8).

Calcium (+)-α,α-dimethylpanthothenate. Benzyl α,α-dimethyl-β-alaninate hydrochloride (15 g) in water (25 ml) was made strongly alkaline with 6 *N* sodium hydroxide solution. The free amino acid ester was immediately extracted with ether, washed and dried (K_2CO_3). Evaporation of the ether yielded the benzyl ester as a colourless oil (12 g). (—)-Pantholactone (10 g, $[\alpha]_D^{20}$ −50.5°, water, *c* 2 %) was added, and the mixture was heated on the water bath for 5 hours. It was then diluted with water (50 ml), acidified with 2 *N* hydrochloric acid to pH 1—2 and repeatedly extracted with ether. The combined extracts were washed with two 20 ml portions of 2 *N* sodium hydroxide and then with water. After drying (Na_2SO_4) and removal of the ether, a colourless, sticky oil (13.5 g) was obtained. This was dissolved in glacial acetic acid (50 ml), palladium on charcoal (2 g, 10 %) added and the mixture shaken in an atmosphere of hydrogen for 5 hours. The catalyst was filtered off and the solution evaporated *in vacuo* on a water bath. A colourless syrup was obtained, which could not be induced to crystallise. It was dissolved in acetone (75 ml), filtered from a small amount of solid and evaporated to dryness *in vacuo*. The syrup was dissolved in water (300 ml), stirred for 2 hours with an excess of calcium carbonate, filtered, and continuously extracted with ether overnight. The aqueous solution was evaporated to dryness *in vacuo* at 40° and yielded a glass. This was dissolved in methanol (75 ml) and the solution was filtered and evaporated to dryness giving a white solid (5.0 g). This solid was purified by dissolution in methanol (50 ml), pouring into acetone (500 ml, reagent grade), storing overnight in a refrigerator, filtering off a small amount of precipitated material, and evaporation to dryness. The solid salt thus obtained was ground with little acetone (20 ml), filtered off, and washed with a little acetone and with ether. Yield 4.2 g, $[\alpha]_D^{20}$ + 27.2° (water, *c* 0.44 %). The product contained one mole of water of crystallization. (Found: C 48.0; H 7.81; Ca 7.37. $CaC_{22}H_{46}N_2O_{10} \cdot H_2O$ requires: C 48.0; H 7.69; Ca 7.28). The water could be removed by prolonged heating at 120° in high vacuum. (Found: H_2O (by loss of weight) 3.2 %. Calculated: 3.3 %).

The author wishes to acknowledge his indebtedness to Dr. A. Bolinder, Institutionen för livsmedelskemi, for carrying out the microbiological tests, to Dr. Karl Folkers of E. Merck Company, U. S. A., for a generous gift of (—)-pantholactone, to *Statens Tekniska Forskningsråd* for a Fellowship, and to *Cancernämnden* for financial support and to Miss E. Duintjer for skilful assistance.

1. Jönsson, Å. *Acta Chem. Scand.* **8** (1954) 1203.
2. Kuhn, R. and Wieland, Th. *Ber.* **73** (1940) 972.
3. Brit. pat. 601.142 *Chem. Abstracts* **42** (1948) 8818.

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Studies on Antimetabolites

V. Improved Reduction of α-Oximino-β-phenylisovaleric Acids *

E. DUINTJER AND Å. JÖNSSON

Organisk-kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

The demand for relatively large amounts of "*neo*phenylalanine" (I) and "*neo*tyrosine" (II) for physiological tests prompted a reinvestigation of the reactions leading to these compounds in order to improve the yields. A detailed study of the reduction of the intermediate α-oximino acids showed that the use of anhydrous ethanolic hydrogen chloride instead of the lactic acid previously used [1] to control the pH in the reduction with sodium amalgam

* Part IV. *Acta Chem. Scand.* **8** (1954) 1491.

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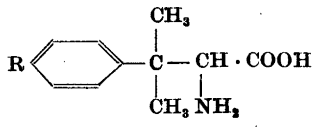

I R = H
II R = OH

gave considerably improved yields of the amino acids. This appeared to be due partly to decreased side reactions leading to nitrogen-free products and partly to easier isolation of the amino acids. Reaction temperature, pH and area of amalgam appeared to be the main factors determining the yields. It was found that at high temperatures (near the boiling point of the mixture) much nitrogen-free material was formed. At room temperature, this side reaction was depressed but the reduction was very slow. At high pH-values (above 7) much nitrogen was lost as ammonia. A large ratio of amalgam area/mole oximinocompound is essential in order to decrease the time required for the reaction, but the high ratio must not be produced by stirring the mixture, since this results in drastically decreased yields (probably by destruction of the molecular orientation at the amalgam surface). By following closely the directions given in the experimental part, it was possible to increase the yield of *neo*phenylalanine to 80—90 % and that of *neo*tyrosine methyl ether to 70—75 %.

In addition to *neo*tyrosine methyl ether, α-hydroxy-β-(*p*-methoxyphenyl)*iso*valeric acid could be isolated from the reaction mixture in about 10 % yield. In the reaction mixture from the reduction of α-oximino-β-phenyl*iso*valeric acid, there was also found some nitrogen-free acid material, probably α-hydroxy-β-phenyl*iso*valeric acid, but this fraction could not be induced to crystallise and was not further investigated.

Experimental: The oximinoacid (0.1 mole) was added to 2 % sodium amalgam (750 g) covered with commercial absolute ethanol (300 ml) in an Erlenmeyer flask of one liter capacity (bottom diameter about 12 cm). Approximately 7 *N* absolute ethanolic hydrogen chloride was added dropwise from a buret to keep the mixture just acid to *p*-bromocresol green (determined by placing a drop of the liquid on a strip of filter paper moistened with a 1 % solution of the indicator). About 75—85 ml acid was required. The temperature of the reaction mixture was kept at 40—50° by appropriate cooling or heating in a water bath. When the reaction ceased, which was indicated by a slowing up and ceasing of the consumption of acid, *N* aqueous hydrochloric acid was added to dissolve the solid precipitate. The solution was separated from the mercury, filtered, evaporated to about 100 ml on the water bath, saturated with hydrogen sulphide, filtered, and the pH adjusted to 6—6.5 by careful addition of ammonia. After storing overnight in the refrigerator the amino acid was filtered off, washed in turn with icecold water (50 ml), acetone (50 ml) and ether (150 ml). The yield of *neo*phenylalanine was 80—90 % and that of *neo*tyrosine methyl ether 70—75 %.

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Synthetic Plant Hormones

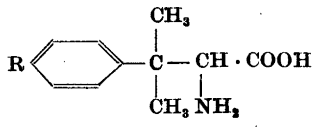
VI. Preparation of some α-Phenoxy and α-1-Naphthoxy fatty Acids * (Addition to Part IV)

E. DUINTJER AND Å. JÖNSSON

Organisk-kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

Some further α-aryloxy fatty acids have been prepared in addition to those reported in Part IV [1] of this series, for investigation of their plant growth activity. All the acids were prepared by condensation of the appropriate sodium phenoxide with the ethyl ester of the α-chloro fatty acid. Physical data for the acids not previously

* Part V. *Acta Chem. Scand.* **8** (1954) 119.



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described are reported in the experimental part. A detailed report on the plant growth activity of the compounds will be published shortly elsewhere by Dr. B. Hansen, University of Lund.

*Experimental.** *General method.* Sodium (0.1 mole) was dissolved in absolute ethanol (50 ml) and this solution was added to a solution of the phenol (0.1 mole) in a little absolute alcohol. The appropriate ethyl α-chloro fatty ester was added and the mixture refluxed overnight. A solution of sodium hydroxide (0.2 mole) in water (20 ml) was added and the whole refluxed for two hours and then poured into water, filtered and made strongly acid using hydrochloric acid. With the readily oxidisable 2,4-dichloro-1-naphtol, the condensation reaction was carried out in a nitrogen atmosphere.

D,L-α-*4-Chlorophenoxyisovaleric acid*, colourless needles from benzene-petrol. M. p. 97—98°. (Found: Cl 15.4; neutr. equiv. 229. $C_{11}H_{13}ClO_3$ requires: Cl 15.5; neutr. equiv. 228.4).

D,L-α-*2,4-Dichlorophenoxyisovaleric acid* separated as an oil. After distillation (b. p. about 200°/10 mm Hg) it crystallised. Needles from petrol, m. p. 65—66°. (Found: Cl 26.7; neutr. equiv. 262. $C_{11}H_{12}Cl_2O_3$ requires: Cl 26.9; neutr. equiv. 263.1).

D,L-α-*2,6-Dimethylphenoxypropionic acid*, needles from petrol. M. p. 62—63°. (Found: C 68.4; H 7.25; neutr. equiv. 196. $C_{11}H_{14}O_3$ requires: C 68.0; H 7.27; neutr. equiv. 194.2).

D,L-α-(*2,4-Dichloro-1-naphthoxy*)*propionic acid*, needles from chloroform-petrol. M. p. 129°. (Found: Cl 24.5; neutr. equiv. 285. $C_{13}H_{10}Cl_2O_3$ requires: Cl 24.8; neutr. equiv. 285.1).

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* All melting points uncorrected. Petrol refers to the fraction b.p. 40—60°.

1. Jönsson, Å. *Acta Chem. Scand.* **7** (1953) 596.

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ACTA CHEMICA SCANDINAVICA 8 (1954) 1495—1501

Studies of Absorption Spectra

III. Absorption Bands as Gaussian Error Curves

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The bands of transition group spectra are shown to be approximated by Gaussian error curves with the wavenumber as the independent variable, and the characteristic parameters (height and wavenumber of the maximum and the halfwidths) are used for the description of several bands with good results (see Figs. 1—5). The resolution of more bands in complicated spectra is discussed, and related to the perturbation theory of crystal fields acting on transition group ions.

An absorption spectrum, *i. e.* the molar extinction coefficient ε as function of the wavenumber $\nu = \frac{1}{\lambda}$ in cm^{-1} (or the wavelength λ), consists very often of one or more bands, symmetrical around their maxima. Various expressions of the exact form of the bands have been proposed. Henri and Bielecki [1] described with success some bands of acetone in the ultraviolet by the formula

$$\varepsilon = a\,\nu\,e^{-b(\nu-\nu_0)^2} \tag{1}$$

where ν_0 is the wavenumber of the maximum and a and b are two constants, determining the height and width of the band. Kuhn and Braun [2] later discussed the far simpler Gaussian error curve as the best approximation of bands:

$$\varepsilon = \varepsilon_0\,e^{-(\nu-\nu_0)^2\vartheta^{-2}} \tag{2}$$

Introducing $\delta = \vartheta\sqrt{\ln 2}$, this can be rewritten

$$\varepsilon = \varepsilon_0\,2^{-\left(\frac{\nu-\nu_0}{\delta}\right)^2} \tag{3}$$

where ε_0 is the extinction coefficient in the maximum, and δ is the halfwidth (since $\varepsilon = \frac{\varepsilon_0}{2}$ for $\nu - \nu_0 = \pm\delta$). If (3) is valid, the half-widths are useful for

characterizing bands, as done by Linhard and Weigel [3,4]. The area of a band will be proportional to the product of δ and ε_0, since

$$\int_0^\infty e^{-kx^2} dx = \frac{\sqrt{\pi}}{2\sqrt{k}} \tag{4}$$

If $\nu_0 \gg \delta$, we therefore get from eq. 3

$$\int_0^\infty \varepsilon \, d\nu = 2\varepsilon_0 \int_0^\infty e^{-(\nu-\nu_0)^2\delta^{-2}} \ln 2 \, d(\nu-\nu_0) = \frac{\sqrt{\pi}}{\sqrt{\ln 2}} \varepsilon_0 \delta = 2.1289 \, \varepsilon_0 \delta \tag{5}$$

This area is proportional to the probability of the transition, causing the band, since the number of classical oscillators per absorbing molecule is

$$P = \frac{1\,000}{N} \cdot \frac{mc^2}{\pi e^2} \; 2.30 \int \varepsilon d\nu = 4.32 \cdot 10^{-9} \int \varepsilon d\nu \tag{6}$$

(N is Avogadro's number, e and m the charge and mass of the electron, and c the velocity of light *in vacuo*, *cf.* Broer, Gorter and Hoogschagen [5]).

Lowry and Hudson [6] proposed the use of a Gaussian formula with λ as independent variable:

$$\varepsilon = \varepsilon_0 e^{-\left(\frac{\lambda-\lambda_0}{\beta}\right)^2} \tag{7}$$

and Mead maintained [7] that bands in the absorption spectra of chromium(III) and cobalt(III) complexes could best be represented by (7).

While the question of the exact congruence of absorption bands with Gaussian ν- or λ-curves will be discussed in the following, it is at least certain at this point that the usual resonance formula:

$$\varepsilon = \frac{\varepsilon_0 \cdot \alpha}{\alpha + (\lambda-\lambda_0)^2} \tag{8}$$

is rarely or never applicable to spectra of solutions, although the formula is useful for the study of absorption lines of gases, *e. g.* Fraunhofer lines. It is seen that (8) implies a much slower decrease of ε for large values of $(\lambda-\lambda_0)^2$ than the Gaussian curves (3) and (7).

This fact is of theoretical interest, since the broadening of bands therefore is not alone due to Heisenberg's uncertainty principle

$$h \sim \Delta E \cdot \Delta t$$

where h is Planck's constant and ΔE and Δt the corresponding uncertainties of energy and time. The observed ΔE would correspond to $\Delta t \sim 10^{-14}$ sec., which seems improbably small, even in these condensed systems.

If the spectrum is not a composite of adjacent, overlapping bands, the graphic representation of log ε as function of ν will show parabolic curves if eq. (3) is valid, and this is quite common according to the atlas of Landolt-Börnstein tables [8]. But the special case of transition group spectra deserve closer investigation, one of the reasons being the crystal field calculations of Ballhausen [9] *et al.*[10].

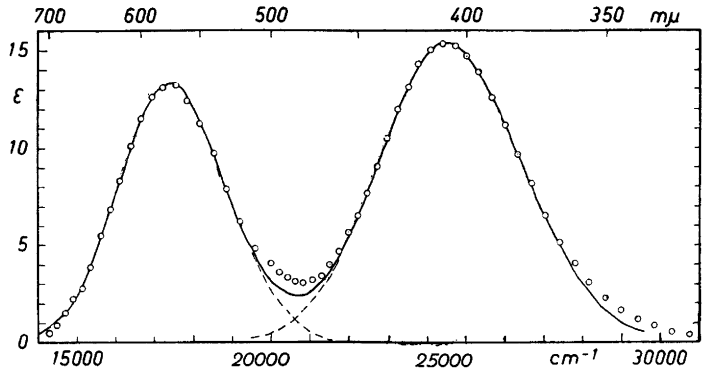

Fig. 1. Absorption spectrum of hexaaquo chromium (III) ions. 0.06 M $Cr(ClO_4)_3$, *0.1* M $HClO_4$. *The calculated curve from eq. 3 with the parameters given in the text is drawn full. The partial bands are punctuated.*

SELECTED EXPERIMENTAL DATA

Fig. 1 gives the absorption spectrum of $[Cr(H_2O)_6]^{+++}$ (in dilute $HClO_4$ to avoid hydroxo-complexes). The two bands resemble closely Gaussian error curves, but it is seen that they are slightly distorted towards high wavenumbers. If two slightly different halfwidths, δ (—) and δ (+) are used in the formula (3) for $\nu<\nu_0$ and $\nu>\nu_0$ respectively, it is seen that the spectrum can be described within the experimental accuracy of about one per cent. It is seen that the two bands of $[Cr(H_2O)_6]^{+++}$ can be described by the four parameters:

$\lambda_0 = \frac{1}{\nu_0}$	ν_0	ε_0	$\delta(-)$	$\delta(+)$
575 mμ	17 400 cm^{-1}	13.3	1 550 cm^{-1}	1 700 cm^{-1}
408	24 500	15.3	2 050	2 250

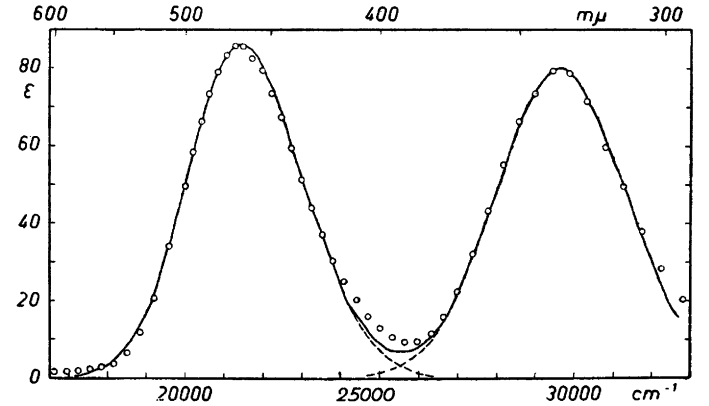

Fig. 2. Absorption spectrum of tris (ethylenediamine) cobalt (III) ions. 0.01 M $[Co\ en_3]Cl_3$. *The calculated curve from eq. 3 with the parameters given in the text is drawn full. The partial bands are punctuated.*

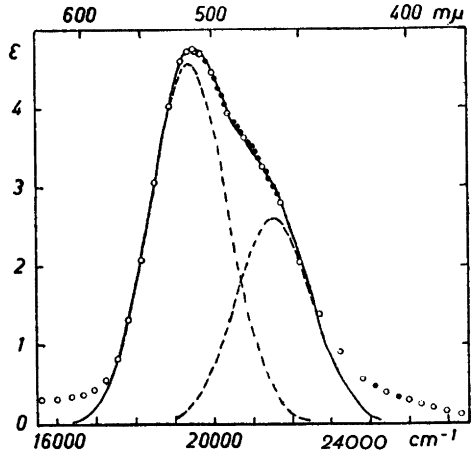

Fig. 3. Absorption spectrum of hexaaquo cobalt(II) ions. 0.1—0.2 M $Co(NO_3)_2$. Open circles measurements by Lene Rasmussen, filled circles by the author. The calculated curve from eq. 3 with the parameters given in the text is drawn full. The partial bands, obtained by resolution, are punctuated.

This method will perhaps be useful for the classification of the steadily increasing material of absorption spectra in the place of explicit tables or figures. The phenomenon that δ (+) is about ten per cent greater than δ (—) corresponds in this case to an approximate validity of formulae as (1) or (7), and it cannot be said with certainty which corresponds best with reality. The following examples will show that there is no reason to think that all absorption bands are of the same form when such small differences are discussed. It is theoretically inconvenient that the band has a discontinuous second differential quotient in the maximum when δ (—) and δ (+) are different. This fact has no influence on the representation of measurements, since their experimental uncertainty is large compared with the small changes of ε with ν near the maximum.

Fig. 2 shows the spectrum of $[Co\ en_3]^{+++}$, which can easily be prepared free of other coloured materials. It is evident from the measurements of Bjerrum and Rasmussen [11] that many ethylenediamine cobalt(III) complexes have bands which resemble Gaussian error curves, and the two bands for $[Co\ en_3]^{+++}$ can be described by:

$\lambda_0 = \nu_0^{-1}$	ν_0	ε_0	$\delta(-)$	$\delta(+)$
467 mμ	21 400 cm^{-1}	86	1 600 cm^{-1}	1 900 cm^{-1}
338	29 600	80	1 900	2 000

The following paper in this series will show that many transition group ions have small bands with intensities (*i. e.* $\varepsilon_0\delta$) only about one per cent of the intensity of ordinary bands, and which also seem to have Gaussian form. Among these are also $[Cr(H_2O)_6]^{+++}$ and $[Co\ en_3]^{+++}$, which each exhibit a small band in red which cannot be seen in Figs. 1 and 2.

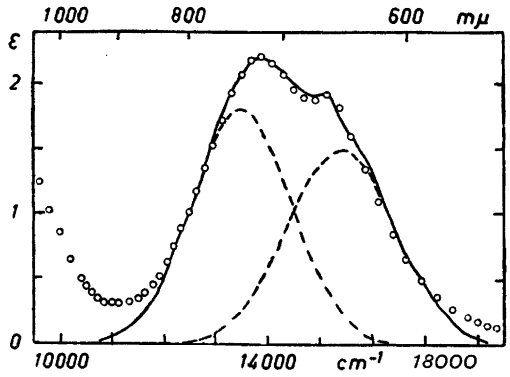

Fig. 4. Absorption spectrum of hexaaquo nickel(II) ions (the red bands). 0.2 M $Ni(NO_3)_2$. *The calculated curve from eq. 3 with the parameters given in the text is drawn full. The partial bands are punctuated.*

In some cases the absorption spectrum seems to consist of two bands with a difference in wavenumber ν_0 of only the order of magnitude of δ. These bands are therefore overlapping and must be found by resolution of the experimental curve. Fig. 3 gives the spectrum of $[Co(H_2O)_6]^{++}$ (from the measurements of Lene Rasmussen [12]) showing the resolution into the two bands

$\lambda_0 = \nu_0^{-1}$	ν_0	ε_0	δ
514 mμ	19 400 cm⁻	4.55	1 150 cm⁻¹
454	21 550	2.6	1 150

(the tail in the red and probably also in the ultraviolet being due to the above mentioned small bands). Only one value of δ is given in order not to strain the experimental material too much. Similarly, Fig. 4 gives the red band of $[Ni(H_2O)_6]^{++}$ analyzed into:

$\lambda_0 = \nu_0^{-1}$	ν_0	ε_0	δ
740 mμ	13 500 cm⁻¹	1.8	1 100 cm⁻¹
650	15 400	1.5	1 200

the effect of the increasing infra-red band being notable at low wave numbers. The violet band of $[Ni(H_2O)_6]^{++}$ is single, as shown in Fig. 5 with the parameters

$\lambda_0 = \nu_0^{-1}$	ν_0	ε_0	$\delta(-)$	$\delta(+)$
395 mμ	25 300 cm⁻¹	5.2	1 500 cm⁻¹	1 500 cm⁻¹

Fig. 5 also gives the spectrum of $[Ni\ en_3]^{++}$, which shows three bands: the middle one is symmetrical and single, in contradistinction to the band in Fig. 4. The parameters are

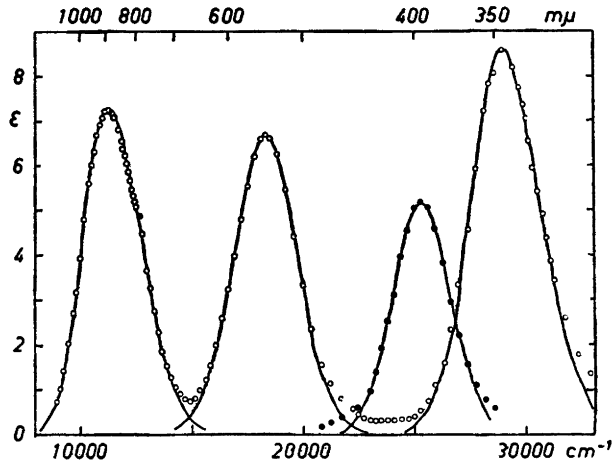

Fig. 5. Absorption spectrum of tris(ethylenediamine)nickel(II) ions (open circles) and violet band of hexaaquo nickel(II) ions (filled circles). 0.1 M $Ni(NO_3)_2$ (0.5 M en added in the former case). Below 350 mμ, $Ni(ClO_4)_2$ and en was used. The calculated curves from eq. 3 with the parameters given in the text are drawn full.

$\lambda_0 = \nu_0^{-1}$	ν_0	ε_0	$\delta(-)$	$\delta(+)$
890 mμ	11 200 cm^{-1}	7.3	1 250 cm^{-1}	1 800 cm^{-1}
545	18 350	6.7	1 650	1 650
345	29 000	8·6	1 700	1 900

According to unpublished measurements by C. J. Ballhausen, the spectra of the mixed ethylenediamine-aquo-nickel(II) ions also have nearly Gaussian form.

The method of analysing spectra into a number of Gaussian curves can of course in some cases lead to results of no physical significance, since it is equally as general as, *e. g*, Taylor or Fourier analysis. It is only when a small number of high bands are obtained corresponding to the experimental curve that the method is reliable for finding the atomic transitions. It was used early by Brode [13], and with success especially in the cases as $CoCl_4^{--}$ or MnO_4^- where there is clearly vibrational structure of the bands, in contrast to the normal bands studied here. Similar analyses were performed by Linhard and Weigel[3,4].

In the cases where the spectra cannot be measured directly, but are obtained from equilibrium mixtures of coloured complexes (analyzed using known mass action constants), it is necessary to take special precautions before Gaussian analysis. But for example the mixed complexes $[Ni(NH_3)_2(H_2O)_4]^{++}$ or $[Ni(NH_3)_3(H_2O)_3]^{++}$ show several bands [14] which are similar in structure to the bands of $[Ni(H_2O)_6]^{++}$, in this case partially due to the two geometrical isomers.

In the copper(II) complexes [10], several applications of the Gaussian analysis have been made. In some cases, as for $[Cu(NH_3)_4(H_2O)_2]^{++}$, the spectrum seems to correspond to only one band with $\delta(-)$ larger than $\delta(+)$. This is

also found in the spectrum of $[Ti(H_2O)_6]^{+++}$ and may be caused by perturbations from fields of lower symmetry [10,15], but more bands cannot be distinguished. The arguments [10] connected with the ratio of the wave numbers of the two bands of, *e. g.*, $[Cu(H_2O)_6]^{++}$, are not altered essentially by the possibility of slightly differing $\delta(-)$ and $\delta(+)$. If the partial bands are not assumed to be exactly symmetrical, the possible ratio of the wave numbers of the maxima is altered from 1.34 to 1.2—1.40. By examination of the observed spectrum [10] of copper(II) aquo ions, it is seen that the lowest band in the infra-red cannot be placed at lower wave numbers than 9 800 cm^{-1}, and therefore the value 1.6, which would correspond to penta-configuration, is excluded.

The form of absorption bands can have considerable theoretical interest, since the oscillations of the ligands and the resulting fluctuation of their perturbation effects on the central ion [10] might be investigated in this way especially if temperature effects are also considered.

Acknowledgments. I am very much indebted to the head of the laboratory, Professor Jannik Bjerrum, for his interest in the work and for valuable discussions.

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Studies of Absorption Spectra

IV. Some New Transition Group Bands of Low Intensity

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A group of bands with low intensities and in some cases with small halfwidths are found in the visible spectra of manganese(II), iron(II), cobalt(II) and other transition group elements. The numbers and positions of the bands are found in agreement with the crystal field theory.

According to the crystal field theory the characteristic absorption spectra of the complex ions of the transition group elements are due to transitions between a small number of discrete energy states. The fields of the nearest other atoms, *i.e.* the ligands of the complex, split the possible levels of the free ion, removing their degeneracy. Fig. 1 gives a qualitative representation of such a splitting. The energy E is given as a function of the effective perturbation strength π (*cf.* Orgel [1]). At $\pi = 0$, *i.e.* the unperturbed ion, the terms are ^4X, ^4Y and ^2Z, known from atomic spectroscopy. They exhibit the total spin quantum number $S = \frac{3}{2}$, $\frac{3}{2}$ and $\frac{1}{2}$, respectively, since the multiplicity is equal to $2S + 1$. They are split into respectively three, one and four levels by the crystal field. To a first approximation, this splitting is proportional to π. The number of levels increases generally by increasing L of the original term and by decreasing the symmetry of the field (*cf.* Bethe [2]). The number arising from a given term cannot exceed $2L + 1$, when comparatively small (L, S) coupling effects are not considered.

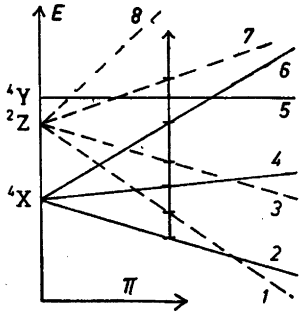

Fig. 1. Orgel diagram. Energy E as function of effective perturbation strength π. Arbitrary choice of terms in the free ion. ^4X, ^4Y and ^2Z. The cross-points between the eight levels and the vertical line represent the possible energy levels of an ion with given π.

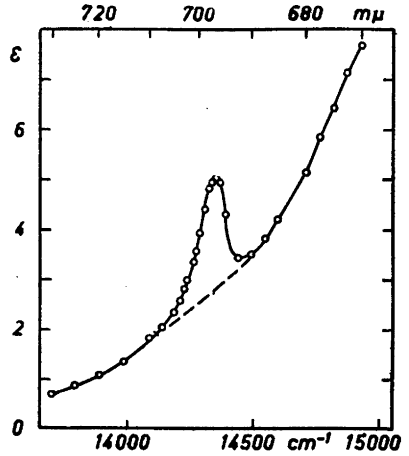

Fig. 2. Small band of tris (oxalato) chromium (III) ions. 0.13 M $K_3[Cr\ ox_3]$.

The vertical line in Fig. 1 represents the energy levels at a given π. The transitions between the ground-state (line 2) and the other levels with the same multiplicity (lines 4, 5 and 6) correspond to bands of ordinary intensity. As Hartmann and Schläfer [3] pointed out, atomic transitions where S changes by 1 correspond to bands having intensities about one percent of the ordinary bands, which occur when S is constant under the transition. The bands mentioned in the present paper are presumably of this type: ions with from two to eight 3d-electrons can exhibit these small bands, since these ions have states of different S.

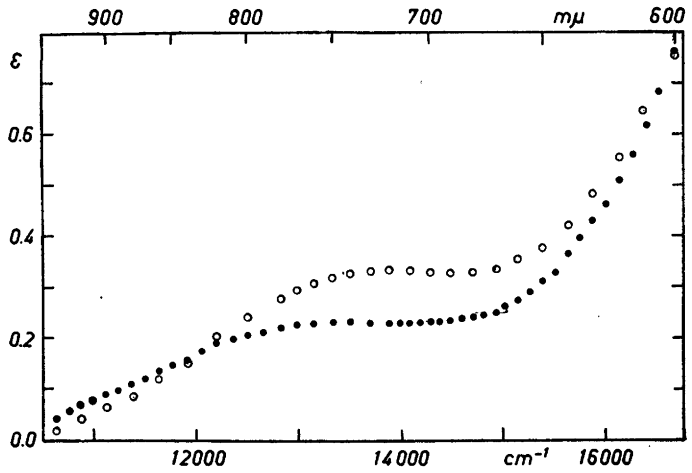

Fig. 3. Small bands of tris (ethylenediamine) cobalt (III) and hexamine cobalt (III) ions. Open circles 0.1 M and 0.8 M $[Co\ en_3]Cl_3$, filled circles 0.15 M $[Co(NH_3)_6]Cl_3$.

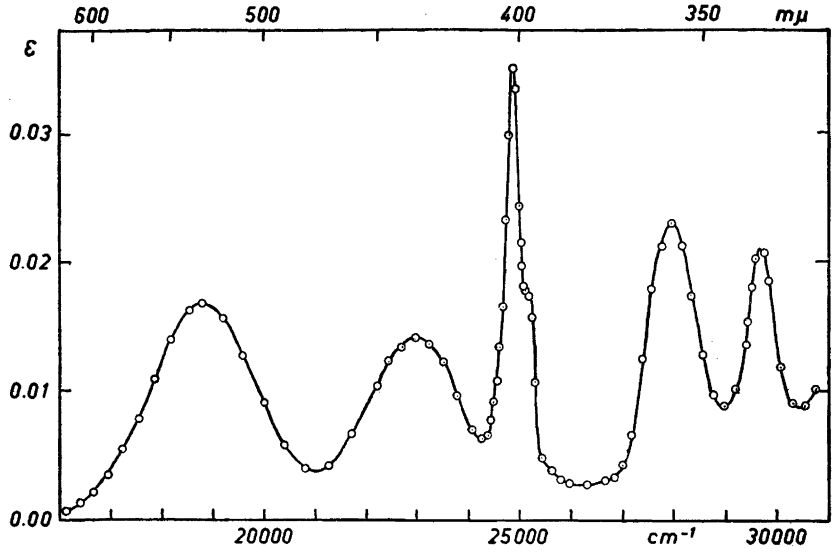

Fig. 4. Absorption spectrum of manganese(II) ions. 2.12 M $MnSO_4$ 0.04 M SO_2.

Several bands of low intensity, known from the literature or measured here for the first time, are discussed in the first section, arranged according to the different transition group ions:

Chromium(III). It was discovered by Etard [4] that chromium(III) complexes besides the usual two strong bands have a narrow band in the red. This was later investigated by several physicists: Bois and Elias [5] for instance studied magnetic and temperature effects. Deutschbein [6] concluded that the band (which also can be found as narrow lines in the spectrum of the ruby) was due to an atomic transition ^4F—^2G in Cr^{+++}.

While the band in $[Cr(H_2O)_6]^{+++}$ can be clearly observed in an ordinary spectroscope, it is not easily measured by a spectrophotometer. Fig. 2 gives the small band of the tris (oxalato) complex $[Cr\ ox_3]^{---}$, which is observable with the resolving power of a Beckman DU. It is seen that it occurs at 697 mμ (14 350 cm^{-1}) superimposed on the tail of the strong band, and with a halfwidth about 3 mμ or ~ 60 cm^{-1}. This is comparable with the halfwidths of the bands of the rare earth ions (see the review by Stewart [7]).

Cobalt (III). This oxidation state is perhaps only known in one complex with $S = 2$. Grey [8] studied the light purple K_3CoF_6, which is hydrolyzed in solution. The reflection spectrum is not known, but it is likely to have one strong band ~ 540 mμ. The high absorption bands of the diamagnetic complexes ($S = 0$) are well known, and it has been found that they also exhibit small bands. Fig. 3 gives the spectra of $[Co\ en_3]^{+++}$ and $[Co(NH_3)_6]^{+++}$ in the red, where each complex has a low and very broad band with maxima at 730 mμ (13 700 cm^{-1}) and 770 mμ (13 000 cm^{-1}), respectively, and with halfwidths about 1 700 cm^{-1}. These bands do not seem to be caused by impurities, since the spectrum does not change by recrystallization of $[Co\ en_3]Cl_3\ 3\ H_2O$,

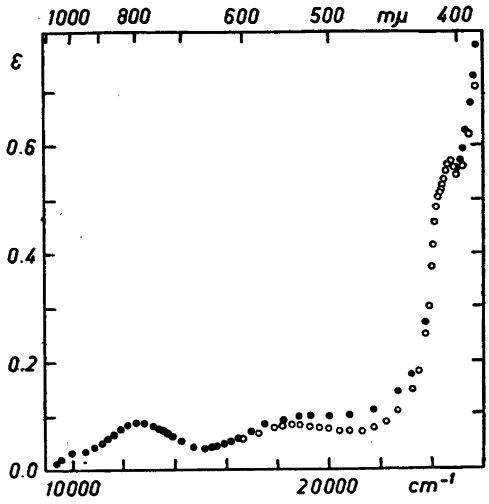

Fig. 5. Absorption spectrum of iron (III) ions. Open circles 0.1 M $Fe(ClO_4)_3$ in 8 M $HClO_4$, filled circles 0.3 M $Fe(ClO_4)_3$ in 2.5 M $HClO_4$.

or to be due to an ion association effect of the type described by Kiss and Czegledy [9], since Beer's law is obeyed on dilution.

Manganese (II). The ground-state of the free ion,[6] S, is not split by any crystal field (*cf.* the theory of Bethe [2]). Since the ion has no other state of $S = \frac{5}{2}$, only bands of small intensity should occur, provided the crystal field does not decrease the energy of one of the states with $S = \frac{3}{2}$ or $S = \frac{1}{2}$ to an extent such that it falls to a lower energy than the original ground-state and thus makes the complex magnetically anomalous (see the cross-point between the lines 1 and 2 in Fig. 1).

Fig. 4 shows the spectrum of 2 M $MnSO_4$ in H_2O and 0.04 M in SO_2 (added in order to suppress hydrolysis and traces of higher oxidation states). The bands, which are so weak that the spectrum had to be measured in 10 cm cells, can be described by the Gaussian parameters [10] ε_n and ν_n, which are the extinction coefficient and wavenumber of the maximum of the n'th band respectively, and $\delta(-)$ and $\delta(+)$, which are the halfwidths toward $\nu < \nu_n$ and $\nu > \nu_n$ for each band. Thus $\varepsilon = \frac{\varepsilon_n}{2}$ for $\nu = \nu_n - \delta(-)$ and for $\nu = \nu_n + \delta(+)$. The wavelengths λ_n of the maxima are also given (Table 1).

Table 1.

Band No.	ν_n	λ_n	ε_n	δ (−)	δ (+)
1	18 800 cm^{-1}	532 mμ	0.017	1 200 cm^{-1}	1 200 cm^{-1}
2	23 000	435	0.014	900	900
3	24 900	402	0.036	200	—
4 (shoulder)	25 150	397.5	~0.018	—	200
5	28 000	357.5	0.023	600	600
6	29 700	337	0.021	400	400

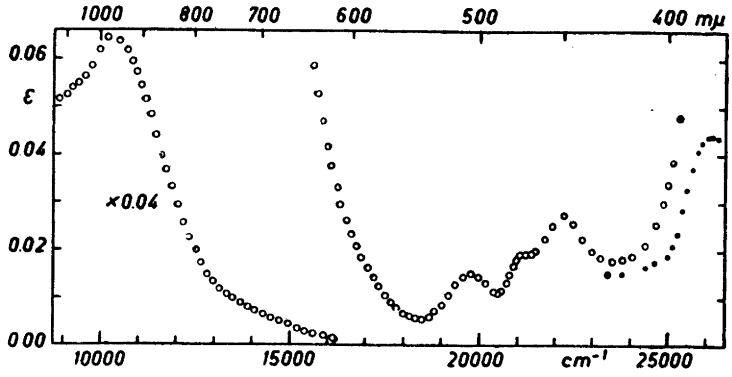

Fig. 6. Absorption spectrum of iron(II) ions. Open circles: 1 M $FeSO_4$ 0.1 M SO_2 0.9 M H_2SO_4. The strongest band multiplied by 0.04. Filled circles: 0.5 M $FeSO_4$ 2 M H_2SO_4 measured in the first 10 minutes after dissolution of $FeSO_4$ 7 H_2O.

Kato [11] photographed bands at 510, 401.5, 358 and 335.5 mμ, which correspond fairly well with the bands Nos. 1, 3, 5 and 6 in Table 1. The theoretical interpretation of these and other bands are given in a later section. Dreisch and Kallscheuer [12] showed that there are no bands of lower wavenumbers.

Iron(III) is isoelectronic with Mn^{++}, having the configuration [A]$3d^5$. Since the hexaquo ion is a fairly strong acid, and $[Fe(H_2O)_5OH]^{++}$ is very highly absorbing, especially in the ultraviolet, the spectrum is not easily obtained pure. Fig. 5 gives the spectrum of $Fe(ClO_4)_3$ in 2.5 *M* and 8 *M* $HClO_4$. Even the strongest possible acid concentration cannot totally suppress the hydroxy-complexes. The bands are given in Table 2.

Table 2.

Band No.	ν_{max}	λ_m	ε_{max}	δ (−)	δ (+)
1	12 600 cm⁻¹	794 mμ	0.1	1 600 cm⁻¹	1 700 cm⁻¹
2	18 500	~540	0.1	~2 300	—
3 (shoulder)	24 300	411	0.4	500	—
4	24 600	406	0.5	—	—

The narrow band discovered by Rabinowitch and Stockmayer [13] has a fine structure (bands Nos. 3 and 4) similar to bands Nos. 3 and 4 of manganese(II). The intense molecular spectra of iron (III) complexes are due to transfer of electrons from the ligands to the central ion. They are more pronounced in "oxidizing" cations such as Fe(III), Cu(II) or Ir(IV) compared with ions of lower electron affinity, *e.g.*, Mn(II) or Co(II). The wavenumbers of these bands are determined by the electron affinity of the central ion and the ionization potential of the ligands, which of course are strongly altered in the polarizing field of the central ion. For this type of band, the explanation of Linhard and Weigel [14] of the Co(III) bands in the far ultraviolet with $\varepsilon \sim 10\,000$ applies very well.

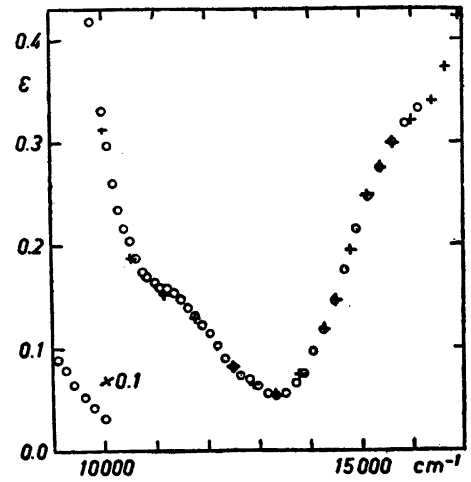

Fig. 7.. Small bands of hexaquo cobalt (II) ions. Open circles 0.9 M $CoSO_4$ 0.2 M H_2SO_4 measured by the author. Cross-points 0.1 M and 0.5 M $Co(NO_3)_2$ measured by Miss Lene Rasmussen, M. Sc.

Iron(II) has six 3d-electrons. Fig. 6 gives the spectrum, which is described in Table 3.

Table 3.

Band No.	ν_m	λ_m	ε_m	δ (−)	δ (+)
1	10 400 cm^{-1}	960 mμ	1.6	—	1 700 cm^{-1}
2	19 800	505	0.014	700 cm^{-1}	—
3	21 100	474	∼0.01	∼ 200	—
4	22 200	450	0.025	∼ 600	∼600
5	25 900	386	∼0.04	∼ 800	—

The first band corresponds to a transition between the two levels of the groundstate ^5D, which is split up by a cubic crystal field. No evidence has been found for the vibrational structure reported by Dreisch and Trommer [15] for the first band, but it is flattened somewhat as in the analogous band of copper (II) complexes [16]. Kato [11] has photographed the spectrum of an iron (II) solution with so low a metal concentration that the wavelengths 520 and 498 mμ given by Kato cannot be very certain.

Cobalt (II). This 3d^7 system also has small bands. Fig. 7 shows the spectrum of $[Co(H_2O)_6]^{++}$ in the range 9—17 000 cm^{-1}. At least two bands are found:

γ_n	λ_m	ε_m	δ (−)	δ (+)
11 300 cm^{-1}	880 mμ	∼0.1	—	1 200 cm^{-1}
16 000	625	∼0.2	1 200 cm^{-1}	—

Dreisch and Trommer [15] measured a stronger band in the infra-red at ∼ 8 000 cm^{-1}, which corresponds to a transition between two levels of the ^4F groundstate. The tail of this band can also be seen in Fig. 7.

Nickel(II). The $3d^8$ system in $[Ni(H_2O)_6]^{++}$ does not give observable small bands. The absorption minimum at 502 mμ has $\varepsilon = 0.04$, while the minimum at 895 mμ has $\varepsilon = 0.29$, which can be explained by superposition of the strong bands alone [10]. $[Ni\ en_3]^{++}$ has flatter minima at 405—430 mμ ($\varepsilon = 0.28$) and $\sim$ 670 mμ with $\varepsilon = 0.7$. The strong bands are discussed in the following section.

THEORETICAL DISCUSSION

In cubic crystal fields such as originate from six octahedrally placed ligands the energy of the central ion can be expressed by

$$E = E\ (\text{free ion}) + NE_1 + (n-N)E_2 \qquad (1)$$

where E (free ion) is the energy of the corresponding state in the free ion *in vacuo*, while E_1 and E_2 are complicated perturbation energy expressions (see, *e.g.*, Ref.[17]) and (E_1-E_2) is the energy difference between the two states in a cubic field of a one-electron system. n is the number of 3d-electrons in the ion, and N a characteristic constant for each of the levels into which a given term in the free ion is split by the crystal field. This is a first-order approximation; for increasing values of E_1-E_2 second-order contributions to the energy must also be taken into consideration.

The slopes of the lines in the Orgel diagram (*vide* Fig. 1) are given by

$$\frac{dE}{d\pi} = k(N - \frac{2n}{5}) \qquad (2)$$

where k is a universal constant for all complexes of regular octahedral configuration. In the one-electron system, the upper doubly degenerate state has $N = 1$ and the lower triply degenerate state $N = 0$. As Santen and Wieringen [18] pointed out, it is also formally possible in systems with more than one effective electron to consider N as the number of γ_3-electrons and $(n-N)$ as the number of γ_5-electrons (γ_3 and γ_5 are the two possible states of one electron in a cubic field in Bethe's notation [2]). The numbers N are the best first-order approximation at small perturbation strengths π.

In chromium(III) and cobalt(III) complexes the crystal field is so strong that in some cases it changes the numbers N from their first-order values. The two bands of normal intensity arise from the transitions $\gamma_5^3 \rightarrow \gamma_5^2\gamma_3$ ($S = \frac{3}{2}$) in Cr^{+++} and $\gamma_5^6 \rightarrow \gamma_5^5\gamma_3$ ($S = 0$) in Co^{+++}. As shown by Orgel [1] each of the excited states mentioned here is a doublet whose internal energy difference is $\sim$ 8 000 cm^{-1}. In the following, energy differences will for convenience be given in the wavenumber unit, cm^{-1}, corresponding to $\frac{1}{hc}$ erg.

Manganese(II) has in the free ion *in vacuo* the following terms [19] when small multiplet splittings (some hundred cm^{-1}) due to (L,S)-coupling are disregarded.

$[A]3d^5$:	^6S	0 cm^{-1}
	^4G	26 800
	^4P	29 200
	^4D	32 300
	^4F	43 600

According to a theorem of Bethe [2], the centre of gravity of a term in the free ion cannot be displaced except by a constant amount for all terms. In the calculation of the centre of gravity the energy of each level is multiplied by its degeneracy number (*e.g.*, 1, 1, 2, 3 and 3 for Γ_1, Γ_2, Γ_3, Γ_4 and Γ_5 in a cubic field [2]). Thus ^6S and ^4P, which do not split up in a cubic crystal field, must both have $N = 2$ in eq. 1, and their energy difference in the manganese (II) complex must be nearly the 29 200 cm^{-1} of the free ion. The narrow band No. 6 with $\nu_6 = 29\,700$ cm^{-1} is thus identified. The other bands are due to the split levels of ^4G and ^4D which in some cases must have N less than 2, while others have higher N corresponding to bands with wavenumbers higher than the transition in the free ion. In the eighth paper of this series, it will be shown that the deviations of N from 2 are due to second-order effects.

In iron(III) complexes the values of N are identical to those in the isoelectronic Mn^{++}. The observed states of the free ion [19] when ^6S is put at 0 cm^{-1} include ^4G at 32 000 cm^{-1} and higher excited states. The perturbation in $[Fe(H_2O)_6]^{+++}$ must be essentially stronger than in the corresponding $[Mn(H_2O)_6]^{++}$, since $(E_1—E_2)$ is increased for the same values of N. The first four bands in each of the two spectra presumably correspond to the same transitions. The low wavenumber 12 600 cm^{-1} of the first iron(III) band shows that the magnetically anomalous state with $S = \frac{3}{2}$ is not far from crossing the ground-state (see the vertical line in Fig. 1 slightly before the cross-point of lines 1 and 2). The case where increasing perturbation decreases the wavenumber in contrast to most bands is also exemplified by the chloro- and ethylenediaminetetraacetate-complexes of Mn(II), which displace the band at 532 $m\mu$ to 524 and 540 $m\mu$, respectively, while the band at 402 $m\mu$ in the case of the ethylenediaminetetraacetate complex is displaced to 416 $m\mu$. For comparison can be stated that in nickel(II) complexes the bands are displaced in opposite directions as in most other cases — bathochromic effects in the chloro- and hypsochromic effects in the ethylenediaminetetraacetate-complexes.

The identification of the four small bands in Fe^{++} is hindered by the crowding in the free ion of the energies of ^3P, ^3H and ^3F between 19 400 and 21 800 cm^{-1}.

In cobalt(II) the states of the free ion are [19]:

$[A]3d^7$:	^4F	0 cm^{-1}
	^4P	15 400
	^2G	17 300
	^2H	23 000

It may appear anomalous that small bands are observed at wavenumbers as low as 11 300 cm^{-1} and 16 000 cm^{-1} when the excited states of $S = \frac{1}{2}$ are so high in the free ion. But the ground-state ^4F is split into three levels, the lowest having $N = \frac{11}{5}$ and the others having $N = 3$ and 4[18]. The strong band in the infrared at 8 000 cm^{-1} thus corresponds to $(3—\frac{11}{5})(E_1—E_2)$ in the case of $[Co(H_2O)_6]^{++}$, giving $E_1—E_2 = 10\,000$ cm^{-1}. Since the excited states can assume considerably lower values of N (minimally 1, since at most six γ_5-electrons can occur in the system), a perturbation energy of $(1—\frac{11}{5})(E_1—E_2) = —12\,000$ cm^{-1} is possible, thus explaining the low wavenumbers of the observed small bands.

The observed energy differences in nickel(II) complexes are explained very well by the crystal field theory, as expressed in eq. 1. In the free ion [19] ^3P is situated 16 900 cm^{-1} over the ground-state ^3F. If $(E_1—E_2) = 7\ 600$ cm^{-1} in $[Ni(H_2O)_6]^{++}$ and 10 500 cm^{-1} in $[Ni\ en_3]^{++}$, remarkable agreement with the experimental data is obtained (Table 4).

Table 4.

Transition	Energy difference	$[Ni(H_2O)_6]^{++}$ calc.	obs.	$[Ni\ en_3]^{++}$ calc.	obs.
$^3F(\Gamma_2)—^3F(\Gamma_5)$	$1(E_1—E_2)$	7 600 cm^{-1}	~ 8 000 cm^{-1}	10 500 cm^{-1}	11 200 cm^{-1}
$^3F(\Gamma_2)—^3F(\Gamma_4)$	$\frac{9}{5}(E_1—E_2)$	13 700	~14 000	18 900	18 350
$^3F(\Gamma_2)—^3P(\Gamma_4)$	$\frac{6}{5}(E_1—E_2)+16\ 900$ cm^{-1}	26 000	25 300	29 500	29 000

The crystal field quantum numbers of the levels are given in parentheses, using the notation of Bethe [2]. The conclusion drawn by Roberts and Fields [20] that the term scheme of nickel(II) complexes disagrees with the crystal field theory does not seem to be justified. Recently Griffiths and Owen [21] identified the two first bands of the aquo nickel(II) ion with the internal transitions of ^3F.

No cubic crystal field alone can make a nickel(II) complex diamagnetic ($S = 0$), since the ground-state $^3F(\Gamma_2)$ has $N = 2$, which is the lowest number of γ_3-electrons any d^8-system can have. But tetragonal fields of sufficient strength can give one of the states with $S = 0$ lower energy than the original state with $S = 1$, as predicted by Orgel [1]. Calculations of C. J. Ballhausen, to be published later, show that it is one of the three states which in a cubic field degenerate to the level $^1G(\Gamma_4)$ that has strongly decreased energy in the square-planar nickel(II) complexes. Second-order effects in strong crystal fields of tetragonal symmetry can also decrease the energy of one of the two states, forming the cubic level $^1D(\Gamma_3)$.

The copper(II) aquo ion was discussed by J. Bjerrum *et al.*[16] and found to be a distorted octahedral complex $[Cu(H_2O)_6]^{++}$, the absorption spectrum consequently being greatly influenced by tetragonal fields. Another proof of the non-regularity of the coordination sphere of copper(II) is the decreasing value of $(E_1—E_2)$ found in divalent aquo ions of the first transition group except in Cu^{++}:

V^{++}	~13 000 cm^{-1}
Cr^{++}	~12 000
Fe^{++}	10 400
Co^{++}	~10 000
Ni^{++}	7 600
Cu^{++}	13 600

showing that the quantity $(E_1—E_2)$ is not determined by a pure cubic field in the last ion. The values of $(E_1—E_2)$ for trivalent hexaaquo ions are of the order of magnitude 20 000 cm^{-1}, because the ionic radii are smaller and the perturbation effects thus larger.

The first-order approximations of the crystal field theory thus accommodate all observed features of the absorption spectra. In particular the narrow bands

are placed at nearly the same wavenumbers as with the free ions *in vacuo*, because the perturbation energies of the excited and the ground-states are nearly equal (*i.e.* the two lines in the Orgel diagram have equal slopes as the lines 2 and 3 in Fig. 1). In these cases the suggestions of Deutschbein [6] and Kato [11] are thus correct. The distinction between weak and strong crystal fields is only significant in the systems with more effective electrons, *i.e.* from d^2 to d^8, which are also the configurations presenting more values of S.

EXPERIMENTAL DETAILS

The spectra were measured in 10 cm cylindrical or 1 cm rectangular absorption cells at 25° C in a Beckman DU spectrophotometer. The solutions were rendered dust-free by filtration through the new Frisenette filters No. 651. These seem to produce slight absorption in the ultraviolet, but were nevertheless very useful for the measurements made here.

The salts used for the measurements were Merck and AnalaR reagents, selected with special regard to avoiding traces of other coloured materials (*e. g.* Fe in manganese(II) or Ni in cobalt(II) salts).

While most interest in absorption spectra has been concentrated on the maxima of the bands, the minima studied here (in 1 M solutions using 10 cm cells) may also have some analytical interest. Thus coloured impurities at 10^{-4} M concentration can often be directly determined. None of the new bands found are situated where strong bands of other transition group ions might be expected.

In iron(II) solutions containing high excess of SO_2 and only a little H_2SO_4 a straw-yellow colour is observed, presumably from sulphito-complexes. These have a steeply increasing absorption from the blue and outwards into the ultraviolet, due to molecular spectra with high extinction coefficients. But the small quantity of SO_2 added to $FeSO_4$ in addition to the H_2SO_4 had no influence on the small bands. For stoichiometrical reasons, $MnSO_4$ cannot have been disturbed by the addition of SO_2. 6 M HCl alters the spectrum considerably, due to formation of chloro-complexes. A solution of $MnSO_4$ and excess of tetrasodium ethylenediaminetetraacetate was also measured. It is not oxidizable by the air. The difficulties in preparing pure $[Fe(H_2O)_6]^{+++}$ from $Fe(OH)_3$ + $HClO_4$ have been mentioned above. As comparison solutions sulphuric acid and perchloric acid were used at the same concentrations as the total content of sulphates and perchlorates in the measured solutions. This is important in the infra-red, where water is strongly absorbing.

The small bands of $[Co(H_2O)_6]^{++}$ must not be confused with the bands in the orange of chloro-cobalt(II) complexes and other acido complexes. Miss Lene Rasmussen, M. Sc., of this laboratory, has shown that the spectrum of 0.5 M $CoCl_2$ in water does not differ from the usual aquo ion spectrum.

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A Microscopic Investigation of Binary Systems of Long Normal and *Iso*-Chain Carboxylic Acids

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In this paper stearic acid is called *n*-C_{18}, *iso*-palmitic acid *iso*-C_{16} and so on. Mixtures of long normal and *iso*-chain carboxylic acids are of great biological importance and they may also have analytical use.

As a way of identifying long *iso*-chain carboxylic acids Weitkamp [1] suggested the study of binary systems between the unknown *iso*-fatty acid and different normal fatty acids. He found that *iso*-C_{18} gave a simple eutectic mixture with *n*-C_{17} but an incongruently melting molecular compound with *n*-C_{16}, and that *iso*-C_{20} gave the same types of systems with *n*-C_{19} and *n*-C_{18}, respectively. He suggests that *iso*-fatty acids give molecular compounds with normal fatty acids which have two carbon atoms less than the *iso*-acid. He also makes a schematic explanation of this behaviour.

In order to locate a methyl group in a methyl substituted fatty acid Cason and Winans [2] studied the binary systems with normal fatty acids. A part of their paper deals with the systems of *iso*-C_{18} with *n*-C_{17} and *n*-C_{16}. The results are almost the same as those of Weitkamp [1].

In this investigation many more systems of *iso*- and normal fatty acids have been investigated. *Iso*-acids with 14, 15, 16, 17, 18, 20, 25, and 26 carbon atoms have been studied. The contact method described by Kofler and Kofler [3, p. 115] has been used. This method is much faster than the ordinary capillary method, but on the other hand it does not give the compositions of the eutectic points and the molecular compounds. This is not fundamental, however, at the first mapping of the binary systems of *iso*- and normal chain carboxylic acids.

MATERIAL USED

All acids used were obtained from Professor E. Stenhagen and his collaborators. The *iso*-acids are described in Refs. [4–6] and the normal acids in Refs. [7–9].

Normal fatty acids exhibit polymorphism [9]. From melt the even-numbered acids crystallize in form C which has been investigated by Vand, Morley, and Lomer [10]. The unit cell is monoclinic and the crystal form has the orthorhombic

Table 1. Thermal data for binary systems of long normal- and iso-chain

Number of carbon atoms in the *iso*-acid	M. p. of *iso*-acid °C	Eutectic point °C	Incongruent m. p. of molecular compound °C	Congruent m. p. of molecular compound °C	Eutectic point °C	M. p. of *n*-acid °C	Number of carbon atoms in the *n*-acid	Type in Table 2
14	53	31				44	12	E
	53	39				41	13	E
	53	39				54	14	E
	53	37				52	15	E
15	52	32				44	12	E
	52	40				41	13	E
	52	47				54	14	E
	52	44				52	15	E
	52	39				63	16	E
16	62	31	33			44	12	EM
	62	34				41	13	E
	62	42	44			54	14	EM
	62	49				52	15	E
17	60	34				44	12	E
	60	37		38	34	41	13	M
	60	43		44	43	54	14	M
	60	50.5		52	51	52	15	M
	60	55				63	16	E
	60	53				61	17	E
	60	48				70	18	E

packing of the hydrocarbon chains described by Bunn [11] and Vainshtein and Pinsker [12]. In this packing the plane of every second chain is almost perpendicular to the plane of the others. The odd-numbered normal fatty acids crystallize from melt in form B′, except *n*-C_{13} which gives crystal form A′. Form A′ is described by the author [13]. The unit cell is triclinic and the crystal form has the triclinic packing of hydrocarbon chains described by Müller and Lonsdale [14]. In this packing all the chain planes are parallell. Form B′ has been investigated by the author and will soon be described. It has a triclinic unit cell and the same orthorhombic packing as form C of even-numbered acids.

The *iso*-acids used in this investigation seem to appear in only one crystal form [5]. *Iso*-palmitic acid has been investigated by Stenhagen, Vand and Sim [15].

It is well known that mixing two compounds may cause a change of crystal form of one or both components, and this may of course be possible here as well.

THERMAL STUDIES

The binary systems were studied in a polarizing microscope using a heating arrangement ("Heiztisch") designed by Kofler and Kofler [3, p. 2]. The contact method described by the same authors [3, p. 115] was used. According to this

carboxylic acids measured on the "Heiztisch" by Kofler and Kofler [3].

Number of carbon atoms in the *iso*-acid	M. p. of *iso*-acid °C	Eutectic point °C	Incongruent m. p. of molecular compound °C	Congruent m. p. of molecular compound °C	Eutectic point °C	M. p. of *n*-acid °C	Number of carbon atoms in the *n*-acid	Type in Table 2
18	68	32				41	13	E
	68	42	43.5			54	14	EM
	68	45.5				52	15	E
	68	50.5 [a]	52 [b]			63	16	EM
	68	55 [c]				61	17	E
20	74	44				54	14	E
	74		46.5		44.5	52	15	ME
	74	52		53	52	63	16	M
	74	54		55	52.5	61	17	M
	74	59 [d]	60 [e]			70	18	EM
	74	64 [f]				69	19	E
25	82	60				70	18	E
	82		64		62	69	19	ME
	82	68.5		69	68	75	20	M
	82	73.5		75	73	74	21	M
	82	71		73	72.5	80	22	M
	82	78	78.5			79	23	EM
	82	77				84	24	E
	82	75				83	25	E
26	87	62				69	19	E
	87		68		67	75	20	ME
	87	68		69	67	74	21	M
	87	70		73	71.5	80	22	M
	87	73		74.5	73	79	23	M
	87	75	76.5			84	24	EM
	87	80				83	25	E

a) Weitkamp[1]: 52° Cason and Winans[2]: 52.5°
b) Weitkamp[1]: 53° Cason and Winans[2]: 54°
c) Weitkamp[1]: 58° Cason and Winans[2]: 58°
d) Weitkamp[1]: 60.5°
e) Weitkamp[1]: 59.5°
f) Weitkamp[1]: 65°

method the components are melted on an objectglass under a coverslip so that a contact is formed. They are then allowed to diffuse into each other so that the concentration of the components varies continuously from 0 to 100 %. The whole melt is then cooled down and then gently heated on the "Heiztisch" under the microscope. Then the melting parts will appear as black zones, which grow wider and wider. The thermometer was calibrated with the normal fatty acids used, the melting points of which were known accurately [7-9]. For further details of the "Heiztisch" and the contact method Kofler and Kofler [3] should be consulted.

Table 2. Types of binary systems of long normal- and iso-chain carboxylic acids.

Number of carbon atoms	Normal chain													
	12	13	14	15	16	17	18	19	20	21	22	23	24	25
14	E	E	E	E										
15	E	E	E	E	E									
16	EM	E	EM	E										
17	E	M	M	M	E	E	E							
18		E	EM	E	EM	E								
19														
20			E	ME	M	M	EM	E						
21														
22														
23														
24														
25							E	ME	M	M	M	EM	E	E
26								E	ME	M	M	M	EM	E

Iso-chain

E = eutectic mixture
EM = incongruently melting molecular compound with eutecticum between itself and the *iso*-acid.
ME = incongruently melting molecular compound with eutecticum between itself and the *n*-acid.
M = congruently melting molecular compound.

Three different types of binary systems were obtained: the components forming eutectic mixtures, congruently melting compounds and incongruently melting compounds. Alla data obtained are collected in Tables 1 and 2.

DISCUSSION OF RESULTS

As Weitkamp [1] pointed out *iso*-acids form molecular compounds with normal acids, which have two carbon atoms less. However, when the *iso*-acid has 15 of fewer carbon atoms no molecular compounds are obtained (see Table 2). This is probably due to stabilization of the different structure types of the components caused by the comparatively great influence of the carboxylic groups when the chains are short.

For the higher *iso*-acids molecular compounds are obtained with normal acids having two or more carbon atoms less than the *iso*-acid. Thus *iso*-C_{17} forms three molecular compounds and *iso*-C_{26} five (see Table 2). The schematic explanation by Weitkamp [1] for systems *iso*-C_n—*n*-C_{n-2} is very informative, whether the packing of the hydrocarbon chains is of the orthorhombic, triclinic or perhaps another type. When the difference is three or more carbon atoms there will probably be holes in the packing and when these holes are too large the molecular compound will collapse. Of course when the number of carbon atoms in the *iso*-acid increases the largest allowable size of the holes will also increase.

For the shortest even-numbered *iso*-acids and the possible normal acids, the influence of the carboxylic groups on the structure types is still too great so that in some cases no molecular compound is obtained. A regular behaviour does not appear until *iso*-C_{20}.

It is known that if a binary system contains an intermediate phase this often forms a peritectic if the difference between the melting points of the compounds is large. Then the eutectic lies between the intermediate phase and the component with the lowest melting point. The results now obtained show a tendency in this direction for *iso*-C_{20} and longer acids. The system *iso*-C_{20} —*n*-C_{15} is of the type ME, *i.e.* the eutectic lies between the intermediate phase and the phase with the lowest melting point. When the length and the melting point of the *n*-acid increases the type changes to M and later to EM.

SUMMARY

Binary systems of long normal and *iso*-chain carboxylic acids are studied under the microscope. *Iso*-acids with 14, 15, 16, 17, 18, 20, 25, and 26 carbon atoms have been investigated. Those with 16 or more carbon atoms are found fo form molecular compounds with normal acids having two to four, five or six carbon atoms less than the *iso*-acid. Some anomalies in the binary systems for the lower members of the *iso*-acids have been found.

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Über den Abbau von Lignin und Ligninmodellsubstanzen mit Hypochlorit. II. Mitteilung

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Die Einwirkung von Hypochlorit auf verschiedene Typen von Ligninmodellsubstanzen, in welchen ein Guajacolrest ätherartig an das α-, β- und γ-Kohlenstoffatom einer Phenylpropaneinheit gebunden ist, wurde untersucht. Der ätherartig gebundene Guajacolrest wird unter gewissen strukturellen Voraussetzungen in α- und β-Äthern, nicht dagegen in γ-Äthern abgebaut. Das Vorkommen verschiedener Phenoläthergruppierungen im Lignin wird diskutiert.

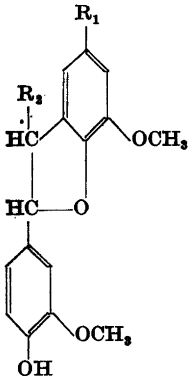
Dehydrierungspolymerisat aus Coniferylalkohol wird ähnlich wie Lignin abgebaut.

Die Synthese einiger Modellsubstanzen wird beschrieben.

In einer vorangehenden Mitteilung [1] wurde über das Verhalten von verschiedenen Ligninpräparaten, Phenolen und Phenoläthern gegen alkalische Hypochloritlösung berichtet. Es hat sich gezeigt, dass die Ligninpräparate in verschiedener Hinsicht (Reaktionsgeschwindigkeit, Hypochloritverbrauch, Veränderungen im UV-Spektrum während des Abbaus, Verhalten nach der Methylierung) mit phenolischen Substanzen vergleichbar sind. Dieses Ergebnis war insofern überraschend, als in den untersuchten Ligninpräparaten — mit Ausnahme des Sulfatlignins — nicht jede Lignineinheit, sondern höchstens jede 2. [2], möglicherweise sogar nur jede 4.—6.[3] Einheit eine freie Phenolgruppe besitzt. Es galt daher, durch weitere Versuche mit teils bekannten, teils neuen Modellsubstanzen festzustellen, ob unter bestimmten strukturellen Voraussetzungen auch nichtphenolische Einheiten abgebaut werden oder ob durch den Abbau vorhandener phenolischer Einheiten neue, ursprünglich verätherte Phenolgruppen freigelegt und dann ebenfalls weiter abgebaut werden können.

Die untersuchten Modellsubstanzen sollen zwei Lignineinheiten darstellen, von welchen die eine phenolätherartig an das α-, β- oder γ-Kohlenstoffatom der Seitenkette der anderen gebunden ist. Neben einfachen Phenoläthern wurden auch solche untersucht, bei welchen die Phenolätherbindung in einen Cumaran-, Cumaron- oder Flavanring eingebaut ist.

Unter den Modellsubstanzen mit einer einfachen Phenolätherbindung am α-Kohlenstoffatom der Seitenkette erwiesen sich Benzyl- und 4-Methoxybenzyl-guajacyläther bei 90—95° stabil gegen alkalisches Hypochlorit. Von Modellen mit einer in einen Cumaranring eingebauten α-Phenolätherbindung wurden untersucht: Dehydrodiisoeugenol (I), Dihydro-dehydrodiisoeugenol (II), der von Freudenberg und Hübner [4] dargestellte Dehydrodiconiferylalkohol (III) sowie die 2-(4-Oxy-3-methoxyphenyl)-3-methyl-cumaran-5-carbonsäure (IV).



I: $R_1 = CH:CH\cdot CH_3$ $R_2 = CH_3$

II: $R_1 = CH_2\cdot CH_2\cdot CH_3$ $R_2 = CH_3$

III: $R_1 = CH:CH\cdot CH_2OH$ $R_2 = CH_2OH$

IV: $R_1 = CO_2H$ $R_2 = CH_3$

Letztere erhält man durch vorsichtige Permanganatoxydation von Dehydrodiisoeugenolacetat in Form ihres Acetats. Dieses wird mit Sodalösung möglichst unter Luftausschluss verseift, um eine oxydative Spaltung unter Bildung von Vanillin zu vermeiden.

Die Verbindung IV lässt sich bereits mit neutraler Sulfitlösung bei 135° sulfitieren, während ihr Methyläther, die sog. Erdtman'sche Säure in saurer Lösung leichter sulfitiert wird [5] als in neutraler oder schwach alkalischer Lösung, worauf in andrem Zusammenhang eingegangen werden soll.

Von diesen Verbindungen verbraucht I anfänglich rasch (innerhalb von 5 Min.) etwa 6 Mol Hypochlorit, worauf die Oxydation nur langsamer weitergeht, ohne nach etwa 2 Stunden bei einem Verbrauch von 11 Mol einen deutlichen Endpunkt erreicht zu haben, wie aus Fig. 1 hervorgeht. Der Anfangsverbrauch von etwa 6 Mol dürfte in Wirklichkeit noch höher sein, da bei Zugabe von I zur Hypochloritlösung zunächst ein Teil Substanz ausfällt und erst allmählich wieder gelöst wird. Die Oxydation von II wird durch die rasche Ausfällung eines harzigen Produkts unübersichtlich. Die Säure IV verbraucht bei 20° sehr rasch ein Mol Hypochlorit unter Bildung eines Monochlorderivats, in welchem das Chloratom vermutlich neben der Phenolgruppe sitzt. Bei 90—95° verbraucht IV rasch 9—10 Mol, worauf die Oxydation nur noch sehr langsam fortschreitet. Die UV-Absorption deutet darauf hin, dass das Abbauprodukt noch aromatisch sein muss. Absorptionsmaximum bzw. -minimum liegen vor der Oxydation bei 263 und 245 mμ, nach der Oxydation bei 268 und 242 mμ. Ist in I und IV die Phenolgruppe methyliert, so werden nur unbedeutende Mengen Hypochlorit verbraucht. Die Cumarangruppierung wird demnach nicht angegriffen. Bei III liegt der Anfangsverbrauch an Hypochlorit sehr hoch (15—16 Mol), was — in Übereinstimmung mit der niedrigen UV-Absorp-

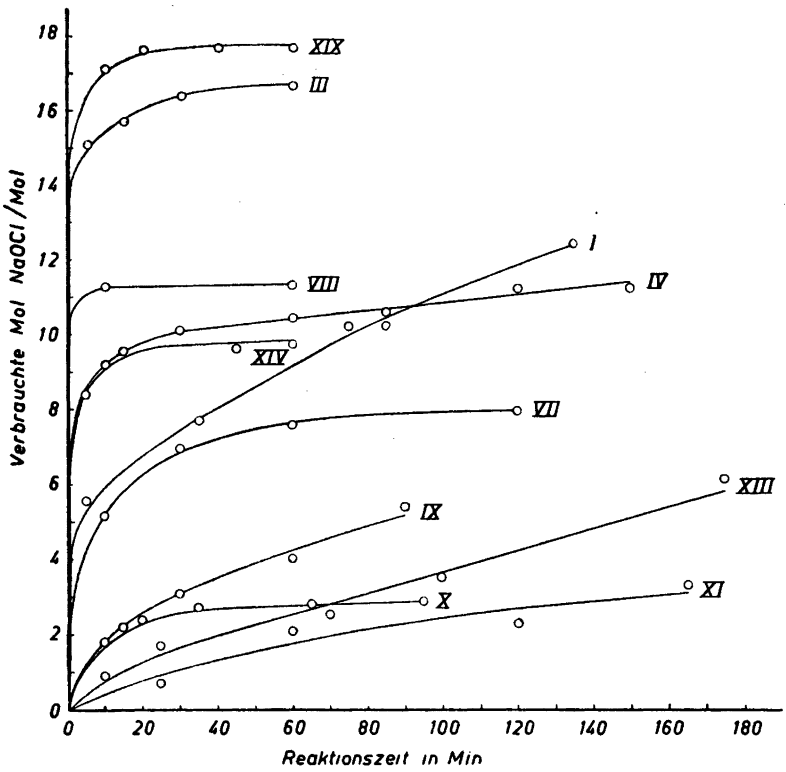
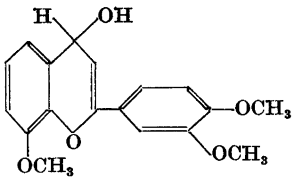


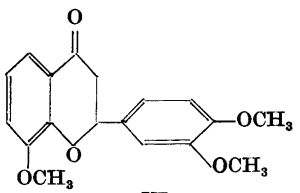
Fig. 1. Hypochloritverbrauch einiger Modellsubstanzen bei 90—95°.

tion nach der Oxydation — darauf hindeutet, dass in diesem Fall die phenolische *und* die phenolätherartig gebundene Einheit abgebaut werden. Die Anwesenheit solcher Dehydrodiconiferylalkohol-Gruppierungen — nach Freudenberg [2,6] sollen sie mit 40—50 % am Aufbau des Lignins beteiligt sein — kann daher bis zu einem gewissen Grad erklären, warum im Lignin ausser den phenolischen Einheiten auch nichtphenolische Einheiten abgebaut werden. Dabei muss allerdings vorausgesetzt werden, dass die phenolische Gruppe in III bei der Weiterdehydrierung zum Lignin nicht verschwindet. Das durch Methylierung mit Diazomethan aus III erhältliche ölige Reaktionsprodukt verbraucht erwartungsgemäss bedeutend weniger Hypochlorit.

Von den Verbindungen, welche eine α-Phenylätherbindung in ein Chromansystem eingebaut enthalten, wurde 2-(3,4-Dimethoxyphenyl)-4-oxy-8-methoxychroman (V) untersucht, welches sich stabil erwies. Das entsprechende Flavanon (VI) wird dagegen wegen der in alkalischer Lösung erfolgenden Aufspaltung zu einem Oxychalkon rasch oxydiert.

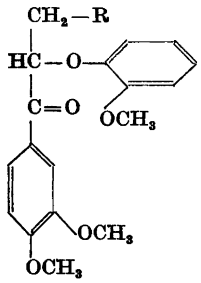


V

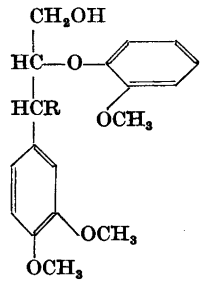


VI

Unter den Modellsubstanzen mit einer vom β-Kohlenstoffatom der Seitenkette ausgehenden Phenolätherbindung erwies sich der 3,4-Dimethoxyphenacyläther des Guajacols [7] ziemlich stabil. (Verbrauch bei 95° etwa 1 Mol im Verlauf von 2 Stunden). Dagegen werden die von Adler, Lindgren und Saedén [7] dargestellten Verbindungen VII—X trotz Abwesenheit phenolischer Gruppen von Hypochlorit in verschiedenem Umfang angegriffen.



VII: R = OH
VIII: R = SO_3H
XI: R = OCH_3

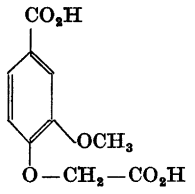


IX: R = OH
X: R = SO_3H

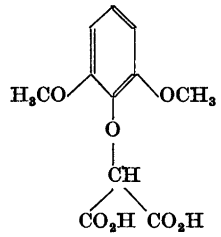
Die gegen Hypochlorit empfindlichste Struktur besitzen die Verbindungen VII und VIII. VII wird trotz seiner Unlöslichkeit relativ rasch oxydiert und verbraucht innerhalb von 2 Stunden etwa 9 Mol Hypochlorit. Bei der wasserlöslichen Sulfosäure VIII verläuft die Oxydation praktisch momentan. In beiden Fällen wird der in β-Stellung gebundene Guajacolrest sowie das β- und γ-Kohlenstoffatom der Seitenkette aboxydiert, sodass als aromatisches Abbauprodukt Veratrumsäure hinterbleibt. Der Abbau des ätherartig gebundenen Guajacolrestes beruht auf einer direkten Oxydation und nicht auf einer primär erfolgenden Alkalispaltung, denn VII wird durch Alkali gleicher Konzentration bei 95° innerhalb von zweieinhalb Stunden nur zu etwa 5 % gespalten. Ist in VII die in β-Stellung befindliche Carbonylgruppe zu einer sekundären Alkoholgruppe reduziert (IX), so verläuft die Oxydation wesentlich langsamer. Möglicherweise ist die Oxydation des α-Hydroxyls in diesem Fall die geschwindigkeitsbestimmende Reaktion. Ersetzt man das α-Hydroxyl durch eine Sulfogruppe, so bleibt die Oxydation der Sulfosäure X nach Verbrauch von knapp 3 Mol Hypochlorit stehen, was nur auf eine Oxydation in der Seitenkette hindeutet.

Aus den Abbauversuchen in dieser Substanzgruppe geht hervor, dass in β-Stellung phenolätherartig gebundene aromatische Reste einer Oxydation mit Hypochlorit zugänglich sind. Bei Vorhandensein einer Carbonylgruppe in α-Stellung kann der Abbau bisweilen ebenso schnell verlaufen wie bei phenolischen Substanzen. Dass im Lignin derartige Gruppierungen wie VII bzw. VIII vorkommen, ist durchaus möglich. Deren Menge kann jedoch

nicht sehr gross sein, denn die Ligninsulfosäure enthält nur auf etwa 12 Einheiten eine Carbonylgruppe, einschliesslich der von Adler und Mitarbeitern [8] nachgewiesenen Coniferylaldehydgruppierungen. Der Hypochloritverbrauch der Ligninsulfosäure sinkt daher auch nicht wesentlich, wenn in ihr die Carbonylgruppen mit Natriumborhydrid reduziert werden. Wesentlich grössere Bedeutung als der Gruppierung VII messen Adler und Lindgren [7,9] der Gruppierung gemäss IX bei, weil deren Vorkommen im Lignin Reaktionen wie Sulfitierung und Alkoholyse recht befriedigend erklären kann. Auch Freudenberg vermutet neuerdings [6], dass solche β-Phenyläther zu etwa 15 % am Aufbau des Lignins beteiligt sind. Der schnelle Abbau des Lignins mit Hypochlorit spricht jedoch nicht dafür, dass das Lignin zu einem wesentlichen Teil aus solchen — nur langsam oxydierbaren — Gruppierungen aufgebaut ist. Ob die in β-Stellung ätherartig gebundenen Einheiten in IX und X leichter abgebaut werden, wenn in p-Stellung zur Seitenkette statt einer Methoxylgruppe eine freie Phenolgruppe steht, bleibt noch zu untersuchen. Wahrscheinlich wird jedoch in diesem Fall die phenolische Einheit bevorzugt oxydiert, ohne dass der ätherartig gebundene Rest abgebaut wird. Er könnte z.B. in Form eines Guajacyläthers der Tartronsäure erhalten bleiben. Substanzen vom Typ der Phenoxyessigsäure oder der Phenoxymalonsäure wie XII und XIII [10] sind gegen Hypochlorit stabil. XII wurde erhalten, indem Vanillin zunächst mit Bromessigester kondensiert und der entstehende Phenoxyessigester verseift und mit Permanganat oxydiert wurde.

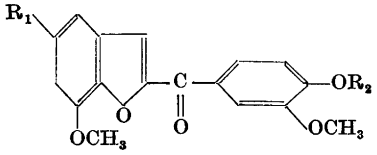


XII



XIII

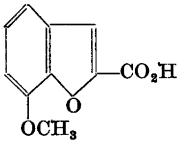
Im Gegensatz zu VII und VIII sind zwei kondensierte β-Phenyläther, nämlich 2-(3,4-Dimethoxybenzoyl)-7-methoxycumaron (XIV) und 2-(3,4-Dimethoxybenzoyl)-5-formyl-7-methoxycumaron (XV) gegen Hypochlorit stabil. Das phenolische Präparat XVI hingegen wird rasch angegriffen. Der Abbau erfasst jedoch nur den phenolischen Rest und nicht den Cumaronanteil, der in Form der 7-Methoxycumaron-2-carbonsäure (XVII) erhalten bleibt.



XIV: $R_1 = H$ $R_2 = CH_3$

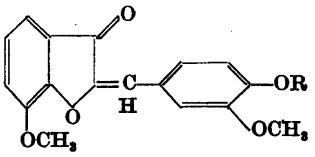
XV: $R_1 = CHO$ $R_2 = CH_3$

XVI: $R_1 = H$ $R_2 = H$



XVII

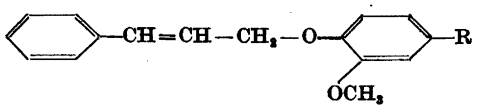
2-(3,4-Dimethoxybenzal)-7-methoxycumaranon-(3) (XVIII) wird von Hypochlorit nur langsam oxydiert, wobei aus dem Dimethoxybenzalrest Veratrumsäure gebildet wird. Die entsprechende phenolische Substanz XIX wird unter Verbrauch von 17 Mol Hypochlorit rasch total abgebaut.



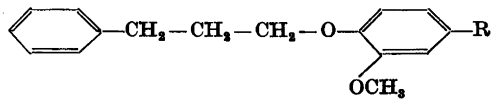
XVIII: R = CH_3
XIX: R = H

Über Synthese und weitere Umsetzungen der Verbindungen V, VI, XIV —XVI, XVIII und XIX wird in andrem Zusammenhang berichtet.

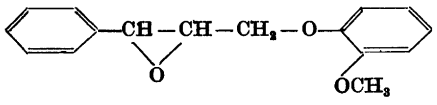
Unter den Verbindungen, die in γ-Stellung phenolätherartig verknüpft sind, erwies sich der Cinnamyläther des Guajacols (XX) [11] sowie der daraus durch Hydrierung erhaltene γ-Phenylpropyl-guajacyläther (XXII) stabil gegen Hypochlorit. Auch der Cinnamyläther des Vanillins (XXI) und dessen Hydrierungsprodukt (XXIII) werden nicht abgebaut. Um wasserlösliche γ-Äther zu bekommen, wurde versucht, an die Doppelbindung in XX Sulfit zu addieren, was jedoch weder mit neutraler noch mit saurer Sulfitlösung gelang. Dagegen erhält man durch Anlagerung von Sauerstoff an XX mittels Benzopersäure das entsprechende, auf der Oxydationsstufe des Phenylglycerins stehende Oxyd XXIV. Dieses lässt sich im Vakuum bei 190° destillieren, ohne dass eine Umlagerung in ein entsprechendes Keton erfolgt. Auch durch Erwärmen mit Toluolsulfosäure findet keine merkliche Umlagerung statt. Der Äthylenoxydring in XXIV lässt sich mit neutralem Sulfit, nicht dagegen mit Bisulfit zu einer Oxysulfonsäure XXV aufspalten, welche in Form ihres β-Naphthylaminsalzes isoliert wurde. Ob in XXV die Hydroxyl- oder die Sulfogruppe in α-Stellung steht, ist noch nicht ermittelt. Weder XXIV noch XXV wird mit Hypochlorit abgebaut.



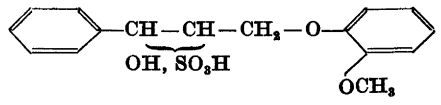
XX: R = H
XXI: R = CHO



XXII: R = H
XXIII: R = CHO



XXIV



XXV

Aus diesen Oxydationsversuchen darf wohl der Schluss gezogen werden, dass γ-Phenyläther durch Hypochlorit nicht abgebaut werden. Die Anschauung von Hibbert und Mitarbeitern [12], dass der Abbau des Lignins durch Hypochlorit auf einer Spaltung von γ-Äthern beruht, kann demnach nicht zutreffen.

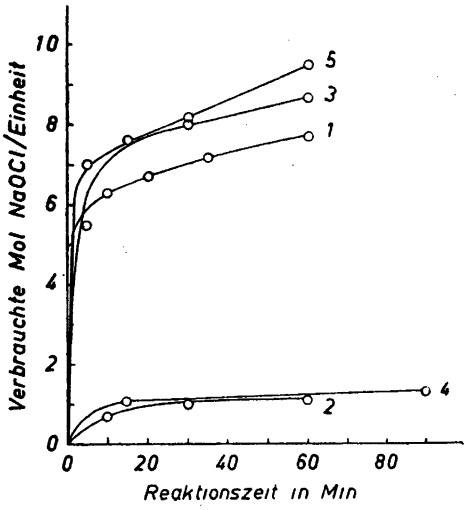
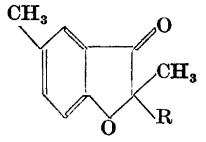


Fig. 2. Hypochloritverbrauch von 1. Ligninsulfosäure, 2. methylierter Ligninsulfosäure, 3. und 5. Dehydrierungspolymerisat aus Coniferylalkohol und 4. mit Diazomethan methyliertem Dehydrierungspolymerisat (entsprechend 3) bei 95°.

Da von Holmberg [13] und Erdtman [14,15] auch die Möglichkeit acetalartiger Gruppierungen im Lignin erörtert wurde, lag es nahe, auch einige Versuche in dieser Richtung zu machen. Es wurde daher geprüft, wie sich das 2-Oxy-2,5-dimethylcumaranolon (XXVI) [16] sowie die daraus von Aulin-Erdtman, Björkman, Erdtman und Hägglund [15] hergestellte Sulfonsäure XXVII gegenüber Hypochlorit verhält. Es zeigte sich, dass XXVI unter Verbrauch von 12—13 Mol sehr rasch abgebaut wird, während die Sulfosäure XXVII stabil ist. Mit der Einschränkung, dass bisher nur diese beiden Verbindungen geprüft wurden, lässt sich folgende Schlussfolgerung ziehen: Würde z.B. Salzsäurelignin solche halbacetalartige, abbaubare Gruppierungen wie XXVI enthalten, Ligninsulfosäure dagegen stabile Gruppierungen gemäss XXVII, so müssten sich die beiden Präparate in ihrem Verhalten gegenüber Hypochlorit deutlich unterscheiden. Dies ist jedoch wie früher gezeigt nicht der Fall.



XXVI: R = OH

XXVII: R = SO_3H

Ausser den genannten Modellsubstanzen wurde auch der Abbau zweier von Freudenberg und Heimberger [17] aus Coniferylalkohol dargestellter Dehydrierungspolymerisate (DHP) untersucht, welche Dehydrodiconiferylalkohol-Gruppierungen neben Pinoresinoleinheiten und einer geringeren Menge von β-Phenoläther-gruppierungen enthalten sollen. Diese Präparate verbrauchen, wie aus Fig. 2 hervorgeht, etwas mehr Hypochlorit als Ligninsulfosäure. Die UV-Absorption der Oxydationslösung lässt vermuten, dass nicht sämtliche aromatische Einheiten vom Abbau erfasst werden. Nach der Methylierung mit Diazomethan verbrauchen die DHP-Präparate nur noch 1 Mol Hypo-

chlorit. Sie werden also durch Methylierung ebenso stabilisiert wie die früher untersuchten Ligninpräparate.

Zur Ergänzung der früheren Mitteilung betreffend die Reaktion von Hypochlorit mit Phenolen seien noch einige Versuche nachgetragen. Durch Chlorierung mit kalter Hypochloritlösung wurde aus Dihydroferulasäure deren 5-Chlorderivat, aus *o*-Vanillinsäure eine vermutlich in der *p*-Stellung zur Phenolgruppe chlorierte Substanz erhalten. Aus 4-Oxy-5-methoxy-isophthalsäure entsteht unter Abspaltung von Kohlensäure 5-Chlorvanillinsäure. Auch aus Dehydrodivanillinsäure entsteht ein chloriertes Reaktionsprodukt. Dagegen wurden bei den zahlreichen Versuchen keinerlei Anzeichen dafür gefunden, dass auch Verbindungen mit verätherten Phenolgruppen durch alkalische Hypochloritlösung chloriert werden, wie von Hopkins und Chisholm[18] beschrieben ist. Auch Verbindungen mit Dioxymethylengruppen wie z.B. Piperonal werden nicht chloriert.

In Zusammenhang mit der Frage, welche Abbauprodukte aus verschiedenen Phenolen entstehen, wurde der Abbau von 4-Oxy-3-methoxy-diphenylmethan untersucht. Diese Verbindung erhält man, indem Benzoylchlorid nach Friedel-Crafts mit Veratrol zu dem früher auf andrem Weg erhaltenen 4-Oxy-3-methoxybenzophenon [19] kondensiert wird, welches nach einem modifizierten Wolff-Kishner-Abbau [20] reduziert wird. Der Hypochloritabbau liefert bei 95° nur Benzoesäure.

EXPERIMENTELLER TEIL

2-(3-Methoxy-4-oxyphenyl)-3-methyl-cumaran-5-carbonsäure (IV)

Zu einer Lösung von 4,35 g Dehydrodiisoeugenolacetat in 100 ml 70 % Aceton werden bei 0—4° im Verlauf von etwa 3 Stunden 4,8 g Kaliumpermanganat und 3,2 g Kaliumbiphosphat in 10 Portionen zugesetzt. Nach beendeter Oxydation wird das entstandene Mangandioxyd abgenutscht und mit wässrigem Aceton gewaschen. Aus dem Filtrat wird das Aceton im Vacuum abdestilliert, wobei sich Substanz ausscheidet, welche in Äther aufgenommen wird. Durch Ansäuern der wässrigen Lösung sowie eines Bicarbonatextraktes der Ätherlösung wird das Acetat von IV ausgefällt. Nach einmaligem Umkristallisieren aus Alkohol erhält man 2,17 g vom Schmp. 194—196°. (Gef. OCH_3 16,74. Ber. für $C_{20}H_{20}O_7$ (372,4): OCH_3 16,67). Diese werden durch einstündiges Erwärmen auf dem Wasserbad mit 35 ml 1 *M* Sodalösung unter Stickstoff verseift. Aus der angesäuerten Lösung wird IV mit Äther extrahiert. Durch Umkristallisieren aus Alkohol erhält man 1,42 g farblose Nadeln vom Schmp. 196—198°. Der Mischschmp. mit dem Acetat liegt bei 164—178°. (Gef. C 65,16; H 5,46. Ber. für $C_{18}H_{18}O_6$ (330,3): C 65,45; H 5,49).

Sulfitierung. 0,22 g IV werden durch zwanzigstündiges Erhitzen mit 10 ml Sulfitlösung (1,1 g Natriumpyrosulfit und 0,4 g Natriumsulfit) bei 135° praktisch vollständig sulfitiert.

Monochlorderivat. Zu 0,32 g IV in 5 ml 2 *N* Natronlauge gibt man 10 ml etwa 0,4 *N* Hypochloritlösung. Nach etwa 15 Min. setzt man 1 g Natriumsulfit zu und säuert mit Schwefelsäure an. Das ausfallende Chlorprodukt wird aus verdünnter Essigsäure (1 : 1) in Form farbloser Nadeln vom Schmp. 216—217,5° erhalten. (Gef. Cl 9,13. Ber. für $C_{18}H_{17}O_6Cl$ (364,8): Cl 9,72).

β-Methoxy-α-(2-methoxyphenoxy)-propioveratron (XI)

Zu einer lebhaft gerührten Suspension von 6 g fein gepulvertem VII in 60 g 25 % Natronlauge werden langsam 15 ml Dimethylsulfat zugetropft. Die ungelöste Substanz wird abfiltriert und nochmals methyliert. Man erhält 5,12 g XI, welches nach Umkristallisieren aus Alkohol bei 88—92° schmilzt. (Gef. OCH_3 35,71. Ber. für $C_{19}H_{22}O_6$ (346,4)-OCH_3 35,79).

2-Methoxy-4-carboxy-phenoxyessigsäure (XII)

Zu einer Lösung von 2,3 g Natrium in 100 ml abs. Methanol gibt man zuerst 15,2 g Vanillin, kurze Zeit nachher 16,7 g Bromessigester. Die Mischung wird am Wasserbad 3 Stunden gekocht. Nach Abdestillieren des Methanols im Vacuum wird der Rückstand mit Wasser versetzt und das Reaktionsprodukt (2-Methoxy-4-formyl-phenoxyessigsäure-äthylester) abgenutscht. Aus Cyclohexan kristallisiert es in farblosen Nadeln vom Schmp. 94°. (Gef. C 60,26; H 6,03. Ber. für $C_{12}H_{14}O_5$ (238,2): C 60,55; H 5,92).

Durch Verseifen mit verdünnter Natronlauge entsteht 2-Methoxy-4-formyl-phenoxyessigsäure vom Schmp. 182°. (Gef. C 57,30; H 5,00. Ber. für $C_{10}H_{10}O_5$ (210,2): C 57,14; H 4,79).

In eine Lösung von 6,3 g der vorstehenden Substanz in 120 ml 1 % Natronlauge werden unter Rühren allmählich 5 g Kaliumpermanganat eingetragen. Nach Abnutschen des Mangandioxyds wird das Filtrat im Vacuum eingeengt und angesäuert. Man erhält XII nach Umkristallisieren aus Wasser in Form farbloser Nadeln vom Schmp. 249—250,5°. (Gef. C 53,01; H 4,62. Ber. für $C_{10}H_{10}O_6$ (226,2): C 53,09; H 4,46).

7-Methoxy-cumaron-2-carbonsäure (XVII)

Zu 0,164 g XVI in 15 ml 0,3 *N* Natronlauge gibt man 60 ml etwa 0,4 *N* Hypochloritlösung bei 95°. Nach einer halben Stunde werden 3 g Natriumsulfit zugegeben und hierauf die Lösung mit Schwefelsäure angesäuert. Das ausfallende Oxydationsprodukt (0,078 g) wird aus Wasser umkristallisiert. Schmp. 213,5—215,5°. (Gef. C 62,42; H 4,34. Ber. für $C_{10}H_8O_4$ (192,2): C 62,48; H 4,19).
Das UV-Spektrum zeigt zwei Maxima bei 228 und 269 mμ und ein Minimum bei 243 mμ.

γ-Phenylpropyl-guajacyläther (XXII)

3 g XX werden in 200 ml Alkohol mit einem Palladium-Kohle-Katalysator hydriert. XXII wird aus Alkohol oder Cyclohexan umkristallisiert und schmilzt bei 72—73°. (Gef. OCH_3 12,62. Ber. für $C_{16}H_{18}O_2$ (242,3): OCH_3 12,81).

Vanillincinnamyläther (XXI)

Zu einer Lösung von 8,2 g Natrium in 150 ml abs. Methanol gibt man zuerst eine Lösung von 54 g Vanillin in 125 ml abs. Methanol. und dann eine Lösung von 70 g Cinnamylbromid in 100 ml abs. Methanol. Nach Stehen über Nacht werden die abgeschiedenen Kristalle abgenutscht und mit kaltem Methanol gewaschen (Ausb. 40 g). Nach Umkristallisieren aus Alkohol oder Tetrachlorkohlenstoff liegt der Schmp. bei 118—119°. XXI ist unlöslich in Alkali, gibt keine Eisenchloridreaktion und ist nicht acetylierbar zum Unterschied von dem gleichzeitig entstehenden Cinnamylvanillin. (Gef. C 75,75; H 6,28; OCH_3 12,04. Ber. für $C_{17}H_{16}O_3$ (268,3): C 76,10; H 6,01; OCH_3 11,57).

γ-Phenylpropyl-vanillinäther (XXIII)

Wird aus XXI durch Hydrierung mit einem Palladium-Kohle-Katalysator in Alkohol erhalten. Schmp. 77—78°. (Gef. OCH_3 11,63. Ber. für $C_{17}H_{18}O_3$ (270,3): OCH_3 11,48).

Cinnamyl-guajacylätheroxyd (XXIV)

3,38 g XX werden mit etwas überschüssiger Benzopersäure in Chloroformlösung bei 0° stehen gelassen. Nach einer Woche wird die Chloroformlösung zur Entfernung der Benzoesäure mit Sodalösung ausgeschüttelt. Die im Chloroform gelöst bleibende Substanz wird in Vacuum destilliert. Bei einer Badtemp. von 190° (0,3 mm) erhält man 1,9 g XXIV vom Schmp. 45—46,5°. (Gef. OCH_3 12,02. Ber. für $C_{16}H_{16}O_3$ (256,3): OCH_3 12,11).

Sulfitierung von XXIV

0,26 g XXIV werden mit 0,26 g Natriumsulfit in 3 ml Wasser 23 Stunden auf 135° erhitzt. Die Lösung, welche nur Spuren ungelöste Substanz enthält, wird nach Katjonenaustausch zur Entfernung von schwefliger Säure im Vacuum eingeengt und dann mit Bariumcarbonat neutralisiert. Aus der Lösung des Bariumsalzes wird durch Zugabe von β-Naphthylaminhydrochlorid das β-Naphthylaminsalz von XXV gefällt. Schmp. 177—179,5°. (Gef. S 6,19; OCH_3 6,28. Ber. für $C_{26}H_{27}O_6NS$ (481,6): S 6,66; OCH_3 6,44).

5-Chlordihydroferulasäure

Zu einer Lösung von 0,74 g Dihydroferulasäure in 10 ml 2 *N* Natronlauge werden 70 ml etwa 0,4 *N* Hypochloritlösung gegeben. Nach 25 Min. werden 2 g Natriumsulfit zugesetzt. Nach einigem Stehen wird die Lösung mit Schwefelsäure angesäuert und wiederholt mit Äther ausgeschüttelt. Der nach Abdestillieren des Äthers hinterbleibende Rückstand wird aus wenig Wasser umkristallisiert. Man erhält 0,48 g Chlordihydroferulasäure. Schmp. 119,5—121°. (Gef. Cl 15,68; OCH_3 13,66. Ber. für $C_{11}H_{11}O_4Cl$ (230,7): Cl 15,37; OCH_3 13,46).

Einwirkung von Hypochlorit auf *o*-Vanillinsäure

Zu einer Lösung von 0,508 g *o*-Vanillin in 5 ml 2 *N* Natronlauge werden 35 ml etwa 0,4 *N* Hypochloritlösung gegeben. Nach 30 Min. werden 2 g Natriumsulfit zugesetzt. Nach einigem Stehen wird die Lösung mit Schwefelsäure angesäuert und ausgeäthert. Man erhält 0,392 g einer chlorierten *o*-Vanillinsäure, welche nach Umkristallisieren aus Wasser bei 186—188° schmilzt. (Gef. C 47,65; H 3,38. Ber. für $C_8H_7O_4Cl$ (202,6): C 47,42; H 3,49).

Einwirkung von Hypochlorit auf 4-Oxy-5-Methoxy isophthalsäure

Zu 0,156 g obiger Säure in 2 ml 2 *N* Natronlauge gibt man 30 ml etwa 0,4-n Hypochloritlösung. Nach einer Stunde werden 2 g Natriumsulfit zugesetzt und mit Schwefelsäure angesäuert. Durch Ausschütteln mit Äther werden 0,073 g 5-Chlorvanillinsäure erhalten. Schmp. und Mischschmp. 237—238°.

4-Oxy-3-methoxy-diphenylmethan

In eine Mischung von 60 g Aluminiumchlorid und 225 ml Schwefelkohlenstoff werden 55 ml Veratrol und dann im Lauf einer Stunde unter Rühren 50 ml Benzoylchlorid eingetropft. Nach weiterem Zusatz von 60 g Aluminiumchlorid in 3 Portionen bleibt die Mischung über Nacht stehen. Nach der üblichen Aufarbeitung wird das Reaktionsprodukt in Neutralteil, sowie einen soda- und alkalilöslichen Anteil zerlegt. Der letztere wird in Vacuum destilliert und liefert nach Umkristallisieren aus Alkohol oder Tetrachlorkohlenstoff 4-Oxy-3-methoxy-benzophenon. Schmp. 97—98°. (Gef. C 73,95; H 5,64. Ber. für $C_{14}H_{12}O_3$ (228,2): C 73,67; H 5,30).

Semicarbazon: Schmp. 225,5—226,5°. (Gef. C 63,14; H 5,54. Ber. für $C_{15}H_{15}O_3N$ (285,3): C 63,15; H 5,30).

15,1 g 4-Oxy-3-methoxy-benzophenon werden mit 7,5 ml Hydrazinhydrat eine Stunde, nach Zusatz von 13 g Kaliumhydroxyd noch weitere zwei Stunden unter Rückfluss in einem Ölbad von 180° erhitzt. Nach Verdünnen mit Wasser wird Kohlensäure eingeleitet und das abgeschiedene Öl in Äther aufgenommen. Nach Ausschütteln der Ätherlösung mit Bleiacetatlösung wird die im Äther verbleibende Substanz in Vacuum destilliert. Man erhält eine Hauptfraktion vom $Kp_{0,5}$ 137—139°. (Gef. C 77,82; H 6,81. Ber. für $C_{15}H_{15}O_3$ (214,3): C 78,48; H 6,59).

p-Nitrobenzoat: Schmp. 84,5—85,5°. (Gef. C 68,78; H 4,77. Ber. für $C_{21}H_{17}O_5N$ (363,4): C 69,41; H 4,72).

Für die freundliche Überlassung verschiedener Präparate sind wir den Herren Prof. *E. Adler, H. Erdtman* und *K. Freudenberg* zu Dank verpflichtet.

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The Composition of the Azeotropes Acetaldehyde — Ethyl Ether, Propylene Oxide — Ethyl Ether and Ethylene Glycol — Diethylene Glycol Monoethyl Ether

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The compositions of three azeotropes with minimum boiling points have been investigated. The azeotrope, acetaldehyde-ethyl ether, boils at 18.9° C at 760 mm Hg and contains 76.5 per cent by weight of acetaldehyde. The azeotrope, propylene oxide-ethyl ether, boils at 32.6° C at the same pressure and contains 49.6 % propylene oxide. At 36 mm Hg the azeotrope, ethylene glycol-diethylene glycol monoethyl ether, boils at 108.5° C and consists of 26.6 % ethylene glycol and 73.4 % diethylene glycol monoethyl ether.

When working on ethylene oxide and products therefrom, mixtures were found which contained the components of the azeotropes mentioned in the heading. The first two azeotropic compositions do not seem to have been published before. The third one has earlier been investigated at 760 mm Hg [1].

EXPERIMENTAL

Distillation apparatus. A Podbielniak Hyper-Cal apparatus, Cat. No. HC-701-A, Series "D", has been used for all distillations. A 25 mm I.D. × 36″ long Heli-Grid packed column was used. All distillations were carried out with a reflux ratio of 200 : 1.

Analyses. Mixtures of acetaldehyde and ethyl ether were analysed by a spectrophotometric method. A suitable amount of the sample was diluted with ethyl ether to 100 ml volume and the extinction of this solution was measured in a Beckman Spectrophotometer, model DU, with light path of the sample holder 1.00 cm. By means of the molar extinction coefficient at 288 mμ of pure acetaldehyde in ethyl ether, which was found to be 17.3 $l \cdot mole^{-1} \cdot cm^{-1}$, the content of acetaldehyde in the sample could be calculated using Lambert-Beer's law.

Mixtures of propylene oxide and ethyl ether were analysed by determining the propylene oxide content using a method given by Lubatti for ethylene oxide [2].

Mixtures of ethylene glycol and diethylene glycol monoethyl ether (the latter henceforth called ethyl diglycol) were analysed by determining the ethylene glycol content using a method given by Pohle, Mehlenbacher and Cook [3]. This method is based on the Malaprade potassium periodate oxidation method [4].

Starting materials. Pure acetaldehyde was prepared by fractional distillation of technical acetaldehyde.

Pure ethyl ether was prepared by drying usual laboratory ethyl ether first with anhydrous calcium chloride and then with metallic sodium. The dry ether was fractionated.

Pure propylene oxide was prepared by fractionation of a technical propylene oxide.

In the experiment for obtaining the azeotrope, ethylene glycol-ethyl diglycol, chemicals of technical quality were used.

AZEOTROPES

In order to obtain the azeotrope of acetaldehyde and ethyl ether a mixture of 75 % acetaldehyde and 25 % ethyl ether was distilled. (All compositions in this paper are given in per cent by weight). The main fraction was analysed to contain 76.5% acetaldehyde. The boiling point of this fraction was measured by boiling it slowly under reflux in a Claisen flask and the temperature was read on a thermometer graduated to 0.1° C. The boiling point of pure acetaldehyde was determined in the same way on the same occasion. The temperature of the boiling azeotrope was 19.9° C and that of the pure acetaldehyde 21.4° C. The boiling point of acetaldehyde is given as 20.4° C at atmospheric pressure [5]. The boiling point of the azeotrope would thus be 18.9° C.

To obtain the azeotrope, propylene oxide — ethyl ether, a mixture of 50% of the former substance and 50 % of the latter was fractionated. The main fraction contained 49.6 % propylene oxide. The boiling point of the fraction was measured in the way described above and was found to be 2.0° C lower than that of pure ethyl ether. The boiling point of ethyl ether is given as 34.6° C [6] and thus the boiling point of the azeotrope would be 32.6° C at 760 mm Hg. The same method gave the boiling point of propylene oxide as 34.2° C whereas the published value is 33.9° C at atmospheric pressure [7].

To obtain the azeotrope, ethylene glycol — ethyl diglycol, a mixture of 50 % technical ethylene glycol and 50 % technical ethyl diglycol was fractionated at 36 mm Hg. The main fraction was redistilled under the same conditions. After the first fractionation the main fraction contained 26.5 % ethylene glycol and, after the second one, 26.6 %. The temperature was measured with an iron-constantan thermocouple, which was calibrated against a standardised thermometer. The thermocouple E.M.F. was measured with a potentiometer from Leeds & Northrup Co. In this way the boiling point of the azeotrope was determined to be 108.5° C at 36 mm Hg.

This azeotrope is reported [1] to boil at 195.0° C at 760 mm Hg and to contain 29.5 % ethylene glycol under those conditions.

The following correlation between vapour pressure and temperature of ethylene glycol is given [8]:

$$\log p = 7.8808 - \frac{1\,957}{193.8 + t}$$

where p is the pressure in mm Hg and t the temperature in ° C. From this equation the boiling point of pure ethylene glycol is calculated to be 115.6° C at 36 mm Hg.

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Enzymatic Hydrolysis of Organophosphorus Compounds

IV. Specificity Studies

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The enzymatic hydrolysis of a series of organophosphorus compounds was studied with rabbit plasma and a purified preparation (Fraction IV—1) from human serum as enzyme sources. Most of the compounds studied are split by these enzymes.

Amongst the homologues of tabun, the methoxy compound is split at the highest rate. Amongst the analogues of this compound, the chloride is hydrolysed at a higher rate than the fluoride and the cyanide.

By summation experiments it was shown that the phosphorylphosphatase ("tabunase") of both rabbit plasma and Fraction IV—1 splits tabun, DFP, and mintacol; the latter compound is probably also split by other enzymes. TEPP is hydrolysed by a separate enzyme or enzymes present in these enzyme preparations.

In a previous paper [1] the existence of an enzyme (phosphorylphosphatase, "tabunase") catalysing the hydrolytic breakdown of dimethylamido-ethoxy-phosphoryl cyanide (tabun) was reported. Some properties of the enzyme were also described [2]. A third paper [3] discussed the effect of reversible cholinesterase inhibitors on phosphorylphosphatase and the significance of considering the presence of this enzyme in studies on tabun as a cholinesterase inhibitor. In the present paper the specificity of phosphorylphosphatase is demonstrated.

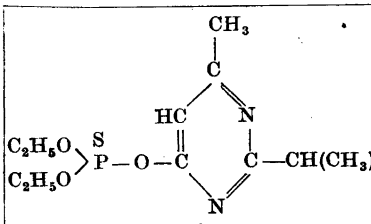
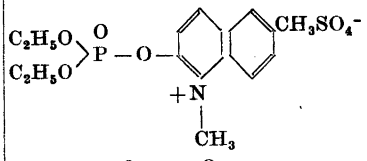
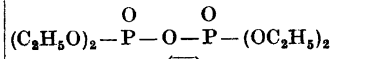
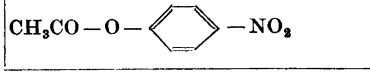
METHODS AND MATERIAL

The Warburg manometric technique was used to follow the enzymatic hydrolysis of various organophosphorus compounds [1]. Rabbit plasma and Fraction IV—1 prepared from human postpartum serum were used as enzyme sources, except as otherwise stated.

The organophosphorus compounds used as substrates are tabulated in Table 1. They were all of high purity, except as otherwise stated. The synthesis and properties of the compounds containing nitrogen were described by Holmstedt [4]. They were, in addition to DFP, TEPP and mintacol, synthesized at the Research Institute of National Defence of Sweden. Chlorothion [5], systox and isosystox [6] were synthesized by Schrader. Malathion and diazinon were commercial products (Geigy, A. G., Basel). Ro3—0422 was prepared

Table 1. Enzymatic hydrolysis of various organophosphorus compounds by rabbit plasma (0.1 ml) and Fraction IV—1 (10 mg) from human serum. Substrate concentration, 5.32 × 10^{-3} M, except for malathion and diazinon (0.2 vol. %), and for chlorothion, systox and isosystox (suspensions). Activity expressed in b_{30} values.

Formula	Name or code	Mol. wt.	Density g/ml 20° C	Spont. hydrol.	Rabbit plasma	Fraction IV—1
$(CH_3)_2N$, C_2H_5O > P(O)—CN	tabun	162.13	1.077	10	315	170
$(CH_3)_2N$, CH_3O > P(O)—CN	dimethylamido-methoxy-phosphoryl cyanide	148.11	1.12	12	≈400	≈450
$(CH_3)_2N$, $(CH_3)_2CHO$ > P(O)—CN	dimethylamido-*iso*propoxy-phosphoryl cyanide	176.16	1.054	11	62	78.5
$(CH_3)_2N$, C_2H_5O > P(O)—Cl	dimethylamido-ethoxy-phosphoryl-chloride	171.57	1.188	very rapid	?	?
$(CH_3)_2N$, CH_3O > P(O)—F	dimethylamido-methoxy-phosphoryl fluoride	141.09	1.155	2	60	25.5
$(CH_3)_2N$, $(CH_3)_2N$ > P(O)—Cl	bis(dimethylamido)-phosphoryl chloride	170.59	1.198	0	—	0
$(CH_3)_2N$, $(CH_3)_2N$ > P(O)—F	bis(dimethylamido)-phosphoryl fluoride	154.10	1.115	0	—	0
$(CH_3)_2CHO$, $(CH_3)_2CHO$ > P(O)—F	DFP	184.15	1.070	1	179	132
C_2H_5O, C_2H_5O > P(O)—O—C$_6$H$_4$—NO_2	mintacol, paraoxon	275.20	1.265	1	208	39
CH_3O, CH_3O > P(S)—O—C$_6$H$_3$Cl—NO_2	chlorothion	297.66	1.433	2	0	0
C_2H_5O, C_2H_5O > P(S)—O—CH_2—CH_2—S—C_2H_5	systox	258.34	1.119	5	0	0
C_2H_5O, C_2H_5O > P(O)—S—CH_2—CH_2—S—C_2H_5	isosystox	258.34	1.132	3	0	0
CH_3O, CH_3O > P(S)—S—CH(CO—O—C_2H_5)—CH_2—CO—O—C_2H_5	malathion	330.36	technical product	3	0	0

	diazinon	304.35	technical product	3	0	0
	Ro 3—0422	406.37		6	75	138
	TEPP	290.20	21.185	12	331	43
	*p*-nitrophenyl acetate (PNPA)	181.14		3	≈370	≈650

by Roche Products, Ltd., England [7]. The synthesis of *p*-nitrophenyl acetate was carried out according to the method described by Huggins and Lapides [8]. The substrates were dissolved in water and their concentrations were in most experiments 5.32×10^{-3} *M* (total volume of the reaction mixture, 2.00 ml).

RESULTS

The enzymatic hydrolysis of various organophosphorus compounds. A series of organophosphorus compounds was tested for their enzymatic hydrolysis by the rabbit plasma and by partly purified phosphorylphosphatase (Fraction IV—1) from human serum. The results are recorded as b_{30} values (initial enzymatic hydrolysis rates) in Table 1 and Fig. 1. Amongst the homologues of tabun, the methoxy compound is split at a higher rate, the *iso*-propoxy compound at a lower rate than tabun. The chloride analogue (dimethylamido-ethoxy-phosphoryl chloride) of tabun is split at a very high, enzymatically not measurable, rate; the spontaneous hydrolysis of this compound in water is likewise very rapid. The dimethylamido-methoxy-phosphoryl fluoride is enzymatically hydrolysed by both enzymes studied at a lower rate than the corresponding cyanide and chloride. From these preliminary studies with amido-substituted alkoxy-phosphoryl compounds, we conclude that the chlorides are enzymatically (and also spontaneously) hydrolysed at a higher rate than the cyanides and these latter derivatives at a higher rate than the fluorides.

The enzymes studied do not split the substituted diamidophosphoryl halogenides.

The three well-known compounds, DFP, TEPP and mintacol were all found to be split by the rabbit plasma and Fraction IV—1. DFP behaves to these two enzyme sources in roughly the same way as do tabun and its analogues. TEPP and mintacol, on the other hand, are hydrolysed at much higher rates by the rabbit plasma compared with the rates found for the Fraction IV—1.

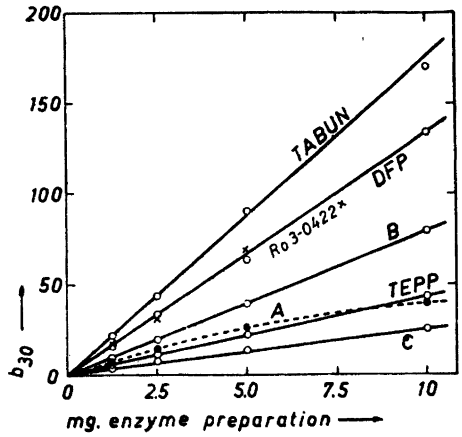


Fig. 1. Enzymatic hydrolysis of various organophosphorus compounds by a purified preparation (Fraction IV—1) of phosphorylphosphatase from human serum. Enzymatic hydrolysis (b_{30}) plotted against enzyme concentration (mg of Fraction IV—1 in the reaction mixture, total volume 2.00 ml). Substrate concentration, 5.32 × 10^{-3} M, except in the case of mintacol which was used in saturated solution. A: dimethylamido-methoxy-phosphoryl cyanide; B: dimethylamido-iso-propoxy-phosphoryl cyanide; C: dimethylamido-methoxy-phosphoryl fluoride.

Ro 3-0422 (diethyl-3-quinolylmethyl-phosphate methylsulphate) is split at a comparatively high rate by Fraction IV—1 and relative to that, at a lower rate by rabbit plasma. Its spontaneous hydrolysis is appreciable in aqueous solution; a 2.66 × 10^{-2} *M* solution is broken down 38 % after 40 minutes at room temperature.

None of a series of compounds recently introduced as insecticides, *viz.* chlorothion, systox, isosystox, malathion, diazinon, were found to be attacked enzymatically. They are all sparingly soluble in water and were therefore used as aqueous suspensions.

Table 2. Enzymatic hydrolysis of tabun, TEPP, and mintacol by various fractions of human postpartum serum. Fractionation carried out by a partly modified (Kabi) method No. 6 of Cohn et al. *Fraction IV-1 was obtained from various fractionation procedures.*

Fraction	b_{30}/10 mg protein		
	tabun	TEPP	mintacol
Original serum	40	13	11
I	8	5	1
II (γ-globulin)	2	9	0
III (β-globulin)	1	4	0
IV-1	135—188	27—43	31—43
IV-4	3	7	0
IV—5 + 6	2	3	0
IV—6 + 9	0	0	0
V (albumin)	2	9	0

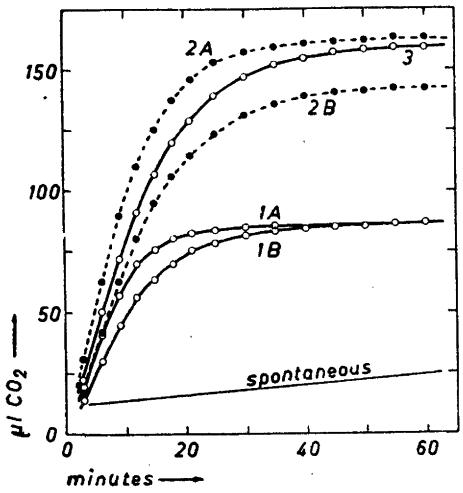


Fig. 2. Enzymatic hydrolysis of tabun and mintacol and a mixture of these substrates by rabbit plasma (0.1 ml). The spontaneous hydrolysis curve refers to a mixture of tabun and mintacol, the concentrations of which are those of the curves 1A and 1B.

Curve	Substrate	$M \times 10^{-3}$	b_{30}
1A	tabun	2.66	175
1B	mintacol	2.66	150
2A	tabun	5.32	290
2B	mintacol	5.32	210
3	tabun + mintacol	2.66 of each	230

In confirmation with the results obtained by other authors (*cf.* below), *p*-nitrophenyl acetate (PNPA) was split by the rabbit plasma. This ester is hydrolysed at a comparatively high rate by Fraction IV—1.

In Fig. 1 the enzymatic hydrolysis rates of some organophosphorus compounds studied are plotted against the concentration of a purified preparation (Fraction IV—1) of phosphorylphosphatase from human serum. In most cases, where the enzyme concentration is not too high, hydrolysis rate is proportional to enzyme concentration.

The phosphorylphosphatase activities of the various fractions of human serum were tested with tabun, TEPP, and mintacol as substrates. As was described in the first paper of this series [1], tabun is almost exclusively split by the Fraction IV—1. These results are compared with those obtained with TEPP and mintacol (Table 2). Mintacol is hydrolysed only by the Fraction IV—1 which splits tabun. TEPP also is destroyed at the highest rate by this fraction, but almost all fractions studied attack this compound at low rates; especially noticeable are the hydrolysis rates for the Fractions II and V. From these results we conclude that the hydrolysis of TEPP by human blood serum is catalysed by at least three factors; one of these is present in the same Fraction IV—1 as the enzyme(s) which hydrolyse(s) tabun and mintacol.

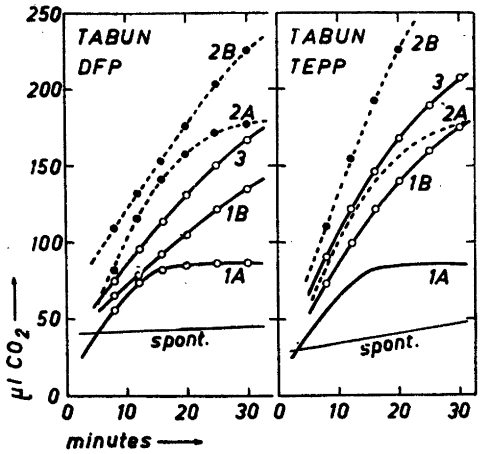


Fig. 3. Enzymatic hydrolysis of tabun mixed with DFP and TEPP. Enzyme: rabbit plasma (0.1 ml). The spontaneous hydrolysis curves refer to the mixtures of substrates.

Curve	Substrate	$M \times 10^{-3}$	b_{30}	μl CO_2 found at total hydrolysis
1A	tabun	2.66	172	86
1B	DFP	2.66	100	195
	TEPP		200	230
2A	tabun	5.32	270	190
2B	DFP	5.32	180	380
	TEPP		330	440
3	tabun + DFP	2.66 of each	120	235
	tabun + TEPP		280	300

Summation experiments. A comparison between the hydrolysis rate, when an enzyme preparation is acting upon a mixture of substrates, with the rates when the substrates are hydrolysed separately, has been frequently used in enzyme specificity studies. Although the results might be misleading in some special cases, as has been pointed out recently by Goldstein [9], they can generally be used as criteria of specificity. Such summation experiments were carried out in the present study as preliminary tests for the presence of phosphorylphosphatases in the rabbit plasma and a purified preparation of human serum (Fraction IV—1).

Figs. 2 and 3 record the results obtained with the rabbit plasma. The experiments performed with tabun and mintacol (Fig. 2) show that there are probably two enzymes present, each splitting each of the substrates. Therefore, the enzyme hydrolysing mintacol, called A-esterase by Aldridge [10], is not identical with the "tabunase" (phosphorylphosphatase) reported recently by the present authors [1]. The possibility remains that the A-esterase of the rabbit plasma also splits tabun or that "tabunase" alsc splits mintacol but no further studies have as yet been performed to elucidate this question (*cf.* below).

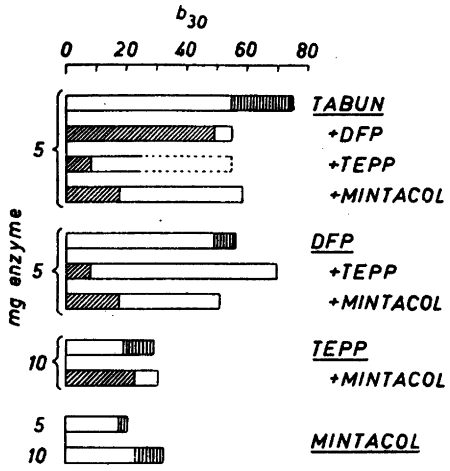


Fig. 4. Summation experiments with Fraction IV—1. Substrate concentrations, 2.66×10^{-3} M; hydrolysis rates, when the concentration of the substrate is twice that value, are marked by vertical shading. Oblique shading marks the enzymatic hydrolysis of the second substrate when present alone.

The results obtained with DFP and TEPP when these substrates are mixed with tabun, are illustrated in Fig. 3. They show that DFP is probably split by the same enzyme as is tabun. There is no additive effect in this case. TEPP, on the other hand, is probably not split by the "tabunase". The effect is not absolutely additive, but the higher hydrolysis rate for the substrate mixture compared with those obtained with separate substrates (tabun and TEPP) is significant. There may be a mutual inhibition by the substrates or their reaction products.

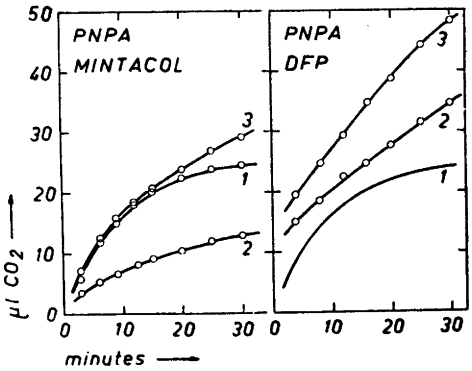


Fig. 5. Enzymatic hydrolysis of p*-nitrophenyl acetate (PNPA) mixed with mintacol and DFP; Fraction IV—1 (2.5 mg per reaction mixture).*
1: PNPA; 2: mintacol or DFP; 3: (PNPA + mintacol) or (PNPA + DFP).

The results in summation experiments with the Fraction IV—1 are illustrated in Fig. 4. They show that the same enzyme in this preparation splits tabun, DFP and mintacol. TEPP, on the other hand, is most probably hydrolysed by a second enzyme. This is demonstrated with all certainty by the DFP-TEPP mixture. The experiments performed with tabun and TEPP are not as conclusive, due to the rapid decline in reaction velocity during the hydrolysis of a mixture of these substrates. There is probably an interaction between the substrates and the two separate enzymes supposed to be present. A similar situation exists for the TEPP-mintacol mixture.

It was demonstrated by Aldridge [10] that the A-esterase of the rabbit plasma splits both mintacol and *p*-nitrophenyl acetate. In summation experiments performed with Fraction IV—1 (Fig. 5), the initial hydrolysis rates obtained show that DFP is probably split by an other enzyme than is *p*-nitrophenyl acetate (PNPA). In the case of mintacol, it is surmised that this substrate is hydrolysed both by an enzyme, which corresponds to the A-esterase and by a phosphorylphosphatase ("tabunase", "DFP-ase").

DISCUSSION

The ability of rabbit plasma and partly purified phosphorylphosphatase from human serum to hydrolyse a series of organophosphorus compounds has been demonstrated. Analogues (halogenides) and homologues of tabun (dimethylamido-ethoxy-phosphoryl cyanide) are all hydrolysed by these enzyme preparations at various rates. The bis(dimethylamido)-phosphoryl derivatives are not attacked, which is of interest, as these compounds are known to undergo another transformation (probably an oxidation) in the organism, to highly active cholinesterase inhibitors. Whether these products are hydrolysed by the phosphorylphosphatase has not been studied. The dialkoxyphosphoryl halogenides are probably split by the same enzyme as tabun, demonstrated in the present paper in experiments with DFP. TEPP, on the other hand, is hydrolysed by an enzyme separate from "tabunase".

It was recently demonstrated by Mounter and Whittaker [11] that human blood plasma contains an "aromatic esterase" hydrolysing phenyl esters. This enzyme was suggested to be probably indentical with the mintacol-insensitive esterase, the so-called A-esterase of Aldridge [10]. The results obtained in the present study allow us to suggest that mintacol is attacked not only by the A-esterase, but also by a phosphorylphosphatase, both these enzymes being present in Fraction IV—1 of human serum. Tabun and DFP are probably not hydrolysed by the A-esterase. Neither of these enzymes appear to hydrolyse TEPP.

We are indebted to Professor Gustaf Ljunggren, Chief of the Research Institute of National Defence, Department 1, for his most generous aid and continual interest in these investigations. Systox, isosystox, and chlorothion were supplied by Dr. Gerhard Schrader, Wupperthal-Elberfeld, to whom we owe our sincere thanks for his generosity. Ro 3-0422 was kindly given by Dr. F. Hobbiger, London. The gift of diazinon and malathion from Messrs. Philips AB, Stockholm, is gratefully acknowledged. We wish also to express our sincere thanks to Ing. Henrik Björling of A/B Kabi, Stockholm, for the preparation of Fraction IV—1.

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Über Molekülverbindungen zwischen α-Aminosäuren und nicht-reduzierenden Zuckern. II *

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Die Synthesen von D,L-, D- und L-γ-(*o*-Oxyphenyl)-α-aminobuttersäure werden beschrieben.

Sowohl die D-, als auch die L-Aminosäure geben eine kristallisierte Molekülverbindung mit Rohrzucker im Mol-Verhältnis 1:1; dagegen erhält man aus einer Lösung von der razemischen Aminosäure und Rohrzucker, nach dem *Dimroth*schen Verfahren, nur die Komponenten zurück.

Bei Desoxokynurenin liegt absolut sterische Spezifität vor; nur die L-Form, nicht aber die D-Form dieser Aminosäure gibt mit Rohrzucker eine kristallisierte Molekülverbindung. Einen Austausch der Kernaminogruppe der Aminosäure gegen OH-Gruppe hebt diese sterische Spezifität auf.

Bei Versuchsbedingungen, unter welchen D,L-Kynurenin und D,L-Tryptophan papierchromatographisch in optische Antipoden gespalten werden, wandern D,L-Dihydrokynurenin, D,L-Desoxokynurenin und D,L-γ-(*o*-Oxyphenyl)-α-aminobuttersäure als einheitliche Flecken. Für die papierchromatographische Spaltbarkeit des D,L-Kynurenins scheint somit die Ketogruppe notwendig zu sein.

In früheren Arbeiten [1,2] wurde mitgeteilt, dass bei der Bildung einer kristallisierten Molekülverbindung zwischen L-Kynurenin und Rohrzucker [3,4] die Ketogruppe des Kynurenins ohne Bedeutung für die Bindung zum Rohrzucker war, dagegen erwies sich die Kernaminogruppe als notwendig. In Übereinstimmung hiermit lieferte L-Desoxokynurenin eine kristallisierte Molekülverbindung mit Rohrzucker im Mol-Verhältnis 1 : 1. Sowohl das synthetische Kynurenin, als auch das synthetische Desoxokynurenin wurden über die kristallisierten Molekülverbindungen der L-Form dieser Aminosäuren mit Rohrzucker in optische Antipoden gespalten [4,2]. Des weiteren ergab sich, dass D-Desoxokynurenin keine kristallisierte Molekülverbindung mit Rohrzucker geben konnte, dass also hier absolut sterische Spezifität vorlag.

* Teil I: Weichert, R. *Arkiv Kemi* **5** (1953) 523.

γ-(o-Oxyphenyl)-α-aminobuttersäure und Rohrzucker

Die vorliegende Arbeit behandelt den Austausch der Kernaminogruppe des Desoxokynurenins durch Diazotierung gegen eine OH-Gruppe und das Verhalten der gewonnenen D,L-, D- und L-γ-(o-Oxyphenyl)-α-aminobuttersäuren gegenüber Rohrzucker, Raffinose und Melezitose. Die Versuche wurden nach dem *Dimroth*schen [5] Verfahren zur Herstellung von kristallisierten Molekülverbindungen im Mol-Verhältnis 1 : 1 durchgeführt.

Es zeigte sich, dass aus einer Lösung von der razemischen Aminosäure und Rohrzucker die Komponenten wieder auskristallisierten, aber dass sowohl die reine L-Aminosäure, als auch die reine D-Aminosäure mit Rohrzucker kristallisierte Molekülverbindungen gaben. In diesem Fall kann also nicht, wie bei Kynurenin und Desoxokynurenin, eine Spaltung des Razemats über eine Rohrzuckerverbindung erzielt werden.

Gibt eine Antipode einer Aminosäure eine kristallisierte Molekülverbindung mit Rohrzucker, so muss folgende Bedingung erfüllt sein, damit aus einer Lösung der razemischen Aminosäure und Rohrzucker die Molekülverbindung auskristallisiert und nicht das Razemat [1]:

$$G_{MV} > \sqrt{G_{DL}},$$

wo G_{MV} nach der *Dimroth*schen Formel die »Stabilitätskonstante» der Molekülverbindung und G_{DL} die des Razemats bedeutet. Bei Desoxokynurenin war diese Bedingung vorhanden. Der Wert $\sqrt{G_{DL}}$ bei D,L-Desoxokynurenin war ca. 2,8. G_{MV} bei der Verbindung L-Desoxokynurenin-Rohrzucker konnte man dadurch abschätzen, dass die Verbindung schon aus einer Lösung auskristallisierte, die in Bezug auf Rohrzucker, bzw. D,L-Desoxokynurenin halbgesättigt war; daraus ergab sich $G_{MV} > 11$. Bei der D,L-γ-(o-Oxyphenyl)-α-aminobuttersäure liegt der Wert $\sqrt{G_{DL}}$ im Vergleich mit anderen Aminosäuren [1] sehr hoch, etwa bei 8,8, und daraus erklärt sich wohl, dass in diesem Fall das Razemat auskristallisiert.

Mit dem Austausch der Kernaminogruppe gegen OH-Gruppe ist die sterische Spezifität, wie sie bei Desoxokynurenin vorliegt, wo nur die L-Form eine kristallisierte Verbindung mit Rohrzucker gibt, aufgehoben. Eine Erklärung hierfür steht noch aus.

Weder mit Raffinose noch mit Melezitose geben L- oder D-γ-(o-Oxyphenyl)-α-aminobuttersäure kristallisierte Molekülverbindungen.

Papierchromatographisches Verhalten des Desoxokynurenins und der γ-(o-Oxyphenyl)-α-aminobuttersäure.

Mehrere razemische Aminosäuren erleiden Spaltungen in optische Antipoden bei Papierchromatographie [6] und als Erklärung hierfür muss man eine Adsorption an der optisch aktiven Cellulose annehmen; eine Adsorption, die aus sterischen Gründen für die eine Antipode der betreffenden Aminosäuren grösser ist als für die andere.

Zu dieser Gruppe papierchromatographisch spaltbarer Aminosäuren gehört auch das D,L-Kynurenin. Da L-Kynurenin in der kristallisierten Molekülverbindung mit Rohrzucker durch L-Desoxokynurenin und durch L-, bzw.

D-γ-(*o*-Oxyphenyl)-α-aminobuttersäure ersetzt werden kann, liegt die Annahme nahe, dass auch die Razemate der letztgenannten beiden Aminosäuren papierchromatographisch spaltbar wären. Dieses scheint aber nicht der Fall zu sein. Bei Durchlaufchromatographie mit Butanol-Wasser (75 ml Wasser mit *n*-Butanol zu 500 ml Volumen), oder mit Methanol-Wasser (3 : 1), wandern die D-, L- und D,L-Formen der resp. Aminosäuren gleich lange Strecken auf Whatman Nr. 4-Papier. Auch findet man keine Verlängerung des Razematflecks, wie man es bei den spaltbaren Aminosäuren beobachtet, wenn die Wegstrecke für vollständige Spaltung zu kurz ist. Unter den gleichen Versuchsbedingungen und Wanderungsstrecken wird D,L-Tryptophan, bzw. D,L-Kynurenin, gespalten, und erscheint auf dem Papier als zwei deutlich von einander getrennte Flecke. D,L-Dihydrokynurenin [3], mit Methanol-Wasser (3 : 1) chromatographiert, zeigt auch keine Aufspaltung in Antipoden. Wie andere aromatische Aminosäuren wandern auch die oben Genannten mit Wasser unter Verzögerung auf dem Papier.

Tabelle 1. Quotient der Wanderungsstrecken der Antipoden.

	Wasser	Butanol-Wasser		Methanol-Wasser	
	a	a	b	a	b
Kynurenin	0,90	0,83	0,82	0,80	0,79
Tryptophan			0,92		0,91

[a] Berechnet aus den optichen Isomeren
[b] Berechnet aus den zwei Flecken des gespaltenen Razemats

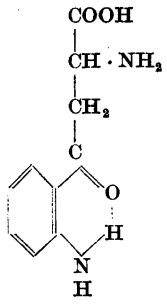
Tabelle 2. R_F-Werte im Wasser.

	L	D,L	D
Kynurenin	0,63	0,62	0,57
Tryptophan	0,53	0,55	
Desoxokynurenin	0,73	0,74	0,74
γ-(*o*-Oxyphenyl)-α-aminobuttersäure	0,76	0,77	0,76
Dihydrokynurenin		0,69	

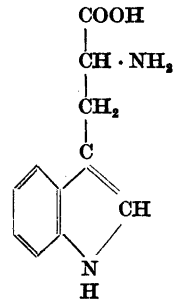
Die Ketogruppe des Kynurenins, die bei der Bildung einer kristallisierten Molekülverbindung mit Rohrzucker ohne Bedeutung ist, scheint also bei der papierchromatographischen Antipodentrennung notwendig zu sein, da eine Veränderung der Ketogruppe zur —CH(OH)— oder —CH_2— diese Spaltung verhindert.

Ein Grund hierfür kann noch nicht gegeben werden. Vielleicht wäre eine Erklärung, dass durch die Veränderung der Ketogruppe zur —CH(OH)—, bzw. —CH_2—, die Lage der α-Aminosäuregruppe im Raum eine andere wird, und für die Bindung zur Cellulose ungünstiger. Diese räumliche Differenz spielt aber bei der Bildung von kristallisierten Molekülverbindungen mit Rohrzucker keine grössere Rolle. Eine andere Erklärung könnte in der starken Wasserstoffbrücke zwischen Ketogruppe und *o*-Aminogruppe im Kynurenin liegen. Die in der Literatur bisher beschriebenen, papierchromatographisch spaltbaren Aminosäuren sind, mit Ausnahme von Kynurenin und 3-Oxykynurenin, sämtlich aromatische β-Derivate des Alanins, nämlich verschiedene Dioxy-

phenylalanine, Tryptophan und Oxytryptophan. Kynurenin mit einer Wasserstoffbrücke könnte man auch als aromatisches Alaninderivat betrachten und es zeigt eine gewisse Ähnlichkeit mit Tryptophan:



Kynurenin



Tryptophan

Das eigentümliche ist, dass bei Kynurenin die D-Form, bei Tryptophan aber die L-Form vom Papier am stärksten adsorbiert wird.

EXPERIMENTELLER TEIL

D,L-*γ*-*(o-Oxyphenyl)*-*α*-*aminobuttersäure*. 2,05 g D,L-Desoxokynureninsulfat in 80 ml 0,07 *N* Schwefelsäure suspendiert und auf 0° abgekühlt, wurden mit etwas mehr als der für *eine* Aminogruppe berechneten Menge Natriumnitrit diazotiert (9 ml von einer 6,7 % Natriumnitritlösung). Nach beendeter Reaktion wurde die klare Lösung 15 Min. gekocht, wobei sie sich braun färbte und eine geringe Menge von einem braunen Niederschlag entstand, der abfiltriert wurde. Das rotbraune Filtrat wurde fünf mal mit Äther ausgeschüttelt, und die Wasserphase mit Kohle gekocht und filtriert. Das Filtrat, das sehr stark gelb gefärbt war, wurde durch Vacuumdestillation auf etwa 45 ml Volumen eingeengt und tropfenweise mit konz. Ammoniak versetzt, wobei die D,L-*γ*-(*o*-Oxyphenyl)-*α*-aminobuttersäure ausfiel. Nach einmaliger Umkristallisation aus Wasser wogen die stäbchenförmigen Kristalle 0,67 g (49 % der Theorie). Die Aminosäure zeigte positive Ninhydrinreaktion und Blaufärbung mit Folin's Reagenz. Umkristallisation aus Wasser: Schmp.: 287—288° (Zersetz.) *. (Gef.: C 61,14; H 6,81; N 7,36. Ber. für $C_{10}H_{13}O_3N$ (195,2): C 61,53; H 6,71; N 7,18).

D-*γ*-*(o-Oxyphenyl)*-*α*-*aminobuttersäure*. 3,76 g D-Desoxokynureninsulfat wurden, entsprechend obiger Beschreibung, in D-*γ*-(*o*-Oxyphenyl)-*α*-aminobuttersäure übergeführt. Ausbeute: 1,68 g (67 % d.Th.). Stäbchen (aus Wasser), die positive Ninhydrin- und Folins Reaktion gaben. Schmp.: 277—278° (Zersetz.). Optische Drehung in 1,0 *N* HCl, $[\alpha]_D^{20} = +26{,}4°$ ($c = 0{,}492$, $l = 2$ dm).

L-*γ*-*(o-Oxyphenyl)*-*α*-*aminobuttersäure*. 2,41 g L-Desoxokynureninsulfat, wie oben behandelt, ergaben 0,91 g L-*γ*-(*o*-Oxyphenyl)-*α*-aminobuttersäure (57 % d.Th.). Schmp.: 277—279° (Zersetz.). Optische Drehung in 1,0 *N* HCl, $[\alpha]_D^{20} = -29{,}7°$ ($c = 0{,}454$, $l = 2$ dm).

D,L-*γ*-*(o-Oxyphenyl)*-*α*-*aminobuttersäure und Rohrzucker*. 12,2 mg D,L-*γ*-(*o*-Oxyphenyl)-*α*-aminobuttersäure und 53,1 mg Rohrzucker wurden in 1 ml heissem Wasser gelöst, mit 17 ml abs. Alkohol versetzt, filtriert und bei +3° aufbewahrt. Nach 3 Tagen wurden die ausgeschiedenen Kristalle (rhombische Blättchen) abgesaugt. Positive Ninhydrinreaktion, negative Anthronreaktion und ein Schmelzpunkt von 283° (Zersetz.)

* Alle Schmelzpunkte sind bei schnellem Erhitzen und mit kurzem Thermometer gemessen.

zeigten, dass die Kristalle aus D,L-γ-(*o*-Oxyphenyl)-α-aminobuttersäure bestanden. In dem Filtrat schieden nach weiteren 3 Tagen Rohrzuckerkristalle aus.

Molekülverbindung zwischen D-γ-(o-Oxyphenyl)-α-aminobuttersäure und Rohrzucker. 25,0 mg D-γ-(*o*-Oxyphenyl)-α-aminobuttersäure und 108,4 mg Rohrzucker wurden in 2 ml heissem Wasser gelöst, mit 34 ml abs. Alkohol versetzt, filtriert und 17 Tage bei +3° aufbewahrt. Es kristallisierten allmählich rosettenförmig angeordnete Nadeln, die positive Ninhydrin- und Anthronreaktion ergaben. Ausbeute: 17,1 mg (25 % d.Th.). Schmp.: 193—194° (Zersetz.). Da die Molekülverbindung sich nicht ohne Zersetzung umkristallisieren liess, wurde sie ohne weitere Reinigung analysiert: (Gef.: N 2,63. Ber. für $C_{22}H_{35}O_{14}N$ (537,5): N 2,60).

Molekülverbindung zwischen L-γ-(o-Oxyphenyl)-α-aminobuttersäure und Rohrzucker. 24,8 mg L-γ-(*o*-Oxyphenyl)-α-aminobuttersäure und 108,3 mg Rohrzucker unter den gleichen Bedingungen behandelt wie oben für die D-Aminosäure und Rohrzucker beschrieben, ergaben 30,0 mg (44 % d.Th.) Molekülverbindung, bestehend aus nadelförmigen Kristallen, in Rosetten geordnet, die positive Ninhydrin- und Anthronreaktion gaben. Schmp.: 196—198° (Zersetz.). (Gef.: N 2,66. Ber. für $C_{22}H_{35}O_{14}N$ (537,5): N 2,60).

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Low-molecular Carbohydrates in Algae

V *. Investigation of *Laminaria cloustoni*

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The brown alga *Laminaria cloustoni* has been investigated with respect to its low-molecular carbohydrates. Mannitol, 1-mannitol β-glucoside, 1,6-mannitol di-(β-glucoside) and 1-mannitol acetate were isolated. All of these substances have been found previously in another brown alga, *Fucus vesiculosus*. In addition to these substances, the disaccharide laminarobiose was isolated in a low yield and the presence of laminarotriose demonstrated by paper chromatography. Finally, a great number of spots, mostly due to unidentified substances, was observed on the chromatograms of the different fractions obtained after separation on carbon and cellulose columns.

In previous papers in this series the investigations of the brown algae *Fucus vesiculosus* (Part I[1]) and *Pelvetia canaliculata* (Part IV) were reported. Both these algae contain D-mannitol, 1-mannitol β-glucoside and 1,6 mannitol di-(β-glucoside), and *P. canaliculata* also D-volemitol and the corresponding volemitol glucosides. 1-Mannitol acetate was isolated from *F. vesiculosus* but not from *P. canaliculata*. In the present paper a similar investigation of a third brown alga, *Laminaria cloustoni*, is reported.

By fractionation of the carbohydrate fraction on carbon and cellulose columns mannitol (7 %), its monoglucoside (0.5 %), diglucoside (0.08 %) and monoacetate (0.01 %) were isolated, *i.e.* all the substances previously found in *F. vesiculosus*. Laminarobiose (0.02 %) was further isolated and characterised as its crystalline β-octaacetate, and the presence of laminarotriose demonstrated by paper chromatography. The carbohydrate fraction was very complex, and the presence of at least twenty unidentified substances was demonstrated by means of paper chromatography. Most of these substances seem to occur in rather small quantities, and attempts to isolate some of them in a state of purity by further separations were not successful. Some of the substances were reducing, others nonreducing carbohydrates, while some after hydrolysis gave a

* IV: *Acta Chem. Scand.* 8 (1954) 817.

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positive ninhydrin reaction. It seems not improbable that some of the substances are artefacts, formed by condensation of sugars with amino compounds.

In Table 1 the relative amounts of sugar alcohol derivatives, calculated as percentage of free sugar alcohol found in the algae, are given. The yields obtained by the fractionation are of course not of analytical accuracy, but the figures obtained for the mono- and di-glucosides are surprisingly similar for the different plants, and it seems possible that these substances are characteristic for at least a group of brown algae.

For the monoacetate, however, the figures differ very much, from 2 % in *Fucus* to nil in *Pelvetia*. The *Laminaria* was richer in mannitol than the other algae and a greater amount of material was extracted, so if the *Pelvetia* contained monoacetates in a relative percentage of the same magnitude as that of the *Laminaria*, this would correspond to rather small amounts (10—20 mg), which might easily be overlooked. It is therefore not improbable that the mannitol monoacetate occurs in the same group of brown algae. It is safer, however, to await generalisations until several further species of brown algae from different families have been investigated.

Table 1. Sugar alcohol derivatives, in percentage of free sugar alcohol isolated.

Alga	Monoglucoside	Diglucoside	Monoacetate
Fucus vesiculosus	10.0	2.4	2.0
Laminaria cloustoni	7.1	1.1	0.1
Pelvetia canaliculata (mannitol)	9.2	1.5	—
» » (volemitol)	10.0	2.2	—

Free reducing sugars, except glucose and fructose, which are probably formed by hydrolysis of the sucrose, have not been found in algae before. Those found here, laminarobiose and laminarotriose, have not previously been found in Nature but are obtained by partial hydrolysis of laminaran, a glucan with 1,3-β-bonds occurring in brown algae. Their occurrence in *L. cloustoni* may be due to either of two causes. Either they are present in the living plant as precedents in the biosynthesis of laminaran or they are formed from this polysaccharide by a post-mortal, enzymatic hydrolysis. As no special precautions were taken to destroy the enzyme systems when the plant was collected, it is impossible to distinguish between these possibilities.

A substance, m.p. 250° (slight decomp.), which travels very fast on the carbon column but slow on paper was also isolated in a yield of 0.03 %. It might be a cyclitol and is now subject to further investigations.

EXPERIMENTAL

(Melting points uncorrected, all evaporations under reduced pressure, 40°).

The air-dried, ground alga (1 045 g), kindly supplied by Dr. E. T. Dewar, Inst. of Seaweed Research, Inveresk, Scotland, was extracted with ether for 3 days and with methanol for 9 + 7 days. The methanol extracts were concentrated to dryness, the residues treated with water and undissolved material removed by filtration. After treatments

Table 2. Fractionation of the low-molecular carbohydrates from L. cloustoni on a Carbon-Celite column.

(Fractions listed in the order of elution from the carbon column. Chromatograms run on Whatman 1 filter paper, ethyl acetate-acetic acid-water, 3:1:1, used as solvent and, unless otherwise stated, silver nitrate-sodium ethoxide [2] as reagent.)

Fraction	Yield, g		Contents, by paper chromatography
	1:st extr.	2:nd extr.	
a	0	0.2	Salts.
b	24.0	23.5	Mannitol, glucose, fructose, strong spot just faster than sucrose, strong spot between mannose and xylose, which gives positive reaction with anisidine phosphate.
c_1, c_2	6.0	7.0	Mannitol, glucose, fructose and faint, unidentified spots. In c_2 only mannitol.
d	1.33	0.66	Mannitol, four intense spots between those of mannose and rhamnose, all with positive anisidin ephosphate reactions and one with positive resorcinol-hydrochloric acid reaction.
e	1.29	—	Eight unidentified spots, one with positive anisidine phosphate reaction, same R_F as glucose.
f	4.28	1.37	Mannitol monoglucoside, trace mannitol monoacetate.
g	0.52	0.31	Laminarobiose, trace mannitol monoglucoside.
h	0.40	0.41	Mannitol diglucoside, two weak, unidentified spots.
i	0.34	0.39	Traces of mannitol diglucoside and of unknown substances.
j_2	—	0.20	Intense spot between mannitol mono- and di-glucosides.
k	0.31	0.28	Trace of laminarotriose, large number of unidentified spots.
l	0.28	0.27	Laminarotriose.
m	0.20	0.44	Unidentified spots
50 %	0.77	0.60	» » , an intense spot of low R_F, strongly reducing with anisidine phosphate reagent.

with lead acetate and hydrogen sulphide the aqueous solutions were concentrated to dryness. Yields, 179 g and 42 g. Ionic material in the first extract was removed by ion exchange (Amberlite resins IR 120 and IR 4B) and the residue (69 g) was dissolved in aqueous ethanol, when mannitol (20 g), m. p. 161—163°, separated. The mother liquors were concentrated and fractionated on a carbon-Celite column (50 × 5 cm), using the gradient elution techniques. Eluants: 12 l aqueous ethanol, 1—45 %, followed by 4 l 50 % ethanol. The eluate was divided into fractions, which were investigated by paper chromatography and similar fractions combined and concentrated to dryness. The second extract, without previous ion exchange and removal of mannitol, was subjected to the same fractionation procedure. The results of these fractionations, which gave very similar results, are given in Table 2.

Fraction b. The fraction was deionised, most of the mannitol removed by crystallisation and the residue concentrated to an orange syrup, which on standing deposited colourless crystals of high m. p. (1.34 g). Part of the syrup was fractionated on a hydrocellulose column, using aqueous butanol as eluant. Mannitol, glucose and a slow moving substance, identical to that which crystallised from the syrup, were isolated. These high melting fractions were combined and recrystallised five times from ethanol. Yield, 0.28 g. M. p.

250° (Slight decomp.). The substance is optically inactive, contains only C, H and O, reduces the silver nitrate-sodium ethoxide reagent and has an R_F-value slightly higher than that of D-inositol.

Fraction c. On recrystallisation pure D-mannitol was obtained, m. p. 164—165°, undepressed on admixture with authentic material.

Fraction e_1. By fractionation of this fraction on thick filter paper (Whatman 3 MM), a chromatographically pure, amorphous substance (100 mg) was obtained. The non-reducing substance had an R_F-value slightly higher than that of 1-mannitol β-glucoside and on hydrolysis yielded a mixture of mannitol and glucose, why the substance might be an isomer to 1-mannitol β-glucoside.

Fractions f_1 *and* f_2. Pure 1-mannitol β-glucoside (1.06 g) was obtained by crystallisation from f_2. M. p. 137—138°, undepressed on admixture with authentic material.

Fraction f_1 was fractionated on a hydrocellulose column (50 × 7 cm) using *iso*propanol-butanol-water (7:1:2) as eluant. 1-Mannitol acetate (180 g), m. p. 119—120, and 1-mannitol β-glucoside (3.4 g), m. p. 136—138°, identical to authentic materials, were obtained. In addition, a compound with colour reactions and R_F-values in different solvent systems identical to those of gentiobiose, was obtained in a low yield (5 mg).

Fraction g. Chromatographically pure laminarobiose was isolated from this fraction by separation on thick filter paper. By acetylation with sodium acetate-acetic anhydride at 130—140°, it was converted to β-laminarobiose octaacetate, m. p. 158—159°, undepressed on admixture with authentic material.

Fraction h. The main spot in this fraction was chromatographically indistinguishable from that of authentic 1,6-mannitol di-(β-glucoside). Partial hydrolysis yielded glucose, mannitol and 1-mannitol β-glucoside.

Fraction l. This fraction, in colour reactions and R_F-values in different solvents, was indistinguishable from laminarotriose. Glucose was the only substance detected after hydrolysis.

Acknowledgement. The authors are indebted to Dr. W. J. Whelan of Bangor for samples of laminarobiose, laminarotriose and their acetates.

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Sedimentation and Viscosity Studies on Micelles of the Cesium Salt of Hexadecane-dicarboxylic acid-(1,16)-monomethyl ester

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Sedimentation and viscosity measurements on $CH_3 \cdot OOC \cdot (CH_2)_{16} \cdot COOCs$ in 0.10 *M* CsCl show that this solution has the character of a gel. The constant *k* in Freundlich's formula was determined to be 4.6.

The dibasic soaps $CsOOC \cdot (CH_2)_{16} \cdot COOCs$ and $CsOOC \cdot (CH_2)_{12} \cdot COOCs$ also were investigated, but here no macro aggregates were found.

This paper deals with an investigation of the properties of $CH_3 \cdot OOC \cdot (CH_2)_{16} \cdot COOCs$ in a salt solution, studied by sedimentation and viscosity measurements.

The reason for choosing the cesium soap was that the sodium and potassium soaps were too insoluble. (The solubility at 20° C for the potassium soap in 0.10 *M* KCl is about 0.1 % by weight; the corresponding value for the cesium soap in 0.10 *M* CsCl is 0.5 %).

The soap was prepared by neutralizing hexadecane-dicarboxylic acid-(1,16)-monomethyl ester with cesium hydroxide. The ester was kindly made available by Professor Einar Stenhagen of the Medico-chemical Institute of the University.

PARTIAL SPECIFIC VOLUME

Notations: $\bar{V}_1$ = the partial specific volume[1] of the soap solution
v = specific volume of the soap solution
c_1 = concentration of soap.

For a binary system we have

$$\bar{V}_1 = v + (1-c_1)\frac{dv}{dc_1} \qquad (1)$$

For the system soap + electrolyte + water eq. (1) is valid if the concentration of salt is constant. The specific volume v was determined with a 10 ml

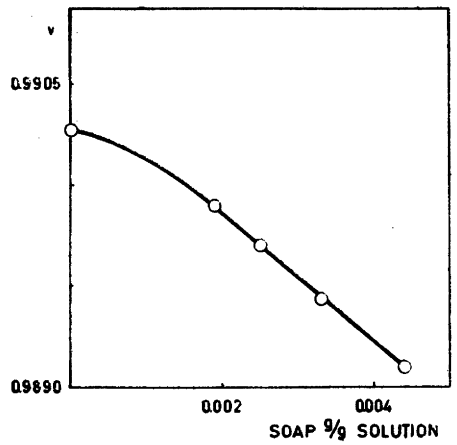

Fig. 1. Specific volume of the soap solution vs. *concentration of soap.*

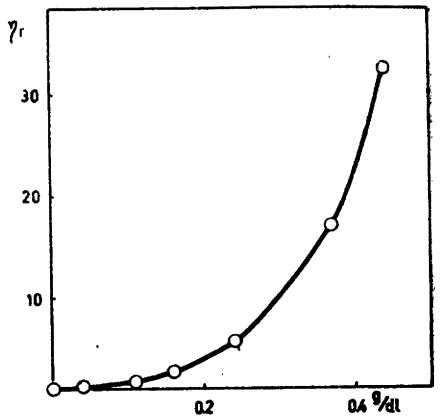

Fig. 2. Relative viscosity as a function of concentration.

pycnometer at 25° C for different soap concentrations using 0.10 *M* CsCl as solvent (Fig. 1).

Result: $\overline{V}_1 = 0.668 \pm 0.002$.

VISCOSITY

The viscosities of the soap solutions were studied at a temperature of 25° C in an Ostwald viscometer with an effluent time for water of 28.7 sec. at this temperature. 0.10 *M* CsCl was used as solvent.

From Fig. 2 it is evident that the relative viscosity, η_r, of the soap solutions increases rapidly with increasing soap concentration.

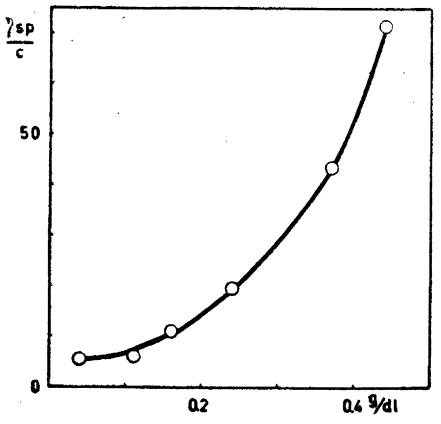

Fig. 3. Dependence of η_{sp}/c upon concentration.

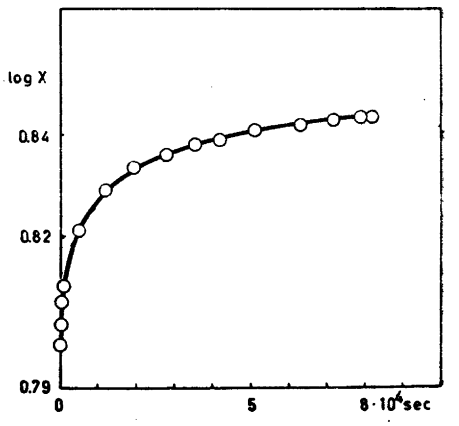

Fig. 4. log x = f (t) for r.p.s. = 300.

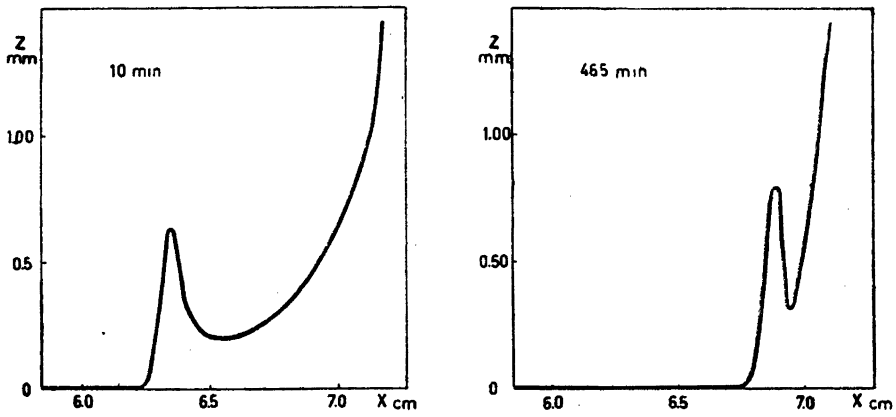

Fig. 5. Sedimentation diagrams for r.p.s. = 300.

Fig. 3 shows that the concentration dependence of the reduced viscosity, η_{sp}/c (in dl/g), is very pronounced.

SEDIMENTATION

In the sedimentation runs [2] the following equipment was used

1) A low-speed ultracentrifuge ($<$ 250 r.p.s.) and a cell distance of 5.2 cm from the axis of rotation,
2) A high-speed ultracentrifuge ($>$ 250 r.p.s.) and a cell distance of 6.5 cm from the axis.

0.10 *M* CsCl was used as solvent. The temperature varied between 20° and 24° C.

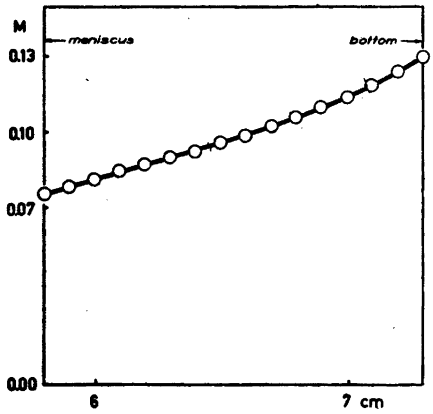

Fig. 6. Concentration of the original 0.10 M CsCl solution vs. the distance from the axis. r.p.s. = 800, length of run = 390 min.

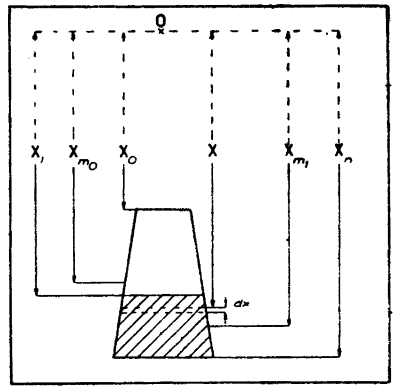

Fig. 7. The cell.

The sedimentation constant, s, is given by

$$s = \frac{\frac{dx}{dt}}{\omega_2 x} \tag{2}$$

where

x = the distance of the boundary (solvent/solution) from the axis of rotation

t = time after full speed

ω = angular velocity.

Thus from (2)

$$\log x = \frac{\omega^2 s}{\ln 10} \cdot t + c$$

and $\log x = f(t)$ is represented by a straight line if s is a constant.

Sedimentation runs were made for a soap concentration of 0.29 % (by weight) at rotor speeds of 150, 200, 300, 600 and 800 r.p.s. The boundary (solvent/solution) was very sharp. From Figs. 4 and 5 it is evident that the sedimentation is very much retarded which may mean that the solution has the character of a gel [3]. Naturally the retarded sedimentation also depends on the sedimentation of the CsCl solvent. From the refractive index gradient, the concentration of the cesium chloride was easily determined in every point of the cell at different times from the start of the run (Fig. 6).

For a rotor speed of 600 r.p.s. and t = 150 min. the concentration of the cesium chloride at the meniscus was 0.0866 M, while that at the bottom was 0.1143 M.

For r.p.s. of 300, no sedimentation of CsCl was detected. Thus from the above, it follows that for r.p.s. $\leq$ 300, the cause of the retardation is due only to the gel character of the solution.

GEL PRESSURE

The gel pressure was studied by sedimentation measurements.

Notations:

c_0 = the original concentration of the gel (at start)

c = » » » » (during run)

V = partial specific volume » » »

ς_s = density of the solvent

ω = angular velocity.

Notations, illustrated in Fig. 7:

O = the axis of rotation

x_0 = the distance of the meniscus from the axis of rotation

x_n = » » » » bottom » » » » »

x_1 = » » » » boundary » » » » »

$$x_{m_0} = \frac{x_0 + x_n}{2}$$

$$x_{m_1} = \frac{x_1 + x_n}{2}$$

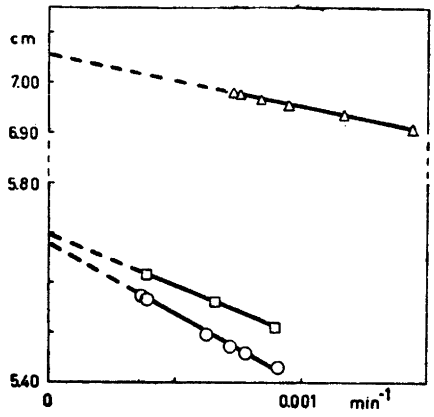

Fig. 8. $x = f\left(\frac{1}{t}\right)$. ○: *r.p.s.* = *150*, □: *r.p.s.* = *200*, △: *r.p.s.* = *300.*

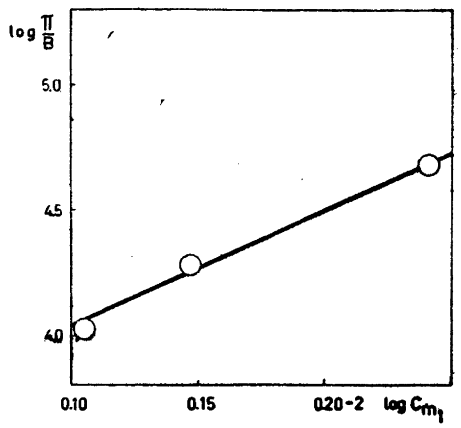

Fig. 9. $log\ \frac{\pi}{B} = f\ (log\ c_{m_1})$.

The gravitational force acting on the volume element (base-area = 1, height = dx) is

$$dP = c(1—V\varsigma_s)\omega^2 x dx$$

The pressure on any area with the distance x_{m_1} from the axis is

$$P_{m_1} = \int_{x_1}^{x_{m_1}} c(1—V\varsigma_s)\omega^2 x dx \qquad (3)$$

Let the gel pressure be denoted by π. Then at equilibrium the gel pressure at x_{m_1} is given by $P_{m_1} = \pi$.

The concentration at x_{m_1} is to a good approximation given by

$$c_{m_1} = c_0 \frac{x_{m_0}}{x_{m_1}} \cdot \frac{x_n—x_0}{x_n—x_1}$$

In eq. (3) $c \approx c_{m_1}$ and $1—V\varsigma_s = B$ = constant.

At equilibrium:

$$\frac{\pi}{B} = \frac{c_{m_1}\omega^2}{2}(x_{m_1}^2 — x_1^2)$$

By plotting the position (x) of the gel/solvent boundary *vs.* $\frac{1}{t}$ (t = time after full speed) the equilibrium position (x_1) was determined by extrapolation to infinite time (Fig. 8).

Thus c_{m_1} and $\frac{\pi}{B}$ could be readily calculated (Table 1).

Freundlich and Posnjak [4] have shown that

$\pi = Ac^k$

where

π = gel pressure
c = gel conc.
A = constant
k = constant $(2 < k < 5)$

thus $\frac{\pi}{B} = \frac{A}{B} \cdot c^k$

whence $\log \frac{\pi}{B} = k \log c + \text{constant}$

and k is easily determined if $\frac{\pi}{B}$ and c are known.

The constant k was found to be 4.6 (Fig. 9).

Table 1.

c_0	ω	x_0	x_n	x_1	$\log c_{m_1}$	$\log \frac{\pi}{B}$
$2.9 \cdot 10^{-3}$	300 π	4.432	5.995	5.677	0.105−2	4.015
$2.9 \cdot 10^{-3}$	400 π	4.368	5.995	5.697	0.147−2	4.280
$2.9 \cdot 10^{-3}$	600 π	5.828	7.276	7.056	0.242−2	4.686

NOTES ON DIBASIC SOAPS

Sedimentation runs were also done with the dicesiumsalts of hexadecane-dicarboxylic-acid-(1,16) and dodecane-dicarboxylic acid-(1,12) in various media:

1) H_2O
2) CsBr (0.1—0.6 *M*)
3) 0.1 *M* CsBr + 0.1 *M* Cs_2CO_3.

The solubility of the soaps was about 4 % by weight in water and about 2 % in 0.1 *M* CsBr.

The soap concentrations used were 0.4—2.8 % (by weight); the rotor speed was 1 000 r.p.s. and the temperature varied between 21° and 23° C.

No larger aggregates (micelles) were found in these solutions.

The author wishes to acknowledge his indebtness to Professor Stig Claesson for his very kind interest and for the many facilities which were made available.

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On the Exchange of Sulphur between Dithionite and Thiosulphate and between Tetrathionate and Sulphate Ions

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The exchange of sulphur atoms between dithionite and thiosulphate ions as well as between tetrathionate and sulphate ions has been investigated at room temperature by a tracer method using ^{35}S as a radioactive indicator.

The former system was investigated in two different solutions, an alkaline (pH ~13), and a slightly acid one (pH ~6), the time of exchange being varied with an upper limit of 44 h in the alkaline solution and 1.25 h in the weakly acid one. No exchange of sulphur atoms could be detected.

The system tetrathionate-sulphate was studied in neutral to weakly acid solutions with a maximum time of exchange of 44 h. No exchange of sulphur atoms could be detected.

In a previous paper [1] it has been reported that the exchange of sulphur atoms between dithionite and thiosulphate as well as between tetrathionate and sulphate ions was being studied. The present paper gives a full report of the experimental procedure and the results of the investigation.

EXPERIMENTAL

Syntheses. The isotopic exchange reactions were performed using ^{35}S as labelled atoms. The ^{35}S preparations were supplied by A.E.R.E., Harwell, Berkshire, England.

The following active ions were prepared: $S^*SO_3^{2-}$, $SS^*O_3^{2-}$, $O_3SS^*SSO_3^{2-}$, $O_3S^*SSSO_3^{2-}$, and $S^*O_4^{2-}$; S* representing the radioactive isotope ^{35}S. The two different radioactive thiosulphate ions were prepared according to conventional methods [2], *i. e.* boiling an alkaline sulphite solution together with solid sulphur, one of the reactants being labelled with ^{35}S. The following reactions take place:

$$SO_3^{2-} + S^*_{(s)} = S^*SO_3^{2-} \quad (1)$$

$$S^*O_3^{2-} + S_{(s)} = SS^*O_3^{2-} \quad (2)$$

* The author, unfortunately, died suddenly before completing this investigation. This paper presenting the results obtained by him, has been written by Mr. B. Mueller.

After the boiling, the solutions were filtered in order to separate any solid sulphur that might remain. It was checked that the solutions did not contain any unreacted sulphite ion. To a small part of the solution was added a strontium solution. If no precipitate appeared all sulphite ion had been used up in reactions (1) and (2).

The radioactive sulphite solution used for reaction (2) was produced by combusting radioactive sulphur in a stream of air which was sucked into a wash bottle containing a dilute sodium hydroxide solution.

The two tetrathionate ions mentioned above were prepared according to reactions (3) and (4).

$$S^*SO_3^{2-} + I_2 + S_2O_3^{2-} = O_3SS^*SSO_3^{2-} + 2I^- \quad (3)$$

$$SS^*O_3^{2-} + I_2 + S_2O_3^{2-} = O_3S^*SSSO_3^{2-} + 2I^- \quad (4)$$

These preparations were made partly according to a method described by Sander [3] and modified by Martin and Metz [4] resulting in a solid product, and partly by pouring active thiosulphate solution into an excess of iodine solution. When using the latter method the preparations were made immediately before starting an exchange experiment.

The radioactive sulphate ions were made by oxidation of sulphur dioxide in a 5 % hydrogen peroxide solution.

The commercially available preparations of sodium dithionite are highly contaminated by decomposition products, *e. g.* sulphite, thiosulphate, sulphate. These preparations had thus to be purified. From their solution in dilute sodium hydroxide, the unstable hydrate $Na_2S_2O_4 \cdot 2H_2O$ was precipitated by addition of solid sodium hydroxide. On heating to about 60° C, the hydrate lost its water of hydration. The product thus formed was freed from the mother-liquid by washing with absolute alcohol and finally dissolved in about 0.1 *M* sodium hydroxide solution, this solution being stored in a stoppered bottle in a nitrogen atmosphere.

Other chemicals used were of analytical reagent grade quality.

Analyses. The analyses of sodium dithionite were performed according to Zocher and Saechtling [5]. The metallic silver was separated and determined by Volhard titration.

The tetrathionate preparations are analyzed according to a method given by Kurtenacker [6]. The solid preparations contained 99 % sodium tetrathionate and about 1 % thiosulphate.

PROCEDURE USED IN EXCHANGE EXPERIMENTS

1. The system $S_2O_4^{2-} - S_2O_3^{2-}$. The isotopic exchange reactions were performed at room temperature — some in an alkaline solution (0.1 *M* NaOH, pH ~ 13) and some in a slightly acid solution (acetate buffer, pH ~ 6). In the alkaline medium, a radioactive thiosulphate solution was mixed with an inactive sodium dithionite solution, the total concentrations of the two ions being both 0.015 *M*. The mixture was stored in a closed glass bottle in a nitrogen atmosphere to avoid unnecessary oxidation. The exchange was interrupted by oxidizing the solution with iodine. If the oxidation is carried out in a slightly acid solution the following well known reactions take place:

$$S_2O_4^{2-} + 3I_2 + 4H_2O = 2SO_4^{2-} + 6I^- + 8H^+ \quad (5)$$

$$2S_2O_3^{2-} + I_2 = S_4O_6^{2-} + 2I^- \quad (6)$$

To obtain an oxidation at the right acidity, an iodine-iodide solution containing an acetate buffer (pH = 5) was prepared. The exchange solution was poured into an excess of this solution. The sulphate ions produced according to reaction (5) were precipitated by the addition of an excess of barium ions. The barium sulphate precipitate, No. 1, was separated from the mother liquor by centrifugation. The tetrathionate ions formed according to (6) were oxidized by boiling the remaining solution with bromine-water. The sulphate ions now formed were precipitated as barium sulphate, No. 2. The sulphur atoms in ppt. No. 1 thus originated exclusively in the dithionite ions while ppt. No. 2 only contained sulphur atoms which existed in the thiosulphate ions at the end of the exchange.

In the acid solution the procedure was slightly altered. Owing to the rather rapid decomposition of dithionite in such a medium the pH of the solutions containing dithio-

nite were lowered to *ca.* 6 immediately before starting the exchange experiments. In these solutions the dithionite ions decompose giving thiosulphate, hydrogen sulphite, sulphate and sulphur. According to Van Der Heijde [7] there is an instantaneous and complete exchange of sulphur atoms between dithionite and hydrogen sulphite ions in neutral and acid solutions. He could not detect any exchange in alkaline solutions. In order to avoid errors caused by an exchange of sulphur atoms with hydrogen sulphite this component was separated from the dithionite solutions by adding an excess of strontium ions. All of the strontium sulphite precipitate was removed by filtration before adding the radioactive thiosulphate solution and starting the exchange. As the dithionite decomposes continually, fresh precipitate is formed in the exchange solution all the time. To avoid mixing this precipitate with the strontium sulphate formed by the oxidation of dithionite ions with iodine at the end of the exchange, the exchange solution was made to pass through a filter paper directly into the iodine-iodide solution. The strontium sulphate thus formed (ppt. No. 1) contained only the sulphur atoms from the dithionite ions still present at the end of the exchange. The mother liquor was treated in the same way as described above, the only difference being that ppt. No. 2 as well as No. 1 consisted of strontium sulphate instead of barium sulphate.

Due to the greater rate of decomposition of dithionite ions in the slightly acid solution than in the alkaline one, the time of exchange had to be shortened, the time used being 75 minutes instead of up to 44 hours in the latter medium.

2. The system $S_4O_6^{2-} - SO_4^{2-}$. These exchange experiments were made at room temperature in a neutral medium. In such a solution the tetrathionate ions disintegrate, partly forming sulphate ions [8]. It is therefore necessary to remove the sulphate ions so formed before mixing the radioactive tetrathionate solution with the inactive sulphate solution. This is done by the addition of a known amount of barium ions. All sulphate ions as well as the thiosulphate ions that might be present are thus precipitated. Finally inactive sulphate ions are added in such a quantity that the concentration ratio, tetrathionate:sulphate, will be about 1:2 in the solution. The precipitates were separated from the mixture by filtering. The time of exchange in these experiments varied between 10 min. and 44 h. The exchange was interrupted by addition of a surplus of barium ions. The precipitated sulphate was separated from the mother liquor by centrifugation. The tetrathionate ions in the mother liquor were oxidized to sulphate ions and precipitated as barium sulphate by the same method as previously described. These two precipitates were recovered for subsequent activity measurements.

To check the results obtained, one experiment was carried out using radioactive sulphate ions and inactive tetrathionate ions.

Sample preparation and Counting Techniques. The sulphate precipitates, the radioactivity of which had to be determined were prepared according to Hevesy [9]. In order to avoid the necessity of correcting the measured activities for selfabsorption all samples were of "infinite thickness". The measured activities of different samples are hereby directly comparable. All activity measurements were performed over a period of at least five minutes, using a bell-jar counter with a very thin window connected to a "SC 33 1000 Scaler" from Tracerlab. Inc., Boston, Mass., U. S.

RESULTS

1. The system $S_2O_4^{2-}$—$S_2O_3^{2-}$

Owing to the inequality of the sulphur atoms in thiosulphate [10], separate isotopic exchange experiments were carried out with the two different thiosulphate ions synthesized as described above. Table 1 presents some detailed experimental results.

All activity values in Table 1 as well as in Table 2 are the measured activities minus the natural background activity.

Table 1.

Expt. No.	Active ions used	Time of exchange min	pH	Counts per min for ppt. No. [a] 1	2	3	Fraction of original activity for ppt. No. [a] 1	2
1	$S^*SO_3^{2-}$	2 640	13	109	3 829	4 061	0.03	0.95
2	$SS^*O_3^{2-}$	0.67	13	263	5 977	5 787	0.05	1.03
3	—»—	286	13	286	5 564	5 787	0.05	0.96
4	—»—	1 380	13	256	5 657	5 787	0.04	0.98
5	$S^*SO_3^{2-}$	75	6	3	3 529	4 256	0.00	0.83

The results indicate that under the prevailing circumstances no exchange of sulphur atoms between dithionite and thiosulphate ions occurs. The rather small activities recorded for ppt. No. 1 are presumably, at least, partly due to the decomposition of the tetrathionate ion formed by the iodine oxidation, the tetrathionate ions being far from stable in the slightly acid solution [8]. The fact that the measured activities of ppt. No. 1 in experiments Nos 2, 3, and 4 are practically constant although the time of exchange is varied from 40 sec. to 23 h is definite evidence for the absence of a measurable exchange of sulphur atoms between dithionite ions and the central sulphur atom in thiosulphate ions within 23 h. If a slow exchange had occurred the recorded activities for ppt. No. 1 should have changed with time according to a reaction of the first order [11]. In expt. No. 1 the activity of ppt. No. 1 is smaller than in expt. No. 4 although the time of exchange is almost doubled. This indicates that not even the outer sulphur atom in the thiosulphate ion takes part in any exchange with the sulphur atoms of dithionite ion.

The fact that for expt. No. 5 the sum of the activities of the ppts. Nos. 1 and 2 are definitely smaller than for ppt. No. 3, may be explained thus: Certain decomposition products of the dithionite ions, for instance $S_2O_3^{2-}$, are during the separation precipitated together with the initially-labelled thiosulphate, resulting in a reduction of the registered activity. For the other experiments these differences are within the limits of experimental error.

2. The system $S_4O_6^{2-}$—SO_4^{2-}

In these experiments the two radioactive tetrathionate ions formed according to reactions (3) and (4) were used in different experiments. Table 2 gives some results.

From the results tabulated below it is evident that the variations with time of the activities of ppt. No. 1 for expts. Nos. 1, 2 and 3 do not follow a reaction of the first order. This is evidence for the absence of a measurable rate of exchange of sulphur atoms between $O_3SS^*SSO_3^{2-}$ and SO_4^{2-} within 43 h[11]. For the other tetrathionate ion no variation of the time of exchange has

[a] Ppt. No. 1 contains the sulphur atoms of the previously inactive dithionite ions.
Ppt. No. 2 contains the sulphur atoms of the previously active thiosulphate ions.
Ppt. No. 3 contains the sulphur atoms of the originally radioactive thiosulphate directly oxidized to sulphate and precipitated as barium sulphate.

Table 2.

Expt. No.	Active ions used	Time of exchange min.	Exchange solution	Counts per min. for ppt. No. [a] 1	2	3	Fraction of original activity for ppt. No. [a] 1	2
1	$O_3SS^*SSO_3^{2-}$	10	Water	96	1 743	1 757	0.05	1.00
2	—»—	1 620	—»—	80	1 641	1 607	0.05	1.02
3	—»—	2 580	—»—	306	3 829	4 061	0.08	0.94
4	$O_3S^*SSSO_3^{2-}$	2 640	Acetate buffer pH = 6.2	72	5 874	5 890	0.01	1.00
5	$S^*O_4^{2-}$	5	Water	45	1 783	2 611	0.02	0.68

been made. As the activity of ppt. No. 1 is smaller in expt. No. 4 than in expts. Nos. 1, 2 and 3 it is evident that no exchange occurred in this case either, within 44 h.

The considerable loss of activity of the originally-radioactive sulphate ions in expt. No. 5. after the exchange is due to the fact that a certain amount of the tetrathionate had formed sulphate ions by decomposition. In this experiment no separation of sulphate ions in the tetrathionate solution was performed before adding the radioactive sulphate ions to the solution.

DISCUSSION

As no exchange of sulphur atoms between dithionite and thiosulphate ions was found, it is not possible to conclude anything about the structure of the dithionite ions as regards equality or inequality of the chemical bonds between the sulphur atoms and the remaining part of the ion.

Regarding the system tetrathionate-sulphate the results prove once again the great stability of the bonds of the sulphur atom in the sulphate ion. No information about the chemical bonds in the tetrathionate ion, including the possible inequality of the chemical bonds of the different sulphur atoms as anticipated above, is given by the results obtained. *Cf.* a recent determination of the crystal structure of a tetrathionate compound [12].

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[a] Ppt. No. 1 contains the sulphur atoms of the originally inactive ions.
Ppt. No. 2 contains the sulphur atoms of the originally radioactive ions having been separated from the exchange solution.
Ppt. No. 3 contains the originally radioactive sulphur atoms directly oxidized to sulphate and precipitated as barium sulphate for activity measurements.

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Some Considerations Regarding the Nomenclature of Organic Phosphorus Compounds

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The old American, British and German nomenclatures of organic phosphorus compounds and the new Anglo-American proposals are discussed, and the new Swedish proposals are presented. The Swedish nomenclature is based on the designation of all organic derivatives of tervalent phosphorus as *phosphines*, and all derivatives of quinquevalent phosphorus containing oxygen as *phosphine oxides*, or alternatively as derivatives of *phosphoryl* compounds.

The chemistry of organic phosphorus compounds, long a borderline between organic and inorganic chemistry, has in recent years attracted considerable interest. The vast number of compounds synthesized by the German chemist Michaelis [1] during the latter half of the nineteenth century, and by Arbusow [2] in Russia in the beginning of this century, have, for purely academic reasons, once more assumed current interest owing to their applicability as solvents, surfaceactive agents, lubricating-oil additives, plasticizers, insecticides, drugs, and, with regard to the most toxic members of the group, as war gases. In the forefront is the advent of a series of new insecticides, synthesized by Schrader [3], some of which show entirely new properties as chemotherapeutic agents for plants.

The use of these compounds by chemists, biochemists, pharmacologists, and technicians in different countries calls for the use of a readily understandable international nomenclature. However, there is considerable confusion in this respect, not only on the international plane but also in individual countries. To facilitate a survey of the profusion of names in current use, Table 1 has been drawn up containing 37 compounds and their designations in the U.S.A., Britain, and Germany. The U.S.A. is represented not only by Chemical Abstracts [4] but by Kosolapoff [5], who in his book "Organophosphorus Compounds" has proposed a nomenclature somewhat differing from that of Chemical Abstracts. Britain is represented by British Chemical Abstracts, and Germany by Beilstein [6]. As regards Beilstein's nomenclature, only the names

Table 1. Examples of the old American, British and German

Formula	Present name in		
	USA I [4]	USA II [5]	Britain
RPH_2	Alkylphosphine	Alkylphosphine	Alkylphosphine
$RP=O$	—	—	—
$RP=NR$	—	—	—
$RP(OH)_2$	Alkanephosphonous acid	Alkanephosphonous acid	Alkylphosphinic acid
$RP(OR)_2$	Dialkyl alkanephosphonite	Dialkyl alkanephosphonite	Dialkyl alkylphosphinate
$RP(OR)Cl$	Alkyl alkanechlorophosphinite	Alkyl alkanechlorophosphinite	—
$RP(OR)(NR_2)$	Alkyl dialkylamidoalkanephosphinite	Alkyl dialkylamidoalkanephosphinite	—
$R_2P(OH)$	Dialkylphosphinous acid	Dialkylphosphinous acid	Dialkylphosphinous acid
$R_2P(OR)$	Alkyl dialkylphosphinite	Alkyl dialkylphosphinite	Alkyl dialkylphosphinite
R_2PCl	Dialkylphosphinous chloride	Dialkylchlorophosphine	—
$R_2P(NR_2)$	Dialkyl-dialkylphosphinous amide	Dialkyl-dialkylphosphine amide	Dialkyl-dialkylaminophosphine
$(RO)_3P$	Trialkylphosphite	Trialkylphosphite	Trialkylphosphite
$(RO)_2PCl$	Dialkyl chlorophosphite	Dialkyl chlorophosphite	Dialkoxychlorophosphine
$(RO)(R_2N)PCl$	Alkyl dialkylamidochlorophosphite	Alkyl-chloro-dialkylamidophosphite	—
$(RO)P=NR$	Alkoxyalkyliminophosphine	Alkyl-alkylimidophosphite	Alkoxyalkyliminophosphine
R_4PCl	Tetralkylphosphonium chloride	Tetralkylphosphonium chloride	Tetralkylphosphonium chloride
R_3PO	Trialkylphosphine oxide	Trialkylphosphine oxide	Trialkylphosphine oxide
$R_3P=NR$	Trialkyl-alkylphosphine imide	Trialkylphosphine alkylimine	Trialkylphosphine alkylimine
$RPO(OH)_2$	Alkanephosphonic acid	Alkanephosphonic acid	Alkylphosphonic acid
$RPO(OR)_2$	Dialkyl alkanephosphonate	Dialkyl alkanephosphonate	Dialkyl alkylphosphonate
$RPO(OR)Cl$	Alkyl alkanechlorophosphonate	Alkyl chloroalkanephosphonate	Alkylalkoxychlorophosphine oxide
$RPOCl_2$	Alkanephosphonic dichloride	Alkanephosphonyl dichloride	Alkyldichlorophosphine oxide
$RPO(OR)(NR_2)$	Alkyl dialkylamidoalkanephosphonate	Alkyl dialkylamidoalkanephosphonate	Alkylalkoxydialkylaminophosphine oxide
$RPO(NR_2)Cl$	Dialkylamidoalkanephosphonic chloride	Chlorodialkylamidoalkanephosphonate	Alkylchlorodialkylaminophosphine oxide
$R(RO)_2P=NR$	Dialkyl alkylimidoalkanephosphonate	Dialkyl alkylimidoalkanephosphonate	—
$RHPO(OH)$	Alkanephosphinic acid	Alkanephosphonic acid	Alkylphosphonous acid
$R_2PO(OH)$	Dialkylphosphinic acid	Dialkylphosphonic acid	Dialkylphosphonous acid
$R_2PO(OR)$	Alkyl dialkylphosphinate	Alkyl dialkylphosphonate	Alkyl dialkylphosphonite
R_2POCl	Dialkylphosphinic chloride	Dialkylphosphonyl chloride	Dialkylchlorophosphine oxide

nomenclatures and the new Anglo American and Swedish proposals.

Present name in	Suggested name in	
Germany [6]	USA and Britain [7]	Sweden
Alkylphosphin	Alkylphosphine	Alkyl-phosphine
Alkylphosphinigsäureanhydrid	Alkyloxophosphine	Alkyl-oxophosphine
—	Alkyl(alkylimino)phosphine	Alkylimino-alkyl-phosphine
Alkylphosphinigsäure	Alkylphosphonous acid	Alkyl-dihydroxy-phosphine
Alkylphosphinigsäure-dialkyl-ester	Dialkyl alkylphosphonite	Dialkoxy-alkyl-phosphine
Alkylphosphinigsäure-alkylester-chlorid	Alkyl alkylphosphonochloridite	Alkoxy-alkyl-chloro-phosphine
Alkylphosphinigsäure-alkylester-dialkylamid	Alkyl dialkyl-alkylphosphon-amidite	Dialkylamino-alkoxy-alkyl-phosphine
Dialkylhydroxyphosphin	Dialkylphosphinous acid	Dialkyl-hydroxy-phosphine
Dialkylalkoxyphosphin	Alkyl dialkylphosphinite	Alkoxy-dialkyl-phosphine
Dialkylphosphin-chlorid	Dialkylphosphinous chloride	Dialkyl-chloro-phosphine
Dialkyl-dialkylamino-phosphin	Dialkyl-dialkylphosphinous amide	Dialkylamino-dialkyl-phosphine
Trialkylphosphit	Trialkyl phosphite	Trialkoxy-phosphine or trialkyl phosphite
Dialkylphosphorigsäure-chlorid	Dialkyl phosphorochloridite	Dialkoxy-chloro-phosphine
Phosphorigsäure-alkylester-clhorid-dialkylamid	Alkyl dialkylphosphoramido-chloridite	Dialkylamino-alkoxy-chloro-phosphine
Phosphorigsäure-alkylester-alkylimid	Alkyl alkylphosphenimidite	Alkylimino-alkoxy-phosphine
Tetralkylphosphonium chlorid	Tetralkylchlorophosphoran	Tetralkylphosphonium chloride
Trialkylphosphinoxyd	Trialkylphosphine oxide	Trialkyl-phosphine oxide
Trialkylphosphin-alkylimid	Trialkyl-alkylphosphinimide	Trialkyl-phosphine-alkylimide
Alkylphosphonsäure	Alkylphosphonic acid	Alkyl-dihydroxy-phosphine oxide
Alkylphosphonsäure-dialkylester	Dialkyl alkylphosphonate	Dialkoxy-alkyl-phosphine oxide
Alkylphosphonsäure-alkylester-chlorid	Alkyl alkylphosphonochloridate	Alkoxy-alkyl-chloro-phosphine oxide or alkoxy-alkyl-phosphoryl chloride
Alkylphosphonsäure-dichlorid	Alkylphosphonic dichloride	Alkyl-dichloro-phosphine oxide or alkyl-phosphoryl dichloride
Alkylphosphonsäure-alkylester-dialkylamid	Alkyl-alkyl-dialkylphosphon-amidate	Dialkylamino-alkoxy-alkyl-phosphine oxide
Alkylphosphonsäure-chlorid-dialkylamid	Alkyl-dialkylphosphonamidic chloride	Dialkylamino-alkyl-chloro-phosphine oxide or dialkylamido-alkyl-phosphoryl chloride
Alkylphosphonsäure-dialkyl-ester-alkylimid	Dialkyl alkyl-alkylphosphon-imidate	Dialkoxy-alkyl-phosphine-alkylimide
Alkylphosphinigsäure	Alkylphosphinic acid	Alkyl-hydroxy-phosphine oxide
Dialkylphosphinigsäure	Dialkylphosphinic acid	Dialkyl-hydroxy-phosphine oxide
Dialkylphosphinigsäure-alkyl-ester	Alkyl dialkylphosphinate	Alkoxy-dialkyl-phosphine oxide
Dialkylphosphinigsäure-chlorid	Dialkylphosphinic chloride	Dialkyl-chloro-phosphine oxide or dialkyl-phosphoryl chloride

Continued Table 1.

Formula	Present name in		
	USA I [4]	USA II [5]	Britain
$R_2PO(NR_2)$	Dialkyl-dialkylphosphinic amide	Dialkyl-dialkylphosphone amide	Dialkyl-dialkylaminophosphine oxide[6]
$R_2(RO)P=NR$	Alkyl alkylimidodialkylphosphinate	Alkyl alkylimidodialkylphosphonate	—
$(RO)_3PO$	Trialkyl phosphate	Trialkyl phosphate	Trialkyl phosphate
$(RO)_2POCl$	Dialkyl chlorophosphate	Dialkyl chlorophosphate	Dialkyl chlorophosphonate
$(RO)_2(R_2N)PO$	Dialkyl dialkylamidophosphate	Dialkyl dialkylamidophosphate	—
$(RO)(R_2N)POCl$	Alkyl dialkylamidochlorophosphate	Alkyl chlorodialkylamidophosphate	—
$(R_2N)POCl_2$	Dialkylamidophosphoric dichloride	Dichlorodialkylamidophosphate	Dichlorodialkylaminophosphine oxide
$(RO)_3P=NR$	Trialkyl alkylimidophosphate	Trialkyl alkylimidophosphate	—

proposed in Ergwk. are included in the table in cases where Hptwk. and Ergwk. have different nomenclatures. Furthermore, the new Anglo-American proposals [7] are incorporated in the table.

In attempting a systematic designation of organic phosphorus compounds containing one phosphorus atom, acids of phosphorus have generally been taken as a basis. Consequently the following eight acids, regardless of whether they exist as such or not, have to be employed:

$(HO)_3P$	$(HO)_3PO$
$HP(OH)_2$	$HPO(OH)_2$
$H_2P(OH)$	$H_2PO(OH)$
$(HO)PO$	$(HO)PO_2$

These acids, however, do not cover all possibilities, and it has been necessary to designate the remaining compounds as derivatives of the following hydrides:

$$H_3P \qquad H_3PO \qquad H_5P$$

The possibility also exists of basing a nomenclature entirely on the last three compounds, as was already attempted by Michaelis [1], whose nomenclature unfortunately never gained universal recognition. This principle is to be preferred, because so many substituents have been introduced into some compounds that they can scarcely be regarded as derivatives of phosphorus acids.

The naming of the above-mentioned acids has already led to considerable confusion. One example is that compounds of the structure $XPO(OR)_2$, where X = *halogen*, and R = *alkyl* or *aryl*, have in the U.S.A. been named *phosphates* but in Britain *phosphonates*. The reason for this is that the British nomenclature has extended *phosphonic acids* to include compounds symbolized

Continued Table 1.

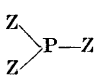
Present name in	Suggested name in	
Germany [6]	USA and Britain [7]	Sweden
Dialkylphosphinigsäure-dialkyl-amid	Dialkyl-dialkylphosphinic amide	Dialkylamino-dialkyl-phosphine oxide
Dialkylphosphinigsäure-alkyl-ester-alkylimid	Alkyl dialkyl-alkylphosphin-imidate	Alkoxy-dialkyl-phosphine-alkyl-imide
Trialkylphosphat	Trialkylphosphate	Trialkoxy-phosphine oxide or trialkyl phosphate
Phosphorsäure-dialkylester-chlorid	Dialkyl phosphorochloridate	Dialkoxy-chloro-phosphine oxide or dialkoxy-phosphoryl chloride
Phosphorsäure-dialkylester-dialkylamid	Dialkyl dialkylphosphoramidate	Dialkylamino-dialkoxy-phosphine oxide
Phosphorsäure-alkylester-chlorid-dialkylamid	Alkyl dialkylphosphoramido-chloridate	Dialkylamino-alkoxy-chloro-phosphine oxide or dialkylamido-alkoxy-phosphoryl chloride
Phosphorsäure-dichlorid-dialkyl-amid	Dialkylphosphoramidic di-chloride	Dialkylamino-dichloro-phosphine oxide or dialkylamido-phosphoryl dichloride
Phosphorsäure-trialkylester-alkylimid	Trialkyl phosphorimidate	Trialkoxy-phosphine-alkylimide

by $XPO(OH)_2$ and not limited the term to compounds of the structure $RPO(OH)_2$, as in the American nomenclature. A much more serious discrepancy is demonstrated by the use of the two acids $R_2PO(OH)$ and $RP(OH)_2$. In the American nomenclature *phosphinic acid* has been used for the former whereas the same name has been used for the second one in the British nomenclature; the British designation for the first acid has been *phosphonous acid*, which in the U.S.A. has been used for the second acid. The German nomenclature in general adheres to the use of names derived from corresponding acids, but is not consistent.

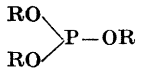
The nomenclature recently proposed in Britain and the U.S.A. [7] by the Organic Division's Advisory Committee on the Nomenclature of Organic Phosphorus Compounds constitutes an attempt to devise an entirely logical system covering all possibilities and all possible structures, whether existing now or not. In so doing, the committee invented a number of new names and endings, some of which may create further confusion. Quite apart from the linguistic point of view this nomenclature seems to be too perfectionistic, and the great number of endings makes it difficult and, indeed, unpractical.

To avoid the illogicalities apparent in earlier British and American nomenclature, and to exclude new unfamiliar names and endings, we have for several years used our own nomenclature, which has a simple construction and obviates misunderstanding. This nomenclature is set forth in the last column of Table 1. A few very simple rules make it readily comprehensible. Our nomenclature can be set forth according to the following simple principles.

I. All compounds containing tervalent phosphorus of the type



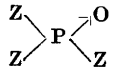
where Z is any univalent radical, are named *phosphines*. If all radicals are linked to phosphorus with oxygenbridges,



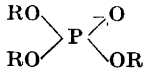
(R = *alkyl* or *aryl*)

the old designation *phosphite* may alternatively be used.

II A. Compounds containing quinquevalent phosphorus of the structure



where Z is any univalent radical, are named *phosphine oxides*. This does not exclude the use of ordinary designations, as *phosphates*, for compounds of the general structure



(R = *alkyl* or *aryl*)

Furthermore, the use of *phosphoryl* is recommended wherever practicable, for example in compounds containing *halide*, *cyanide*, etc., although it does not cover all possibilities. The employment of *phosphoryl* is justified by the common knowledge of that radical and by the ease with which it can be used in designating and pronouncing of a large number of compounds.

II B. The well-known designation of *phosphonium* compounds

$$R_4PX \qquad (R = \textit{alkyl} \text{ or } \textit{aryl} \text{ and } X = \textit{halide} \text{ etc.})$$

has not been considered necessary to change.

Structures formed by the operation of replacing one or two H's in H_3P or H_3PO respectively O in H_3PO are designated by the incorporation of appropriate affixes. The list of affixes is given in Table 2.

Table 1 does not show how compounds in which oxygen is substituted for sulphur, are named. Some authors use *thio* in such cases irrespective of how the sulphur atom is linked, though most of them use *thio* or *thiolo* only for compounds in which sulphur forms a bridge between phosphorus and another atom, whereas compounds with sulphur linked only to phosphorus is named by means of the prefix *thiono*. According to the new Anglo-American nomenclature the location of substituents in esters containing sulphur is denoted by the symbols O- and S-prefixed to the radical name; the symbol S is used in conjunction with the affix *thio*. The affixes *thiolo* and *thiono* are used only where ambiguity arises. Our proposal in this case appears from Table 2.

The radical R_2N- is designated as *dialkylamino* in *phosphines* and *phosphineoxides*, but in compounds denominated with the aid of *phosphoryl* the radical is named *dialkylamido-* to preserve the analogy to the well-known name *phosphoryltriamide*.

As to the order of the various radicals in denoting a compound, we suggest the following: *alkylimino-*, *alkylamino-*, *alkoxy-*, *alkyl-*, *hydroxy-*, *oxo-*, *halo-*

Table 2. List of affixes.

Replacement of	Affix in		
	H_3P	H_3PO According to	
		Phosphine oxide designation	Phosphoryl designation
Two H's by RN=	alkylimino-	alkylimino-	alkylimido-
O by RN=	—	-phosphine-alkylimide	—
H by R_2N-	dialkylamino-	dialkylamino-	dialkylamido-
H by RO-	alkoxy-	alkoxy-	alkoxy-
H by RS-	alkylmercapto-	alkylmercapto-	alkylmercapto-
H by R-	alkyl-	alkyl-	alkyl-
H by HO-	hydroxy-	hydroxy-	hydroxy-
H by HS-	mercapto-	mercapto-	mercapto-
Two H's by O=	oxo-	oxo-	oxo-
O by S=	—	-phosphine sulphide	-thiophosphoryl
H by F-	fluoro-	fluoro-	fluoride
H by Cl-	chloro-	chloro-	chloride
H by Br-	bromo-	bromo-	bromide
H by I-	iodo-	iodo-	iodide
H by NC-	cyano-	cyano-	cyanide
H by NCO-	cyanato-	cyanato-	cyanate

geno-. In Table 2 the radical R is consistently denoted as *alkyl*, but it may of course, also be an aromatic radical, *i. e. aryl*.

We are indebted to the Director of the Research Institute of National Defence, Dept. 1, Professor G. Ljunggren, for the kind interest he has shown in this work.

Our thanks are also due to Professors A. Fredga, K. Myrbäck and L. G. Sillén and D ipl. Eng. E. Engel for valuable discussions.

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A Study of the Phosphorus-Containing Proteins of Cells

II. The Turnover Rate of P^{32}-labelled Phosphoserine of the Schneider Protein Residues of Several Rat Organs

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The method for the study of acid-stable protein phosphorus of cells described previously [1] has been extended, and used in a study of protein phosphorus turnover. Of the several organs studied, liver and kidney were found to have the highest specific activity values for protein phosphoserine. The maximum incorporation for all organs investigated occurred at approximately three hours after injection of radioactive phosphate. The acid hydrolysate of the Schneider protein residue has been subdivided in about 50 separate fractions all containing phosphopeptides. The methods described have been of great aid in this procedure.

In the first paper of this series [1] a method was presented for the study of the more acid-stable phosphorus of cellular proteins. Using this method it was possible to isolate crystalline phosphoserine from calf liver protein. Preliminary experiments where the activity of the phosphoserine phosphorus of the rat liver protein was determined in animals killed ½, 4, and 24 hours after injection of radioactive inorganic phosphorus also indicated that the method was of use for tracer studies. The phosphorus bound to serine exhibited a high turnover rate. In a short communication [2] we have described the further extension of the method and its application to a tracer study of the rat liver protein phosphorus *.

METHODS

Groups of 10 male rats from the departmental colony averaging 300 g weight were used for each experiment. These rats are inbred and free of infection. Their care has been described by Hallgren [4]. They were fasted for approximately 18 hours before injection with P^{32}-phosphate (1 μC subcutaneously per g body weight corresponding to $3.05 \cdot 10^5$

* After these two articles had been submitted for publication a paper appeared by Kennedy and Smith describing a similar method and its use in demonstrating the presence of phosphoserine in mouse ascites tumor cells and in liver mitochondria.

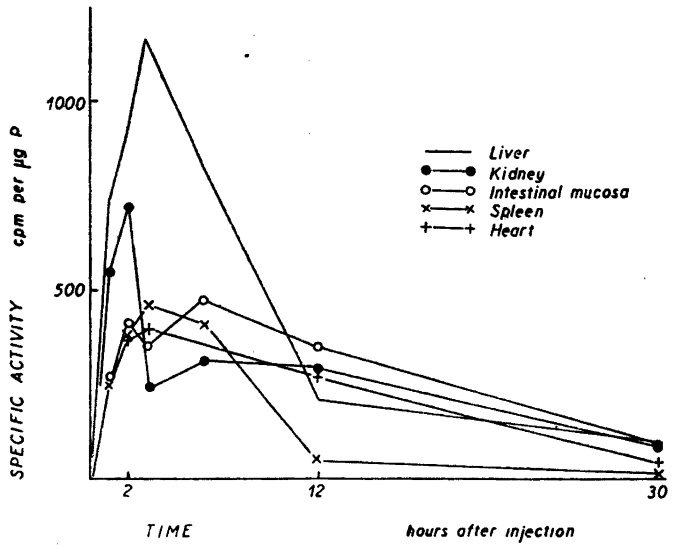

Fig. 1. Specific activities of phosphoserine fractions obtained from the Dowex 50 column after injection of 1 µC $H_3P^{32}O_4$ per g body weight.

cpm with the scaler used). After varying intervals of time the animals were killed and the Schneider protein fractions of various organs prepared and treated as described previously [1]. Subsequently, the Dowex 50 fractions were further resolved using the procedure of Busch, Hurlbert, and Potter [5] on Dowex 1 formate, 2 % DVB.

Paper chromatography of the phosphorus-containing fractions was carried out as before, except that when relatively large amounts were to be run Whatman No. 3 paper, washed according to Hanes, Hird, and Isherwood [6], was used. Scanning of the chromatograms was done according to the following procedure: the chromatogram is taped to an aluminium or brass strip, which is then mechanically moved under an end window Geiger tube (20th Century Electronics, type EW3H). The tube is enclosed in a lead chamber and is shielded from the chromatogram by an exchangeable slotted mask, generally having a slot width of 5 mm. The Geiger tube is connected to a counting rate meter and recorder as described in another paper [7] except that a Leeds and Northrup Speedomax Recorder 0–6 mV is used instead of a Micromax. The roller feeding the strip is connected to the roller in the chart recorder by a synchronous link, both having the same diameter to facilitate evaluation of the chromatogram.

For analysis of the small amounts of substances isolated, it was necessary to use a more sensitive method for phosphorus determination than that used previously. For digestion prior to phosphorus determination it was convenient to use the sealed tube method of Grunbaum, Schaffer and Kirk [8]. Subsequently, inorganic phosphorus was determined using a procedure based on that of Martin and Doty [9], but modified in so far as smaller volumes were used with micropipettes and the colorimetric determinations made in 5 cm cuvettes in a Beckman model B spectrophotometer. This method was not as sensitive as that described by Schaffer, Fong, and Kirk [10], but it allowed the determination of less than 0.1 µg phosphorus and it did not require special equipment.

Radioactivity measurements were made in duplicate on stainless steel planchettes; at least 1000 impulses were counted.

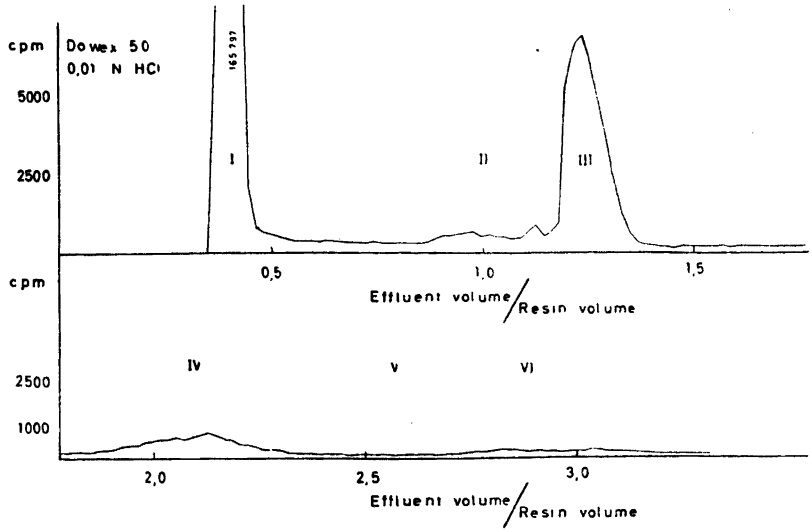

Fig. 2. Radioactivity curve from a hydrolysate of the rat liver protein fraction. The animals killed 3 hr after injection of 5 μC P^{32} per g body weight.

RESULTS

Preliminary data [2] showed that the phosphoserine fraction of a rat liver protein hydrolysate as isolated on a Dowex 50 column had a high specific activity with a maximum at approximately three hours after injection of radioactive phosphate. As is evident from Fig. 1, where the specific activity values of rat liver, kidney, spleen, intestinal mucosa, and heart phosphoserine fractions are compared, the only other organ having a comparatively high activity is the kidney.

Subsequent experiments indicated that each of the phosphorus-containing fractions obtained from Dowex 50 columns could be further subdivided on Dowex 1 columns. When this was done, it became apparent that while inorganic phosphate and phosphoserine dominate the respective Dowex 50 fractions, the peptide fractions are decidedly complex. Moreover, most of the peptide subfractions could be further subdivided using paper chromatography with benzene-propionic acid or *iso*butyric acid-ammonia.

In order to determine whether the phosphorus of the many fractions which could be obtained using the two types of ion exchange resin differed in specific activity, 10 rats were injected with 5 μc P^{32}-phosphate per g body weight. The animals were killed after three hours and the liver and kidney phosphopeptides fractionated. Fig. 2 is a photograph of the radioactivity curve of the rat liver protein hydrolysate obtained by plotting the number of impulses per minute in 1 ml aliquots of each tube from the fraction collector. To facilitate a comparison between different runs the ordinata has been expressed as parts of the volume of the resin bed. Only six comparatively well defined peaks can be

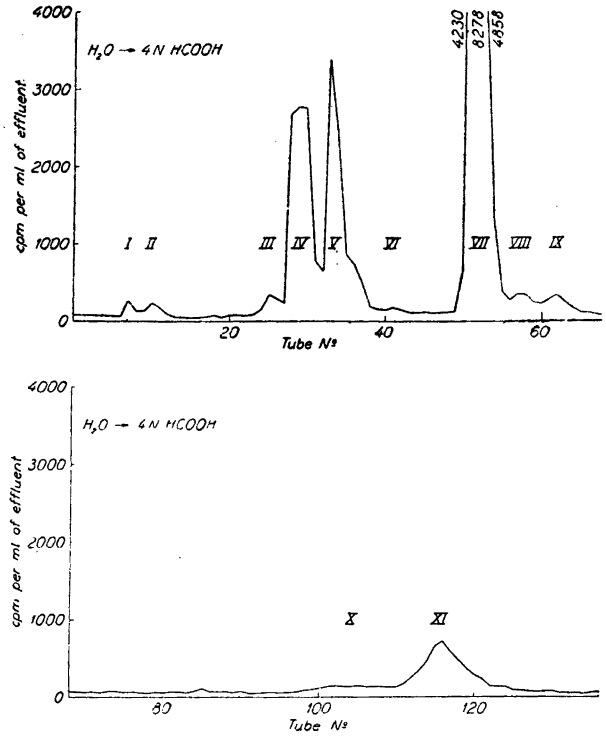

Fig. 3. Peak IV from the Dowex 50 column (Fig. 2) separated on a Dowex 1 column. Column dimensions: 11 × 185 mm.

seen, the first containing inorganic phosphorus, the second peptide bound phosphorus as well as peaks Nos. IV, V, and VI. The third peak mainly consists of phosphoserine. About 92 % of the radioactivity can be eluted from the column with 0.01 *N* HCl.

A qualitative amino acid analysis was also run on each of the fractions isolated in continuation of the study of the peptides present. Whereas the specific activity value of the pure phosphoserine was about 4 500 cpm per μg P the value of the other fractions ranged from 350 to 13 000. Fraction II from the Dowex 50 column (Fig. 2) yielded 15 fractions when Dowex 1 was used in conjunction with paper chromatography. Serine and glutamic acid were found to dominate most of these. About 89 % of the activity of fraction III from Dowex 50 was found to be located in one Dowex 1 peak. The material from this peak moved as one spot on chromatograms run in benzene-propionic acid Moreover, the only amino acid present after a further hydrolysis with 2 *N* HCl for 20 hours at 120° C was serine. This subfraction will henceforward be designated "chromatographically pure phosphoserine".

Like fraction II, fraction IV from the Dowex 50 column was also a complex mixture of peptides. Fig. 3 shows the Dowex 1 elution curve of this frac-

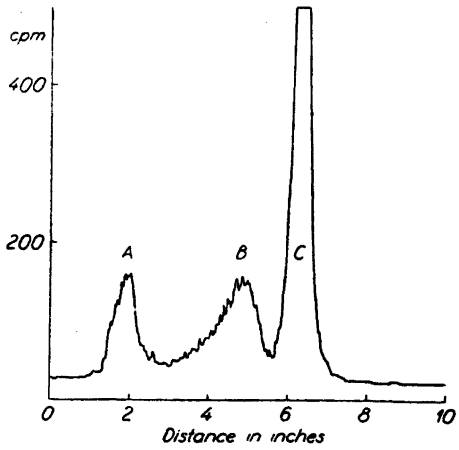

Fig. 4. A paper chromatogram from peak V (Fig. 3) scanned through an automatic device described in the text. Solvent system: benzene-propionic acid.

tion. Of special interest is peak No. VII which on further subdivision using paper chromatography could be shown to contain a fraction which after hydrolysis gave only threonine and traces of glycine (spec. activity 6 160).* Fig. 4 is a photograph of the radioactivity curve obtained when a one-dimensional (benzene-propionic acid) chromatogram of peak No. V was run through the scanning device. When the sections of the chromatograms corresponding to peaks A, B, and C were cut out and eluted, and the phosphopeptides hydrolysed, several amino acids were found to be present in each: alanine, glutamic acid, serine, and valine. In addition A contained glycine and leucine; B contained glycine; C contained threonine and tyrosine, with relatively larger quantities of glutamic acid than the two other.

The experiment where larger amounts of radioactive phosphate were injected also made it possible to detect two additional peaks, V and VI, emerging from the Dowex 50 column. Of these, VI was the strongest, and further fractionation indicated that it contained greater amounts of a peptide of serine, glutamic acid, and leucine. It was of interest that the specific activity of this peptide, 4 500, approximated that of chromatographically pure phosphoserine.

In this experiment it has also been possible to separate the first peak from the Dowex 50 column into seven fractions by the Dowex 1 procedure. About 84 % of the activity from the column moves as orthophosphoric acid. It has a specific activity of 955, the other fractions range from 782 to 2 395. These have not been hydrolysed and analysed for amino acids.

For comparison the TCA-soluble phosphorus-containing compounds from the same experiment have been fractionated according to the system described by Busch *et al.*[5] A part of the curve obtained is shown in Fig. 5. 26 fractions were collected and the specific activities of these were determined. They

* Since this paper was submitted for publication it has been possible to isolate crystalline phosphothreonine from a partial hydrolysate of Schneider protein from sheep liver.

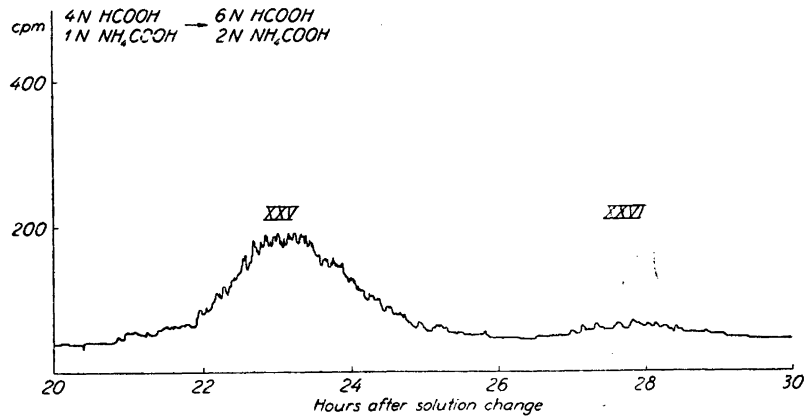

Fig. 5. The last part of the radioactivity curve obtained from the TCA-soluble substances from rat liver. The animals killed 3 hr after injection of 5 μC P^{32} per g body weight. Separation through a Dowex 1 (2 % DVB) column. Column dimensions: 26.7 × 280 mm. Automatic registration according to [7].

ranged from 62.1 to 148 000, most values lying between 3 000—8 000. So far it has not been possible to show the presence of free phosphoserine in this extract.

Having shown that the "phosphoserine fraction" from the Dowex 50 column was not completely homogeneous, it was felt that the determination of the specific activity values of chromatographically pure phosphoserine at different periods after injection was necessitated. In Table 1 some of these values are given. The Table also contains the specific activity values of the inorganic phosphate fraction (peak I in Fig. 2) and in some cases the phosphate purified through Dowex 1. Calculated from the peaks emerging from the Dowex 50 column, the total amount of phosphoserine phosphorus is only about 1/20th as great as the total amount of inorganic phosphorus. If the phosphate present in peptides is included this figure increases to about 1/10th. At the time for maximal incorporation, the specific activity of the phosphoserine values is 2.7 to 10 times greater than that of the inorganic phosphorus. The former value refers to the kidney, the latter to the spleen.

DISCUSSION

Using the present methods it is possible to isolate pure phosphoserine from the cell protein residue. Nevertheless, the limitations of the methods for the study of protein phosphorus turnover discussed previously should be kept in mind. In addition there is the possibility that not all the phosphoserine isolated was originally present as such before hydrolysis. Although it is unlikely that inorganic phosphate becomes esterified during the course of hydrolysis (this does not happen when casein is partially hydrolysed in the presence of P^{32} and we have not been able to observe it in experiments with organ protein)

Table 1. Specific Activities cpm/μg P.

	Phosphoric acid	Phosphoric acid purified on Dowex 1	Phosphoserine	Phosphoserine purified on Dowex 1
Liver				
1 hr	164		836	
1	220	90	643	715
2	156		929	1 078
3 *	299	191	1 152	911
3	400	104	1 204	669
6	143		827	
12	111	349	210	306
30	54		84	
Kidney				
1 hr	256		691	556
1	359	269	398	
2	271		725	441
3*	195	182	328	795
3	164	220	153	
6	191		316	351
12	81	72	297	371
30	44		89	50
Spleen				
1 hr	32		253	
2	73		387	
3 *	55		479	
3	44		439	
6	90		415	
12	54		44	
30	32		7	
Intestinal mucosa				
1 hr	61		255	
1	46		297	
2	71		417	
3 *	22		350	
3	118		401	
6	125		478	
12	70		348	
30	81		96	
Heart				
1 hr	45		259	
2	90		373	
3 *	99		232	
3	90		568	
12	61		270	
30	22		42	
Brain				
6 hr	10		201	

* The animals in this group were injected with 5 μC per g body weight. Values divided by 5 for comparison.

it may be that some phosphorus may have migrated from a phosphoamide linkage in serine to the hydroxyl group. Plapinger and Wagner-Jauregg [11] have shown this to occur when N-di*iso*propyl phosphorylserine is hydrolysed with aqueous hydrochloric acid. Little can be said at the moment of this possibility.

Even if the phosphoserine isolated does not come exclusively from protein-bound phosphoserine, it goes without saying that a measurement of the P^{32} turnover such as we have made can only give an average value for presumably different kinds of phosphoproteins at different loci within the cell. It is clear that work with protein preparations of the various cell organelles is called for. Moreover, it is possible that even if a single phosphoprotein could be isolated from a given cell component, the phosphorus bound to different serine residues, *e. g.* diester phosphorus contra monoester phosphorus, might differ in specific acitivity.

The phosphoric acid in the first peak from the Dowex 50 column must have different origins. Partly it comes from the phosphoproteins but most probably also from not completely extracted nucleic acids and phospholipids. This may explain the differences in values between different organs.

It must also be kept in mind that some of the subfractions isolated contain substances adsorbing ultraviolet light at 260 mμ pointing to the fact, that some nucleotides have withstood the strong acid hydrolysis.

With all these reservations in mind and the future experiments they suggest, the results of the present investigation would seem to be of value in narrowing the search to two organs, kidney and liver. It is of interest that our results tend to confirm those of Johnson and Albert [12] who, using a modified Schneider procedure for the determination of the "phosphoprotein phosphorus", found higher values for maximum specific activity in kidney and liver.

Since we have employed rats in these studies it has not been feasible to study the P^{32} turnover of phosphoserine in pancreas. Such a study would be of interest, however, in view of the high protein synthesis activity of this organ [13]. Further study of the brain, preferably using intracisternal radioactive phosphate injection [14], should prove interesting since the specific activity of brain phosphoserine is relatively high even with subcutaneous injections.

Another point of fundamental importance concerns the source and destination of the phosphoserine phosphorus. But before this problem can be elucidated many more studies of the turnover rates of purified phosphorus compounds of the cell are needed. It would be of interest to compare the specific activities of some of the lower energy compounds of the TCA-soluble fraction, especially those that are not directly involved in glycolysis. Also it would be interesting to compare the activities of phosphoserine with the phosphoinositol which Dawson [15] has shown to have a much greater specific activity than the other phosphorus-containing compounds of the lipid fraction.

This investigation was supported by a grant from the *Swedish Medical Research Council.* The technical assistance of Miss I. Andersson, Mr. T. Fransson, and Mr. T. Persson is gratefully acknowledged. We also wish to thank Mr. C. I. Sjöberg and Mr. P. Olsson for technical constructions.

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Apparatus for Laboratory Batch Distillation

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In the course of work on liquid furan compounds, mixtures, in volumes of 2—200 ml, containing 2—5 components were regularly obtained. These mixtures were separated by distillation into their components in a reasonably pure state (97—99 %) by using one of the four setups described in this article. Two of the setups are for simple distillation, of small and of large charges respectively, and two are for fractional distillation. Since it may be inferred from observations by Rossini, Mair and Streif [1] that most organic compounds decompose too rapidly above 200° C to make prolonged fractionation possible, the setups for fractional distillation are only intended for distillation temperatures up to 200° C.

A. SETUPS FOR SIMPLE DISTILLATION

Mixtures of components boiling more than 100° C apart were separated by using one of the Claisen-type setups shown in Figs. 1 and 2. In both stills the tube leading from the still pot to the condenser has a rather large inner diameter so that the stills can be used for distillations at pressures down to 0.1 mm. During the past five years more than one thousand distillations have been carried out by us in one or the other of these setups, including all simple distillations reported in earlier publications on furan compounds [2] from this laboratory.

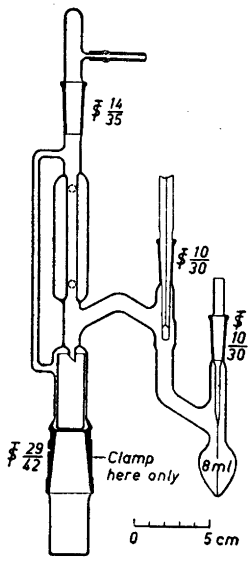

Fig. 1. Setup for simple distillation of 0.5—6.0 ml.

B. SETUP INCLUDING A 5 CM COLUMN

Components boiling 30—100° C apart were separated by fractional distillation using the setup shown in Fig. 3, consisting of a Widmer-type column and a fraction collector for four flasks holding 8, 8, 32 and 125 ml, respectively. The column is packed with 1/16-in. Dixon gauze rings [3] or with 1/8-in. glass helices. The efficiency of the column may be roughly regulated, partly by regulation of the boilup rate, and partly by

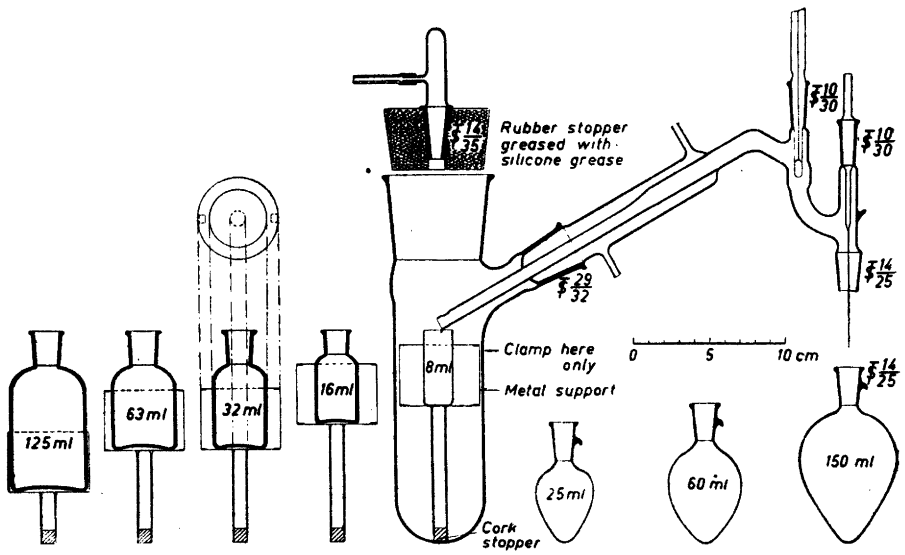

Fig. 2. Setup for simple distillation of 5—120 ml.

regulation of the reflux ratio by cooling (with a stream of air) or insulating (with a cloth) the vertical air condenser at the top of the column.

With this setup, which also has been in regular use for five years, all previously published distillations [2], where the use of a 5 cm column was reported, were carried out.

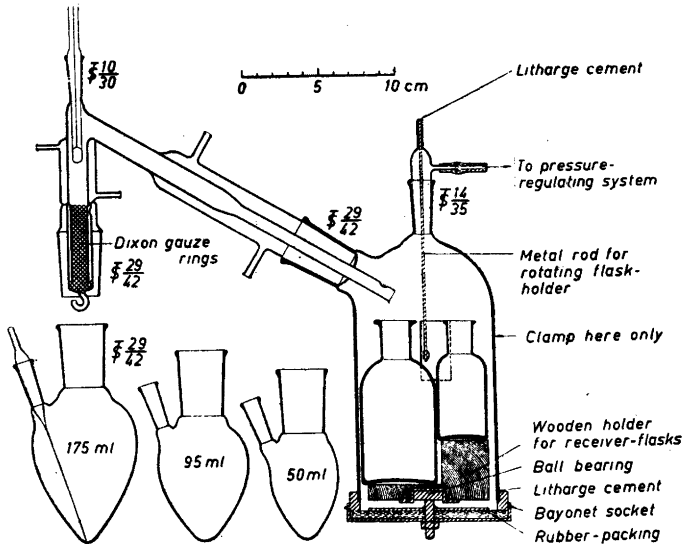

Fig. 3. Setup including a 5 cm Widmer-type column.

C. SETUP INCLUDING LARGER COLUMNS

Mixtures of components boiling 3—30° C apart were separated by using a new type of particularly well insulated stills. The distilland in the still pot is heated by an internal heater, and the efficient insulation makes it possible to carry out an entire distillation without changing the heat input. The stills are connected with a pressure-regulating system and an automatic fraction collector, so that the whole setup becomes automatic and needs no attendance by the operator.

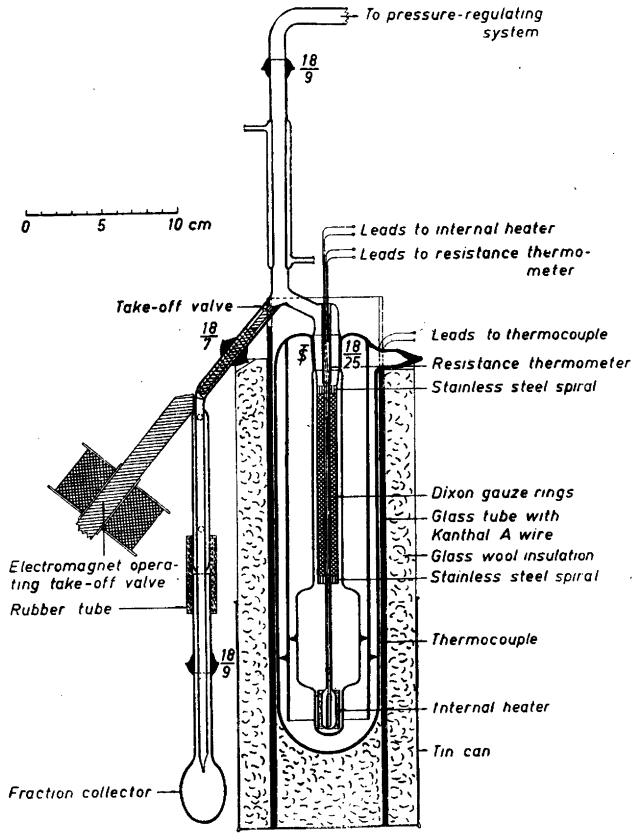

Fig. 4. Still including a 12 cm column.

The stills

Three stills of similar design but of different sizes were found sufficient to meet all our requirements. The dimensions of the stills are summarized below.

Still No.	Rectifying section		Volume of cylindrical part of still pot ml	Maximum charge of still pot ml
	Area cm²	Length cm		
I	0.97	12	70	60
II	0.97	25	140	120
III	0.97	50	280	240

A detailed drawing of the smallest still (I) is shown in Fig. 4. In the following the construction and use of the different parts are described.

The still pot, the wall of which is a vacuum jacket with four silvered surfaces, is constructed of the thinnest possible pyrex glass. The pot, which with its long neck also insulates the rectifying section of the still, thus has a small heat capacity but is at the same time efficiently insulated. It will be seen that all parts of the still pot inside of the outer wall of the vacuum jacket are sealed to the outer wall only at their upper end, *so that stills of any length may be constructed without risk of breakage due to expansion.*

The silvering and sealing of the vacuum jacket were carried out after the directions of Barr and Anhorn [4]. Prior to sealing, the jacket was evacuated with a mercury vapor pump (single stage jet pump T/Sl/626 from *The Thermal Syndicate Ltd.*, Wallsend, Northumberland, England) to 1.0×10^{-6} mm and baked, by wrapping with electrical glass-cloth heating tape, at about 400° C under this pressure for 24 hours.

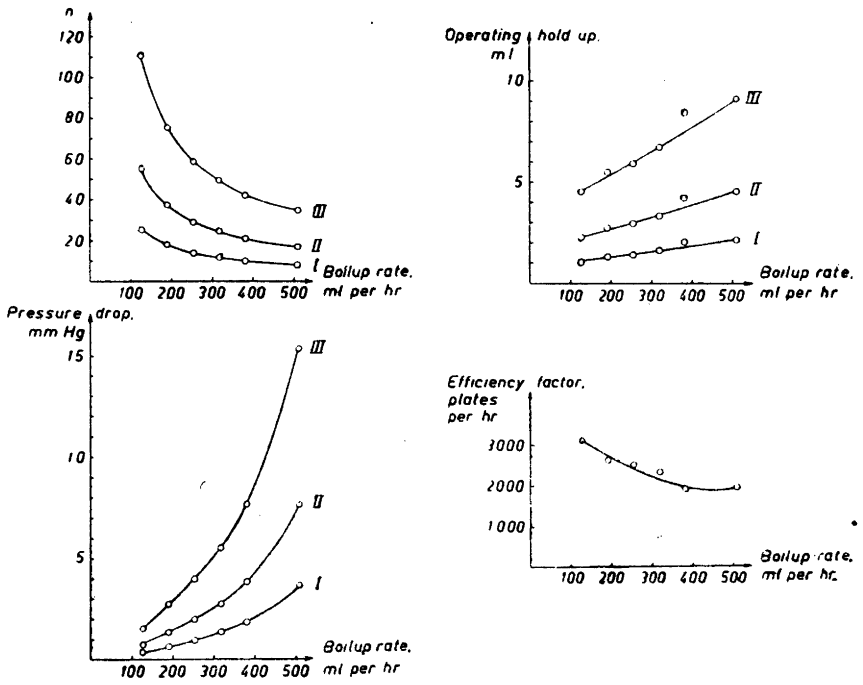

Fig. 5. Performance data for columns I—III.

The rectifying section (the column) consists of a 12 mm (ID) tube packed with 1/16-in. Dixon gauze rings. A 4.5 mm (OD) tube supporting the internal heater runs concentrically through the column. Two spirals of stainless steel keep the packing in place. It is a simple matter to change the packing as well as the spirals, if they should become corroded.

Taking into account all important factors characterizing a column packing, *viz.* the efficiency factor, the H.E.T.P., the pressure drop and the ease of manipulation, Dixon's packing is in our opinion the best reported. Dixon tested his packing with a mixture of 20 parts by volume of *n*-heptane and 80 parts of methyl*cyclo*hexane under atmospheric pressure (2,2,4-trimethylpentane was used in the holdup experiments). Performance data for our columns, calculated from Dixon's data [5], are shown in Fig. 5.

The internal heater is filled with a silicone oil (DC 550 from *Dow Corning Corporation*, Midland, Michigan, U.S.A.) in which a small heating coil of kanthal A wire (0.15 mm, 70 ohm, maximum load 70 watt) wound on a porcelain tube is placed. The two leads to the coil, one of which is insulated by a thin-walled capillary glass tube, are of silver wire (0.7 mm). The coil is fed from a *variable transformer* connected with the network through a *constant voltage device*. The heat input may vary from 2—8 watt, for distillations under 10—20 mm, to 12—50 watt for distillations under atmospheric pressure.

The internal heater is surrounded by a slightly larger glass tube which is pressed over a layer of loosely packed glass wool. This arrangement efficiently prevents bumping under all distillation conditions. The glass wool is usually renewed after each distillation. If the heat input is large, say above 10—25 watt, the glass wool layer is no longer necessary and should be omitted to avoid thermal destruction of the distilland.

The tube supporting the internal heater widens out immediately above the column packing to make place for the small *resistance thermometer (cf.* Willingham and Rossini [6]) (100 ohm, 3 × 30 mm from *Degussa Hanau*, Hanau, Germany), which registers the distillation temperature continuously by means of a *recorder*. Changes of 0.1° C can be read on the chart. The leads to the thermometer are insulated by capillary glass tubes.

The distillate is taken off intermittently from the still head into the take-off line through a 2—3 mm hole. The *take-off valve* consists of a spring, a 4-in. nail shortened to about 7 cm and a 5/32-in. steel ball, which fits into the spherically ground outer opening of the take-off hole. At the upper end of the tube leading from the still head to the fraction collector a spherical ground joint is interspaced, enabling the metallic parts of the take-off valve to be put in place. No special care was taken to protect these metallic parts against corrosion, since they all are cheap and easily replaceable, but they may well be coated with teflon. The valve is opened intermittently for about one second at a time, which is sufficient to allow the small volume of liquid (about 0.1 ml) accumulated above the steel ball to run off.

The strong *electromagnet* operating the valve is activated by an *electrical timer*, which was chosen in preference to a clockwork because of the ease with which the time interval between two activations can be adjusted. With a take-off of 0.1 ml per activation, time intervals of 10 seconds (minimum interval) and of 30 minutes (maximum interval) correspond to product rates of 36 and 0.2 ml per hour, respectively. When a pure compound is distilled at constant boilup rate and a fixed setting of the timer, the product rate remains constant within 3 %.

The electrical heating mantle insulating the still pot consists of a glass tube wound with kanthal A wire (0.40 mm, 500 ohm) and insulated by 20 mm thick tubing of glass wool, the lower end of which is fitted snugly into a tin can, so that the still can stand on a platform without support. The glass tube extends at the upper end about 35 mm above the glass wool insulation and is slotted here to receive the take-off line and the seal-off tube of the vacuum jacket of the still pot (*cf.* Todd [7]). The extension is used to support a 25 mm broad electrical glass cloth *heating tape* (250 ohm, length 40 cm) not shown in Fig. 4, which is wound loosely around the lower part of the still head and the outer surface of the glass tube. The upper end of the glass tube is then covered by an asbestos plate. The heating tape and the asbestos plate serve to insulate the lower part of the still head, so that the distillate reaches the take-off hole even at minimum boilup rate (about 20 ml/hour). The heating tape and the heating mantle surrounding the still pot are connected in series, both being fed from a *variable transformer*. At a distillation temperature of 150° C, the effect of the tape is about 13 watt and of the mantle about 26 watt.

The temperature between the still pot and the inner surface of the glass tube is measured by means of a *thermocouple*. If the temperature is adjusted to the boiling point of the distillate at the beginning of a distillation, there will be practically no heat exchange between the still pot and its surroundings. If, at the end of the distillation, the distillation temperature has risen 30° C, a certain amount of heat will be lost from the still pot. Judging from crude measurements of heat losses at larger temperature differences, we believe that this loss does not exceed 0.4 watt. Since the lowest boilup rate used in these stills corresponds to a heat input of 2.0 watt, the maximum decrease in boilup rate during a distillation is 20 %. This is an extreme case, and in general the decrease is only 1—10 %. It follows that the insulation of the still is such that, provided the compounds to be separated have approximately the same heat of evaporation, no increase of the heat input of either the outer heating or of the internal heater is necessary to keep the boilup rate almost constant during an entire distillation. This is also true of the two larger stills, which, while having a larger heated surface, at the same time have a smaller rise in distillation temperature during a distillation (maximally about 15° C and 7° C, respectively).

The pressure-regulating system

At the top of the reflux condenser the stills are connected by means of a spherical ground joint with a 10 mm (ID) glass tube leading through a cooling trap to the pressure-regulating system. Two branches of the tube carry vacuum rubber tube connections to the fraction collector and to a manometer showing the distillation pressure. The pressure-regulating system, which is situated in an air thermostat (accuracy ± 1° C), consists of a 10 l flask, serving as surge tank, and a sintered glass disk manostate of new design (Fig. 6). The manostate is tilted about 45° during evacuation and reverted into vertical position when the desired pressure has been reached. The low pressure side of the manostate is connected with an oil pump, which is stopped automatically at 1—2 mm. During a distillation under 10—20 mm the pump operates less than one per cent of the time (*cf.* Cox [8]).

In order to evacuate the system rapidly it is convenient to by-pass the manostate by a tube leading directly from the oil pump to the surge tank. To make sure that the by-pass tube can be efficiently interrupted, an ordinary stopcock as well as a three-way stopcock is inserted, so that the tubing between the stopcocks can be opened to the atmosphere. At the same time the three-way stopcock serves to let air into the system after a distillation.

The vacuum rubber tubing used for the by-pass tube has an inner diameter of 5 mm. All other rubber tube connections are made of thinner tubing (2 mm ID, 6 mm OD), fitting 4 mm (OD) glass tubes.

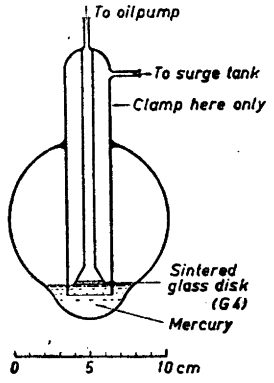

Fig. 6. Sintered glass disk manostate.

The above pressure-regulating system keeps the pressure constant within 0.3% (or better) at any pressure between 10 mm and atmospheric pressure. In particular there is no drifting of the pressure over long periods of time.

The fraction collector

The fraction collector is automatic and can collect 48 fractions during a single distillation, so that distillation curves based on other properties than the boiling point may be drawn. The collector is shown in Fig. 7. The distillate, coming from the tube leading to the take-off valve, drops directly into the small receiver tubes, in which it later is to be stored. At intervals the three solenoids (1—3) are energized in rapid succession by a system of relays accentuated by an electrical clockwork, allowing a new fraction to be collected.

The clockwork, which was chosen in preference to other timing devices because of its ability to accurately reproduce long time intervals, is adjustable to intervals from 5 minutes to 24 hours.

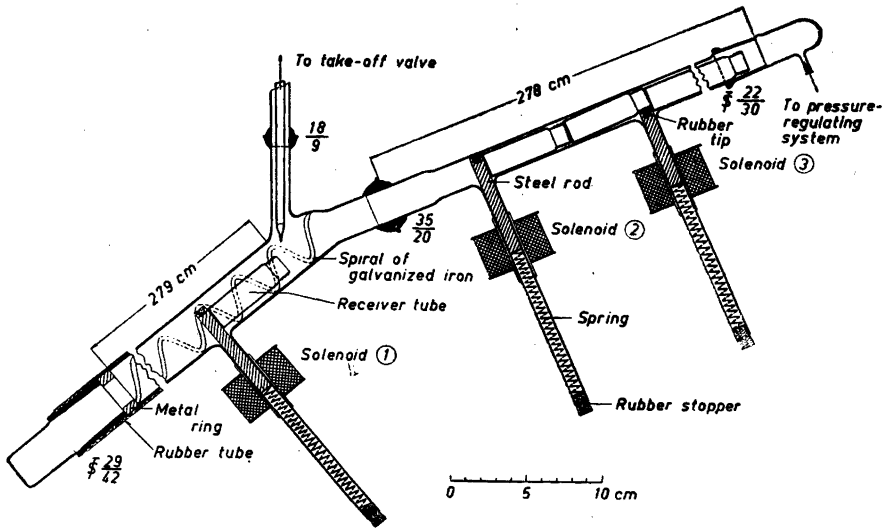

Fig. 7. Automatic fraction collector.

The spiral of galvanized iron wire (3.0 mm) in the lower end of the collector makes the filled receiver tubes fall smoothly. The tubes hold 8—9 ml when filled to the neck, but should only contain a maximum of 5—6 ml in order to prevent the distillate from splashing over during the fall. The filled tubes stand on top of each other, thus minimizing distillation from one tube to another.

An electrical fan is placed directly in front of the take-off tube in order to prevent warmth from the still from heating the middle section of the collector.

The volume of the collector is about 2 liter. Since the volume of the surge tank of the pressure-regulating system is 10 liter, sudden fluctuations in room temperature of ± 5° C will only cause the distillation pressure to change ± 0.3 %, corresponding to a change in boiling point of ± 0.1° C.

Assembly and operation

Assembling the setup presents no special problems. All rubber to glass connections are greased with Dow Corning Silicone Stopcock Grease as are the conical ground joints at both ends of the fraction collector and the large spherical joint in the middle. The conical joint connecting the still head and the column with the still pot is greased with Dow Corning Silicone High Vacuum Grease. The two spherical joints on the take-off line and the one at the top of the still head are greased with Cello-Grease No. 14—637 (*Fisher Scientific Company*, New York, U.S.A.).

When the fraction collector is charged with receiver tubes, all openings should be closed, so that the falling tubes are checked by the air.

Starting a distillation is rapid and simple. After charging, the system is evacuated to the distillation pressure and the outer heating adjusted roughly to the distillation temperature. The column is flooded and the input to the internal heater then reduced to give the desired boilup rate. The boilup rate may be calculated accurately from the heat of evaporation of the distilland, but it will usually be sufficient to reckon 1 watt equivalent to 10 ml per hour.

After equilibrium is reached, the take-off begins. If there is a small fore-run, the take-off may usually be started shortly after the flooding, in particular when the smallest

still is used. We have often started charging the small still for a distillation under 10—20 mm just before closing time and left it distilling without attention overnight.

Using maximum still pot charges the yield of distillate is 97—98 % of the distilland. After a distillation the setup should be taken apart and cleaned immediately. We have never experienced sticking of the conical joint of the still pot. A tar-like residue in the still pot may easily be removed with chromic acid. Examples of the use of the setup are given in another article [9] in this journal. Further examples will possibly appear in later articles.

SUMMARY

The construction and use of four new setups for laboratory batch distillations under reduced or atmospheric pressure are described. The setups, which we mainly have used for the separation of mixtures of furan compounds, are easy to operate and are intended for general use.

Technical assistance. The glass blowing was carried out by Erik Christensen of *Dansk Glasapparatur*, Kongens Tværvej 5, Copenhagen F. The vacuum jackets were silvered by Zdeněk Tyle. The drawings were in part made by Niels Grue-Sørensen.

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Studies on Copper(II) Periodates

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The first and second ionization constants of periodic acid in aqueous solutions at 25° C were determined. By mixing copper perchlorate, sodium periodate and sodium hydroxide solutions the compounds Cu_2HIO_6, $Cu_5(IO_6)_2$ and Cu_2NaIO_6 were precipitated as indicated by the results of chemical analysis and the form of the potentiometric precipitation curve. The solubility products and the limits of existence of these compounds were determined. The hydrates of these compounds were preliminarily investigated and the existence of the mono-, di-, and trihydrates of Cu_2HIO_6 and the pentahydrate of $Cu_5(IO_6)_2$ was confirmed. Dehydrated Cu_2NaIO_6 was also obtained.

The aim of the present investigation was to check the compositions of the copper periodates reported in the literature [1] and particularly to determine the limits of existence for these compounds. With this purpose in view varying amounts of sodium periodate, copper perchlorate and sodium hydroxide solutions were mixed and after equilibrium was reached the precipitates formed and the solutions were analyzed and their pH's determined.

EXPERIMENTAL

The stock solution of sodium periodate was prepared from commercial $NaIO_4$ and that of copper perchlorate from copper oxide and perchloric acid. The sodium hydroxide, carbonate free, was prepared and stored in the usual way. All chemicals were of analytical grade (Merck, Darmstadt).

The pH was measured with a glass electrode using a Radiometer PHM 3 pH meter.

For determination of copper the precipitates were treated with hydrochloric acid and thereafter the copper of the copper chloride so formed was titrated iodometrically. The sum of the copper and periodate was determined iodometrically.

Copper sodium periodate was converted to chlorides by means of hydrochloric acid and then analyzed iodometrically (copper) and using Dowex 2 ion exchanger (sodium) [2].

RESULTS

For the quantitative treatment of the equilibrium the ionization constants of periodic acid were necessary. The first ionization

$$H_5IO_6 + H_2O = H_3O^+ + H_4IO_6^- \quad (1)$$

has been many times investigated [3]. Our results for the constant

$$\frac{(H^+)[H_4IO_6^-]}{[H_5IO_6]} = K_1' \qquad (2)$$

are presented in Table 1. In equation (2) the denotation $[H_4IO_6^-]$ and $[H_5IO_6]$ may be considered as total concentrations of the negatively univalent ions and the neutral constituents respectively. Besides the ion $H_4IO_6^-$, at least IO_4^- should exist corresponding to the equilibrium[3]

$$H_4IO_6^- = IO_4^- + 2\ H_2O \qquad (3)$$

For our purposes the single hydration can be neglected. In Table 1, c_{IO_4} denotes

Table 1. Determination of the first ionization constant of periodic acid at 25° C.

$\sqrt{I}$	$c_{IO_4} \cdot 10^3$	$c_A \cdot 10^3$	pH	pK_1'
0.080	4.72	1.895	2.821	1.58
0.082	4.70	2.36	2.719	1.54
0.085	4.69	2.82	2.635	1.47
0.087	4.68	3.28	2.567	1.46
Mean	$pK_1' = 1.51 \pm 0.03$,		$\sqrt{I} = 0.084$	
Thermod. constant	$pK_{1,0} = 1.55 \pm 0.03$			

the total concentration of $NaIO_4$ and c_A that of the perchloric acid added. The equation

$$pK_1' = pH + \log\{(c_A - [H^+])/(c_{IO_4} - c_A + [H^+])\} \qquad (4)$$

was used in the calculation. The hydrogen ion concentration was obtained from the pH values using the activity coefficient values of Näsänen, Lumme and Mukula [4]. The thermodynamic constant was calculated in the usual way using the Debye-Hückel equation.

The second ionization

$$H_4IO_6^- + H_2O = H_3IO_6^{=} + H_3O^+ \qquad (5)$$

has been studied recently by Crouthamel *et al.*[3] They seem to be the only ones who have determined the second ionization constant

$$\frac{(H^+)[H_3IO_6^{=}]}{[H_4IO_6^-]} = K_2' \qquad (6)$$

The possible single hydration is neglected in this case also. The spectrophotometric investigation of Crouthamel *et al.*[3] seems to show the absence of polyacids such as $H_4I_2O_9$ at least in dilute solutions. Our results are presented

Table 2. Determination of the second ionization constant of periodic acid at 25° C.

$\sqrt{I}$	$c_{IO_4} \cdot 10^3$	$c_B \cdot 10^3$	pH	pK_2'
0.078	4.74	0.679	7.376	8.153
0.086	4.71	1.344	7.760	8.160
0.093	4.69	2.007	8.017	8.144
0.100	4.67	2.666	8.264	8.139
Mean	$pK_2' = 8.149 \pm 0.005$;	$\sqrt{I} = 0.089$		
	$pK_{2,0} = 8.269$			

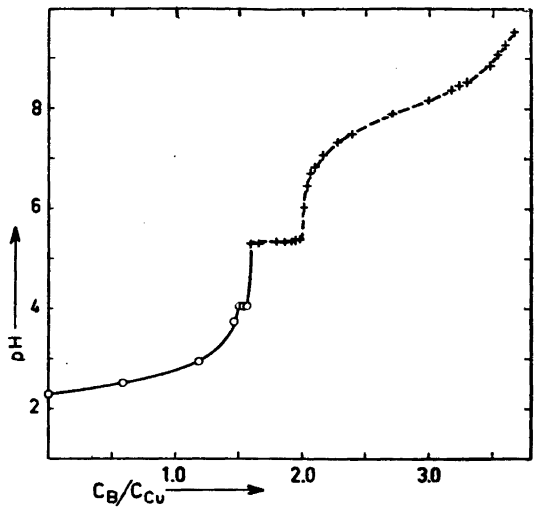

Fig. 1. Titration of a mixed solution of sodium metaperiodate and copper perchlorate with sodium hydroxide. ○ 5 ml 0.1 M $NaIO_4$, 10 ml 0.0475 M $Cu(ClO_4)_2$, 50 ml H_2O. + 10 ml 0.1 M $NaIO_4$, 10 ml 0.0475 M $Cu(ClO_4)_2$, 50 ml H_2O.

in Table 2. In the calculation the equation

$$pK_2' = pH + \log\{(c_{IO_4}-c_B)/c_B\}$$

was used. Here c_B is the stoichiometric concentration of sodium hydroxide in solution.

The results on copper periodate are presented in Fig. 1 and in Tables 3, 4 and 5. In Fig. 1 the pH of the equilibrium solutions as a function of c_B/c_{Cu} is presented. Here c_{Cu} is the stoichiometric concentration of the copper perchlorate added. Between 0 and 1.5 for c_B/c_{Cu} the composition of the precipitate corresponds to the formula $Cu_2HIO_6 \cdot x\ H_2O$. This was confirmed by analysis of the copper and the sum of the copper and periodate in the precipitate. Also the jump near $c_B/c_{Cu} = 1.5$ is in agreement with the reaction

$$2\ Cu^{++} + H_4IO_6^- = Cu_2HIO_6\ (s) + 3\ H^+ \qquad (7)$$

From 1.5 to 1.6 $Cu_5(IO_6)_2 \cdot x\ H_2O$ exist simultaneously with $Cu_2HIO_6 \cdot x\ H_2O$. When $c_B/c_{Cu} = 1.6$ the analysis showed that only $Cu_5(IO_6)_2 \cdot x\ H_2O$ exists. The sudden increase of pH at $c_B/c_{Cu} = 1.6$ is in agreement with the reaction

$$5\ Cu^{++} + 2\ H_4IO_6^- = Cu_5(IO_6)_2(s) + 8\ H^+ \qquad (8)$$

Between 1.6 and 2 a mixture of $Cu_5(IO_6)_2 \cdot x\ H_2O$ and $Cu_2NaIO_6 \cdot x\ H_2O$ exists. The jump of pH at $c_B/c_{Cu} = 2$ is in agreement with the reaction

$$2\ Cu^{++} + H_4IO_6^- + Na^+ = Cu_2NaIO_6(s) + 4\ H^+ \qquad (9)$$

When $c_B/c_{Cu} > 2$ only Cu_2NaIO_6 exists, as was confirmed by analysis of the precipitate and by powder diagrams. That between 1.5 and 1.6 a mixture of

Cu_2HIO_6 and $Cu_5(IO_6)_2$ exists and between 1.6 and 2 a mixture of $Cu_5(IO_6)_2$ and Cu_2NaIO_6, seems to be confirmed by the powder diagrams.

Some preliminary determinations of water of crystallization were also made. After drying in a desiccator for one night $Cu_2HIO_6 \cdot 2\ H_2O$ was obtained. This seems to absorb water at high vapor pressures so that $Cu_2HIO_6 \cdot 3\ H_2O$ is formed. When heated at 150° C in air $Cu_2HIO_6 \cdot H_2O$ was obtained. When heated at 100° C in vacuum $Cu_5(IO_6)_2 \cdot x\ H_2O$ gave $Cu_5(IO_6)_2 \cdot 5\ H_2O$. At higher temperatures in vacuum it began to decompose. Possibly higher hydrates exist in this case too. When heated at 150° C in air dehydrated Cu_2NaIO_6 was obtained.

The quantitative treatment of this equilibrium may be based on the reactions (3), (5), (7), (8) and (9). The case is, however, formally analogous with the equilibrium of metal hydroxy salts[5]. Therefore instead of reactions (7), (8) and (9) the following reactions may be used:

$$Cu^{++} + 1.5\ OH^- + 0.5\ H_4IO_6^- = Cu(OH)_{1.5}(H_4IO_6)_{0.5}(s) \quad (10)$$

$$Cu^{++} + 1.6\ OH^- + 0.4\ H_4IO_6^- = Cu(OH)_{1.6}(H_4IO_6)_{0.4}(s) \quad (11)$$

$$Cu^{++} + 2\ OH^- + 0.5\ Na^+ + 0.5\ H_4IO_6^- = CuNa_{0.5}(OH)_2(H_4IO_6)_{0.5}(s) \quad (12)$$

We obtain therefore

$$[Cu^{++}](OH^-)^{1.5}[H_4IO_6^-]^{0.5} = S_{1.5} \quad (13)$$

$$[Cu^{++}](OH^-)^{1.6}[H_4IO_6^-]^{0.4} = S_{1.6} \quad (14)$$

$$[Cu^{++}][Na^+]^{0.5}(OH^-)^2[H_4IO_6^-]^{0.5} = S_2 \quad (15)$$

where bracketed symbols designate respective concentrations and (OH^-) hydroxyl ion activity. When $c_B/c_{Cu} < 1.5$ we have in addition to equation (13)

$$[H_4IO_6^-] = \frac{c_{IO_4} - 0.333\ (c_B + [H^+])}{1 + 1.333(H^+)/K_1'} \quad (16)$$

where c_{IO_4} is the stoichiometric concentration of sodium periodate. Further

$$[Cu^{++}] = c_{Cu} - 2\ c_{IO_4} + 2[H_4IO_6^-]\{1 + (H^+)/K_1'\} \quad (17)$$

With the aid of (16) and (17) the solubility product S_1 may be calculated using the pH values measured, the stoichiometric concentrations and the value of K_1' known. These results are presented in Table 3.

Table 3. Determination of the solubility product of Cu_2HIO_6 $(Cu(OH)_{1.5}(H_4IO_6)_{0.5})$ *at 25° C.*

pH	$c_{IO_4} \cdot 10^3$	$c_B \cdot 10^3$	$c_{Cu} \cdot 10^3$	$[Cu^{++}] \cdot 10^3$	$[H_4IO_6^-] \cdot 10^3$	$pS_{1.5}$	$\sqrt{I}$
2.314	8.33	0.00	8.03	3.81	5.41	21.07	0.146
2.536	8.07	4.60	7.77	2.23	4.86	21.00	0.144
2.939	7.81	8.89	7.52	0.64	4.22	20.97	0.137
					mean value	21.02	0.142

When c_B/c_{Cu} is between 1.5 and 1.6, reactions (13) and (14) are simultaneously valid. Consequently

$$pS_{1.6} = pS_{1.5} + 0.1\ pOH - 0.1\ pH_4IO_6 \quad (18)$$

From electroneutrality we obtain

$$[H_4IO_6^-] = c_B + c_{IO_4} - 2\,c_{Cu} + [H^+] + 2\,S_{1.5}/(OH^-)^{1.5}[H_4IO_6^-]^{0.5} \quad (19)$$

with sufficient accuracy. In equation (19) the two last terms may generally be neglected. By means of (18) and (19) it is thus possible to calculate the solubility product $S_{1.6}$. These results are summarised in Table 4.

Table 4. Determination of the solubility product of $Cu_5(IO_6)_2$ $(Cu(OH)_{1.6}(H_4IO_6)_{0.4})$ *at 25° C.*

pH	$c_B \cdot 10^3$	$c_{IO_4} \cdot 10^3$	$c_{Cu} \cdot 10^3$	$\sqrt{I}$	$pS_{1.5}$	$pS_{1.6}$
4.072	11.17	7.69	7.39	0.184	20.95	21.71
4.139	11.17	7.69	7.39	0.184	20.95	21.71
4.173	11.37	7.67	7.38	0.184	20.95	21.71
4.224	11.37	7.67	7.38	0.184	20.95	21.70
4.207	11.57	7.66	7.37	0.184	20.95	21.70
4.325	11.57	7.66	7.37	0.184	20.95	21.69
4.241	10.03	13.33	6.33	0.189	20.96	21.74

When c_B/c_{Cu} is between 1.6 and 2.0 equations (14) and (15) are simultaneously valid and we obtain

$$pS_2 = pS_{1.6} + 0.4\ pOH + 0.5\ pNa + 0.1\ pHI_4O_6 \quad (20)$$

From the electroneutrality and stoichiometric relations we further obtain

$$[Na^+] = c_{IO_4} + 2\,c_{Cu} - 0.25\,c_B - 2\,S_{1.6}/(OH^-)^{1.6}[H_4IO_6^-]^{0.4} \quad (21)$$

and

$$[H_4IO_6^-] = c_{IO_4} - 0.25\,c_B \quad (22)$$

Here only the constituent $H_4IO_6^-$ has been taken into consideration, the other terms being negligible. With the aid of equations (20), (21) and (22) we obtain the solubility products S_2. These results are summarized in Table 5.

Table 5. Determination of the solubility product of Cu_2NaIO_6 $(CuNa_{0.5}(OH)_2(H_4IO_6)_{0.5})$ *at 25° C.*

pH	$c_B \cdot 10^3$	$c_{IO_4} \cdot 10^3$	$c_{Cu} \cdot 10^3$	$\sqrt{I}$	$pS_{1.6}$	pS_2
5.289	10.04	13.28	6.31	0.153	21.77	26.27
5.289	10.39	13.24	6.29	0.152	21.77	26.27
5.356	11.26	13.17	6.25	0.151	21.77	26.25
5.322	11.60	13.12	6.23	0.151	21.77	26.26
5.339	11.95	13.08	6.22	0.150	21.77	26.26
5.356	12.12	13.07	6.21	0.150	21.77	26.25
5.356	12.28	13.06	6.20	0.150	21.77	26.25
				0.151	mean value	26.26

From the values obtained for the solubility product the thermodynamic values were estimated by means of the Debye-Hückel equation

$$p(S/S^\circ) = \frac{A\sqrt{I}}{1 + 1.5\sqrt{I}} \quad (23)$$

where A has the values 2.29 ($S_{1.5}$), 2.24 ($S_{1.6}$) and 2.54 (S_2). Hence we obtain for the thermodynamic constants:

$$pS^\circ_{1.5} = 21.29,\ pS^\circ_{1.6} = 22.02,\ \text{and}\ pS^\circ_2 = 26.57.\quad (25^\circ\,C)$$

Besides the measurements reported in Tables 3, 4 and 5 some additional measurements were performed under other conditions. Their results were generally in good agreement with the above results. However for S_2 a series with a considerably smaller value for c_{IO_4} did not give very good results and the values for pS_2 were somewhat smaller than the values reported above. A series with a high sodium perchlorate concentration gave reasonable results and the pH between 1.6 and 2 (*cf.* Fig. 1) was lower to the extent expected.

DISCUSSION

The value obtained here for the first ionization constant ($pK_{1,0} = 1.55$) is in relatively good agreement with the value of Crouthamel *et al.*[3] ($pK_{1,0} = 1.6$). The agreement between the values for the second constant is still better. Here $pK_{2,0} = 8.27$, Crouthamel *et al.*[3] have obtained potentiometrically $pK_{2,0} = 8.25$.

The existence of Cu_2HIO_6 and $Cu_5(IO_6)_2$ was reported long ago [1], but Cu_2NaIO_6 seems not have been reported before. The limits of existence and the solubility products of these compounds seem not have been determined before. The hydrates found in the present investigation $Cu_2HIO_6 \cdot 3\ H_2O$ and $Cu_5(IO_6)_2 \cdot 5\ H_2O$ have been reported long ago by Rammelsberg [1] but $Cu_2HIO_6 \cdot 2\ H_2O$ and $Cu_2HIO_6 \cdot H_2O$ seem not to have been reported before. Further removal of water from $Cu_2HIO_6 \cdot H_2O$ and $Cu_5(IO_6)_2 \cdot 5\ H_2O$ seems not to be possible by heating in air or vacuum because of the decomposition.

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Electron Diffraction Studies on Fluoroderivatives of Biphenyl

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2,2′-Difluorobiphenyl, 2-fluorobiphenyl and 4,4′-difluorobiphenyl have been investigated. For comparison fluorobenzene was also studied. The bond distances found are: C—F 1.34 Å, aromatic C—C 1.40 Å and C—C in biphenyl bridge 1.52 Å. The angles between the phenyl rings are the following: in 2,2′-difluorobiphenyl 60° ± 5°, in 2-fluorobiphenyl 49° ± 5°, and in 4,4′-difluorobiphenyl 44° ± 5°.

Electron diffraction studies have led to the conclusion that biphenyl and biphenylderivatives are non-planar in the gaseous state [1–3]. The non-*ortho* substituted derivatives and biphenyl itself exhibit a deviation from planarity of approximately 45°. The *ortho* substituted derivatives show a somewhat greater deviation, the value of which depends upon the size of the substituent. The substituents studied so far are chlorine, bromine and iodine. In the present work fluorine derivatives have been investigated. The electron diffraction diagrams were taken with the new Oslo sector apparatus. The experimental intensity curves of the four compounds under investigation are given in Fig. 1.

2,2′-Difluorobiphenyl. The radial distribution curves ($\frac{\sigma(r)}{r}$) of 2,2′-difluorobiphenyl and fluorobenzene calculated by Fourier transformation of the corresponding intensity curves are reproduced in Fig. 2. A study of the $\frac{\sigma(r)}{r}$ curve and the intensity curve of fluorobenzene including calculation of theoretical curves led to the following parameters of this molecule: C—C: 1.40 Å, C—F: 1.34 Å. This is in fairly good agreement with values obtained by microwave studies [4] (C—C: 1.3916 Å and C—F: 1.348 Å). In the following a difference procedure, earlier described [2,3], has been applied:

The upper curve of Fig. 3 represents the difference of the two curves of Fig. 2. Before the subtraction was carried out the curves were normalised using the inner part ($1 < r < 3$) as the basis for the normalisation. By studying the upper curve of Fig. 3 the C—C bridge distance was found to be 1.52 Å. The lower curve of Fig. 3 is a theoretical $\frac{\sigma(r)}{r}$ difference curve containing contribu-

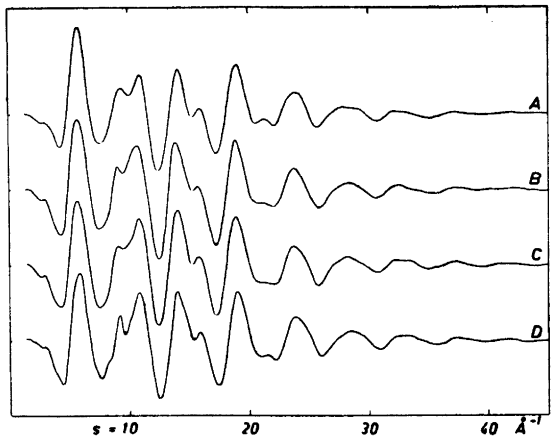

Fig. 1. Intensity curves. A: fluorobenzene, B: 2-fluorobiphenyl, C: 2,2'-difluorobiphenyl, D: 4,4'-difluorobiphenyl.

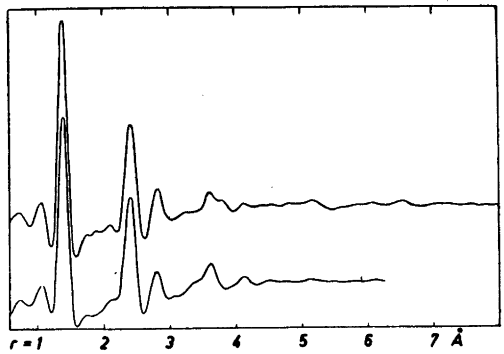

Fig. 2. $\frac{\sigma(r)}{r}$ *curves. Upper curve: 2,2'-difluorobiphenyl, lower curve: fluorobenzene.*

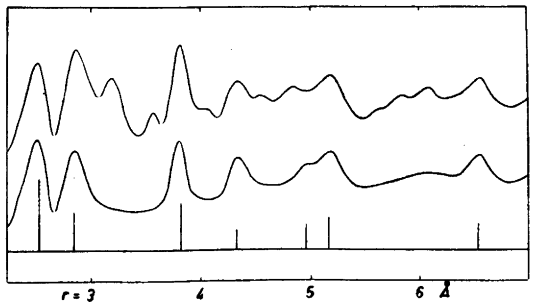

Fig. 3. Upper curve: difference between the two curves of Fig. 2. Lower curve: Theoretical $\frac{\sigma(r)}{r}$ *curve for all the unvariable distances between the two rings of 2,2'-difluorobiphenyl.*

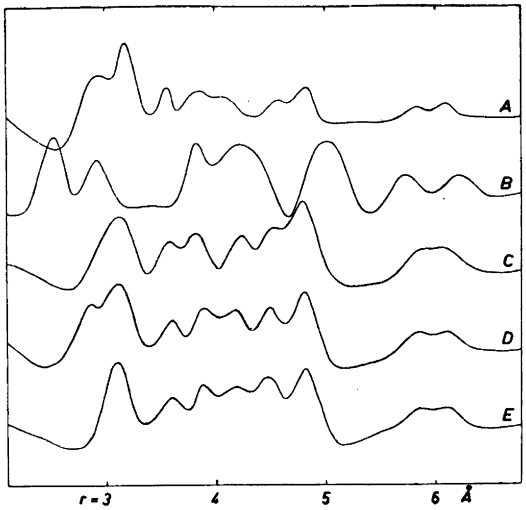

Fig. 4. Curve A: difference between the two curves of Fig. 3. B, C, D, and E: corresponding theoretical curves for various structures.

tions from all distances between the two rings of the 2,2′-difluorobiphenyl which are independent of the angle between the two rings. If we now take the difference between the two curves of Fig. 3 we get a $\frac{\sigma(r)}{r}$ difference curve with contribution only from distances that vary with internal rotation of the molecule about the central C—C bond. This curve, reproduced as curve A of Fig. 4, should be well fitted for the determination of the angle between the two ring planes. Curve B of Fig. 4 is the corresponding theoretical curve for a planar molecule, *trans* form (the *cis* form gives very much the same curve for the r region studied). Curves C, D, and E are calculated for models with the angle φ between the rings equal to 65°, 60°, and 120° respectively. (φ is equal

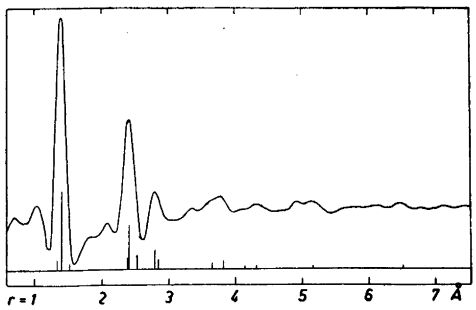

Fig. 5. $\frac{\sigma(r)}{r}$ *curve of 2-fluorobiphenyl.*

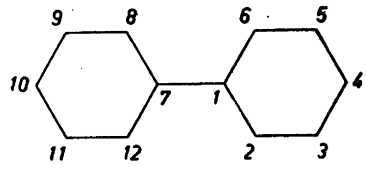

Fig. 6. The numbering of the carbon atoms in biphenyl derivatives applied in Tables 1 and 2.

to 0° for the *cis* position and 180° for the *trans* position). Curve D is no doubt the one of the theoretical curves which is in best accordance with curve A. Studies of the position of the maxima in the various curves of Fig. 4 lead us to the conclution that the φ value is $60° \pm 5°$.

The discrepancy that still exists between curve A and D can easily be explained. First of all the normalisation necessary for carrying out the subtraction introduces uncertainties. Secondly, the form of the normal curves applied is somewhat uncertain. Thirdly, oscillations in the molecule from the equilibrium position will influence the curves, and moreover the influence on the various distances are different. The $\frac{dr}{d\varphi}$ values are considerably greater in the case of the variable C—F distances than in the case of the variable C—C distances. This can for instance easily explain the fact that the maxima at approximately 3.9 Å and 4.2 Å are broader and less pronounced in curve A than the corresponding maxima in curve D.

2-Fluorobiphenyl. In Fig. 5 the $\frac{\sigma(r)}{r}$ curve of 2-fluorobiphenyl is reproduced. In the line diagram we have given all C—C and C—F distances that do not vary with the angle φ. Apparently practically all details in the curve are explained by these distances. However, by correcting for the unvariable distances in the usual way applying the proper normal curves, peaks corresponding to variable distances have been found. The position of these peaks are tabulated in Table 1 together with φ values calculated from the corresponding distances. The numbering of the atoms in the molecule is seen from Fig. 6. This procedure leads us to the value of angle φ of $49° \pm 5°$. The variable distances which have not been included in Table 1 were either not reproducible or not separable.

Table 1. Variable distances in the 2-fluorobiphenyl molecule and φ values calculated from these distances.

C_2-C_8	3.67 Å	46.4°	
C_2-C_{11}	4.46 Å	54.6°	mean value 49.0°
C_2-C_9	4.86 Å	46.2°	
C_3-C_9	6.13 Å	48.6°	

For instance, the distance C_2—C_{12} can not be reproduced by subtraction because of the rather pronounced peaks in the neighbourhood of the r values at which this distance is supposed to occur. The distances F_2—C_8 and F_2—C_{11} are very close together and can therefore not be separated. However, correcting for the neighbouring distances we find that the mean value of the two distances in question should be 4.07 Å. The calculated mean distance assuming 49° gives 4.04 Å which must be regarded as a good correspondance.

4,4'-Difluorobiphenyl. Fig. 7 represents the $\frac{\sigma(r)}{r}$ curve of 4,4'-difluorobiphenyl. In Fig. 8 the difference between the curve of Fig. 7 and the $\frac{\sigma(r)}{r}$ curve of fluorobenzene is reproduced. The line diagram in Fig. 8 describes the position of the distances between the rings that do not vary with φ. The positions of the peaks corresponding to the variable distances are found in the same

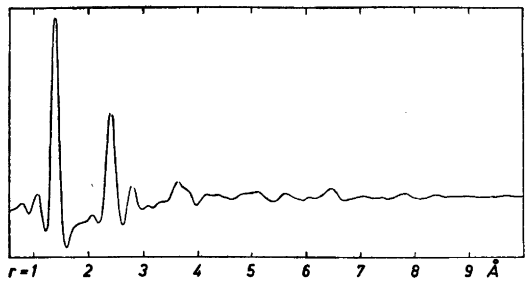

Fig. 7. $\frac{\sigma(r)}{r}$ *curve of 4,4'-difluorobiphenyl.*

Table 2. Variable distances in the 4,4'-difluorobiphenyl molecule and φ values calculated from these distances.

C_2-C_{12}	3.07 Å	45.6°	
C_2-C_8	3.69 Å	42.9°	mean value 44.2°
C_2-C_{11}	4.41 Å	43.4°	
C_2-C_9	4.87 Å	45.0°	

way as for 2-fluorobiphenyl and the resulting values are given in Table 2. We have studied the influence of the shape of the theoretical normal curve used in this procedure, as the shape in this case was believed to influence the determination rather much. We did find a certain variation for the single observed φ values. The mean value did, however, never vary more than a few tenths of a degree. In spite of the rather high reproducibility we believe that the total error in the determination of φ is about $\pm$ 5°.

DISCUSSION OF THE RESULTS

The results obtained are based upon the assumption also applied in earlier work of this kind, that the carbon rings in biphenyl derivatives do not deviate from the hexagonal symmetry. This assumption is probably not strictly correct. As a matter of fact detailed studies of the $\frac{\sigma(r)}{r}$ curve of 2,2'-difluorobiphenyl and of fluorobenzene seem to give indications of the existance of a small deviation, however, too small to influence the result very much. The determination of the φ values is further dependent upon the indirectly determined

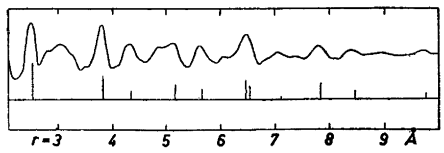

Fig. 8. Difference between the $\frac{\sigma(r)}{r}$ *curves of 4,4'-difluorobiphenyl and fluorobenzene.*

C—C bond linking the two rings together. Both these two sources of error have been taken into consideration in estimating the error range of $\pm$ 5°.

The deviation from coplanarity found in the three biphenyl derivatives under investigation is in good qualitative agreement with what one might expect from earlier studies in this field. The 4,4′-difluorobiphenyl should be expected to deviate from the coplanarity to the same extent as biphenyl and 1,3,5-triphenylbenzene, since the *ortho* hydrogen-hydrogen interaction is primarily believed to be responsible for the deviation. The φ values of 44.2° found for 4,4′-difluorobiphenyl is certainly in very good agreement with the values 45° and 46.4° found for biphenyl and 1,3,5-triphenylbenzene, respectively [1,3]. The φ value found for 2,2′-difluorobiphenyl is considerably smaller than the values of the corresponding chloro, bromo, and iodo compounds [2]. Going from the iodo towards the fluoro compound the following values were found: 79°, 75°, 74°, and 60°. The great gap between the chloro and the fluoro compound demonstrates that the steric effect introduced by fluorine is considerably less than for the other halogens. This is apparently still more pronounced in the case of 2-fluorobiphenyl the deviation of which is hardly greater than that of the non-*ortho* substituted biphenyls. If we apply the angles 45°, 49°, and 60° for biphenyl, 2-fluorobiphenyl, and 2,2′-difluorobiphenyl, respectively, we find the following closest packings: For H—H 2.47 Å, for H—F 2.55 Å and for F—F 2.85 Å. The values are in fair agreement with the van der Waals distances of Pauling which give the following values: 2.4 Å, 2.55 Å, and 2.70 Å.

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Studies on Vanadium Oxides

I. Phase Analysis

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The phase relations in the composition range $VO-VO_{2.50}$ of the vanadium-oxygen system have been investigated. Phases of the following (approximate) compositions were found to exist at temperatures between 650—1 000° C:

VO (homogeneous within the range $VO_{0.75}-VO_{1.20}$), $VO_{1.50}$, $*VO_{1.67}$, $*VO_{1.75}$, $*VO_{1.80}$, $*VO_{1.84}$, $*VO_{1.86}$, $*VO_{1.87}$, $VO_{2.00}$, $VO_{2.17}$ (at 650° C), and $VO_{2.50}$; the formulae marked with an asterisk represent previously unknown oxides.

The $VO_{1.67}$ phase is monoclinic with the C centered unit cell dimensions:

$$a = 9.983 \text{ Å}; \; b = 5.031 \text{ Å}; \; c = 9.835 \text{ Å}; \; \beta = 138.80°$$

The formula of this phase is probably V_3O_8 and the unit cell thus contains 4 V_3O_8.

Powder photographs of the phases $VO_{1.75}-VO_{2.00}$ suggest pronounced structural relationships to exist between these oxides. They may represent oxides belonging to a series with the general formula V_nO_{2n-1}.

The VO_2 phase is monoclinic with the unit cell dimensions:

$$a = 5.743 \text{ Å}; \; b = 4.517 \text{ Å}; \; c = 5.375 \text{ Å}; \; \beta = 122.61°$$

and the observed reflections are in accordance to the space-group $P2_1/c$. The unit cell contains 4 VO_2.

The V_6O_{13} phase has been found to decompose into VO_2 and V_2O_5 at 700° C and higher temperatures.

PREVIOUS WORK

The system vanadium-oxygen has previously been the object of several studies. Thus, Mathewson, Spire, and Samans [1] investigated the lower vanadium oxides by means of X-ray powder photographs and microscopic examinations. They found that VO has a structure of the sodium chloride type with an extended homogeneity range and that this phase can exist in equilibrium with vanadium metal. They also supported the findings of Goldschmidt, Barth, and Lunde [2] and of Zachariasen [3] who found V_2O_3 to have a corundum type of structure.

Klemm and Grimm [4] studied the vanadium-oxygen system for compositions between V and V_2O_3. According to these authors the vanadium phase can dissolve up to 0.4 oxygen atoms per metal atom at 1 450—1 600° C. They also reported that the face-centered cubic VO phase exists between $VO_{0.9}$ and $VO_{1.3}$ at high temperatures but that this phase at lower temperatures decomposes into the metal phase and a higher oxide (not V_2O_3).

Seybolt and Sumsion [5] recently reported the existence of a metallic oxide with the approximate limits of homogeneity $VO_{0.15}$—$VO_{0.25}$. Schönberg [6] confirmed the existence of this oxide. He also demonstrated the VO phase to be stable with an extended homogeneity range at low temperatures.

For compositions between V_2O_3 and V_2O_5 a phase analysis was performed by Hoschek and Klemm [7]. They found some evidence for the existence of a new oxide within each of the ranges V_2O_3—VO_2 and VO_2—V_2O_5 but did not express a definitive inference concerning the number of phases existing. They also stated that the VO_2 phase is homogeneous between $VO_{1.8}$ and $VO_{2.0}$.

Measurements of the electric conductivity of vanadium oxides as well as X-ray studies performed by Klemm and Pirscher [8] were thought to confirm the existence of the phases suggested by Hoschek and Klemm [7].

Goldschmidt [9] reported that VO_2 has a tetragonal structure of the rutile type.

The composition range VO_2—V_2O_5 was studied at this Institute by Aebi [10] who demonstrated the existence of an intermediate phase $VO_{2.17}$. He determined the crystal structure of this phase by means of X-ray methods and found the formula to be V_6O_{13}.

The crystal structure of V_2O_5 was recently determined by Byström, Wilhelmi, and Brotzen [11].

PRESENT INVESTIGATION

As the knowledge of the system especially for compositions between V_2O_3 and VO_2 seemed to be rather incomplete a new phase analysis was thought to be desirable. This article will present an account of investigations of the system within the composition range VO—V_2O_5 at temperatures between 650 and 1 000° C *. For compositions between V and VO a phase analysis has simultaneously been performed at this Institute by Schönberg [6].

EXPERIMENTAL

The starting materials for the preparations were vanadium metal (Vanadium Corporation of America, 99.7 % Grade) and vanadium pentoxide (Schering-Kahlbaum and L.K.B., Sweden, *pro analysi*) which had been heated at 400° C. Vanadium trioxide of high purity obtained by reduction of vanadium pentoxide with hydrogen at 800° C was also used for several syntheses. The vanadium content of this oxide was determined by titration with permanganate [13].

Preparations of vanadium oxides with compositions between VO and V_2O_5 were generally obtained by heating weighed mixtures of vanadium metal powder (or in some cases vanadium trioxide) and vanadium pentoxide in evacuated silica tubes at temperatures between 650 and 1 000 °C. The heating time varied between 2 days and 20 days. For lower oxides it was generally necessary to use a longer heating time to attain equilibrium

* A short note concerning this work has recently been published [12].

than for oxides rich in oxygen. The samples were mostly quenched in cold water from the heating temperature. At temperatures above 700° C samples richer in oxygen than the dioxide appreciably affected the silica tubes with a greenish layer appearing on the inside of the walls of the tubes.

The samples obtained in this way were investigated under the microscope and by taking X-ray powder photographs in a Guinier focussing camera with monochromatized Cr-$K\alpha$ radiation. In the cases where single crystals could be obtained rotation and Weissenberg photographs were taken with Cu-K radiation.

PHASES OBSERVED

The present phase analysis has revealed the existence of eleven discrete oxides with the following approximate composition: VO, $VO_{1.50}$, $VO_{1.67}$, $VO_{1.75}$, $VO_{1.80}$, $VO_{1.84}$, $VO_{1.86}$, $VO_{1.87}$, $VO_{2.00}$, $VO_{2.17}$, and $VO_{2.50}$. (The existence of an oxide phase with the approximate homogeneity range $VO_{0.15}$

Table 1. Results of the phase analysis of vanadium oxides.

Composition of the samples	Temperature of preparation and quenching	Appearance of the samples	Phases observed
$VO_{1.07}$	~ 900° C	Dark or medium gray powder	VO
$VO_{1.15}$	»	—»—	VO
$VO_{1.20}$	»	—»—	VO
$VO_{1.25}$	»		VO(+V_2O_3)
$VO_{1.35}$	»		V_2O_3+VO
$VO_{1.45}$	»		V_2O_3(+VO)
$VO_{1.50}$	»	Grayish powder	V_2O_3
$VO_{1.55}$	»		V_2O_3(+$VO_{1.67}$)
$VO_{1.65}$	»		$VO_{1.67}$(+V_2O_3)
$VO_{1.67}$	»	Dark gray powder or very small crystals	$VO_{1.67}$
$VO_{1.72}$	»		$VO_{1.75}$ + $VO_{1.67}$
$VO_{1.75}$	»	Dark lustrous powder or aggr. of very small crystals	$VO_{1.75}$
$VO_{1.77}$	»		$VO_{1.75}$ + $VO_{1.80}$
$VO_{1.80}$	»	Nearly black lustrous powder or aggr. of small crystals	$VO_{1.80}$
$VO_{1.82}$	»		$VO_{1.80}$ + $VO_{1.84}$
$VO_{1.84}$	»	Lustrous powder or aggr. of blue-black crystals	$VO_{1.84}$
$VO_{1.85}$	»		$VO_{1.84}$ + $VO_{1.86}$
$VO_{1.86}$	»	Strongly lustrous blue-black powder or very small crystals	$VO_{1.86}$
$VO_{1.865}$	»		$VO_{1.87}$ + $VO_{1.86}$
$VO_{1.87}$	»	—»—	$VO_{1.87}$
$VO_{1.92}$	»		VO_2 + $VO_{1.87}$
$VO_{2.00}$	»	Needle- or rodshaped blue-black crystals	VO_2
$VO_{2.15}$	≥ 700° C		VO_2(+V_2O_5)
$VO_{2.30}$	»		V_2O_5 + VO_2
$VO_{2.10}$	~ 650° C		VO_2 + V_6O_{13}
$VO_{2.17}$	»	Black lustrous powder or small crystals	V_6O_{13}
$VO_{2.25}$	»		V_6O_{13} + V_2O_5
$VO_{2.50}$	»	Brownish yellow powder or brownish crystals	V_2O_5

Table 2. X-ray diffraction data of the phases $VO_{1.67}$, $VO_{1.75}$, $VO_{1.80}$, $VO_{1.84}$, $VO_{1.86}$, $VO_{1.87}$, and VO_2. Cr-Ka radiation.

$VO_{1.67}$

hkl	I_{rel}	$\sin^2\Theta_{obs}$	$\sin^2\Theta_{calc}$
$\bar{2}$,0,2	m	.0609	.0610
$\bar{1}$,1,1	w	.0671	.0671
2,0,0	st	.1214	.1214
0,0,2	w	.1250	.1250
$\bar{1}$,1,1	st	.1598	.1598
$\bar{3}$,1,3	st	.1892	.1892
$\bar{0}$,2,0	m	.2074	.2073
$\bar{3}$,1,1	vw	.2171	.2171
$\bar{1}$,1,3	m	.2244	.2245
$\bar{4}$,0,4	vw	.2442	.2442
$\bar{2}$,2,1	m+	.2674	.2673
$\bar{2}$,2,2	m	.2688	.2684
$\bar{3}$,1,4			.2690
1,1,2	m	.3000	.2999
2,2,0	m−	.3287	.3287
$\bar{0}$,2,2	vw	.3325	.3324
$\bar{5}$,1,5	m+	.4332	.4333

VO_2

hkl	I_{rel}	$\sin^2\Theta_{obs}$	$\sin^2\Theta_{calc}$
$\bar{1}$,1,1	w	.1198	.1198
$\bar{0}$,1,1	vst	.1283	.1283
$\bar{1}$,0,2	w	.1830	.1830
$\bar{2}$,0,2	w+	.2220	.2220
$\bar{2}$,1,1	m+	.2234	.2234
$\bar{2}$,0,0	w	.2241	.2242
$\bar{2}$,1,2	m	.2864	.2863
$\bar{2}$,1,0	m	.2887	.2885
$\bar{1}$,2,1	vw	.3124	.3127
0,1,2	w	.3207	.3203
$\bar{0}$,2,1			.3212
$\bar{3}$,0,2	vw+	.3732	.3732
$\bar{3}$,1,1	vw+	.4395	.4391
$\bar{1}$,2,2			.4402
$\bar{2}$,1,3	w	.4771	.4772
$\bar{2}$,2,2	w	.4788	.4792
2,2,0	m+	.4814	.4814
$\bar{2}$,1,1			.4816
$\bar{1}$,1,3	vw	.5027	.5027

$VO_{1.75}$

$\sin^2\Theta_{obs}$	I_{rel}
.0358	vw
.0494	w
.0744	m
.0960	w−
.1187	st
.1223	w
.1414	w
.1485	m+
.1607	w
.1711	m+
.1746	m−
.1975	m
.2123	vw
.2142	m−
.2244	m−
.2324	w+
.2550	w
.2756	st
.2832	m−
.2964	m−
.2991	w+

$VO_{1.80}$

$\sin^2\Theta_{obs}$	I_{rel}
.0220	vw
.0503	w
.0832	m+
.1173	vw
.1191	st
.1220	vw
.1310	vw
.1393	vw+
.1432	m+
.1565	vw
.1602	st
.1847	m−
.2022	m
.2166	m−
.2239	m−
.2357	w
.2469	vw
.2768	w+
.2788	m+
.2822	w
.2882	w+

$VO_{1.84}$

$\sin^2\Theta_{obs}$	I_{rel}
.0513	vw
.0554	w
.0895	m+
.1197	st
.1293	w+
.1400	st
.1533	st
.1687	w
.1908	m−
.1990	w
.2056	m+
.2180	m
.2234	m−
.2382	w
.2422	w
.2708	w+
.2782	m
.2796	w+
.2812	m
.2924	m−
.2936	m−

$VO_{1.86}$

$\sin^2\Theta_{obs}$	I_{rel}
.0496	vw
.0610	w−
.0805	vw
.0942	m+
.1204	st
.1244	w+
.1380	st
.1489	st+
.1768	vw
.1789	vw
.1954	w+
.1989	w−
.2083	m
.2188	m
.2235	m
.2388	w−
.2402	w−
.2633	w+
.2794	m
.2807	m
.2825	m

$VO_{1.87}$

$\sin^2\Theta_{obs}$	I_{rel}
.0666	w
.0983	m
.1214	st+
.1369	st
.1461	st−
.1994	w
.2005	w
.2109	m
.2201	m
.2242	m+
.2376	w
.2584	w+
.2812	st
.2822	m
.2840	m
.2931	st
.2987	w
.3126	w+
.3211	vw
.4143	w−
.4646	m+

vst = very strong; st = strong;
m = medium; w = weak; vw = very weak.

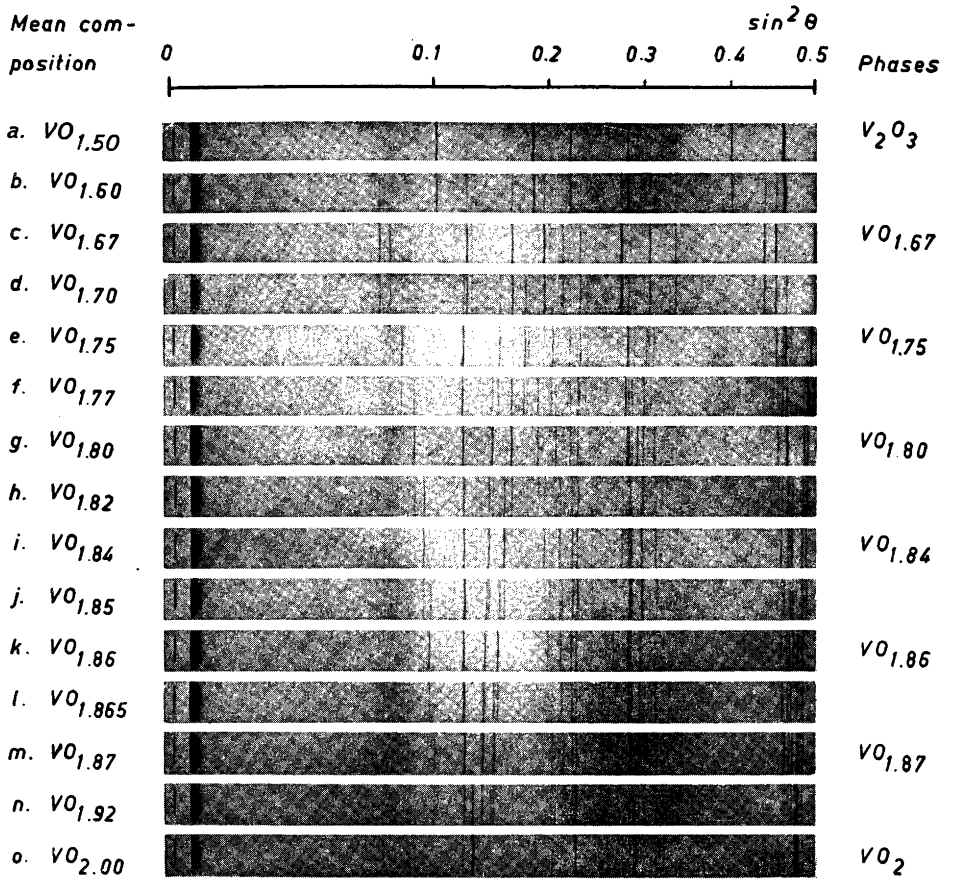

Fig. 1. X-ray powder photographs of samples VO_x (Cr-*Ka radiation*).

—$VO_{0.25}$ has recently been demonstrated by Seybolt and Sumsion [5] and by Schönberg [6].) In the following these approximate formulae will be used as symbols for the various intermediate phases.

The results of the phase analysis are summarized in Table 1 while X-ray diffraction data for the oxides $VO_{1.67}$—VO_2 are listed in Table 2. Fig. 1 represents a selection of powder photographs for compositions $VO_{1.50}$—$VO_{2.00}$.

In agreement with the observations of Klemm and Pirscher [8] measurements of the electric conductivity have shown the oxides VO to $VO_{2.17}$ inclusively to be semiconductors.

The VO phase. As stated by previous authors [1,4,6] the monoxide VO of sodium chloride structure shows a wide range of homogeneity. It was found rather difficult to reach a state of equilibrium in samples of this phase. However, after prolonged heating at 900° C it was possible to obtain several samples

$VO_{1.07}$—$VO_{1.20}$ the powder patterns of which did not show any traces of phases other than VO. The lattice constants derived from powder photographs are given in Fig. 2 together with lattice constants obtained by Schönberg (private communication). Contrary to the statement of Klemm and Grimm [4] the unit cell dimensions thus were found to increase with increasing oxygen content. The upper and the lower limits of the phase range were found to be $VO_{1.20}$ and $VO_{0.75}$ with the axial lengths $a = 4.128$ Å ($VO_{1.20}$) and $a = 4.043$ Å ($VO_{0.75}$) respectively. The axial length at the composition $VO_{1.00}$ was found to be $a = 4.09$ Å.

The V_2O_3 phase. Powder photographs of samples with compositions around $VO_{1.25}$ (heated at 900° C) showed weak lines of the V_2O_3 phase while for compositions between $VO_{1.40}$ and $VO_{1.55}$ the V_2O_3 lines dominated the patterns. Variations in the positions of the reflections could not be detected for samples containing this phase indicating the homogeneity range to be very narrow. The corundum structure [2,3] of the phase was verified, and the following dimensions of the rhombohedral unit cell were derived in reasonable accordance to the values given by Zachariasen [3]:

$$a = 5.467 \text{ Å}; \ \alpha = 53.74°$$

The $VO_{1.67}$ phase. Powder photographs of the samples $VO_{1.55}$—$VO_{1.65}$ showed lines of V_2O_3 together with lines of another phase. The latter lines appeared alone in photographs of the sample $VO_{1.67}$ (Fig. 2 c) and closely around this composition (about $\pm$ 1 atom-%). The range of homogeneity was found to be very narrow. The phase was investigated by single crystal as well as powder methods (Table 2).

The crystal structure was found to be monoclinic with $C2/c$ or Cc as probable space-groups. The unit cell dimensions were:

$$a = 9.983 \text{ Å}; \ b = 5.031 \text{ Å}; \ c = 9.835 \text{ Å}; \ \beta = 138.80°$$

The density of the sample was determined to be 4.55 which corresponds to 11.6 $VO_{1.67}$ per unit cell and suggests a cell content of 4 formula units of V_3O_5. A determination of the crystal structure is in progress.

The $VO_{1.75}$, $VO_{1.80}$, $VO_{1.84}$, $VO_{1.86}$, and $VO_{1.87}$ phases. Powder photographs of samples with the compositions $VO_{1.70}$ and $VO_{1.72}$ clearly showed the phase $VO_{1.67}$ together with the phase denoted $VO_{1.75}$ while in photographs of the samples $VO_{1.77}$ and $VO_{1.79}$ the $VO_{1.75}$ lines appeared together with lines of the $VO_{1.80}$ phase. Samples of the composition $VO_{1.75}$ gave reflections of only one phase, *viz.* the $VO_{1.75}$ phase (Fig. 1 e). Crystals suitable for single crystal methods have not yet been obtained of this phase.

From powder photographs of samples with compositions $VO_{1.77}$—$VO_{1.82}$ the next phase was found to be homogeneous very close to the composition $VO_{1.80}$ (Fig. 1 g). Attempts to produce single crystals by prolonged heatings have, not yet been successful.

The powder photographs of the three following phases which seem to be homogeneous at compositions about $VO_{1.84}$, $VO_{1.86}$, and $VO_{1.87}$ respectively (Fig. 1 i, k, m) resemble each other very much as well as the photographs of the two preceding oxides (Table 1). After prolonged heatings at higher temperatures very small strongly reflecting crystals without characteristic shape could

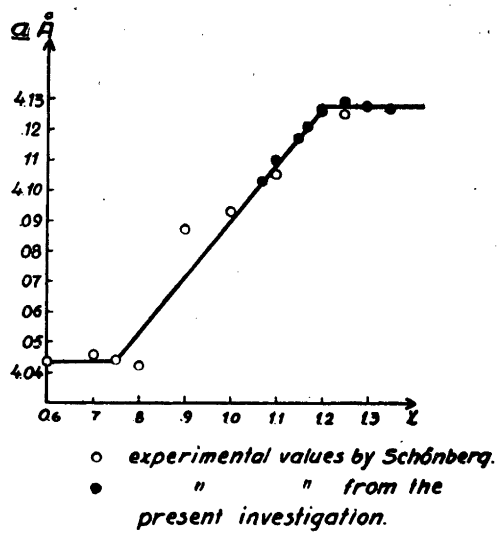

Fig. 2. The axial length (a) as a function of the composition (VOx) at 900 °C.

be observed under the microscope but so far no crystals suitable for single crystal investigations have been obtained.

Extended homogeneity ranges have not been demonstrated for any of the oxides $VO_{1.75}$, $VO_{1.80}$, $VO_{1.84}$, and $VO_{1.86}$. In some powder photographs of samples $VO_{1.90}$—$VO_{1.96}$ a few weak lines were observed besides the powder pattern of $VO_{1.87}$ and together with the pattern of VO_2 (Fig. 1 n). This fact possibly indicates that the $VO_{1.87}$ phase has a variable composition or possibly that still another phase may be present here.

The close similarity between the powder patterns of all the phases from $VO_{1.75}$ and to VO_2 suggests pronounced structural relationships between these phases (Fig. 1 e—o and Table 2). As their powder photographs are rather complicated further interpretation seems to require single crystal methods.

It has been pointed out to the author by Dr. A. Magnéli that these oxides within the limits of error have compositions corresponding to the general formula V_nO_{2n-1}, *viz.* $VO_{1.75}$ ($V_4O_7 = VO_{1.750}$), $VO_{1.80}$ ($V_5O_9 = VO_{1.800}$), $VO_{1.84}$ ($V_6O_{11} = VO_{1.833}$), $VO_{1.86}$ ($V_7O_{13} = VO_{1.857}$), $VO_{1.87}$ ($V_8O_{15} = VO_{1.875}$), and VO_2 (V_nO_{2n-1}, $n \rightarrow \infty$). This conformity together with the pronounced structural relationship suggested by the similarity of the powder patterns might indicate the existence of a "homologous series" of vanadium oxides structurally based on the rutile type structure in a way similar to the homologous series of molybdenum tungsten oxides $(Mo, W)_nO_{3n-1}$ derived from the ReO_3-type structure [14–16].

The VO_2 phase. At first sight single crystal photographs seem to indicate a tetragonal unit cell with the *a* and *c* axes twice as long as those corresponding to the structure of rutile type reported by Goldschmidt [9]. However, the multiplet structure of several powder lines (Fig. 2 o) shows that the actual symmetry is lower, *viz.* monoclinic. Observed and calculated positions and

relative intensities of the powder reflections are recorded in Table 2. The dimensions of the primitive unit cell were from powder photographs found to be:

$$a = 5.743 \text{ Å}; \; b = 4.517 \text{ Å}; \; c = 5.375 \text{ Å}; \; \beta = 122.61°$$

This cell contains four units VO_2. The space-group is probably $P\,2_1/c$. No displacements of the powder lines could be observed either in photographs taken of samples of the VO_2 phase together with the lower oxide phase or together with higher oxide phases. This indicates that the homogeneity range of this phase must be very narrow. A determination of the crystal structure of VO_2 is in progress.

The V_6O_{13} and V_2O_5 phases. Powder photographs of samples with compositions between $VO_{2.05}$ and $VO_{2.15}$ quenched from about 650° C showed VO_2 lines together with lines of V_6O_{13}. Samples $VO_{2.10}$—$VO_{2.40}$ quenched from 700° C and higher temperatures, however, only gave the patterns of VO_2 and V_2O_5 (Table 1) showing the V_6O_{13} phase to be unstable at these temperatures. The unit cell dimensions and symmetry of the V_6O_{13} phase previously found at this Institute by Aebi [10] were verified.

The unit cell dimensions observed for V_2O_5 in the present investigation agree with those derived by Byström *et al.*[11] and by Magnéli and Blomberg [17].

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Studies on the Hydrolysis of Metal Ions

VIII. Methods for Deducing the Mechanism of Polynuclear Hydrolysis Reactions

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It is a well-known fact that a large number of metal ions Me^{z+} may react with water in such a way that hydrogen ions are set free, while the solution remains clear and no visible precipitate is formed. Common examples are Fe^{3+}, Al^{3+}, and Hg^{2+}. Leaving out the water of hydration, we may write the general formula for a reaction of this kind in the form:

$$q\ Me^{z+} + p\ H_2O \rightleftharpoons Me_q(OH)_p^{(qz-p)+} + p\ H^+ \qquad (1)$$

Many earlier attempts have been made to formulate such hydrolysis reactions. Among the products that have been proposed we may mention:

1) the uncharged hydroxide, in molecular or colloidal solution, without intermediate steps, *e.g.* $Al^{3+} + 3\ H_2O \rightleftharpoons Al(OH)_3 + 3\ H^+$

2) one or more mononuclear complexes, formed by splitting off protons from aquo complexes [10], *e.g.* $Al(H_2O)_6^{3+} \rightleftharpoons Al(H_2O)_5OH^{2+} + H^+ \rightleftharpoons Al(H_2O)_4(OH)_2^+ + 2\ H^+$.

3) one or a few polynuclear complexes, *e.g.* $Bi_2(OH)_4^{2+}$ and $Bi_2(OH)_5^+$ [11], or Pb_2OH^{3+} and $Pb_4(OH)_4^{4+}$ [12].

In Part I of the present series [1—7] an attempt was made to explain the data for Bi^{3+} with still another mechanism: the formation of a series of polynuclear complexes, in this case $Bi(OBi)_n^{n+3}$, $n = 1, 2, 3 \ldots$

It may be said that many previous data on metal ion hydrolysis do not permit one to distinguish between various types of mechanism. Generally the total concentration (M) of the metal ion, and the hydrogen ion concentration h of the solution, have been varied either within only narrow limits, or not at all. The old practice of measuring the pH of solutions of "pure salts" is especially very likely to give erroneous results because of impurities: for an example, see part VI[6,p.467]. Moreover, the ionic strength has generally not been kept constant, so that variations in the activity factors cause large uncertainties.

Our research group has tried to study each metal ion over a not too narrow concentration range. We have been working with solutions in a constant ionic medium (usually 1.0 or 3.0 C $NaClO_4$) and have measured the hydrogen ion concentration and, if possible, the free metal ion concentration by electrometric methods [1–7]. Similar investigations are also available from other laboratories [13–15].

Even in our limited experience the ions have shown such a variety of individual behavior that we think it very improbable that one single type of product could account for the hydrolysis of all different metal ions.

For two of the ions studied by our group, namely Hg^{2+} and Tl^{3+}, the main products of the reaction proved to be *mononuclear:* $HgOH^+$ and $Hg(OH)_2$, part II; $TlOH^{2+}$ and $Tl(OH)_2^+$, part V. For a number of other ions, the main products are obviously *polynuclear*, and various polynuclear mechanisms have also been proposed, both by our group (Bi^{3+} part I; Fe^{3+} part VII) and by others (UO_2^{2+}, Ahrland [13]; Al^{3+}, Brosset,[14]; Sc^{3+}, Kilpatrick and Pokras [15]). Now, once one knows that polynuclear complexes are formed, the number of possible products becomes very great. So, even if one polynuclear mechanism seems to explain the data, one may reasonably doubt whether the same data could not equally well be explained by several other mechanisms, perhaps radically different from the one chosen.

Recently, one of us has developed some general equations for the formation of polynuclear complexes between two reactants A and B. It was also shown how the data may be treated in order to find out the nature of the reaction products [8,9]. In the following, these papers will be denoted by ABI and ABII. We shall not repeat here the whole argument in ABI and ABII, but shall instead give a "key" for transforming the equations for the general case to the special case of the hydrolysis of a metal ion. We shall then give some of the main conclusions of ABI and ABII for the special case of hydrolysis equilibria, and finally illustrate their use by application to a few ions.

List of symbols*

A	total concentration of A* (ABI, 2)
a	concentration of free A (ABI, 1)
B	total concentration of B (ABI, 2)
b	concentration of free B (ABI, 1)
c_n	concentration of n:th complex (30)
f	$= \Sigma k_n u^n$ (32)
g	parameter in (37) and (38)
H	total analytical excess concentration of hydrogen ions assuming Me^{z+} not to be hydrolyzed; H may be negative (5)
h	free concentration of H^+ ions (2)
k_n	equilibrium constant for the formation of the n:th complex (30)
k_0	constant, defined by (36) for each of the hypotheses IIIa, IIIb and IIIc
k	equilibrium constant, defined by (36) for IIIa—c, and by the text above (36) for hypotheses II and I

* *N. B.* Chemical symbols in Roman, *concentrations* in *italic* type!

k_{pq} equilibrium constant for formation of $Me_q(OH)_p$ from Me^{z+} and OH^- (12)
k_s solubility product in hypothesis I
k_w ionic product of water
M total concentration of Me in the solution (4)
m concentration of free Me^{z+} (2)
N unique value for n, in hypothesis II
n number of links in complex (15) or (19)
p number of A, or OH, units in complex
q number of B, or Me, units in complex
R partial derivative in (14)
r number of OH in "core", writing the complex as $(OH)_r((OH)_tMe)_n$ (19)
S complexity sum (9)
s number of Me in "core", writing the complex as $Me_s((OH)_tMe)_n$
t number of OH units per Me in "link" (15) or (19)
u $= mh^{-t}$ (31)
v $= ku$ (ABII, 6); v_0 = value of v for $\xi = 0$ (39)
x $= \log M - t \log h$ (17)
X $= x + \log 2 - x_{\frac{1}{2}}$ (ABII, 10)
$x_{\frac{1}{2}}$ value of x for $y = \frac{1}{2}$ (ABII, 10)
y $= Z/t$ (18)
Z Average number of OH^- bound, or H^+ split off, per Me (6)
z charge of Me^{z+} ion
η $= \log M/m$ (10); η_0 = value of η for $\xi = 0$ (39)
$\varkappa_{pq}$ equilibrium constant for the formation of $Me_q(OH)_p$ from Me^{z+} and H_2O (3)
ξ $= x +$ constant, chosen either to make the asymptote of $\eta(\xi)$ pass through the origin (ABII 51, 53) or to make all curves pass through $\eta = \xi = \log 2$ (ABII 69, III c).

Hydrolysis equilibria; general equations

Let us assume that the metal ion Me^{z+} reacts with water to form one or several products. If, say, the ionic medium is aqueous $NaClO_4$, the most general formula for the soluble complexes would be

$$Me_q(H_2O)_\alpha(H^+)_{-p}(Na^+)_\beta(ClO_4^-)_\gamma^{(qz-p+\beta-\gamma)+}$$

However, equilibrium data in an ionic medium do not allow us to distinguish between species containing varying amounts of the solvent H_2O, or of the medium ions (say, Na^+ and ClO_4^-). Thus we suffer no loss in generalization if we write the general formula of the complexes as

$$Me_q(OH)_p^{(qz-p)+}$$

remembering that they may contain, in addition, an unknown amount of H_2O, Na^+ and ClO_4^-. One O^{2-} in an oxo complex would count as 2 OH^- ($= O^{2-} + H_2O$) in the formula.

Let $\varkappa_{pq}$ denote the equilibrium constant of the reaction

$$q\ \mathrm{Me}^{z+} + p\ \mathrm{H_2O} \rightleftharpoons \mathrm{Me}_q(\mathrm{OH})_p^{(qz-p)+} + p\ \mathrm{H}^+ \tag{1}$$

In the following we shall often leave out the charges of the complexes.

We shall introduce the symbols m and h for the concentrations of the "free" Me^{z+} and H^+ (free = bound to water of hydration and inert medium ions only)

$$[\mathrm{Me}^{z+}] = m; \qquad [\mathrm{H}^+] = h \tag{2}$$

From (1) we have the concentration of each complex

$$[\mathrm{Me}_q(\mathrm{OH})_p] = \varkappa_{pq} m^q h^{-p} \tag{3}$$

The total concentration M of metal ion, free or complexed, is

$$M = [\mathrm{Me}^{z+}] + \Sigma q\ [\mathrm{Me}_q(\mathrm{OH})_p] = m + \Sigma q \varkappa_{pq} m^q h^{-p} \tag{4}$$

and the analytical excess H of hydrogen ions (proton excess in the water minus the proton deficiency in the complexes) is:

$$H = [\mathrm{H}^+] - [\mathrm{OH}^-] - \Sigma p[\mathrm{Me}_q(\mathrm{OH})_p] = h - k_w h^{-1} - \Sigma p \varkappa_{pq} m^q h^{-p} \tag{5}$$

Here k_w is the ionic product of water, and the summations are taken over all sets of (p,q), except (0,1) and (1,0) which correspond to Me^{z+} and OH^-.

A useful quantity, which can in general be obtained directly from the experimental data, is the average number of OH^- groups bound per Me atom, or the average number of H^+ split off per Me

$$Z = \frac{\Sigma p[\mathrm{Me}_q(\mathrm{OH})_p]}{M} = \frac{h - H - k_w h^{-1}}{M} \tag{6}$$

Equations (4) and (6) may also be written in the form

$$M - m = m \left(\frac{\partial S}{\partial m}\right)_h \tag{7}$$

$$ZM = h^{-1}\left(\frac{\partial S}{\partial h^{-1}}\right)_m = -h\left(\frac{\partial S}{\partial h}\right)_m \tag{8}$$

where S, the complexity sum, is

$$S = \Sigma[\mathrm{M}_q(\mathrm{OH})_p] = \Sigma \varkappa_{pq} m^q h^{-p} \tag{9}$$

Experimental data

Of the four quantities M, H, m, and h, the first two should always be known from the analytical data for each equilibrium solution studied. Generally, h is also known with some accuracy, whereas m can be measured only in certain favorable cases.

M is the total concentration of the metal in question, eq. (4), whether present as free ion Me^{z+} or bound in complexes. As a rule it is advantageous

to plan the experiments in such a way that M is constant in each group of measurements. Thus M has only a limited number of values: M_1, M_2, M_3 *etc.*, whereas H is varied freely.

H is the analytical excess of hydrogen ions, which may be calculated neglecting the hydrolysis reactions and assuming the solution to be made up of only H_2O, H^+, and Me^{z+}, in addition to the inert medium ions. In solutions with a deficiency of protons, H is negative, see *e.g.* (5).

m is the concentration of "free" metal ions Me^{z+} and can in some cases be measured by means of a metal, amalgam, or redox electrode.

h, the concentration of "free" hydrogen ions can be measured by means of a glass, hydrogen, or quinhydrone electrode. If Me^{z+} forms complexes with quinone or quinol, the quinhydrone electrode should be avoided. If Me^{z+} enters into redox reactions with hydrogen or quinhydrone, one may have to resort to the glass electrode in spite of its lower accuracy.

Z, the average number of H^+ split off from (OH^- bound per) Me atom is easily calculated from (6) if k_w has been determined by separate experiments — often the small correction $k_w h^{-1}$ can be neglected. Z is a much more convenient quantity than H for the following calculations.

If data (M,H,h) are available, it is convenient to transform them to a diagram giving $Z(\log h)_M$, *i.e.* to plot Z as a function of $\log h$ and join all points with the same value of M by a separate curve.

If data concerning m are also available, one may calculate the quantity

$$\eta = \log \frac{M}{m} \tag{10}$$

and draw a diagram $\eta(\log h)_M$.

Our experimental data can thus be represented in one or two diagrams: the family of curves $Z(\log h)_M$, and in favorable cases also $\eta(\log h)_M$. We may then try to use the methods given in ABI and ABII to find the composition of the complexes and, if possible, the details of the reaction mechanism.

Transformation of A—B equations

Let us first compare the formulas derived for the special case of Me^{z+} hydrolysis with the equations derived in ABI for the general case of polynuclear complex formation between A and B. Our equations (1,2,3) may be considered as special cases of (ABI, 3 and 1) transforming the reactants and the free concentrations as follows:

$$B \rightarrow Me^{z+} \; ; \; A \rightarrow -H^+ \tag{11 a}$$

$$b \rightarrow m \; ; \; a \rightarrow h^{-1} \tag{11 b}$$

The total concentration M in (4) corresponds to B in (ABI, 2)

$$B \rightarrow M \tag{11 c}$$

The amount of A bound in complexes in (ABI, 5 and 7) corresponds to the amount of H^+ split off, in (6) and (8)

$$A-a = BZ \rightarrow MZ = h - H - k_w h^{-1} \tag{11 d}$$

Except for a, b, A and B, all quantities Z,η etc. will be denoted by the same symbols as used in ABI and ABII.

The fact that the reactant $-H^+$ is involved in an equilibrium with the solvent H_2O is expressed by the term $k_w h^{-1}$ in (11 d) — similar correction terms would be necessary for any other reactant A which reacts with the solvent.

Some readers may find it over-sophisticated to consider the "proton deficiency" as a reactant and may prefer to use the reactant OH^-. Denoting the equilibrium constant for the formation of the complex $Me_q(OH)_p$ by k_{pq}, one would then replace (3) by

$$[Me_q(OH)_p] = k_{pq} m^q [OH^-]^p = k_{pq} k_w^p m^q h^{-p} \qquad (12)$$

The new set of constants k_{pq} are related to the $\varkappa_{pq}$ by the equation (*cf.* 3):

$$\varkappa_{pq} = k_{pq} k_w^p \qquad (13)$$

but the treatment of the data will be analogous to that given here, and in ABI and ABII. Since, however, the measurements generally give $[H^+]$ directly we have preferred the formulation using H^+.

We shall now use (11 a—d) to transform the main conclusions of ABI and ABII to a form applicable to measurements of metal ion hydrolysis. For proofs and further discussion see the general papers ABI and ABII.

DEDUCTION OF THE GENERAL FORMULA OF THE COMPLEXES

Coinciding curves

It may result that the quantities Z and η depend only on h and are independent of M. One then gets a single curve $Z(\log h)$ and a single curve $\eta(\log h)$, irrespective of the value of M. This implies that only *mononuclear* complexes, of the general formula $Me(OH)_n$, are formed in appreciable amounts.

In the case where only Z ($\log h$) data are available and m is negligible compared with M in the whole range studied, there is an additional theoretical possibility that all complexes are "homonuclear", *i. e.* of the general formula $Me_Q(OH)_n$, with Q constant, and n variable (ABI, p. 311—312).

To find the equilibrium constants, when only mono- (or homo-)nuclear complexes are formed, one may apply methods previously worked out by N. and J. Bjerrum, Leden, Fronæus and others for studying complex equilibria. For examples, the reader is referred to part II and part V, on the hydrolysis of the ions Hg^{2+} and Tl^{3+}.

Parallel curves

It has been found in a number of cases that the curves $Z(\log h)_M$ for different M values are parallel within the experimental error, and that the horizontal spacing between two curves, $\Delta \log h$, is proportional to the difference between the two $\log M$ values, $\Delta \log M$. This may be expressed in the mathematical form:

$$\left(\frac{\partial \log M}{\partial \log h}\right)_Z = -R = \text{constant} \qquad (14)$$

Hence

$$Z = \text{function of } (\log M + R \log h) \qquad (14\text{ a})$$

First let us suppose that m is not negligible compared with M, at least in some part of the range where (14) is valid. (The opposite case will be dealt with in the text accompanying equations (19—21) below.) Then it follows from (14) (see deduction of equations ABI, 19 and 19 a from ABI, 16) that all complexes formed in appreciable amounts can be written in a "core + links" form.

$$\mathrm{Me((OH)_{\mathit{t}}Me)_{\mathit{n}}},\ \text{with}\ t = -R \tag{15}$$

Thus t is the constant obtained from (14), whereas the value(s) of n are as yet undetermined. It follows from (14 a) and (15) that

$$Z = \text{function of } x \text{ only};\ x = \log M - t \log h \tag{15 a}$$

If measurements of m are also available, the curves $\eta(\log h)_M$ often prove to be parallel with a displacement represented by the equation:

$$\left(\frac{\partial \log M}{\partial \log h}\right)_{\eta} = t = \text{constant} \tag{16}$$

Hence

$$\eta = \text{function of } x \text{ only};\ x = \log M - t \log h \tag{16 a}$$

As shown in (ABI, 40—44), equation (16), like (14), implies that all complexes formed in appreciable amounts can be written in the "core + links" form $\mathrm{Me((OH)_{\mathit{t}}Me)_{\mathit{n}}}$ where t is a constant but n may vary.

To determine with what accuracy equations (15 a) and (16 a) are valid, one may plot Z and η as functions of the variable (ABI, 26)

$$x = \log M - t \log h \tag{17}$$

This is the same as to displace the curves $Z(\log h)_M$ or $\eta(\log h)_M$ along the $\log h$ axis to see whether they coincide. This method can also be used if data are available for varying values of M, and not for a discrete set of M values. From a few points one can easily find an approximate value for t; one may then use tentative values 2, 3, *etc.* for t, plotting Z or η against $\log M - 2 \log h$, $\log M - 3 \log h$ *etc.* or, if one prefers, against $\log h - \frac{1}{2} \log M$, $\log h - \frac{1}{3} \log M$ *etc.* If data from a sufficiently broad M range are available, all values for t except one can easily be ruled out.

Before discussing the details of the mechanism (see below) it is convenient to divide Z by t (ABI, 25).

$$y = \frac{Z}{t} \tag{18}$$

and to consider the curve $y(x)$.

There is one special case we should treat separately, namely that where only Z data are available and m is negligible within the whole range studied. Let us suppose that the curves are parallel so that (14) and (14 a) are still valid. In this case the general formula of the complexes is (ABI, p. 308)

$$\mathrm{(Me(OH)_{\mathit{t}})_{\mathit{n}}(OH)_{\mathit{r}}},\ \text{with}\ r = R; \tag{19}$$

Thus r is the constant obtained from the spacing, and t is another constant which may be deduced from the limiting values for $Z: t + r/n_{max}$ and $t + r/n_{min}$ (ABI, 14)

In this case it is convenient to consider the smallest possible complex $Me(OH)_{r+t}$ as the core and $Me(OH)_t$ as the link. The functions to be plotted take the form (ABI, 33 and 34):

$$x = \log M + r \log h \tag{20}$$

$$y = \frac{r + t - Z}{r} \tag{21}$$

This treatment will be exemplified in forthcoming papers on Al^{3+} in the alkaline range and on silicate equilibria.

Equation (14) is closely related to one given by Byé (Ref. [16], eq. 22) and applied by him to the reactions of molybdate ions, by Carpéni and Souchay [17] to other anion systems, and by Schaal and Faucherre [18] to the hydrolysis of Th^{4+}, UO_2^{2+} and other ions. There are, however, two differences: 1) Byé discussed the case where only two species need be considered (two complexes, or Me^{z+} and one complex) and did not formulate the implications of (14) in their most general form. 2) Byé used the function $(-H/M)$, or rather $-H/2M$, instead of Z. In cases where h and $[OH^-]$ can be neglected, this makes no difference — in other cases his equations are not exact.

Other cases

Even if the curves are not parallel, one may tentatively assume that the reaction products can be written in some other "core + links" form, in general $(Me(OH)_t)_n(OH)_r$ [or $Me_s(Me(OH)_t)_n$, where $r = -ts$; AB I, 8]. From equations to be given presently, one can then find the most probable values for r and t; subsequent calculation will show whether or not the "core + links" formulation is a useful approximation.

Data (h, M, Z). Provided m is not negligible in the whole range, the *limiting values for Z* should be (AB I, 15):

$$\left.\begin{array}{ll} Z \to 0 & \text{for increasing } \log h \ (m \approx M) \\ Z \to t + r/n_{max} & \text{for decreasing } \log h \ (m \ll M). \end{array}\right\} \tag{22}$$

From the spacing of the curves $Z(\log h)_M$, (ABI, 35 and 36)

$$\left(\frac{\partial \log M}{\partial \log h}\right)_Z \begin{array}{ll} \nearrow & \dfrac{r + n_{min}t}{n_{min}-1} \text{ for } m \approx M \\ \searrow & -r \text{ for } m \ll M \end{array} \tag{23}$$

The curves $Z(\log h)_M$ will thus tend to coincide (the horizontal spacing tends to zero) for $m \approx M$, provided $n_{min} = 1$, and $r + t \neq 0$.

To test a possible value for r one may calculate h^rMZ and plot it as a function of $(\log h)_M$. From the spacing of these curves we should find (ABI, 37)

$$\left(\frac{\partial \log M}{\partial \log h}\right)_{h^rMZ} \begin{cases} \nearrow & t \text{ for } m \approx M \\ \searrow & -r \text{ for } m \ll M \end{cases} \tag{24}$$

If one suspects that the general formula of the complexes is $(Me(OH)_t)_n$, *i.e.* that $r = 0$, one may plot MZ as a function of $[\log (M - \frac{MZ}{t}) - t \log h]$. Then all points should fall on the same curve irrespective of the value of M (ABI, p. 313).

If m data are not available, and m is always $\ll M$, as is the case with many polyanion equilibria, it is convenient to replace Me^{z+} in the equations of this paper (or in general B in any treatment founded on ABI and ABII) by the smallest possible complex. An example is uranium(VI): as U^{6+} does not seem to exist in solution it is convenient to use the smallest complex, UO_2^{2+}, instead of U^{6+}.

Data (m, h, M, Z). The limiting slopes of the curves $\eta(\log h)_M$ will be (ABI, 39):

$$\left(\frac{\partial \eta}{\partial \log h}\right)_M = -\left(\frac{\partial \log m}{\partial \log h}\right)_M \begin{cases} \nearrow & 0 \text{ for } m \approx M \\ \searrow & -(t + \frac{r}{n_{max}}) \text{ for } m \ll M \end{cases} \tag{25}$$

The spacing of these curves gives the limiting values (ABI, 46)

$$\left(\frac{\partial \log M}{\partial \log h}\right)_\eta \begin{cases} \nearrow & \frac{r + n_{min}t}{n_{min}-1} \text{ for } m \approx M \\ \searrow & \frac{r + n_{max}t}{n_{max}-1} \text{ for } m \ll M \end{cases} \tag{26}$$

Thus the horizontal spacing between the curves $\eta(\log h)_M$ will tend to disappear for $m \approx M$, provided $n_{min} = 1$ and $r + t \neq 0$.

If one wants to test a special value for s, one may plot $m^{-s}(M-m)$ as a function of $(\log h)_M$ or $(\log h)_m$, and see whether the spacing gives acceptable values for t and $r = -ts$, (ABI, 47 and 48):

$$\left(\frac{\partial \log M}{\partial \log h}\right)_{m^{-s}(M-m)} \begin{cases} \nearrow & t \text{ for } m \approx M \\ \searrow & ts = -r \text{ for } m \ll M \end{cases} \tag{27}$$

$$\left(\frac{\partial \log m}{\partial \log h}\right)_{m^{-s}(M-m)} = t \text{ in the whole range} \tag{28}$$

The methods now mentioned here may give plausible values for r and t, provided a core + links formula is at all applicable. More rigid tests can then be devised depending on the accuracy of the data. For instance, one may plot $h^r(M—m)$ as a function of $\log m - t \log h$, which should give a single curve. This curve could then be analysed to find the details of the mechanism (ABI, p. 316).

Depending on the accuracy of the data other functions may prove useful for finding the general formula of the complexes, and the details of the mechanism, *e.g.* $MZ/(M—m)$ or perhaps $(M—m)/H$ (ABI, p. 316).

Applications to data for certain ions

The treatment described above has been applied to the experimental data on the hydrolysis of a number of metal ions. In two cases, as mentioned above, mononuclear complexes were indicated. In all the others, the $Z(\log h)_M$ curves were found to be parallel — application of (14) and (15) gave integral values of t, either 2 or 3. In those cases where $\eta(\log h)_M$ curves were available, they were also parallel and indicated the same t value as did the $Z(\log h)_M$ curves. The results are summarized in Table 1.

Table 1.

Metal ion	Ref.	Data	$-R$	General formula of complexes
Bi^{3+}	1	Z, η	2	$Bi((OH)_2Bi)_n^{3+n}$ or $Bi(OBi)_n^{3+n}$
Fe^{3+}	7	Z, η	2	$Fe((OH)_2Fe)_n^{3+n}$
UO_2^{2+}	13	Z	2	$UO_2((OH)_2UO_2)_n^{2+}$ or $UO_2(OUO_2)_n^{2+}$
Al^{3+} acid	14	Z	3	$Al((OH)_2Al)_n^{3+}$
Al^{3+} alkaline	14	Z	−1	$OH((OH)_2Al)_n^-$, from eq (19)
Th^{4+}	19	Z	3	$Th((OH)_2Th)_n^{4+n}$ or $Th(OOHTh)_n^{4+n}$

In Figs. 1 and 2, Hedström's data for Fe^{3+} (Part VII) are represented as plots of Z(or rather $y = \frac{1}{2}Z$) and η as functions of $x = \log M - 2 \log h$. The points for $M = 5$—50 mC are seen to be independent of M within the limits of error, whereas those for 1 and 2 mC differ a little. For the lowest concentrations, 0.1 and 0.2 mC, the η values differ considerably from those for higher M (no Z values are given for low M because of the large uncertainty). Hedström's reasonable explanation is that at these low M values the formation of mononuclear complexes becomes appreciable.

We may for the moment neglect the lowest M values and consider the range $M = 1$—50 mC, *i.e.* a range of 1.7 log M units. It is seen that good agreement is only obtained for $t = 2$; if Z or η were plotted as functions of, say, $\log M - \log h$ or $\log M - 3 \log h$, the points for different values of M would be systematically spread out along the x axis.

The same is true for the Bi^{3+} curves, Figs. 3 and 4. The range of concentrations is not quite as wide (0.7 log M units); — however, it is clear that reasonable agreement would not be obtained with any value of t except 2.

The remaining ions will be discussed in following papers in this series.

SELECTING A "CORE + LINKS" MECHANISM

Method

Let us suppose that (15 a) and (16 a) are valid within a certain range, *i.e.* that Z and η have proved to be functions of $x = \log M - t \log h$. This indicates that we need primarily consider only complexes of the general formula $Me((OH)_tMe)_n$ (excepting the special case of eq. 19—21). However, we do not know whether only one complex, or a few, or a great number of complexes is formed. To attack this problem we must consider the shape of the experimental curve $y(x)$ and, if available, of $\eta(x)$.

With "core + links" complexes (15), the only reactions we need consider are of the following type:

$$Me^{z+} + tn\ H_2O + n\ Me^{z+} \rightleftharpoons Me((OH)_tMe)_n + tn\ H^+ \qquad (29)$$

Denoting the equilibrium constant of (29) by k_n, we have (AB I, 21):

$$c_n = [Me((OH)_tMe)_n] = k_n m(h^{-t}m)^n = k_n\, m\, u^n \qquad (30)$$

with the abbreviation (ABI, 10)

$$u = m\, h^{-t} \qquad (31)$$

If moreover, we put (ABI, 22)

$$f(u) = \sum k_n u^n \qquad (32)$$

we may prove (ABI, 26, 25, 44)

$$x = \log\ u + \log(1 + f + uf') \qquad (33)$$

$$y = uf'(1 + f + uf')^{-1} \qquad (34)$$

$$\eta = \log(1 + f + uf') \qquad (35)$$

Thus x, y and η are all functions only of the variable u.

Curves calculated for a few mechanisms have been drawn in ABII, giving y and η as functions of x, or rather of X and ξ, which are $= x +$ a constant, containing one of the equilibrium constants, which we shall call k.

The mechanisms considered involve either one equilibrium constant (k) or two (k and k_0) and are, for the special case of the hydrolysis of metal ions:

I. No soluble hydroxo complex is formed; solid $Me(OH)_t$ precipitates *, solubility product $k_s = mh^{-t} = k^{-1}$

II. Only one soluble complex, $Me((OH)_tMe)_N$ is formed, with the equilibrium constant $k_N = k^N$.

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* As before, "$Me(OH)_t$" may also contain water and the medium ions (say Na^+, and ClO_4^-) and may thus have the general formula $Me(H_2O)_\alpha(OH)_tNa_\beta(ClO_4)_{z+\beta-t}$.

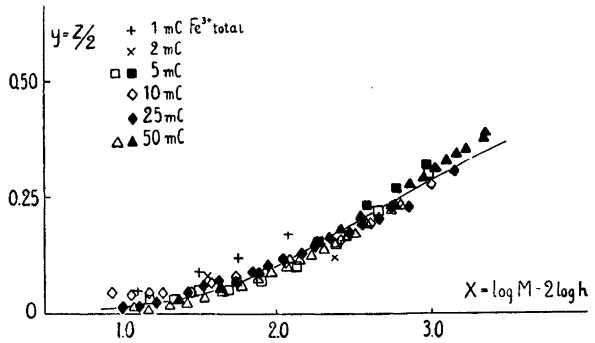

Fig. 1. Hydrolysis of Fe^{3+}. Points: $y(x)$, from Hedström's data[7]. *Curve: calculated for mechanism II, $N = 1$, $\log k = -2.80$.*

III. By repeated reaction, an unlimited series of complexes is formed where n may have any positive integral value.

Three equations expressing the variation of k_n with n (ABII, 28) were considered

$$\left.\begin{aligned} &\text{IIIa}: k_n = k_0 k^n \\ &\text{IIIb}: k_n = k_0 n k^n \\ &\text{IIIc}: k_n = \frac{k_0 k^n}{\lfloor n \rfloor} \end{aligned}\right\} \quad (36)$$

Hypotheses III a—c are similar to but not identical with three cases of polymerization discussed by Connick and Reas [20].

Hypothesis I gives only one possible shape for $y(X)$(limiting curve in ABII, Figs. 1—3), and one for $\eta(\xi)$ (limiting curve in ABII, Figs. 4 and 5). Hypothesis II gives families of curves $y(X)_N$ and $\eta(\xi)_N$, with N as parameter (ABII, Figs. 1 and 4). With hypotheses IIIa, IIIb and IIIc one finds families of curves $y(X)_{k_0}$ and $\eta(\xi)_{k_0}$ (ABII, Figs. 2 a—c and 5 a—c) with k_0 as parameter.

For comparison with the experimental data, the calculated curves should be drawn on not too small a scale.

Let us now suppose that the experimental data in the form $y(x)$ and perhaps also $\eta(x)$ are plotted on the same scale as the calculated curves. By moving them along the x axis an attempt can be made to make the data fit in with some of the curves. Hypotheses I and II, which involve the formation of only one hydrolysis product, and allow only a discrete set of shapes of the curves, are then rather easy to recognize or to rule out.

With IIIa, IIIb, and IIIc, such a clear decision is hard to make. In any real case, the assumption that the equilibrium constants may be represented by one of the simple equations (36) is certainly a very rough approximation. Thus small deviations from the ideal calculated curves are to be expected.

At any rate, after some study of the data and the curves, one may state which of these mechanisms can be definitely excluded, and whether one may get an acceptable agreement with any of them, by choosing an appropriate value for N or k_0. If so, one may find the equilibrium constant k from the position of the experimental curves.

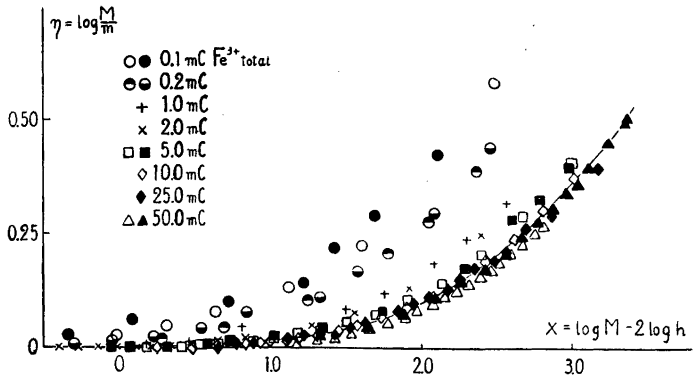

Fig. 2. Hydrolysis of Fe^{3+}. Points: $\eta(x)$, from Hedström's data[7]. *Curve: calculated for mechanism II, $N = 1$, $\log k = -2.80$.*

Application to the iron(III) ion

Let us again consider Figs. 1 and 2, which give the experimental data $y(x)$ and $\eta(x)$ for Fe^{3+}, calculated with $t = 2$, *i.e.* assuming the general formula $Fe((OH)_2Fe)_n^{3+n}$. We shall compare them with the calculated curves, disregarding the points for $M = 0.1$, 0.2 and 1 mC where the mononuclear complexes begin to exist in appreciable concentrations.

Hypothesis I would involve the precipitation of, say, very fine-grained $Fe(OH)_2ClO_4$. This mechanism would require a much steeper rise of both curves than is actually found, and can thus be immediately excluded. In the choice between the remaining mechanisms, it is a drawback that data are available only for a limited range in y ($0 < y < 0.4$, corresponding to $0 < Z < 0.8$) and also in η ($0 < \eta < 0.5$). However, it was hard to extend the range because of the precipitation of FeOOH.

When the experimental data are superimposed on the family of curves for hypothesis II (only one polynuclear complex formed), a remarkably good agreement is obtained with $N = 1$, both for $y(x)$ and for $\eta(x)$ — no other N value gives an acceptable agreement.

With IIIa—c a good agreement with the experimental points $y(x)$ and $\eta(x)$ was obtained for all sufficiently high k_0 values: with IIIa for $k_0 \geqq 5$, with IIIb for $k_0 \geqq 10$, and with IIIc for $k_0 \geqq 2$. On comparing the calculated curves it will be found that with increasing k_0, the lower parts of all the curves $y(x)$ and $\eta(x)$ for these three mechanisms tend to the same shape, namely that of the curve for II with $N = 1$. The explanation is that if k_0 is high, the first complex $Me(OH)_2Me$ will predominate over higher complexes in the first stages of hydrolysis, as may be realized intuitively and also proved, starting from equations (30) and (36).

In Figs. 1 and 2, the curves are calculated for hypothesis II with $N = 1$ — they would almost coincide with the curves for IIIa with $k_0 = 10$, 5 or 1. With the higher values for k_0, the polynuclear complexes after the first are only a small correction in the part of the curves that was available from the

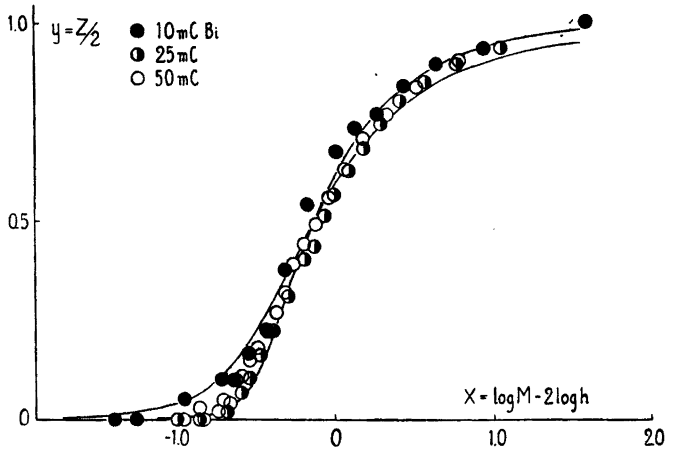

Fig. 3. Hydrolysis of Bi^{3+}, Points: y (x), from Granér and Sillén [1]. Curves: hypothesis IIIa with log k = 0.48, k_0 = 0.01 and 0.1.

experiments. Since the data do not give any clear proof of the existence of higher polynuclear complexes, we shall assume that the only polynuclear complex formed is $Fe((OH)_2Fe)_N$ with $N = 1$, *i.e.* $Fe_2(OH)_2^{4+}$. This is the same mechanism as that found by Hedström [7] by another approach.

To find the equilibrium constant k, we may try to fit the curves calculated for $N = 1$ to the experimental data as well as we can — as we shall see, the points with $y = 1/4$ and $\eta = \log 2$ are especially useful.

Equations (ABII, 22, 23, 58) give, for $N = 1$:

$$y = \frac{g}{1 + 2g}\ ;\ x + \log k = \log g + \log(1 + 2g);\ \eta = \log(1 + 2g) \quad (37)$$

The significance of g (here $= ku$) need not concern us here.
Setting $y = 1/4$ we find:

$$y = 1/4;\ g = \tfrac{1}{2};\ \eta = \log 2;\ x = -\log k \quad (38)$$

From the best fitting curves, disregarding the very low M values we may read off:

$$y(x);\ y = 1/4 \text{ for } x = 2.85 \pm 0.15, \text{ thus } \log k = -2.85 \pm 0.15$$

$$\eta(x);\ \eta = \log 2 \text{ for } x = 2.80 \pm 0.10, \text{ thus } \log k = -2.80 \pm 0.10$$

With our approach we have thus deduced the following main reaction:

$$Fe^{3+} + 2\ H_2O + Fe^{3+} \rightleftharpoons Fe_2(OH)_2^{4+} + 2\ H^+;\ \log\ k = -2.80 \pm 0.10$$

This is substantially the same result as deduced by Hedström [7], whose value was $\log k = -2.91 \pm 0.04$. In addition, Hedström could give equilibrium constants for the formation of mononuclear $FeOH^{2+}$ and $Fe(OH)_2^+$ at the very low Fe

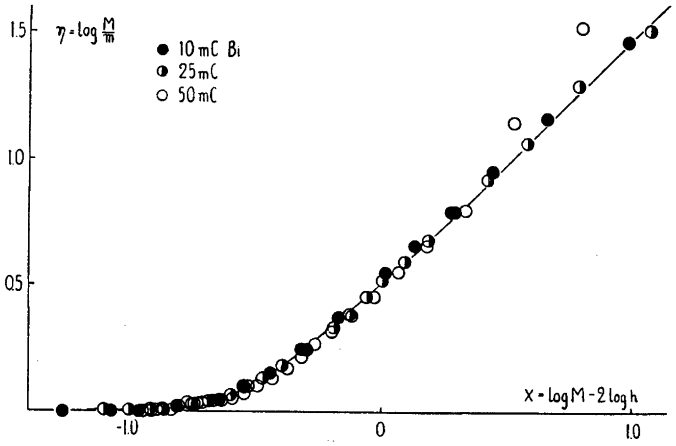

Fig. 4. Hydrolysis of Bi^{3+}. Points: $\eta(x)$ from Granér and Sillén [1]. Curve: hypothesis IIIa with log $k = 0.48$, $k_0 = 0.025$.

concentrations where the points fall off the $y(x)$ and $\eta(x)$ curves. The small difference between our values for log k and his may be due to the fact that we have made no corrections for mononuclear complexes.

Application to the bismuth(III) ion

Figs. 3 and 4 give the experimental data of Granér and Sillén (Part I) for Bi^{3+}, recalculated to $y(x)$ and $\eta(x)$ using the value $t = 2$ previously deduced. By comparison with the curves in ABII we found that *hypothesis I*, which would have implied the formation of solid $BiOClO_4$ (+ perhaps an unknown amount of H_2O and $NaClO_4$) is ruled out. Nor could a reasonable agreement be obtained with any of the curves calculated for *hypothesis II* and various values of N. Thus we found, as did Granér and Sillén, that the data cannot be explained by the formation of only one complex.

With *hypothesis IIIa*, the experimental points $y(x)$ were found to fall in between the curves for $k_0 = 0.01$ and 0.1. Values for k_0 in the range 0.03 ± 0.02 were obtained both by using values of the slope at $y = \frac{1}{2}$ and equation (ABII, 35), and by using values of the distance $(x—x_{\frac{1}{2}})$ for various y, together with equation (ABII, 37).

To find k, one may use (ABII, 33), $x_{\frac{1}{2}} = \log 2 - \log k$. In practice, when the family of calculated curves $y(X)$ is moved across the experimental points, one reads the x value corresponding to the point $X = 0$, $x = -\log k$ in the position where a good fit is obtained (see (ABII, 34)). We thus found $\log k = 0.43 \pm 0.08$.

When the family of calculated curves $\eta(\xi)$ for IIIa was moved along the experimental points $\eta(x)$, a good fit was obtained with k_0 values ≈ 0.025. One may find $\log k$ from the $\eta(x)$ plot by reading the value of x corresponding to $\xi = 0$ at the best fit (equation ABII, 63: when $\xi = 0$, $x = -\log k$).

We thus found $\log k = 0.48 \pm 0.02$; this value is chiefly determined by the points with high values of η, which are close to the asymptote and insensitive to changes in k_0.

To find k_0, one may then read the value η_0 for $\xi = 0$, and calculate v_0 and k_0:

$$\xi = 0; \eta = \eta_0 \; ; \; v_0 = 10^{-\eta_0}$$
$$k_0 = (1-v_0)^3 v_0^{-2} (2-v_0)^{-1} \qquad (39)$$

The equations (39) can be derived from (ABII, 64 and 63). In this way we found $k_0 = 0.025 \pm 0.010$.

With *hypothesis IIIb*, the low $y(x)$ values agree fairly well with the calculated curves for $k_0 = 0.01$, whereas the higher y values deviate somewhat ; from the $y(x)$ curve one finds $\log k = 0.43 \pm 0.08$. The $\eta(x)$ curve for IIIb and $k_0 = 0.01$ also agrees rather well with the experimental data except at the highest values. From this curve one finds $\log k = 0.43 \pm 0.03$.

Finally, with *hypothesis IIIc*, the lower half of the $y(x)$ or $\eta(x)$ data give a tolerable agreement with $k_0 \approx 10^{-4}$, but for higher y or η the calculated curves definitely do not fit the experimental data.

To sum up, we find that with our present approach we have established the general formula of the complexes formed to be $Bi(OBi)_n^{3+n}$, and seem to have ruled out explanations assuming only one polynuclear complex. Of the "repeated" mechanisms tested, the best agreement was obtained by assuming the addition of new links after the first to be either equally easy for all n (hypothesis IIIa), or somewhat easier for higher n (hypothesis IIIb), whereas the agreement was not as good, when it was assumed that the formation of higher complexes is disfavored (hypothesis IIIc).

Assuming meachanism IIIa, the data would be explained by the following reactions:

	Equilibrium constant
$Bi^{3+} + H_2O + Bi^{3+} \rightleftharpoons BiOBi^{4+} + 2\,H^+$	$k_0 k$
$BiOBi^{4+} + H_2O + Bi^{3+} \rightleftharpoons Bi(OBi)_2^{5+} + 2\,H^+$	k

in general:

$$Bi(OBi)_n^{3+n} + H_2O + Bi^{+3} \rightleftharpoons Bi(OBi)_{n+1}^{4+n} + 2\,H^+ \qquad k$$

with $\log k = 0.48 \pm 0.02$, $k_0 = 0.025 \pm 0.010$

It may be noted that Granér and Sillén, who considered only the mechanism IIIa, found $\log k = 0.47 \pm 0.03$ and $k_0 = 0.022 \pm 0.006$. It is especially gratifying that with our present representation the $\eta(x)$ data for high η are also in good agreement with hypothesis IIIa; in the graphical representation chosen in part I, these values came close to the m/M axis, and the agreement was therefore not so easy to check.

We shall not discuss the bismuth equilibria in detail since we hope to have data available for a wider range of concentrations, both with emf and ultracentrifuge methods, in the near future.

DISCUSSION

The $y(x)$ and $\eta(x)$ curves contain in a compact form the information one can get out of the emf data. The examples given here and in the following few papers may indicate what conclusions one may draw from them and what their limitations are.

First of all, the fact that the $Z(\log h)_M$ curves are parallel, so that Z (and η) are functions of $x = \log M - t \log h$, indicates strongly that the formula of all complexes can be written in the form $Me((OH)_tMe)_n$ but it implies nothing about how many and which n values are actually represented.

If the $y(x)$ and $\eta(x)$ curves rule out the simple hypotheses I and II, with only one product, then there are certainly several kinds of polynuclear complexes present. In such cases we have resorted to the hypotheses IIIa—c, where all n values are represented and about equivalent — this however arises from sheer ignorance. We simply do not know any argument that could guide us in choosing three or four values for n and stating that these are the only possible ones occurring among all conceivable n values. On the other hand we have no doubt that if one chooses at random some combination such as $n = 1, 3, 7$, and 13, or 1, 2, 4, 8, and 16, one might adjust the equilibrium constants to get an agreement which is about as good as that obtained with "unlimited" mechanisms.

Since the crystal structures of bismuth oxide compounds contain $(BiO)_n$ sheets, it is tempting to consider the soluble bismuth complexes, with formulas close to $Bi(OBi)_n^{3+n}$, as fragments of such sheets.[1] The crystal structure gives no indication of certain values for n being preferred to all others; this may be one argument for treating all n as roughly equivalent.

An additional support may be found in the work of Kraft and Ryumshin [21] who have prepared a series of bismuth oxide salts of α-phenylbutyric acid (HA), to which they ascribe the general formula "$A_2Bi(OBiA)_nOBiA_2$", or $Bi(OBi)_nA_{3+n}$, with n varying continuously from 0 to ∞. Molecular weights and analytical compositions seem to agree well with this formula for three specimens with n ranging from 3 to 12. From the low viscosity of the solutions of these compounds in organic liquids, Kraft and Ryumshin concluded that the molecules are round rather than thread-like; this agrees well with the picture of a fragment of the $(BiO)_n$ sheet from a crystal structure, surrounded on both sides by α-phenylbutyrate groups.

From emf data one may thus conclude, by applying the methods given in this paper, and in ABI and ABII, that many inorganic ions form a series of hydrolysis products of varying molecular weight, in equilibrium with each other. It would be desirable to check this conclusion by some quite independent method. As already discussed in ABI, solubility data give too limited information when polynuclear complexes are formed. With distribution or ion exchange data one will have to introduce a number of new assumptions and unknown constants. Moreover, just as with the emf data, they give more accurate information on the very smallest complexes rather than on the higher ones. Diffusion data will be hard to interpret, since the complexes are continually breaking up and reforming.

Equilibrium ultracentrifugation is not an altogether ideal method since the complexes have a rather low molecular weight; due regard must also be paid to the variation of ionic strength, equilibrium constants, and activity factors with the distance from the axis of rotation. However the method may give some new useful information, if due consideration is taken of its limitations. Dr. Kurt Kraus, of Oakridge, has already done some work in this field. We are planning to start work on ultracentrifugation in the near future, in collaboration with Professors Ole Lamm and Hugo Theorell.

Whichever mechanism one proposes for a hydrolysis reaction, one must demand that it should be able to explain with reasonable accuracy the rather detailed data obtained by methods founded on the law of mass action.

SUMMARY

Experimental data for the hydrolysis of a metal ion Me^{z+} are conveniently summarized in the form of diagrams of $Z(\log h)_M$ and $\eta(\log h)_M$. (Z = average number of H^+ set free per Me, M = total concentration of Me, $m = [Me^{z+}]$, $h = [H^+]$, $\eta = \log M/m$.)

The formation of polynuclear hydrolysis products may be treated formally as a complex formation between the hydrated Me^{z+} and the reactant "$-H^+$". The general methods given in the papers ABI[8] and ABII[9] for treating complexes between two reactants A and B have been transformed to this special case and applied to a number of systems.

The general formula of the complexes is obtained from the spacing of the Z and η curves, as described in ABI. Of the ions for which data are available in a wide concentration range, only two (Hg^{2+}, Tl^{3+}) give chiefly mononuclear hydrolysis products. A number of others (Fe^{3+}, Al^{3+}, Th^{4+}, UO_2^{2+}, *etc.*) give a set of parallel Z (or η) curves which implies that the complexes can be written in the general "core + links" form $Me((OH)_tMe)_n$ with t a constant = 2 or 3, and n variable.

To find the details of the reaction mechanism, the experimental data for Fe^{3+} and Bi^{3+} have been treated by methods analogous to those in ABII. The data for Fe^{3+} indicate only one polynuclear complex, $Fe_2(OH)_2^{4+}$ while for Bi^{3+}, a series of complexes $Bi(OBi)_n^{3+n}$ with $n = 1, 2, 3, 4 \ldots$ is indicated. The results are thus in agreement with the conclusions in part I and part VII of this series, and the equilibrium constants obtained with the new methods also agree rather well with those obtained earlier.

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In the following papers, the hydrolysis of the ions Th^{4+}, UO_2^{2+}, Al^{3+}, In^{3+}, and others will be discussed.

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Studies on the Hydrolysis of Metal Ions

IX. The Hydrolysis of the Thorium Ion, Th^{4+}

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PREVIOUS WORK ON Th^{4+} HYDROLYSIS

Denham [1] measured $[H^+]$ in various $Th(SO_4)_2$ solutions with a hydrogen electrode. He found that E varied with time — in one case it changed by 17 mV in the course of 11 days.

Hildebrand [2] and Britton [3] titrated various thorium salt solutions with OH^-, using hydrogen electrodes. Their chief aim was to study the precipitation of the hydroxyde or of basic salts. In their measurements, both the ionic strength and the total concentration of thorium varied, so that it is hard to make calculations on the basis of their data.

Chauvenet and Tonnet [4] measured the changes in conductivity and the amount of heat evolved on mixing Th^{4+} (as chloride) and OH^- in various proportions. They found a not too distinct bend at the ratio 1 Th per 2 OH, from which they concluded that the reaction was: $ThCl_4 + 2\ H_2O \rightarrow Th(OH)_2Cl_2 + 2\ HCl$. Chauvenet and Souteyrand-Franck [5] similarly concluded that $Th(OH)_2(NO_3)_2$ is formed in nitrate solutions.

Schaal and Faucherre [6] measured pH in 0.12 C, 0.25 C, and 0.5 C thorium perchlorate and nitrate solutions and varied the ratio Th/OH by adding alkali. The ionic strength was kept constant by means of sodium perchlorate or nitrate. They assumed *a priori* that the following reaction occurs:

$$n\ Th^{4+} + 2n\ OH^- \rightleftharpoons (ThO)_n^{2n+} + n\ H_2O$$

From their data they concluded $n = 4$ and thus the reaction would be:

$$4\ Th^{4+} + 8\ OH^- \rightleftharpoons Th_4O_4^{8+} + 4\ H_2O$$

Souchay [7] claimed that his measurements on the freezing-point depression of thorium nitrate solutions also indicate the presence of this tetramer.

From investigations on thorium nitrate solutions, Kasper [8] concluded that thorium forms mononuclear hydroxyl complexes according to the reaction

$$Th^{4+} + H_2O \rightleftharpoons ThOH^{3+} + H^+$$

and that the equilibrium constant for this reaction is about $10^{-3.4}$.

It has thus sometimes been assumed that thorium forms mononuclear complexes and sometimes that a polynuclear complex is formed.

The present work was started in 1947 to throw more light on the nature of the complexes and if possible to determine the equilibrium constants involved; the greater part of the experimental work was completed in 1948, but a few complementary measurements were made as late as in 1953. The results, and the main conclusions given here, were presented at the Symposium on coordination chemistry in Copenhagen, August 1953.

When the measurements and calculations to be given in the following had already been completed, Dr. Kurt A. Kraus of Oak Ridge was kind enough to send us a manuscript by Kraus and Holmberg [9]. They have studied the hydrolysis of thorium in perchlorate and chloride solutions by EMF measurements with a glass electrode, using either the same ionic medium as ourselves, 1 C $NaClO_4$, or 1 C NaCl, at 25° C.

Kraus and Holmberg assumed the following reactions, and approximate equilibrium constants:

$$Th^{4+} + 4\ H_2O \rightleftharpoons Th(OH)_2^{2+} + 2\ H_3O^+, \quad k = 3.4 \cdot 10^{-8}\ C^2$$

$$2\ Th^{4+} + 4\ H_2O \rightleftharpoons Th_2(OH)_4^{6+} + 2\ H_3O^+, \quad k = 2.6 \cdot 10^{-5}\ C$$

Since these reactions could not explain the data completely, they concluded that other complexes are also formed. As will be seen their data agree excellently with the present work, although our conclusions differ considerably.

METHOD

The present investigation was carried out as a series of potentiometric titrations at 25° C, adding NaOH to thorium perchlorate solutions containing initially a slight excess of $HClO_4$. All solutions were made to contain 1 C ClO_4^- by adding $NaClO_4$. In each titration the total thorium concentration M was kept constant in order to make the calculations simpler. Since the hydrogen ion and thorium concentrations were kept low compared with the concentration of perchlorate ion, we may assume that the activity factors remained constant, and hence that concentrations could be used instead of the activities in the calculations [10].

To study the mechanism of hydrolysis, it would have been desirable to know the concentrations of both H^+ and Th^{4+}. It seems, however, that nobody has ever been able to produce an electrode reversible to Th^{4+}, so we must be content with measuring

$$[H^+] = h \qquad (1)$$

The hydrogen ion concentration h could be measured with a glass, quinhydrone, or hydrogen electrode. The quinhydrone electrode was used in the majority of the experiments because it is accurate and easy to work with. To check that no error is introduced by complex formation between quinhydrone and thorium, the hydrogen electrode was used in a few titrations.

The following two types of reference electrodes were used:

$$CE\| = Hg,\ Hg_2Cl_2 \mid 4\ C\ NaCl \mid 1\ C\ NaClO_4 \mid$$

$$SE\| = Ag \mid 10\ mC\ AgClO_4,\ 990\ mC\ NaClO_4 \mid 1\ C\ NaClO_4 \mid$$

A calomel electrode was used for the first titrations but, later on, silver electrodes were used since they proved more stable. The salt bridge always contained 1 C $NaClO_4$.

The cells for measuring the hydrogen ion concentration may thus be written:

$$CE \parallel H^+,\ QH_2,\ Q \mid Pt$$
$$SE \parallel H^+,\ QH_2,\ Q \mid Pt$$
$$SE \parallel H^+ \mid H_2(g),\ Pd$$

For any of these cells, E (in mV) can be written in the form

$$E = E_0 + E_j + 59.16 \log h$$

Here E_0 is a constant, different for each type of cell, h is the hydrogen ion concentration, and E_j the liquid junction potential [10], which is an approximately linear function of h.

An example of a titration will be given below. Each titration generally started with an acid-base titration (without any thorium present) from which E and $E_j(h)$ were obtained. Then thorium ions were added, first in one large amount, and then simultaneously with the addition of NaOH, so that the total thorium concentration M was kept constant.

For each point of the following titration one could calculate h from E, using E_0, E_j and equation (2). From the composition of the solution, the analytical hydrogen ion excess H (assuming all Th to be present as Th^{4+}) was known. Then one could calculate (*cf.* VIII, 6; $[OH^-]$ is negligible in all Th solutions studied here)

$$Z = \frac{h - H}{M} \qquad (3)$$

Z is the average number of protons split off per Th, or of OH bound per Th. For the following discussion, the experimental data are conveniently plotted in the form $Z(\log h)_M$.

For convenience, the concentrations h and M will generally be expressed in mC, except in the final equilibrium constants, where C is the unit.

EXPERIMENTAL

Reagents

Thorium perchlorate solutions were prepared as follows: Thorium nitrate (Kebo puriss.) was moistened with water and then heated with 70—72 % perchloric acid (Kebo p.a.) on a sand bath at 150—180° C until no trace of nitric acid could be detected in the residue with the $Fe^{2+} + H_2SO_4$ reaction. Water was added to make the Th concentration about 0.1 C. For analysis thorium was precipitated with oxine [11] and determined as ThO_2. In the determination of ClO_4^-, the solution was passed through an ion exchanger saturated with H^+. The total amount of ClO_4^- was then found by titrating the eluate ($H^+ClO_4^-$) with standard sodium hydroxide.

Perchloric acid solutions, about 0.1 C, were prepared from $HClO_4$ p. a. 70—72 %.

Sodium hydroxide solutions, 0.1, 0.5, or 1 C, were prepared by adding water, free from carbon dioxide, to NaOH (p. a.); actually a small amount of carbonate would not affect the measurements very much, since at the low pH studied it would be present chiefly as H_2CO_3. The NaOH solutions were kept in polyethene bottles (in the first experiments in

glass bottles with an inner coating of paraffin) and analyzed either by evaporating a known volume with a small excess of HCl, drying at 360° C in an oven, and weighing the residue as NaCl, or by titration against KIO_3 according to Oesper [12], or against standard HCl.

Quinhydrone (Merck p. a.) was at first recrystallized from 50 % ethanol-water. Later on, the commercial preparation was used directly since no positive reaction was found for iron or acid impurities.

Silver perchlorate solutions were prepared by adding an excess of Ag_2O to a boiling solution of $HClO_4$. The undissolved material was filtered off and the content of Ag^+ in the clear solution was measured by Volhard's method employing a silver wire as primary standard.

Sodium perchlorate was prepared either by recrystallizing commercial $NaClO_4$ repeatedly from alcohol or by mixing $HClO_4$ and $NaHCO_3$ solutions. The stock solutions were analyzed by evaporating and drying at 125° C or 250° C.

The solutions S_0 *etc.* for the titrations (see below) were prepared by mixing appropriate amounts of these stock solutions.

Apparatus

The *cell* was of the same construction as described in previous papers from this Institute [13]. It was kept in a paraffin oil thermostat, 25.0 ± 0.1° C, which was placed in a thermostat room at approximately 25° C.

When a quinhydrone electrode was used, the solution was stirred with nitrogen gas.

Table 1. Summary of titrations.

No	Concentrations in mC					Volumes added (ml)					Electrodes	
	M	S_0	S_1	S_2		S_0	S_1	S_2	S_2 + S_1		for h	ref.
		H^+	OH^-	H^+	Th^{4+}							
1	1.00	49.95	70.00	0.72	2.00	30.00	17.00	47.00	9.00	9.00	Q	SE
2	1.00	29.78	50.00	0.72	2.00	30.00	15.00	45.00	10.00	10.00	Q	SE
3	1.00	29.70	50.00	0.72	2.00	49.00	24.00	72.00	15.00	15.00	H	SE
4	2.00	29.75	50.00	1.44	4.00	49.00	24.00	73.00	31.00	31.00	H	SE
5	2.00	29.75	50.00	1.44	4.00	35.00	16.00	51.00	23.00	23.00	Q	SE
6	5.00	49.93	70.00	3.60	10.00	33.00	20.00	53.00	45.00	45.00[a]	Q	SE
7	5.00	30.00	49.80	3.60	10.00	30.00		30.00	68.00	68.00	Q	CE
8	5.00	30.31	95.62	−9.35	20.00	90.00		30.00	17.00	51.00	Q	CE
9	5.00	30.31	50.00	−9.35	20.00	90.00		30.00	41.50	124.50	Q	CE
10	10.00	30.00	80.00	7.20	20.00	80.00		80.00	110.00	110.00	Q	CE
11	10.00	50.16	95.62	−9.35	20.00	40.00		40.00	83.00	83.00	Q	CE
12	10.00	50.16	95.62	−9.35	20.00	40.00		40.00	73.00	73.00	Q	CE
13	10.00	50.00	94.09	−9.35	20.00	40.00		40.00	48.00	48.00	Q	CE
14	10.00	b	198.6	7.20	20.00	b			20.00	20.00	H	SE
15	10.00	c	198.6	7.20	20.00	c			20.00	20.00	Q	SE
16	20.00	50.00	156.2	−18.70	40.00	40.00		40.00	100.00	100.00	Q	CE
17	20.21	50.00	158.9	14.10	40.42	30.00		30.00	112.00	112.00	Q	CE
18	20.00	30.00	159.5	13.93	40.00	30.00		30.00	100.00	100.00	Q	CE
19	20.00	30.05	202.79	13.93	40.00[d]	30.00		30.00	50.00	50.00[d]	Q	CE

[a] Titration 6: At the end of the usual titration, 49.63 ml of the solution was removed and a back-titration was started, adding 40.40 ml S_2 + 40.40 ml S_3 (49.93 mC H^+) in portions. Another 49.63 ml was removed, and 20.00 ml S_2 + 20.00 ml S_3 was added in portions.

[b] Titration 14 started with 69.63 ml (15.00 mC H^+, 10.00 mC Th) to which (S_1+S_2) was added in portions.

[c] Titration 15 started with 70.00 ml (15.00 mC H^+, 10.00 mC Th) to which (S_1+S_2) was added in portions.

[d] Titration 19: At the end of the usual titration, 70.00 ml S_3(89.79 mC H^+) and 70.00 ml S_2 were added in portions.

Table 2. Data for titration 5, see also Table 1.

Acid-base titration

S_1 ml	$H = h$ mC	E mV	E'_0 mV
0.00	29.75	76.07	163.24
1.00	27.53	77.95	163.13
4.00	21.57	83.85	162.76
6.00	18.08	88.19	162.56
8.00	14.91	93.02	162.44
12.00	9.39	104.67	162.21
16.00	4.73	122.07	162.00

Thorium titration

$S_1=S_2$ ml	H mC	E mV	log h (mC)	h mC	Z
0.00	3.08	131.97	0.506	3.20	0.06
1.00	2.56	136.22	0.433	2.71	0.07
3.00	1.56	146.86	0.252	1.78	0.11
5.00	0.64	160.54	0.021	1.04	0.20
6.00	0.20	167.83	−0.103	0.70	0.29
7.00	−0.22	173.90	−0.205	0.62	0.42
8.00	−0.62	178.03	−0.275	0.53	0.57
9.00	−1.02	180.67	−0.320	0.48	0.75
11.00	−1.77	185.54	−0.402	0.39	1.08
12.00	−2.13	187.74	−0.439	0.36	1.24
14.00	−2.81	192.04	−0.512	0.31	1.56
16.00	−3.40	197.52	−0.604	0.25	1.82
18.00	−4.05	204.19	−0.717	0.19	2.12
20.00	−4.62	212.32	−0.855	0.14	2.38
22.00	−5.16	224.48	−1.060	0.09	2.62
23.00	−5.42	232.38	−1.194	0.06	2.74

Hydrogen (nitrogen) gas was obtained from a cylinder and freed from oxygen by passing the gas through a column filled with activated copper [14]. It was then saturated with water vapor by bubbling it through 1 C $NaClO_4$.

In the cells with quinhydrone, two Pt foils were used as inert electrodes. They were first cleansed in nitric acid (1 : 1), washed with water, and ignited in an alcohol flame. The hydrogen electrodes were lightly palladinated Pt foils [15]. Ag electrodes were prepared and coated with AgI as usual in this laboratory [10].

The E of the cells were measured with a Jensen compensator (type 10 A) and a Multiflex galvanometer, or with a valve potentiometer (Radiometer PHM 3).

PROCEDURE AND SURVEY OF THE DATA

The basis of the calculations are the 19 titrations which are summarized in Table 1. M gives the total thorium concentration which was constant in the "thorium part" of each titration. For a titration, three solutions S_0, S_1, and S_2 were usually prepared: besides Na^+ and ClO_4^-, S_0 contained H^+, S_1 OH^-, and S_2 Th^{4+} and H^+. Columns 3—6 in Table 1 give for each solution the concentrations of the ions, leaving out Na^+ and ClO_4^-, which were added to make all solutions contain 1 000 mC ClO_4^-.

The following columns give the amount of each solution which was added in the course of a titration. As an illustration, titration 5 will be described —

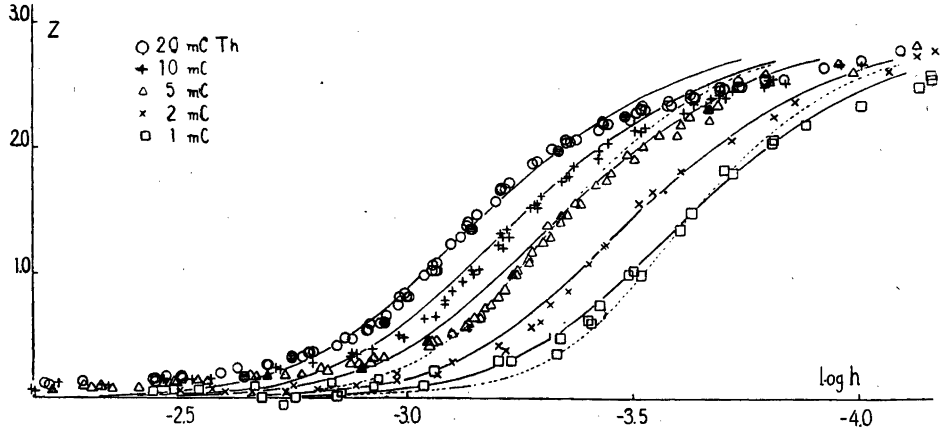

Fig. 1. Hydrolysis of Th^{4+}. Vertical axis: Z = average number of OH^- bound per Th. Horisontal axis: log h *(C scale). The symbols for experimental points at various values for* M *(total Th concentration) are given to the left in the figure. Shaded circles and triangles: reverse titrations. Curves: Z (log* h*) calculated for the various* M *values with hypothesis IIIa, log* $k_0 = -7.50$ *and* $k = 1.0$ *(full-drawn) or* $k_0 = 0.5$ *(broken).*

the detailed data for it are given in Table 2. Titrations 1—6, which were really the most recent ones, were done in essentially the same way.

Titration 5 started with an acid-base titration. The titration vessel initially contained 35.00 ml of the acid solution S_0. To this solution, an almost equivalent amount of hydroxide solution S_1 was added in portions from a buret and E was measured after each new addition. Then $E'_0 = E - 59.16 \log h$ (2) could be calculated and plotted against h. Here, of course, the analytical hydrogen ion excess H was equal to $[H^+] = h$. The diagram $E'_0(h)$ was used in the latter part of the titration for calculating h, the quantity sought for, from E. For instance, in the latter part of titration 5 the equation $E'_0 = 161.77 + 0.049\,h$ was used.

For all the acid-base titrations in experiments 1—6, the points $E'_0(h)$ fell upon parallel straight lines with the slope 0.048 mV/mC. In similar experiments in this laboratory, Biedermann found the slope 0.064 ± 0.002, whereas the present author in 1948 found values ranging between 0.070 and 0.082 in sixteen acid-base titrations (average 0.077). Whatever the reason for this discrepancy, it makes little difference in the curve $Z(\log h)$. In the calculations the value 0.048 or 0.049 was used for the recent titrations (1—6, 14, 15) and 0.077 for the older ones (7—13, 16—19).

At the end of the acid-base titration in experiment 5, the total volume was 51.00 ml. An equal volume, 51.00 ml, of the thorium solution S_2 was now added. The pH of the mixture was then successively increased by addition of small portions of NaOH (S_1). To keep the thorium concentration M constant = 2.00 mC throughout the experiment, an equal volume of S_2 was added for each addition of S_1. After each addition of ($S_1 + S_2$), E was measured repeatedly until it became constant, usually within 15—20 min. The titration was interrupted when a precipitate appeared, as was indicated by the fact that E began to drift.

In titrations 7—13 and 16—19, first 30 or 40 ml of acid (S_0) and then an equal volume of Th solution S_2 were added. Finally the solution was titrated with (S_1 + S_2) just as in the latter part of titrations 1—6. The value for E_0 was calculated either from the E obtained with only S_0 or from E after the first addition of S_2, neglecting the hydrolysis of Th^{4+} in this rather acid solution.

In titrations 14 and 15, the initial solution already contained Th^{4+} and H^+ (see footnote, Table 1).

Reversibility. Two special titrations, 6 and 19, were carried out in both directions: first a basic solution S_1 was added as usual, and then acid and, of course, thorium were added in portions (see footnotes in Table 1). The points from the back titrations are shaded in Fig. 1. They are seen to fall on the same curves as the forward titration points, so one may conclude that reversible equilibria have been studied.

Precipitation. When the total thorium concentration M was 20 mC, a visible precipitate was formed at log h values between —3.80 and —3.90 (C scale) while in this range, E also became irregular. With M = 2 mC, the solution did not get distinctly opaque until log h = —4.70. However E began to drift already at log h = —3.80.

The value of E_0 for SE or CE proved to be quite constant, if measurements were made on the same day or with a few days' interval were compared. However, a slow change of the E_0 was often observed with the CE. On one occasion, a change as great as 4.0 mV was observed with a CE after the lapse of a few months. Since our calculations contain only the differences E_0—E in a single titration, this long-time variation in E_0 did not influence the results.

With hydrogen electrodes (experiments 3, 4, 14), the Z(log h) curves agreed very well with those obtained with quinhydrone electrodes and thus there is no reason to assume an appreciable error from thorium-quinhydrone complexes. With thorium solutions of the lowest M value (1 mC) and low h (1—5 mC), the E with the hydrogen electrode kept drifting indefinitely and would not become stable, whereas the quinhydrone electrode, as usual, gave reproducible potentials very quickly. Remarkably enough it was found that if one waited only about ten minutes after each addition, measured the E (without waiting for the drift to stop) and then made a new addition, the points obtained agreed satisfactorily with those from the quinhydrone titrations.

Table 3 gives log h and Z for one titration at each of the five M values used (1, 2, 5, 10, and 20 mC). The complete data are given in Figures 1 and 3 — titrations with the same M are seen to agree very well in Fig. 1.

GENERAL COMPOSITION OF THE COMPLEXES

The present data are condensed in Fig. 1, which gives $Z(\log h)_M$. If only mononuclear complexes were formed, Z would be a function of h only, and independent of M, and all points in Fig. 1 would fall on the same curve. Obviously this is not so, and one may conclude that Th^{4+} hydrolyses with the formation of polynuclear complexes. If there are mononuclear complexes they do not seem to be important in any part of the curves except perhaps for very low M and Z values — here the data are, however, not accurate enough to permit any certain conclusions.

Table 3. Z *(log* h*) for six titrations.*

Titration 1 (1 mC Th)		*Titration 4* (2 mC Th)		*Titration 6* (5 mC Th)		*Titration 12* (10 mC Th)		*Titration 16* (20 mC Th)	
log *h*	*Z*	log *h*	*Z*	log *h*	*Z*	log *h*	*Z*	log *h*	*Z*
0.567	0.05	0.410	0.045	0.661	0.09	1.232	0.08	1.169	0.08
0.520	0.07	0.347	0.05	0.600	0.11	1.120	0.09	1.008	0.086
0.465	0.07	0.271	0.045	0.531	0.12	0.902	0.10	0.797	0.10
0.341	0.09	0.199	0.07	0.446	0.14	0.683	0.13	0.538	0.18
0.168	0.12	0.114	0.10	0.274	0.19	0.494	0.16	0.416	0.20
0.063	0.16	0.023	0.14	0.179	0.23	0.294	0.24	0.313	0.27
−0.061	0.23	−0.067	0.20	0.084	0.29	0.124	0.36	0.232	0.33
−0.205	0.32	−0.218	0.38	−0.049	0.43	0.019	0.50	0.122	0.47
−0.338	0.50	−0.296	0.62	−0.101	0.51	−0.083	0.78	0.060	0.61
−0.426	0.74	−0.358	0.86	−0.164	0.68	−0.144	1.03	0.020	0.74
−0.491	1.01	−0.436	1.22	−0.217	0.89	−0.208	1.32	−0.038	0.98
−0.628	1.55	−0.540	1.66	−0.244	1.02	−0.270	1.57	−0.098	1.24
−0.811	2.07	−0.809	2.34	−0.276	1.17	−0.359	1.78	−0.155	1.47
−1.156	2.58	−0.166	2.78	−0.311	1.40	−0.517	2.14	−0.228	1.73
				−0.372	1.58	−0.659	2.36	−0.321	1.97
				−0.486	1.94	−0.838	2.54	−0.424	2.15
				−0.604	2.19	−1.004	2.68	−0.538	2.30
				−0.983	2.62			−0.635	2.40
								−0.733	2.49
								−0.832	2.57
								−0.920	2.64
								−1.008	2.71
								−1.091	2.78
								−1.194	2.85
								−1.284	2.90
								−1.345	2.93

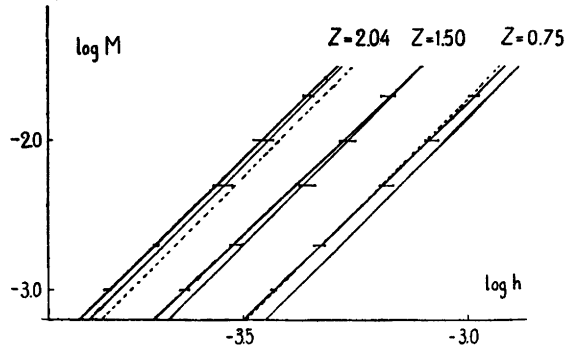

Fig. 2. Calculation of curve spacing: corresponding values for log h *and log* M *are given for three* Z *values. Thick lines: "best" line drawn through experimental data. Thin lines: calculated for hypothesis IIIa, log* $k = -7.50$, $k_0 = 1.0$ *(full-drawn) or 0.5 (broken).*

One may try to apply to these data the general methods for treating polynuclear complexes that have been given by Sillén (ABI[16], ABII[17]) and transformed by Hietanen and Sillén (part VIII[18]) to a form suitable for hydrolysis reactions.

An inspection of the curves in Fig. 1 suggests that the curves $Z(\log h)$ for different M values are parallel. To determine the quantity (*cf.* VIII, 14)

$$\left(\frac{\partial \log M}{\partial \log h}\right)_Z = -R \tag{4}$$

the diagram Fig. 2 was constructed. For each of three Z values (2.04, 1.50, and 0.75) a horizontal line was drawn in Fig. 1, and the log h values were read where the line intersected the $Z(\log h)$ curves for the five different M values. The bars in Fig. 2 indicate the inaccuracy.

As seen from Fig. 2, a straight line can be drawn through the data for each Z value. The slopes of the "best" lines were found to be 2.95, 2.80, and 2.93, and thus equal to 3 within the limits of error. Now if the derivative in (4) is constant and equal to 3 this means (*cf.* VIII, 15a, $t = -R = 3$) that Z should be a function of

$$x = \log M - 3 \log h \tag{5}$$

To check whether this is so, the data were recalculated, plotting (*cf.* VIII, 18)

$$y = \frac{1}{3} Z \tag{6}$$

as a function of x. As seen from Fig. 3, the points fall very nicely on a single curve, within the limits of experimental error. (The choice of $y = \frac{1}{3} Z$ instead of Z is for convenience when comparing our data with the equations and curves calculated for y in ABII[17].)

It is gratifying that the data of Kraus and Holmberg [9] fit in very well with the same curve as the present data if they are plotted in an $y(x)$ diagram.

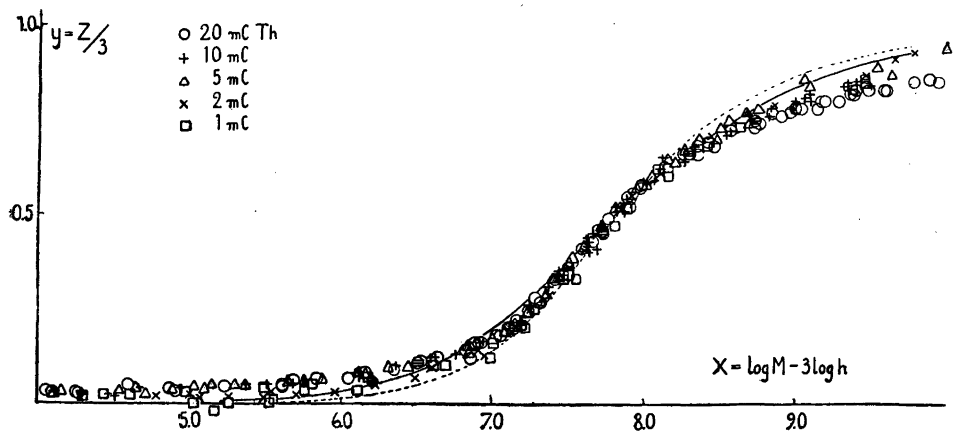

Fig. 3. Data in Fig. 1, recalculated to the form y(x), *assuming* t = 3: x = *log* M — 3 *log* h *(C scale),* y = *Z/3. Symbols for experimental points as in Fig. 1. Curves calculated for hypothesis IIIa, log* k = — 7.50, k_0 = 1.0 *(full-drawn) or 0.5 (broken).*

In Fig. 4 only a part of the points from each investigation, chosen at random, have been plotted so as not to overcrowd the diagram.

Now, as proved in (ABI, 16—19; *cf.* VIII, 15) the fact that Z (or y) is a function of $x = \log M - 3 \log h$ implies that all complexes formed in appreciable amounts can be written in the form

$$Th((OH)_3Th)_n^{(4+n)+} \tag{7}$$

This conclusion seems to be reliable within the range of M and h studied, to judge from the good coherence of the points in fig. 3 or 4. However, to decide whether only one complex (one value for n) or several are formed, we must analyze the curve $y(x)$.

DIRECT ANALYSIS OF $y(x)$

As shown in (ABII, 14, 15) one may use the function $y(x)$ to calculate

$$\log(1 + g) = \int_{-\infty}^{x} y \, dx + \log(1-y) + y \log e \tag{8}$$

$$\log u = x - y \log e - \int_{-\infty}^{x} y \, dx \tag{9}$$

Here (VIII, 31, 32; ABII, 2, 3, 3a)

$$u = mh^{-t} = [Th^{4+}]h^{-3} \tag{10}$$

$$g = f(u) = \Sigma \, k_n u^n \tag{11}$$

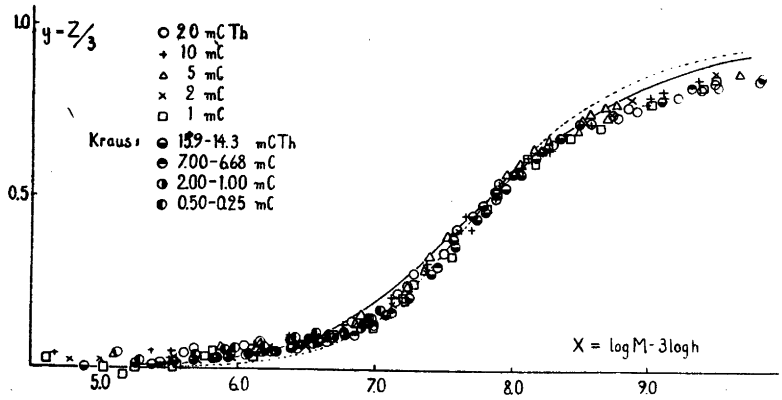

Fig. 4. Comparison of present data with those of Kraus and Holmberg [9], recalculated to the form y(x) *assuming* t = 3. *Symbols: see left side of figure. Curves calculated for hypothesis IIIa as in Fig. 3.*

and k_n is the equilibrium constant for the formation of the n:th complex from the metal ion and water, in this case for the reaction (VIII, 29)

$$(n+1)\ Th^{4+} + 3n\ H_2O \rightleftharpoons Th((OH)_3Th)_n^{(4+n)+} + 3n\ H^+, \text{ equil.const.} = k_n \quad (12)$$

An average curve was drawn through the points of Fig. 3 (in a recalculation, Fig. 4 was used; but this made very little difference). The y values were read at 0.2 intervals of x (Table 4), and the integral $\int y\,dx$ calculated by trapezoidal integration. Thus u and g could be calculated from (8) and (9).

This procedure involves no extra assumption apart from the one — strongly corroborated by the data in Figs. 3 and 4 — that only complexes of formula $Th((OH)_3Th)_n^{4+n}$ need be considered.

One might think of using the data $g(u)$ for calculating the various k_n in (11). It may not be too hard to find a set of k_n that gives an acceptable agreement with $g(u)$. However, because of the limited accuracy of the data, this can

Table 4. Average points y(x) *and primary values for "direct analysis".*

x	y	$\log u + \delta$	$\log(1+g) - \delta$	x	y	$\log u + \delta$	$\log(1+g) - \delta$
5.2	0.012	5.1948	-0.0000_3	7.6	0.394	7.1721	0.2104
5.4	0.017	5.3897	0.0029	7.8	0.484	7.2452	0.2674
5.6	0.021	5.5842	0.0066	8.0	0.568	7.3035	0.3320
5.8	0.030	5.7752	0.0116	8.2	0.640	7.3514	0.4049
6.0	0.040	5.9638	0.0185	8.4	0.694	7.3946	0.4911
6.2	0.050	6.1505	0.0272	8.6	0.736	7.4334	0.5882
6.4	0.062	6.3341	0.0381	8.8	0.765	7.4707	0.7004
6.6	0.080	6.5121	0.0517	9.0	0.792	7.5032	0.8149
6.8	0.110	6.6800	0.0694	9.2	0.816	7.5320	0.9328
7.0	0.155	6.8340	0.0929	9.4	0.840	7.5560	1.0481
7.2	0.216	6.9704	0.1239	9.6	0.860	7.5773	1.1688
7.4	0.300	7.0826	0.1625	9.8	0.875	7.5973	1.2996

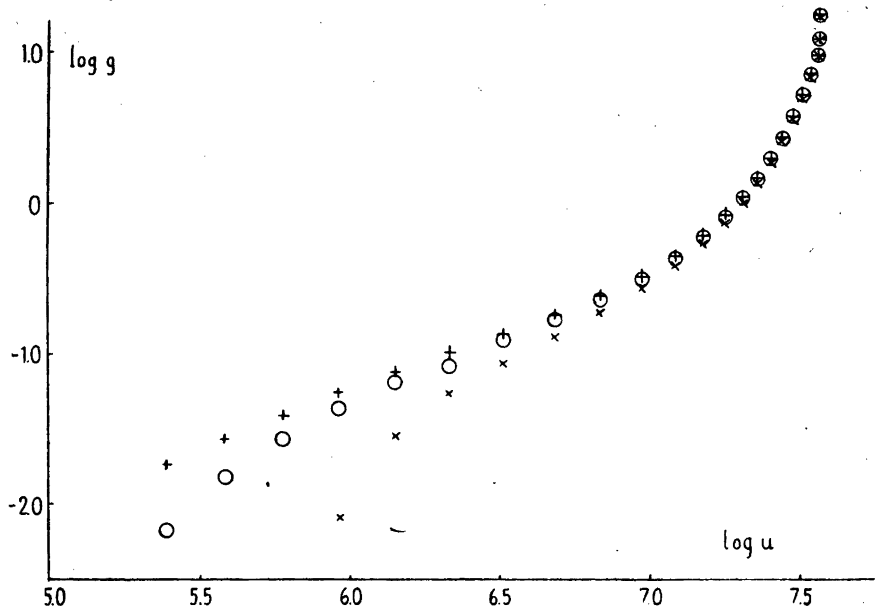

Fig. 5. Direct analysis of y(x) *curve: log* g *as a function of log* u. *The residual integral* δ *was assumed to be 0.0050 (+), 0 (○), or — 0.0150 (×).*

probably be achieved in several ways. We have hence preferred another way of approach. From (ABII, 73) we have

$$\frac{\mathrm{d} \log g}{\mathrm{d} \log u} = \bar{n} = \frac{\Sigma\, n c_n}{\Sigma\, c_n} \tag{13}$$

where $\bar{n}$ is the average number of $Th(OH)_3$ "links" per complex.

Table 4 gives the values for log $(1 + g)$ and log u primarily obtained assuming the residue integral

$$\delta = \int_{-\infty}^{5.2} y \, \mathrm{d}x \tag{14}$$

to be zero. In the following calculations, the values $\delta = 0.0050$ (crosses +), $\delta = 0$ (circles ○) and $\delta = -0.0150$ (×) were used; Fig. 5 shows log g as a function of log u, and Fig. 6 $\bar{n}$ as a function of x, with these three values.

For x values below 7.0, the curves for the three δ values are seen to differ considerably. One would expect $\bar{n}$ to approach the value 1 asymptotically for low x, but this is not achieved with any δ value. It should be remembered that the $\bar{n}$ values in this range are very sensitive to small errors in y.

For $x > 7$, a reasonable variation of δ, which may also include the error in the integral between $x = 5.2$ and 7.0, does not influence the general course of the curves very much.

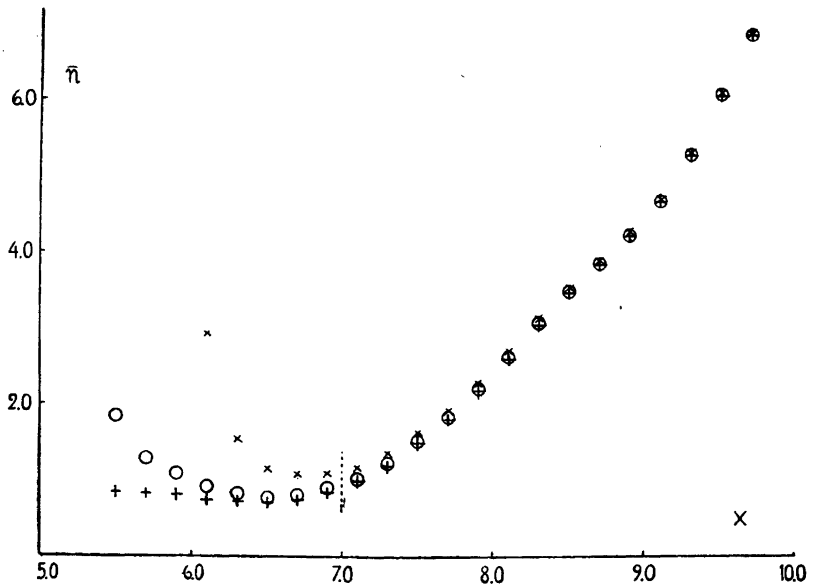

Fig. 6. Direct analysis of y(x) *curve:* $\bar{n}$ *as a function of* x. *Symbols as in Fig. 5.*

If only one complex had existed, one would expect the $\bar{n}$ curve to be a horizontal line at the corresponding n value. If two complexes had predominated over all others, $\bar{n}$ would give two almost horizontal shelves, with a transition curve between them. Finally, if there were a maximum value for n, $\bar{n}$ should approach this n_{max} with increasing x.

As a matter of fact, as far as the data go, $\bar{n}$ is seen to increase with x, and even the slope increases. Thus there is no indication that one or two complexes are more important than all others and if there is a maximum value for n it must be much greater than 6.

The $\bar{n}$ curve may be compared with the results of the preliminary ultracentrifugations of thorium perchlorate solutions quoted by Kraus and Holmberg [9]: "aggregates were found in the vicinity of $Z = 2$ with tetramers possibly occurring for Z just less than 2, and considerably larger polymers for Z larger than 2". (Kraus' symbol has been replaced by Z to conform with the present paper). It should be noted that a "tetramer" with 4 Th would correspond to $n = 3$ (*cf.* eq. 7).

Fig. 6, which follows from the present equilibrium data, and from Kraus' and Holmberg's own equilibrium data, gives however no indication of a sudden change at $Z = 2$ ($x \approx 8.3$ according to Fig. 3), nor of the prevalence of tetramers.

TESTING SIMPLE MECHANISMS

The independent determination of, say, even the first 6 or 8 k_n values would require a greater accuracy than the present data allow. It was then tested whether the $y(x)$ curve could not be approximately described by some of the simple one- or two-parameter mechanisms discussed in ABII and part VIII.

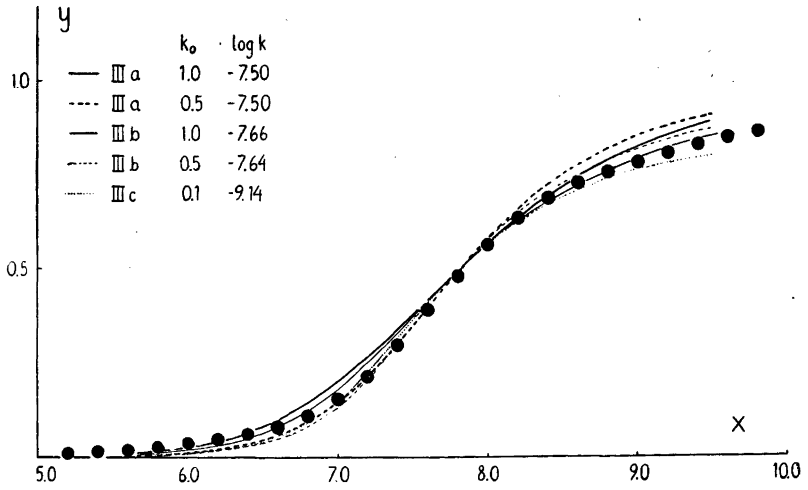

Fig. 7. Curves y(x) *calculated for the simplified mechanisms indicated to the left in the figure. Dots: smoothed values from experimental data, Figs. 3 and 4.*

The hypotheses denoted as I (precipitate formed) and II (only one complex formed) could immediately be ruled out since no acceptable fit could be found with the $y(x)$ curves calculated according to ABII. Thus, more than one polynuclear complex must be formed in the solution — the same conclusion was reached from the "direct analysis" above.

With the hypotheses involving the formation of an unlimited series of complexes: IIIa, IIIb, and IIIc, the fit was considerably better. The three hypotheses assume the following formulas for k_n (ABII 28; VIII 36)

$$\text{IIIa: } k_n = k_0 k^n; \quad \text{IIIb: } k_n = k_0 n k^n; \quad \text{IIIc: } k_n = \frac{k_0 k^n}{\lfloor n} \tag{15}$$

We may remember that the ratio k_{n+1}/k_n is the equilibrium constant for the addition of a new link:

$$\mathrm{Th((OH)_3Th)_n^{n+4} + Th^{4+} + 3\ H_2O \rightleftharpoons Th((OH)_3Th)_{n+1}^{n+5} + 3\ H^+};$$
$$\text{Equil.const} = k_{n+1}/k_n \tag{16}$$

With IIIa this ratio is a constant k. With IIIb, $k_{n+1}/k_n = k(1 + n^{-1})$; it decreases somewhat with increasing n but tends to a limiting value k. With IIIc, the ratio is equal to $k(n + 1)^{-1}$ and thus tends to zero with increasing n.

The best fits with the experimental points were obtained with the following sets of constants:

IIIa: $k_0 = 0.5$ to 1.0; $\log k \approx -7.50$

IIIb: $k_0 = 0.5$ to 1.0; $\log k \approx -7.65$

IIIc: $k_0 =$ about 0.1; $\log k \approx -9.15$

Fig. 7 gives five curves calculated with these constants; the dots are taken from the average experimental curve (Table 4). All the calculated curves in Fig. 7 are fairly close to the experimental points although each deviates slightly in some part of the x range.

Details of calculations

For *IIIa*, $\log k$ was read off to be about -7.5 directly from the $y(x)$ diagram, Fig. 3 or 4, using equation (ABII, 33): $\log k = \log 2 - x_{\frac{1}{2}}$. To study the spread, $\log k$ was also calculated separately for each curve in Fig. 1, using the equation $\log k = \log 2 - \log M + 3 \log h_{\frac{1}{2}}$; $h_{\frac{1}{2}}$ and $x_{\frac{1}{2}}$ are the values at $y = \frac{1}{2}$. For M = 1, 2, 5, 10, and 20 mC, $\log k = -7.60$, -7.55, -7.45, -7.49, and -7.54 was found; average $\log k = -7.53 \pm 0.07$. The average curve gave $\log k = -7.54$.

k_0 was found using equation (ABII, 37): $k_0 = y(1-10^X(1-y))^2(10^X(1-2y)^2)^{-1}$. The abscissa x was measured for y = 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8, and for each value $10^X = 2 \cdot 10^{(x-x_{\frac{1}{2}})}$ was calculated (ABII, 36). There was a trend in k_0 for high y: 0.62, 0.82, 0.85, —, 0.76, 1.00, (2.19).

For *IIIb*, equation (ABII, 40) was used: $\log k = \log(1+v_{\frac{1}{2}}) - x_{\frac{1}{2}}$, where $v_{\frac{1}{2}}$ is calculated from $2k_0 v_{\frac{1}{2}}^2 = (1-v_{\frac{1}{2}})^3$. For $k_0 = 0.5$, $\log k = -7.64$ and for $k_0 = 1.0$, $\log k = -7.66$ were found.

For *IIIc*, equations (ABII, 44) were similarly used.

The comparison of the experimental data with the calculated curves for unlimited mechanisms suggests that the ratio k_{n+1}/k_n approaches a value around $10^{-7.65}$ with increasing n. This is the value for k found with IIIb; moreover, in the "direct analysis", u seems to tend to a limiting value about $10^{7.65}$ ($v \to 1$, $u \to k^{-1}$ for IIIa and IIIb, cf ABII, 31, 38, 6). The ratio k_{n+1}/k_n for low n, and the first constant k_1 may be somewhat higher, perhaps in the neighbourhood of $10^{-7.50}$.

From the experimental data one might try to calculate the first k_n more exactly. We shall not do so at present.

A remarkably good approximation is obtained with the very simplest hypothesis, IIIa with $k_0 = 1$, implying that k_1 and all consecutive constants k_{n+1}/k_n (16) are alike. One then gets the very simple equations (*cf.* ABII, 34a):

$$x + \log k = \log y - 2 \log (1-y) \tag{17}$$

thus for Th^{4+}

$$\log M - 3 \log h - 7.50 = \log \frac{3Z}{(3-Z)^2} \tag{18}$$

STRUCTURE OF THE COMPLEXES

The equilibrium data seem to indicate that a series of complexes $Th((OH)_3Th)_n$ is formed with n = 1, 2, 3 *etc.* Although these data permit no conclusions on the shape of the complexes, it seems very plausible to assume that they are chain-like. It is tempting to compare these complexes with the chains $(Th(OH)_2^{2+})_n$ found by Lundgren and Sillén [19,20] in the crystal structures of thorium hydroxide sulfate and chromate (Fig. 8). Such a chain contains two parallel rows of hydroxide groups, with Th atoms on alternate sides so that

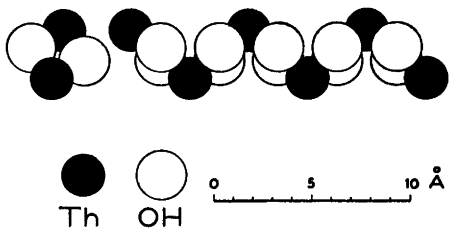

Fig. 8. $(Th(OH)_2^{2+})_n$ chain from thorium hydroxide sulfate or chromate[19,20]

every Th is in contact with 4 OH, and every OH with 2 Th. If, in the pH range studied, one OH group out of two has lost its proton, we would have chains

$$Th\overset{O}{\underset{OH}{}}Th\overset{O}{\underset{OH}{}}\cdots\cdots Th\overset{O}{\underset{OH}{}}Th \text{ or } Th(\overset{O}{\underset{OH}{}}Th)_n$$

which would be equivalent to $Th((OH)_3Th)_n$. By emf methods in constant ionic medium we cannot distinguish between 1 O^{2-} and 2 OH^-.

The assumption that half of the OH^- have lost a H^+ is not so unreasonable if it is remembered, for instance, that for each of $PO_2(OH)_2^-$ and PO_3OH^{2-} (*i.e.* $H_2PO_4^-$ and HPO_4^{2-}), there is a pH range of about 5 units within which this species is predominant.

The assumption that the hydrolysis products of Th^{4+} are chain-like complexes is further supported by the work of Dobry, Guinand, and Mathieu-Sicaud [21] on colloidal thorium hydroxide, obtained by dialysis of dilute thorium chloride solutions. From observations on viscosity, light diffusion, and stream double refraction, and especially from electron microscope pictures, they concluded that the particles are thread-like, of diameter about 18 Å, and of average length 700 Å. The cross-section corresponds to bundles of perhaps some 20 or 30 of the simple chains of Fig. 8. Perhaps the simple chains are joined to bundles by O or OH during some of the later stages of the hydrolysis.

SUMMARY

The hydrolysis of the ion Th^{4+} in 1 C perchlorate medium at 25° C has been studied by electrometric titrations, using quinhydrone or hydrogen electrodes. The data were treated by the methods given in previous papers, ABI[16], ABII[17], and part VIII[18].

Within the experimental accuracy, Z (the number of H^+ split off per Th), proved to be a function of the single variable $x = \log M - 3 \log h$ (M = total Th concentration, $h = [H^+]$). Hence it follows that all complexes formed in appreciable amounts can be written in the form $Th((OH)_3Th)_n^{4+n}$.

From a direct analysis of the experimental $y(x)$ curve ($y = \frac{1}{3} Z$) it is concluded that complexes with a number of different n values are formed, and that no one n value predominates. No upper limit for n was found and it was concluded that there must be n values considerably greater than 6.

A quite good approximation to the experimental data is obtained with the very simple assumption that the equilibrium constants for all successive steps ($n = 0, 1, 2, 3 \ldots$):

$$Th((OH)_3Th)_n^{4+n} + Th^{4+} + 3\ H_2O = Th((OH)_3Th)_{n+1}^{5+n} + 3\ H^+$$

are the same, $k = 10^{-7.50}$. ("IIIa, $k_0 = 1$" in Figs 1, 3, 4, 7). The deviations indicate that the constant is really somewhat lower, perhaps about $10^{-7.65}$, for the higher n values.

In view of X-ray and electron microscope data [19,20,21] it seems plausible that the complexes are chain-like, of formula $Th(\overset{O}{\underset{OH}{}}Th)_n^{4+n}$

The data agree well with those recently published by Kraus and Holmberg[9], although our conclusions differ considerably.

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The Influence of Water on the Autoxidation of Methyl Linoleate

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In the presence of water the autoxidation of methyl linoleate has a short induction period. This is supposed to depend on water interferring with the reactions initiating the autoxidation, while peroxides have not yet formed, rather than on an inhibition of the autoxidation reactions. The pH of the water has no effect on the length of the induction period in the range of pH 5.1—9.2.

Many foods and technical products based on fats contain water which may influence their stability against autoxidation. In the literature divergent views are found on the existence of such an influence. Thus Greenbank and Holm [1] state that water increases the stability of fat, whilst French *et al.*[2] and Mukherjee [3] consider water to have no influence at all.

This lack of agreement may depend upon the fact that these authors used different fats for their investigations. It therefore seemed to be of interest to study the autoxidation of a pure unsaturated fatty acid ester in the presence of water.

EXPERIMENTAL

The investigation method

The investigation was carried out by measuring manometrically the oxygen absorption of methyl linoleate in contact with borate buffers at 40° C.

For investigations of this type vegetable fats are unsuitable, because of their content of natural antioxidants. The influence of water on the autoxidation rate may then be indistinguishable from an effect on the antioxidants. Animal fats have a very low antioxidant content but show, like the vegetable fats, considerable variations also for the same type of fat, which renders difficult the comparison of results obtained with different fats.

As linoleic acid is the most unsaturated acid of several valuable fats, thus strongly affecting their stability (*cf.* Gunstone and Hilditch [4]), methyl linoleate is a suitable model substance for fats in autoxidation studies. It does not possess the disadvantages of natural fats mentioned above, as linoleic acid is easily obtained in a sufficiently pure state. Furthermore its autoxidation reactions have been extensively studied (*e. g.* by Farmer [5] and Bolland and Gee [6]). Methyl linoleate also autoxidizes rapidly enough to permit the investigation to be carried out at a low temperature at conditions similar to those of "normal" storage.

Table 1. The oxygen absorption in ml at NTP of 1 g methyl linoleate.

Dry		pH 5.1		pH 6.5		pH 8.0		pH 9.2	
min.	ml O_2	min.	ml O_2	min.	ml O_2	min.	ml O_2	min.	ml O_2
67	0.06	113	0.04	138	0.06	96	0.06	144	0.01
157	0.12	193	0.09	228	0.07	176	0.09	189	0.10
207	0.18	258	0.13	333	0.18	405	0.16	339	0.24
262	0.25	422	0.17	608	0.50	549	0.38	559	0.44
477	0.66	563	0.42	1 338	2.29	1 291	1.98	1 339	2.27
537	0.73	1 308	2.14	1 478	2.90	1 431	2.46	1 419	2.66
1 267	3.39	1 448	2.70	1 523	3.10	1 476	2.62	1 474	2.95
1 352	3.80	1 553	3.17	1 573	3.36	1 536	2.88	1 524	3.14
1 403[a]	4.00	1 663	3.70	1 635	3.70	1 667	3.68	1 609	3.57
		1 711[a]	4.00	1 689[a]	4.00	1 709[a]	4.00	1 705[a]	4.00

[a] Interpolated value

Materials

Methyl linoleate. Tetrabromostearic acid was prepared as described by McCutcheon [7] and was recrystallized from ligroin until a melt ng point of 115.0—115.3° C was reached. It was then debrominated in boiling methanol acidulated with sulphuric acid to which zinc dust was added in portions. After decantation from the zinc residue more sulphuric acid was added and the linoleic acid was esterified to methyl linoleate by boiling under reflux (*cf.* Ref. [8]). The ester was then transferred into an ether solution and deacidified with alkali. At last the methyl linoleate was fractionally distilled under reduced pressure, the fraction having a b. p. of 149—151° C at 1 mm Hg was collected and sealed in ampullae.

From the debromination step onwards all operations were carried out in an atmosphere of oxygen-free nitrogen. The distillation apparatus and the ampullae were carefully cleaned.

The methyl linoleate thus obtained contained no detectable amounts of free fatty acid, peroxides or heavy metal soaps.

Water. The water used had been doubly distilled in an all-glass apparatus.

Boric acid and sodium tetraborate p. a. The solutions were prepared according to Palitzsch [9]. The pH-values were measured with the solutions in equilibrium with an equal amount of fatty acid ester at 25° C.

Apparatus

The oxygen absorption was measured with a Barcroft apparatus. This consists essentially of a U-tube manometer having both ends connected to flasks. One flask contains the sample and the other one acts as a compensating vessel. For details of construction and application of this apparatus *cf.* Dixon [10].

EXPERIMENTAL RESULTS

The autoxidation of dry methyl linoleate was first studied. Fig. 1 gives the oxygen absorption curve as constructed from the values given in Table 1. The oxygen absorption is initially very slow but increases as the oxygen absorption increases due to autocatalysis *.

Experiments were then made with methyl linoleate to which an equal amount of borate buffer solutions of the pH-values 5.1, 6.5, 8.0 and 9.2 respectively had been added. The oxygen absorption curves obtained were identical and the times necessary for the sorption of 4 ml O_2 at NTP per g methyl linoleate differ only by a few minutes. The oxygen absorption is very slow and fairly constant for about 300 min., but then takes the same course as in the experiment with dry methyl linoleate.

* For a discussion of autoxidation curves of this type see *e. g.* Robertson and Waters [11].

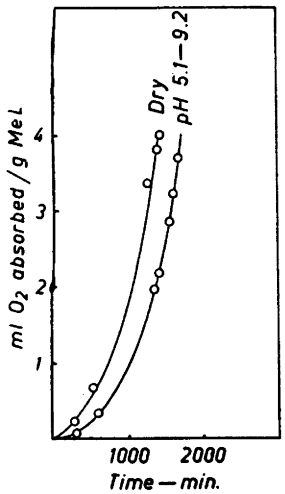

Fig. 1. The autoxidation of methyl linoleate in absence and in presence of water.

DISCUSSION

The experimental results presented above show that water retards the autoxidation reaction of the methyl linoleate but it has no influence on the chain reaction, when this has once started. Such a behaviour is typical of an inhibitor. Water can, however, not act as an inhibitor, since reactions between radicals and water, involving liberation of a hydrogen atom, are generally highly endothermic, because of the high dissociation energy of the reaction $H_2O \rightarrow H + OH$[12]. If water had been an inhibitor the induction period should have lasted until all water had been consumed. The only reasonable explanation of the induction period seems to be that water interferes with the reactions by which the autoxidation is initiated while peroxides have not yet formed. However, these reactions are not known with any degree of certainty (*cf.* however Farmer [5]).

The pH of the water had no effect on the length of the induction period in the pH-range 5.1—9.2 (*cf.* Ref.[13]).

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Ascorbic Acid and Ascorbyl Palmitate as Synergistic Antioxidants for Methyl Linoleate in Presence of Water

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The efficiency of ascorbic acid and ascorbyl palmitate as synergistic antioxidants in methyl linoleate containing 0.0025 % phenyl-α-naphthylamine in the presence of water decreases with increasing pH-value. At pH 5.1 ascorbyl palmitate is very efficient and, due to its higher fat-solubility, more active than ascorbic acid. Ascorbic acid is more effective in dry methyl linoleate. At pH 9.2 neither substance has any effect.

Ascorbic acid and its fatty acid ester have been proposed as antioxidants for fats [1,2] and shown to be highly effective in dry fat (*e.g.* by Golumbic and Mattill [3], Riemenschneider *et al.*[4]). In presence of water ascorbic acid has been reported to be efficient in some cases [5].

Ascorbic acid is known to autoxidize readily in water solution, especially if the water is alkaline [6]. Its efficiency as a synergistic antioxidant may therefore be supposed to be influenced by the presence of water and vary with the pH-value. This has earlier been shown to be the case for some autoxidizable, polyphenolic antioxidants [7].

EXPERIMENTAL

A system of methyl linoleate and borate buffers under oxygen in darkness at 40° C (*cf.* Ref.[8]) has been studied. Phenyl-α-naphthylamine was used as primary antioxidant, since it has been proved active within the investigated range of pH 5.1—9.2.

Chemicals: Methyl linoleate and borate buffer solutions. (Preparation *cf.* Ref.[8]).

Phenyl-α-naphthylamine (Eastman).

Ascorbic acid (Hoffmann-la Roche).

Ascorbyl palmitate. Ascorbic acid was esterified with palmitic acid according to Riemenscheider and Turer [10] and gave ascorbyl palmitate, m. p. 116° C.

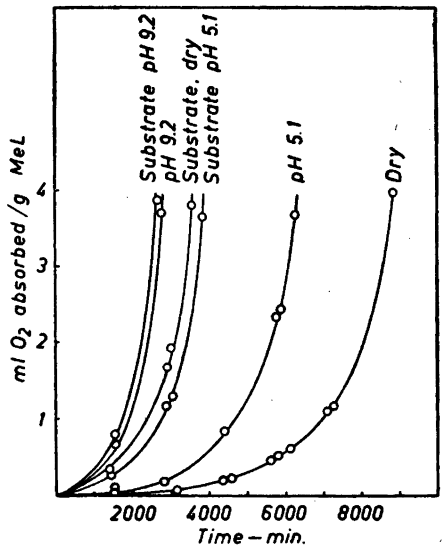

Fig. 1. The influence of pH on the stability of methyl linoleate + 0.0025 % phenyl-α-naphthylamine (substrate) + 0.0025 % ascorbic acid.

RESULTS

Figs. 1 and 2 give the oxygen absorption curves for the two systems methyl linoleate + 0.0025 % phenyl-α-naphthylamine + 0.0025 % ascorbic acid, and methyl linoleate + 0.0025 % phenyl-α-naphthylamine + 0.0025 % ascorbyl palmitate, in a dry state and after addition of equal amounts of buffers of pH 5.1 and 9.2, respectively. For comparison the corresponding curves for the system methyl linoleate + 0.0025 % phenyl-α-naphthylamine [9] have been added.

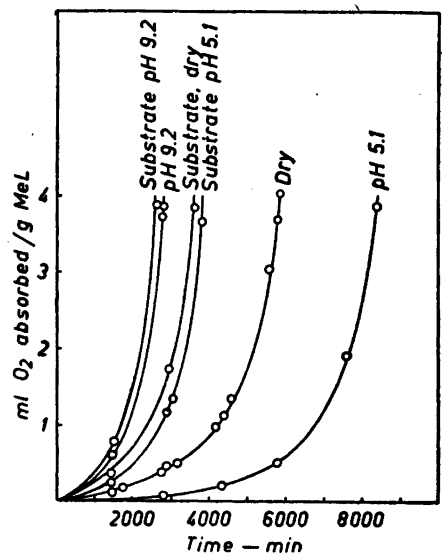

Fig. 2. The influence of pH on the stability of methyl linoleate + 0.0025 % phenyl-α-naphthylamine (substrate) + 0.0025 % ascorbyl palmitate.

Both ascorbic acid and ascorbyl palmitate are powerful antioxidants. In dry methyl linoleate ascorbic acid is most efficient due to its lower molecular weight. In presence of a water phase of pH 5.1, ascorbyl palmitate is, however, the more active. At pH 9.2 both substances are inefficient.

DISCUSSION

The experimental results show that ascorbic acid and ascorbyl palmitate are affected by water, and its pH-value, in the same way as are the autoxidizable polyphenolic antioxidants. The two substances have their maximal effect at pH 5.1. Ascorbyl palmitate is most effective because of its higher fat solubility resulting in a higher concentration in the ester phase. Like the fat-soluble, primary antioxidants it is actually more active in the presence than in the absence of water. Both ascorbic acid and ascorbyl palmitate are inactive at pH 9.2, their autoxidation products then being without stabilizing effect.

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Kinetics and Equilibria in Flavoprotein Systems

II. The Effects of pH, Anions and Temperature on the Dissociation and Reassociation of the Old Yellow Enzyme

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The effects of pH, anions and temperature on the dissociation of the old yellow enzyme (O.Y.E.) and its resynthesis from FMN and protein have been investigated. The results suggest that primary amino groups of the protein may serve as binding sites for FMN. Only doubly anionic FMN couples rapidly with the protein.

With respect to the binding of FMN, the apoprotein of O.Y.E. can be compared to an anionic exchanger of the weakly basic type. Thus, only anions of strong acids are able to dissociate the enzyme. Multivalent anions strongly decrease the association velocity of FMN and apoprotein. This effect is counteracted by univalent anions like chloride or acetate.

A study of the reactions between riboflavin and the old yellow apoenzyme has also been carried out. The association reaction of the riboflavin-protein complex is roughly one-tenth as rapid, and the dissociation one hundred thousand times as rapid as the corresponding reactions in the FMN-protein system.

The possible physiological role of anions in regulating the activity of coenzyme-protein systems has been discussed.

The fluorimetric technique developed in previous studies [1,2] of the dissociation and association velocities in the system

$$\text{FMN} + \text{apoprotein} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{FMN-Protein (O.Y.E.)}$$

offered a possibility of making a systematic and detailed study of the kinetics and equilibria in this system under varied conditions. The effect of pH and temperature on the dissociation and association of O.Y.E. has yielded information regarding the chemical groups involved in the binding of FMN to the apoenzyme, and our observation that riboflavin combines with this apoenzyme gave us the opportunity to investigate the role that the phosphoric ester group of FMN plays in this binding. The discovery, previously reported, that

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O.Y.E. was readily dissociated by chloride ions led to a more complete investigation of the effect of different anions on the dissociation of the enzyme, and it was found that anions also influence the association reaction. We have reason to believe that anions may play a general role in the regulation of the activity of coenzyme-protein systems.

MATERIALS AND METHODS

O.Y.E. was prepared as described previously[2]. Using this procedure, Dr. C. S. Vestling in this laboratory was able to prepare an approximately 90 % pure product without making use of electrophoresis. The fluorescence of this preparation was 1 % of the fluorescence of an equimolar solution of FMN, whereas the preparations which were 25 % pure showed 4 % fluorescence. Neither the fluorescence of the enzyme nor the apoenzyme was quenched by chloride or by alkali, as is the fluorescence of FMN. This small residual fluorescence of the enzyme is in all probability due to impurities.

A commercial preparation of FMN can be purified by paper electrophoresis, as shown in a previous paper [3]. A more convenient method, which allows purification on a larger scale, is to adsorb the commercial preparation on Dowex 2, 200—400 mesh, and elute with formic acid and ammonium formate. Fraction 1 [2], which may be riboflavin or some other non-phosphorylated flavin, can be eluted with water or 1 *M* formic acid, FMN is eluted with 4 *M* formic acid, and the diphosphate fraction, which is inhomogeneous, can be eluted with 4 *M* formic acid plus 2 *M* anmonium formate.

Riboflavin was recrystallized from a commercial preparation. A fresh solution was made each day.

At the time of publication of previous papers [1,2] we had not yet succeeded in preparing FMN which was pure enough to entirely loose its fluorescence on coupling with apoprotein. We have now found that FMN, prepared from O.Y.E. by splitting with HCl in the absence of light, gives a rest fluorescence of only 2 % after combination with apoenzyme. We conclude that pure apoprotein does not fluoresce at all and that it extinguishes completely the fluorescence of an equivalent amount of FMN.

FMN was titrated in the alkaline range by following the disappearance of the fluorescence. The pK' was found to be 10.2 at 23.5°, the same as for riboflavin [3]. At 13.5° we found pK = 10.29. 0.4 *M* sodium chloride did not affect the pK'-values to any appreciable degree.

In the association experiment described in detail in a previous paper, FMN was added to a solution of apoenzyme. The concentration of apoenzyme was relatively high (1.57×10^{-6} *M*), and there was no serious deviation from a second order reaction. However, when the concentration of apoenzyme is of the order of 0.2×10^{-6} *M* or less, it is rather unstable and should be added to a solution of FMN in the cuvette in order to minimize the time for inactivation in the dilute condition. Otherwise deviation from a second order reaction and lower titration values for the apoenzyme may occur. This procedure also has the advantage that the level of deflection for free FMN at $t = 0$ is determined in advance, and has to be corrected only for the small fluorescence of the apoenzyme itself. If, in contrast, FMN is added to a solution of apoenzyme, small errors in the pipetting of FMN may cause appreciable errors when the reaction is very rapid.

With this modification, the association velocity curves in hundreds of experiments were found to coincide strictly with the requirements for a second order reaction.

The inactivation of the apoenzyme in very dilute solutions can be diminished by the addition of serum albumin to the solution (0.3 mg/ml). Serum albumin did not disturb the reaction between FMN and apoenzyme. The inactivation of dilute solutions of apoenzyme has also been frequently noted in dissociation reactions, especially when the dissociation proceeds slowly. The inactivation is observed as a fluorescence drift, no definite equilibrium being reached.

The inactivation of the apoenzyme in high dilutions is probably not due to metal ions or to other SH-inhibitors, since the addition of Cu^{2+} ions (3×10^{-5} *M*) or *p*-chloromercuribenzoate (6×10^{-5} *M*) did not cause any inactivation.

RESULTS

Dissociation of the old yellow enzyme

The rate of dissociation of the old yellow enzyme in acetate and glycine buffers between pH 3.7 and pH 11 is very slow; the rate constant, k_2, is less than 10^{-5} sec.$^{-1}$. In the presence of certain anions the enzyme dissociates readily. The effect of different anions appears to be related to the ionization constant of the acid formed by each ion. Only the anions of strong acids have a strong dissociating effect. Some results are shown in Table 1.

Table 1. Dissociation of O.Y.E. by different anions at pH 4.40 23.5° C.

Salt	Conc. *M*	$k_2 \times 10^4$ sec^{-1}
Na formate	0.1	0
Na acetate	0.1	0
Na F	0.1	0
NaH_2PO_4	0.1	1
Na_2SO_4	0.1	9
NaCl	0.1	33
$NaNO_3$	0.1	63
NaBr	0.1	99

The effect of pH on k_2 in the presence of 0.4 *M* NaCl is shown in Fig. 1. In the acidic range, between pH 4.7 and 6, k_2 is proportional both to the hydrogen and the chloride ion concentration. Thus at 23.5°, $k_2 = 1\,000\,[H^+][Cl^-]$.* Below pH 4.7, some inactivation may take place, resulting in a k_2, which is somewhat higher than expected from the formula. k_2 increases roughly four-fold from 13.5° to 23.5° over the whole pH range, and the energy of activa-

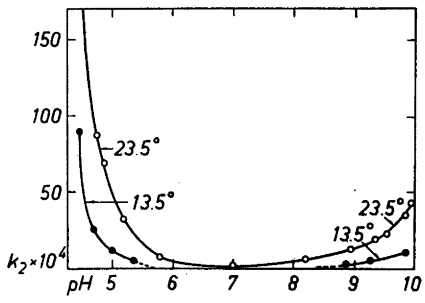


Fig. 1. k_2 *for O.Y.E. as a function of pH in 0.4* M *NaCl.*

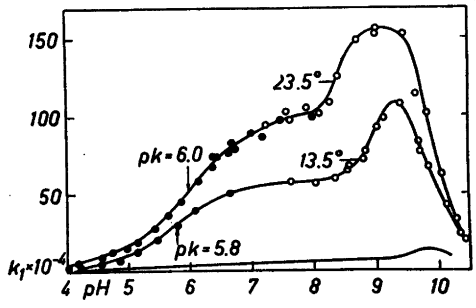


Fig. 2. k_1 *for FMN + protein as a function of pH.* ●●● *in 0.1* M *acetate buffer* ○○○ *in 0.1* M *glycine buffer. The lower curve, experimental points omitted, is* k_1 *for riboflavin + protein in 0.1* M *acetate at 13.5°.*

* One of us (H. T.) dissociated the O.Y.E. reversibly for the first time by dialysis against weak HCl. As indicated by the formula, this was a fortunate choice.

tion, μ, may be calculated to be $\mu = 23\,400$ cal. k_2 is proportional to the chloride concentration also in the alkaline range. This is shown in Tables 2 and 3.

Table 2. Effect of varied [NaCl] *on* k_2 *at pH = 4.93. 0.1* M *sodium acetate buffer, 23.5° C.*

[NaCl]	Q.f.	$k_2 \times 10^4$ sec.$^{-1}$	$\frac{k_2}{[NaCl]}$
0.1	1.06	10.5	105
0.4	1.22	48	120
1.0	1.55	104	104
2.0	2.11	222	111

Association of FMN and apoprotein

In Fig. 2, k_1, the association velocity constant, is plotted as a function of pH, at 23.5° and 13.5°. Acetate and glycine buffers were used in these experiments, since they did not interfere with the reaction velocity. In the alkaline range (glycine buffer), k_1 has a maximum at pH 9.1 at 23.5° and 9.3 at 13.5°, and falls rapidly at higher pH values. This is not due to denaturation of the apoenzyme. Correct titration values of the apoenzyme were obtained between pH 4.6 and 10.5. Below pH 7, k_1 coincides with the dissociation curves of a monovalent acid of $pK' = 6.0$ (23.5°) and $pK' = 5.8$ (13.5°). Since the second dissociation constant of many phosphoric acid esters is usually around $pK' = 6.2$, it was of interest to carry out an acid-base titration of FMN in this region. The results, which are given in Table 4, are somewhat uncertain because of the low solubility of FMN.

In Fig. 3, k_1 is plotted as a function of pH in the presence of 0.4 *M* NaCl. As can be seen from the figure, the maximum of k_1 in the alkaline range does not appear in 0.4 *M* chloride. Instead, the curve drops continuously above pH 7 with increasing pH following a dissociation curve of a monovalent base of p*K* 9.1. As can be seen from the figures, k_1 increases twofold between 13.5° and 23.5°, giving $\mu = 11\,700$ cal/mole. In the pH region around 6 it is seen that k_1 is somewhat higher in glycine than in acetate. In the presence of chloride the negatively charged acetate ions cause an inhibition of k_1: this inhibition does not occur with glycine which is a Zwitterion at this pH.

From the data in Figs. 2 and 3 it may be seen that chloride decreases k_1 in the alkaline range, has no effect around pH 7, and a slightly increasing effect in the acidic range.

Table 3. Effect of varied [NaCl] *on* k_2 *at pH 9.45. 0.05* M *glycine buffer, 23.5° C.*

[NaCl]	Q.f.	$k_2 \times 10^4$ sec^{-1}	$\frac{k_2}{[NaCl]}$
0.1	1.43	4.0	40
0.4	1.61	15	37.4
1.0	2.20	35	35

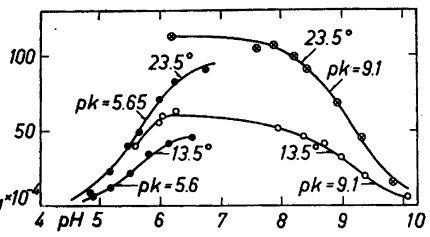


Fig. 3. k_1 *as a function of pH in 0.4* M *NaCl.* ● ● ● *0.1* M *acetate buffer* ⊙ ⊙ ⊙ *and* ○ ○ ○, *0.1* M *glycine buffer.*

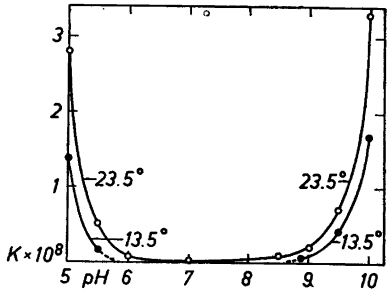


Fig. 4. Dissociation constant, K, for O.Y.E. as a function of pH in 0.4 M *NaCl.*

In Fig. 4, the dissociation constant, K, in 0.4 M NaCl, is plotted as a function of pH at 23.5° and 13.5°. The values are calculated from the results shown in Figs. 1 and 3. The heat of dissociation

$$\triangle H = -4.57 \cdot \log \frac{K_2}{K_1} \left(\frac{T_1 \times T_2}{T_2 - T_1} \right)$$

is approximately —11 700 cal over the entire pH range.

The association reaction is strongly inhibited by polyvalent anions. Some results are shown in Table 5. Monovalent ions generally have no effect or a very slight one on the association reaction. The effect of monovalent anions as compared to the effect of polyvalent anions, is illustrated in the following examples: Whereas secondary phosphate, pH 8.2, $\Gamma/2 = 0.1$, decreased k_1 about 4 fold, there was no measurable effect of primary phosphate at pH 4.23. Divalent anions of stronger acids, like sulfuric acid, however, were found still to inhibit in the acidic range. In the alkaline range, 0.1 M bicarbonate at pH 8.48 decreased k_1 from 125×10^4 M^{-1} sec^{-1} to 95×10^4 M^{-1} sec^{-1}. At pH 9.79, where a greater percentage of the salt is in the form of carbonate ions, it decreased k_1 from 106×10^4 to 25.3×10^4.

The inhibiting effect of polyvalent anions is partially counteracted by monovalent anions. In the presence of polyvalent anions, monovalent anions thus have an effect of increasing the association velocity see Table V.

Table 4. The second dissociation constant of the phosphoric acid group of FMN under different conditions.

Temp. °C	No salt added pK'	In 0.4 M NaCl pK'
23.5	6.2	5.9
13.5	6.2	6.0

Table 5. The inhibition of k_1 *by polyvalent anions and its counteraction by monovalent anions. pH = 7.0. 23.5° C.*

Salts		$k_1 \times 10^{-4}$ M^{-1} sec^{-1}
None		90
Na-phosphate, $\Gamma/2 = 0.1$		14
—»—	+ 0.4 *M* NaCl	64
0.033 *M* Na_2SO_4		50
0.05 *M* Na citrate		10
—»—	+ 0.4 *M* NaCl	40
—»—	+ 0.4 *M* Na acetate	25
0.01 *M* Na versene		19
—»—	+ 0.4 *M* NaCl	63

The reaction between riboflavin and apoenzyme

Riboflavin has been shown to be able to substitute for FMN to some degree [4]. In the system: O.Y.E. apoenzyme, TPN, Zwischenferment and glucose-6-phosphate, a large excess of riboflavin gave a considerable oxygen uptake. It was, therefore, of interest to investigate whether riboflavin could form a nonfluorescent compound with the apoprotein. This was found to be the case, and the rate of the reaction followed the equation

$$-dc/dt = k_1 \text{ [Riboflavin] [Apoenzyme]}$$

A plot of k_1 as a function of pH is included in Fig. 2. k_1 was calculated using the formulas for a second order association reaction with an opposing first order dissociation reaction [2]. As in the FMN-apoenzyme system, a maximum of k_1 was observed in the alkaline range, although the peak in this system was 0.5 pH unit higher, namely pH 9.8. Although k_1 decreased with decreasing pH below pH 7, it did not decrease as rapidly as did k_1 for the FMN-protein system.

The kinetic data for riboflavin-protein could not be measured with the same degree of accuracy as for the FMN-protein system. It may suffice to mention, that k_2 in 0.1 *M* acetate at pH 7 is around 10^{-1} sec^{-1}, that is at least one hundred thousand times higher than k_2 for the old yellow enzyme. Since k_1 for riboflavin + protein is around one tenth of k_1 for O.Y.E. (see Fig. 2), the dissociation constant of riboflavin-protein is at least one million times greater than for O.Y.E. in the absence of dissociating anions.

We have not investigated in detail the effect of anions on the riboflavin-protein system. The dissociation constant, K, seems to be sensitive to small changes in ionic strength of the medium in dilute solutions. For example, in water it is roughly twice as high as in 0.1 *M* acetate. In 0.1 *M* acetate, polyvalent anions like sulfate and phosphate were found to have practically no effect on k_1 and k_2. Chloride ions had some dissociating effect. At pH 6 and 9, 0.4 *M* sodium chloride increased K by about twofold.

DISCUSSION

With respect to binding the negatively charged FMN the protein of the O.Y.E. may be compared to an anion exchanger. Thus, the dissociation of O.Y.E. by anions may be considered to be a displacement of FMN from the protein. The positive charges of the protein are of the weakly basic type; and it is, therefore, to be expected that anions of weak acids are not able to dissociate the enzyme. The strong inhibitory effect of polyvalent anions on the association velocity of FMN and protein is likely to be due to a tendency of these anions to interact with the positive groups of the protein which are essential for the attachment of FMN. Polyvalent anions are known to be bound more strongly to proteins than are monovalent anions [5]. The counteraction of the inhibitory effect of polyvalent anions on k_1 by chloride and other monovalent anions, could be due to a displacement of the polyvalent anions from their binding sites, just as FMN itself is displaced from the protein by for example chloride ions.

In regard to the fact that polyvalent anions are more strongly attached to the protein than monovalent anions, it must be remembered that FMN itself is a divalent anion at pH-values from 6 to 10. It combines rapidly with the protein in this pH-region. The combination velocity decreases with pH down to practically zero around pH 4, where FMN is a monovalent anion. The direct titration of FMN gave a pK' of 6.2 for the second dissociation constant of the phosphoric acid group. The combination velocity constant decreased along a dissociation curve with pK' = 6.0 (at 23.5°), that is, very close to the dissociation curve of the phosphoric acid group. This indicates that the association velocity is essentially proportional to the fraction of FMN which carries a double negative charge. The fact that 0.4 *M* NaCl displaced both curves by 0.2—0.3 pH units (see Figs. 2 and 3), supports the same conclusion. The 0.2 pH units difference in the titration curve and the velocity curve, if significant, remains to be explained.

Riboflavin, which has no phosphoric acid residue, shows a gradual rise in k_1 from pH 4 to 8. In contrast to FMN, riboflavin gives a k_1 curve that is far too flat to be interpreted as a simple dissociation curve.

Considering the fact that only FMN with two negative charges combines rapidly with the protein, it is tempting to suggest that the phosphoric acid residue might be bound to *two* positively charged groups on the protein. This binding would require the two positive groups to be close to one another in the protein molecule, for instance at the same side of a "helix" in two consecutive turns. Such a coincidence would be rare enough to account at least partly for the specific binding of only one molecule of FMN to the large protein molecule and for the high affinity of FMN for the apoprotein. In addition, there must be required a binding site for the imino group of the isoalloxazine, or a hydroxyl group of a corresponding enol form, at a suitable distance from the binding sites for the phosphoric acid.

The fact that k_1 drops sharply at pH-values above 9.3 could be caused by a discharge of the coupling positive groups of the protein. Terminal amino groups and lysine ε-amino groups have their dissociation constants located in this pH region. In the presence of chloride this descending branch of the k_1-

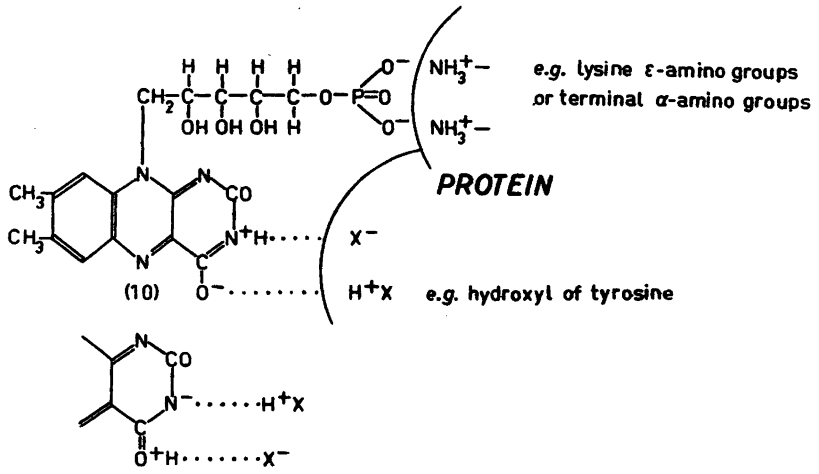
curve on the alkaline side is displaced towards lower pH-values, which might be expected from the fact that salts are known to displace pK'-values towards the isoionic point of the protein [6]. The fact that the pK' = 10.2 of FMN is not influenced by 0.4 M NaCl strongly indicates that the decrease of k_1 above pH 9.1 is not caused by the dissociation of the NH (3) group of FMN. Lowering the temperature from 23.5° to 13.5° C displaces the alkaline part of the k_1-curve towards higher pH-values, by 0.2—0.3 pH units per 10°, see Fig. 2. Primary amino groups are displaced in the same direction by 0.3 pH units per 10°. Thus we have reasons to believe that the phosphoric acid residue in FMN is coupled to one, or more probably, two primary amino groups in the protein.

The increase of k_1 from pH 8.5 to 9 now remains to be explained. In strongly alkaline solution FMN acquires one extra negative charge through the dissociation of the imino group (3) with pK = 10.2. This means that 2 % of the FMN molecules at pH 8.5, 9 % at pH 9.2 are trivalent. If we assume that the association velocity is many times higher for the trivalent FMN molecules than for the divalent ones, this would result in an increase in association velocity in this pH-region. At still higher pH the discharge of the positive protein groups must prevent the association reaction. In the presence of sodium chloride the maximum disappears because the pK':s of the protein amino groups are considerably lowered, whereas the pK' of the isoalloxazine imino group is not. Further experimental investigation must determine whether this tentative explanation is satisfactory or not.

It is interesting to note that Drs. O. and E. Walaas [7] in their measurements of the D-amino acid oxidase activity found a maximum at pH 9.2, that disappeared on the addition of sodium chloride, similar to the curves obtained for k_1 in the FMN-protein system. The maximum of association velocity between protein and flavin nucleotides near pH 9 thus could be a general phenomenon for flavoproteins.

Our data show that riboflavin, as determined by the quenching effect of the protein on the fluorescence, is bound much less firmly than FMN. In the pH range where there is no acid dissociation of the isoalloxazine nucleus (below pH 8), a slow rise of k_1 for riboflavin + protein occurs with increasing pH. The reason for this is not yet clear. The fact that even high concentrations of polyvalent anions did not affect k_1 suggests that cationic group of the protein may not be involved in the binding of riboflavin. The rapid rise in k_1 above pH 9 can perhaps, as for FMN, be understood in terms of increased concentration of the anionic form of isoalloxazine. The decrease above pH 10 could be due to the formation of a negative group on the protein, *e.g.* the dissociation of a tyrosine hydroxyl group. Weber [8] has suggested that the fluorescence might be quenched by tyrosine hydroxyl group. With both riboflavin and the binding site on the protein negatively charged, they would repel each other.

Between pH 4.3 and 9, the linkage between isoalloxazine and the protein could involve hydrogen bonding. As suggested in the formulae below, there are several possibilities for the formation of hydrogen bonds. Geissman [9] has suggested that a hydrogen bond from the protein to nitrogen atom 10 might exist in the leuco form of the isoalloxazine. This extra bond could explain why the reduced complex must be still less dissociated than the oxidized,



as suggested from the oxidation-reduction potential which is higher for O.Y.E. than for riboflavin.

Since phosphate decreases the association velocity, whereas chloride increases the dissociation velocity, it would be possible to prepare two solutions of O.Y.E., one in phosphate, the other in chloride, with the same dissociation constant for the FMN-protein. However, in phosphate with low values for both k_1 and k_2, the equilibrium state would be reached much more slowly than in chloride, where both k_1 and k_2 are high. This difference could be of great importance in such coenzyme-enzyme systems in which the coenzyme is "mobile", thus oxidized when bound to one protein, reduced when bound to another. Under such conditions the anions present in the solution will have influence on the overall reaction velocity. Furthermore, when the anionic influence on the oxidized and reduced complexes are not of equal magnitude, the anions will change the oxidation-reduction potential [10]. Examples of such effects will be given in forthcoming papers.

Finally it might be worth while to point out the interesting anion effects to be expected at the cell membranes, because chloride is often present outside, but not inside the cells. All factors, may it be hormones or others, which change the structure and permeability of the cell membranes will thus be able to influence enzyme reactions through anion effects.

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Hyaluronic Acid

VIII. A Preliminary Diffusion and Sedimentation Study of Potassium Hyaluronate Including a Determination of the Partial Specific Volume *

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Measurements on a 0.1 % solution of potassium hyaluronate gave a diffusion coefficient of 1.0×10^{-7} cm^2sec^{-1}, a sedimentation coefficient of 3.0 *S*, and a partial specific volume of 0.86. From Svedberg's formula the molecular weight was found to be 5.2×10^5, and a calculation of the molar frictional ratio gave a value of 3.8.

Substances giving highly viscous solutions are not ideal for diffusion and sedimentation studies. Hyaluronic acid is known to give very viscous solutions, and the present substance, described in a recent paper by one of the authors [1] has a relative viscosity of about 12 when dissolved in redistilled water at a concentration of 1 g per l.

For a thorough investigation in such a case a series of experiments with decreasing concentration of the solute is normally performed, and the diffusion or sedimentation coefficients found are plotted against the concentration. The values obtained when extrapolated to zero concentration are then assumed to be the best values that can be given for the diffusion — or sedimentation coefficients.

In this preliminary study we have chosen a concentration of about 0.1 % — which is about the lowest that will give reasonable accuracy in the apparatus used for the experiments, and it is assumed to give values which are not too far from those which would be obtained if the above mentioned method of extrapolation was applied.

* Part VII. *J. Colloid Sci.* **9** (1954) 460.

** For earlier publications by the latter author, see Robert Jensen.

DIFFUSION EXPERIMENT

The general equation for free diffusion was adapted by Fick [2] in 1855 from the equation for the conduction of heat. The equation can be written:

$$J = -D\frac{dc}{dx} \text{ (Fick's first law)}$$

where J = amount of substance passing through unit area in unit time
t = time after the beginning of diffusion
D = diffusion coefficient (cm^2/sec)
c = concentration
x = distance in the direction of diffusion.

The minus sign means that the solute diffuses in the direction of decreasing concentration.

This equation combined with $\frac{dJ}{dx} = -\frac{dc}{dt}$ yields at constant D:

$$\frac{dc}{dt} = D\frac{d^2c}{dx^2} \text{ (Fick's second law)}$$

These equations are derived under the condition that the gas-law is valid for the substance in question.

When Fick's second law is integrated with the assumption of the boundary-condition which corresponds to the experimental arrangement, the result is

$$c = \frac{c_0}{\sqrt{\pi}} \int_{-\infty}^{\beta} e^{-\beta^2} d\beta; \quad \beta = \frac{x}{2\sqrt{Dt}}$$

and

$$\frac{dc}{dx} = \frac{c_0}{\sqrt{\pi}} e^{-\beta^2} \frac{d\beta}{dx}$$

or

$$\frac{dc}{dx} = \frac{c_0}{2\sqrt{\pi Dt}} \exp(-x^2/4Dt) \qquad (1)$$

This equation conforms with the well known normal — or Gaussian distribution. Consequently several plots of the concentration gradient $\frac{dc}{dx}$ (or the refractive index gradient $\frac{dn}{dx}$) against x at different times will form a family of smooth Gaussian curves, provided, of course, that the diffusion is ideal. In our experiments the diffusion coefficient is calculated from equation (1) by the "area method" as

$$D_A = \frac{A^2}{4\pi t H^2} \qquad (2)$$

where A = area between the diffusion curve, $\frac{dc}{dx} = f(x)$, and the x axis; H = maximum height of the curve.

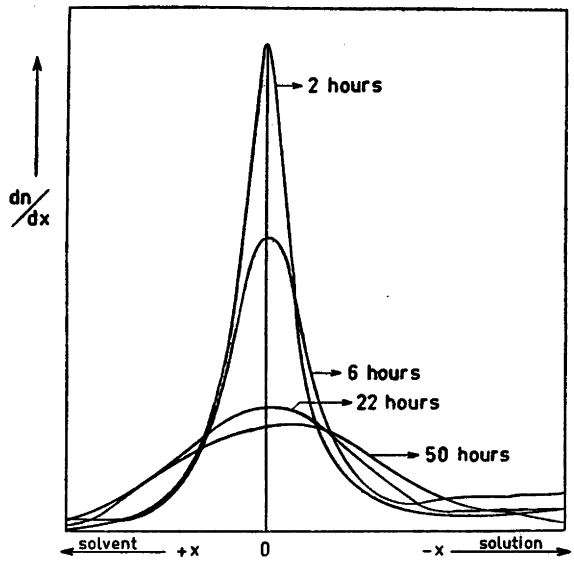


Fig. 1. Diffusion curves of potassium hyaluronate.

The experiment was performed in a Lamm-Polson [3] diffusion cell made from stainless steel, and the Lamm [4] scale method was used for the optical registration. The temperature in the thermostat was 20.0° C and the experiment was run for about 50 hours. The concentration was 0.1 %.

The diffusion curves obtained are seen in Fig. 1. The ordinate is given as $\frac{dn}{dx}$, where n is the refractive index of the solution. During the 50 hours of the experiment the area enclosed by the diffusion curve diminished by nearly 25 % which indicates a corresponding degradation of the preparation during the run, or the presence in the solution of a substance with low molecular weight that diffuses very quickly. The latter alternative is not very probable because the

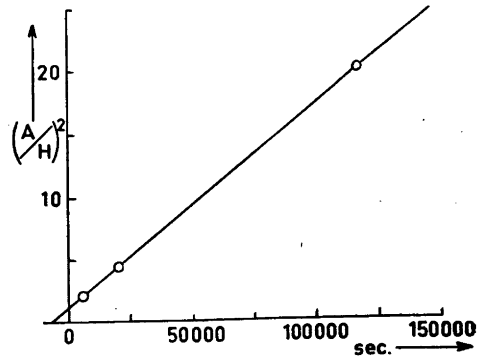


Fig. 2. The graph used for the computation of the diffusion coefficient.

solution was dialyzed for five days against the buffer (0.2 *N* NaCl, 0.025 *N* Na_2HPO_4, 0.025 *N* NaH_2PO_4, pH 6.6) at 5° C before the start of the experiment. In case a degradation has taken place only the segments unable to pass the dialyzing membrane will be left.

In Fig. 2 values of $\left(\frac{A}{H}\right)^2$ are plotted against t. From equation (2) it is seen that in the case of ideal diffusion a linear relationship must be expected. In our experiment a certain mixing of solute and solvent seems to have taken place at the start of the experiment, and therefore the graph does not pass through the zero point. However, when only the slope of the curve is used in the calculation of D this has no influence upon the result. The diffusion coefficient was found to be: $D = 1.0 \times 10^{-7}$ cm^2 sec^{-1}.

The skewness of the diffusion curves in Fig. 1 also indicates that the diffusion coefficient depends on the concentration, and therefore, the value given above should only be taken as an approximate value.

ULTRACENTRIFUGE EXPERIMENT

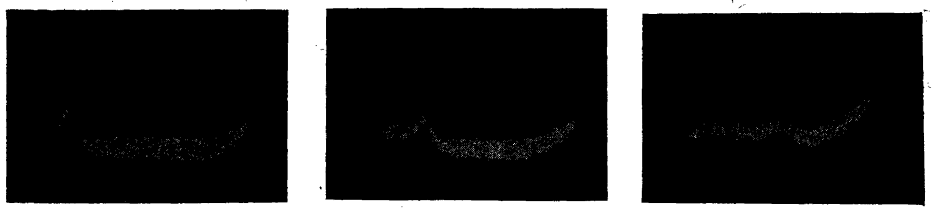
This experiment was performed on the same dialysed solution used for the diffusion experiment. The inner part of the centrifuge cell is made from "Tufnol" (lined bakelite resin), thereby avoiding contact with heavy metals. As shown by one of the present authors [5] ferrous and ferric ions cause a continuous decrease of the viscosity even at a concentration of about 2.5×10^{-5} *M*. The oil driven ultracentrifuge was run at 1 000 r.p.s. for about 2 hours, and the rotor temperature was 28° C. A "Schlieren" system was used for the optical recording of the experiment.

The sedimentation coefficient — the sedimentation rate per unit field of force — is defined by the expression

$$s = \frac{dx/dt}{\omega^2 x}$$

where ω is the angular velocity in radians per second, and x the distance from the axis of rotation.

The sedimentation coefficient was determined at certain intervals of time. Corrected to 20° C the average value for the sedimentation coefficient was found to be $s_{20} = 3.0$ *S*. The sedimentation coefficient is measured in Svedberg units, *S*, one Svedberg unit being 10^{-13} cm sec^{-1}. Fig. 3 gives the sedimentation diagrams 0.5 h, 1 h, and 1.5 h after the beginning of the experiment. These diagrammes show that the main peak was skew during the first hour of sedimentation and then broadened gradually. Possibly a slower moving component is also present. In another experiment an undialysed 0.3 % solution of the same preparation was investigated. In this case the peak behaved as a "Schliere" during all three hours of the experiment. After two hours the diagram was as seen in Fig. 4, and practically no component was visible after the main component. (The sedimentation coefficient was calculated to be $s_{20} = 2.3$ *S* in this case).



½ h 1 h 1½ h

Fig. 3. Sedimentation digrams of 0.1 % potassium hyaluronate. Sedimentation from left to right.



Fig. 4. Sedimentation diagram of 0.3 % potassium hyaluronate.

DETERMINATION OF THE PARTIAL SPECIFIC VOLUME

Besides a knowledge of the diffusion and sedimentation coefficients it is necessary to know the value of the partial specific volume, V, of the substance in order to calculate the molecular weight by Svedberg's formula: $M = \frac{RTs}{D(1-V\varrho)}$

The partial specific volume of potassium hyaluronate was determined from density measurements with two Sprengel-Ostwald pycnometers of about 9 and 5 ml capacity, respectively. The concentration of hyaluronate was about 50 mg dissolved in 10 ml of 0.2 *M* potassium chloride. All measurements were at 20.0° C (Variations 0.005° C). The mean value of three determinations was 0.86 (0.862, 0.859 and 0.860). This value is surprisingly high compared to the specific volume of other substances containing a carbohydrate skeleton where V normally is between 0.5 and 0.6 (*cf.* R. Jensen *et al.*[6]). However, we controlled our method of measurement and calculation by measuring V for a commercial sample of urea and found a value deviating only 2—3 % from the value for urea ($V = 0.738$) given by Drucker [7]. We have assumed that the partial specific volume is independent of the concentration in dilute solution, and therefore, strictly speaking, we have only determined the so-called apparent specific volume.

Insertion of the data given above into Svedberg's formula shows that the molecular weight of our sample of potassium hyaluronate is about 5.2×10^5. This value corresponds closely to that obtained recently by Christiansen and one of the present authors [8] from osmotic pressure measurements, but this

very close correspondence must be taken as accidental, since hyaluronic acid, as has been shown, is not ideal for diffusion and sedimentation analysis. Besides, the molecular weight given by the ultracentrifugation method is a weight-average ($\bar{M}_w = \frac{\Sigma N_i \cdot M_i^2}{\Sigma N_i \cdot M_i}$), while the molecular weight given by the osmotic method is a number-average ($\bar{M}_n = \frac{\Sigma N_i \cdot M_i}{\Sigma N_i}$) — and our hyaluronic acid must be considered a heterogenous, polydisperse substance.

CALCULATION OF MOLAR FRICTIONAL RATIO

The molar frictional ratio f/f_0 is defined as the ratio of the frictional coefficient of the molecule in question to that of a spherical, anhydrous molecule of the same molecular weight. When diffusion and sedimentation analyses are involved this ratio can, according to Svedberg [9], be expressed by

$$\frac{f}{f_0} = \left[\frac{(1-V\varrho)}{D^2 s V}\right]^{1/3} \times 10^{-8}$$

Inserting the experimental data found in this investigation, we find f/f_0 equal to 3.8. Assuming only negligible hydration this indicates threadlike molecules which also corresponds to other findings described in the literature on this substance. (Blix [10]. C. E. Jensen [11,12]).

We would like to express our sincere thanks to Professor J. A. Christiansen for his valuable help and stimulating interest in our problems. For assistance in the experimental work our thanks are due to Miss Emma-Louise Brygmann and Mr. Børge Rasmussen.

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Nucleation Kinetics

VI *. Steady State Treatment of Nucleation and Growth of Crystalline Precipitates

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The irreversible formation of slightly soluble polymers from dissolved monomers is considered. It is assumed that at a certain time t the concentrations of polymers containing n or less monomers are stationary, that the concentration of polymers containing n+1 monomers is increasing and that polymers containing more than n+1 monomers do not exist at the time t.

By means of these assumptions and formerly derived expressions for the steady state rate of nucleation and the steady state distribution of monomers on polymers of different size three equations connecting $1-a$ (the fraction of remaining monomers), $\dot{a}$ (the rate of increase of a), s (the rate of nucleation), a (the initial concentration of monomers) and n are derived.

To solve these equations a relation between w_i (the probability pro unit time that a polymer containing i monomers shall capture a further monomer) $1-a$ and i must be known. If it is known, an equation of the form $\psi(a) = t$ can be derived i.e. $\psi(a)$ is the chronometric integral (the chronomal) of the polymerisaton process. By an appropriate choice of the dependence of w_i on i and $1-a$, *viz.* $w_i = ka^2(1-a)^2\sqrt{i}$ the calculated chronomal for the precipitation of barium-sulphate can be made to coincide with that derived from recent (conductometric) experiments by Turnbull and by Johnson and O'Rourke. In agreement with earlier results by Nielsen and the author the recent experiments thus show that the nucleus in the precipitation of barium-sulphate contains 4 molecules.

The choice mentioned above is based only on the experimental results.

In a recent paper (V) it was shown that attainment of steady state (stationarity) concerning the critical cluster (X_4 in (V)) must probably be considered as practically instantaneous.

We shall now discuss the further evolution of the system considered in (V). Immediately after the concentration of critical clusters (in our example x_4)

* The roman numerals of the preceding papers in this series are indicated in references 1—5.

has become stationary the concentration of nuclei (x_5) is of course not stationary, because the reaction

$$X_1 + X_4 \rightarrow X_5$$

is steadily going on, at first without compensating loss of X_5 either by the reverse reaction

$$X_5 \rightarrow X_1 + X_4$$

or by the capture of a further monomer

$$X_1 + X_5 \rightarrow X_6$$

However, as time goes on x_5 increases and therefore the rates of the compensating reactions will increase. We are asking for the time required for the concentration of nuclei (X_5) to become stationary. We get as before (see V)

$$\begin{aligned} x_4w_4 - x_5w_{-4} &= s_4 \\ x_5w_5 &= s_5 \end{aligned}$$

Multiplication with φ_4 and φ_5, respectively, yields

$$x_4\varphi_4w_4 - x_5\varphi_4w_{-4} = u_4 \quad \text{(a)}$$
$$x_5\varphi_5w_5 = u_5 \quad \text{(b)}$$

Replacing $x_4\varphi_4w_4$ by U which is quasi-constant because x_4 has reached its quasistationary value and because φ_4w_4 varies very slowly with time and remembering furthermore the relation $\varphi_iw_{-i} = \varphi_{i+1}w_{i+1}$ we get, by addition of (a) and (b), $U = u_4 + u_5$. Omitting differentiation of slowly variable functions we get by differentiation with respect to time from the two equations

$$-\dot{x}_5\varphi_4w_{-4} = \dot{u}_4 \quad \text{(a)}$$
$$\dot{x}_5\varphi_5w_5 = \dot{u}_5 \quad \text{(b)}$$

Inserting the obvious relation $-\dot{x}_5 = s_5 - s_4$ in (a) we get

$$s_5\varphi_4w_{-4} - u_4w_{-4} = \dot{u}_4$$

or inserting

$$\varphi_4w_{-4} = \varphi_5w_5$$

$$u_5w_5 - u_4w_{-4} = \dot{u}_4$$

or

$$Uw_5 - u_4(w_5 + w_{-4}) = \dot{u}_4 \quad \text{(c)}$$

whose solution is

$$u_4 = A\ e^{-\lambda t} + Uw_5/\lambda\ ;\ \lambda = w_5 + w_{-4}$$

The constant A may be determined from the condition that at $t = 0$, $x_5 = 0$ and consequently $u_4 = U$. This boundary condition is however of somewhat doubtful validity as it is hard to believe that x_5 is strictly zero when x_4 has become stationary. But the exact value of A is not very important when we consider that the exponential in any case will disappear in a time of the order of magnitude $1/(w_5 + w_{-4})$. According to our assumptions this time is many times larger than the time in which the concentration of critical clusters (X_4) reaches its stationary value, but notwithstanding this it may be small enough

to make the rate s_4 and the concentration x_5 stationary practically instantaneously. In the steady state we get

$$u_4 = Uw_5/(w_5 + w_{-4})$$

or from

$$u_4 = s_4\varphi_4, \quad U = x_4\varphi_4 w_4$$
$$s_4 = x_4 w_4 w_5/(w_5 + w_{-4})$$

or

$$x_4/s_4 = 1/w_4 + w_{-4}/w_4 w_5$$

which is the usual steady state value of x_4/s.

We may now treat the next step in a similar way and so on, that is we are lead to the model that at a certain time the concentrations of polymers X_4, $X_5 \ldots X_n$ are stationary while that of X_{n+1} is not and those of higher polymers are zero (at that time).

At a later time the number n has increased but again we may assume that the concentrations of polymers x_i with $i \leqq n$ are stationary, while the concentration of x_{n+1} is increasing and higher polymers do not exist at that time.

It is therefore a justifiable program to base the calculation on the assumption of steady state.

THE STEADY STATE TREATMENT

By inspection of the expression arrived at for a reciprocal steady state rate it appears that integration of the rate equation always must lead to an equation of the form: $t = \psi(x)$ where t is the time and x the number of advancement of the overall reaction, that is the number of overall reactions which have taken place in unit volume in the interval from $t = 0$ to t. It is often convenient to replace x by α where α is the degree of advancement of the overall reaction, x and α being connected by the relation $x = a\alpha$ where a is the initial concentration of one of the reactants of the overall reaction.

An integral $\psi(x)$ which actually obeys the condition $t = \psi(x)$ may be called a chronometric integral of the reaction or shorter a chronomal. If somehow a chronometric integral of the reaction in question has been found a great advance in the elucidation of its mechanism has been made.

In the present case the overall reaction is

$$X_1 + nX_1 \rightarrow X_{n+1}$$

but it is complicated by the fact that n itself increases with time. However we shall see in the following that also in this case it is possible to arrive at an expression of the form $t = \psi(\alpha)$, where α means the fraction of monomers which have formed polymers at the time in question.

As will appear from papers by the author [6–10] (see especially [10] p. 328) we get by the steady state treatment not only information on the form of the rate expression, but also on the distribution of the intermediates. This distribution can be expressed by means of a matrix which may appropriately be called the distribution matrix. To avoid misunderstandings it must be mentioned that the only operations which we shall perform on this matrix are additions either row for row or column for column or both.

Schematically the matrix can be written as follows:

$$\begin{array}{c|ccccccc} x_1 & M_{11} & M_{12} & M_{13} & M_{14} & M_{15} & \dots & M_{1n} \\ x_2 & & M_{22} & M_{23} & M_{24} & M_{25} & \dots & M_{2n} \\ x_3 & & & M_{33} & M_{34} & M_{35} & \dots & M_{3n} \\ x_4 & & & & M_{44} & M_{45} & \dots & M_{4n} \\ x_5 & & & & & M_{55} & \dots & M_{5n} \\ \cdot & & & & & & & \cdot \\ \cdot & & & & & & & \cdot \\ \cdot & & & & & & & \cdot \\ x_n & & & & & & & M_{nn} \end{array}$$

Denoting the sum of each row by M_i this is intended to mean that the concentration of the polymer x_i is proportional to M_i, the proportionality factor being the rate s which is the steady state rate of nucleation. s is not a constant but it is common to the whole system at a given value of n, which is the number of monomers in the largest polymer whose concentration has become stationary at the time in question. It must be emphasized that it has been assumed that there is no backward reaction, that is the solution is strongly supersaturated with respect to polymer X_n. If that were not so the places to the left of the diagonal would not be empty. We can now calculate 1) the concentration x_1 of free monomers and 2) the concentration c_1 of bound monomers, that is the number of monomers bound in nuclei and their descendants at a certain time.

We get:

$$x_1 = sM_1$$

$$c_1 = s \sum_{\nu}^{n} i\ M_i$$

Furthermore we can calculate the total decrease in concentration by application of the following consideration:

Let the flow-sheet below illustrate the situation:

$$\textcircled{X_1} \rightarrow \textcircled{X_2} \rightarrow \textcircled{X_3} \rightarrow \cdots \rightarrow \textcircled{X_1} \rightarrow \cdots \rightarrow \textcircled{X_n}$$

On account of the stationarity the current s is the same everywhere. Therefore the current s_{12} is the same as the current $s_{n,n+1}$ or the number of dimers formed in unit time from $2X_1$ is the same as the number of polymers of degree $n + 1$ formed in unit time from $(n + 1)X_1$. Consequently the decrease in the concentration of monomers must equal $s(n + 1)$. Dividing the equations by a, the initial concentration of monomers, and replacing x_1/a by $1-\alpha$, c_1/a by α and s/a by A we thus get

$$1 - \alpha = M_1 \tag{1}$$

$$\alpha = A \sum_{\nu}^{n} M_i \tag{2}$$

$$\dot{\alpha} = A(n + 1) \tag{3}$$

Now conforming to our basic assumptions (compare V p. 909) we replace the sum M_1 by its largest term. If the nucleus contains 5 monomers the largest term in M_1 will be of the form $k_1k_2k_3/k_{-1}k_{-2}k_{-3}k_{-4}a^4\ (1-\alpha)^4$ that is $M_1 = M_{14}$* $= 1/B_1\ (1-\alpha)^4$ and generally, if the nucleus contains ν monomers the largest term in M_1 is $M_{1,\nu-1} = 1/B_1(1-\alpha)^{\nu-1}$. It may be added here that if i denotes the row and j the column inspection of the equations in [10] p. 328 shows that

$$M_{i+1,j} = M_{i,j}w_i/w_-$$

or in our case

$$M_{i+1,j} = M_{i,j}k_ia(1-\alpha)/k_-$$

It will be more important for the following to note that for consecutive columns in the same row

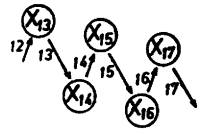
$$M_{i,j+1} = M_{i,j}w_{-j}/w_{j+1}$$

or in our case

$$M_{i,j+1} = M_{i,j}k_{-j}/k_{j+1}a(1-\alpha)$$

We shall now consider the sums M_i beginning with their diagonal terms. In accordance with the general form of the matrix-elements $M_{i,i}$ always equals $1/w_i = 1/k_ia(1-\alpha) = 1/B_i(1-\alpha)$. The next term in the same line becomes $M_{i,i+1} = w_{-i}/w_iw_{i+1}$. From our basic assumption that a nucleus or a crystallite has a much larger tendency to capture a further monomer than to loose one the ratio w_{-1}/w_{i+1} must be small as compared to 1 and the same is true of the following members. Therefore in the case of a non-electrolyte we have good reason to assume that the sum M_i can be represented with sufficient accuracy by its first term $1/B_i(1-\alpha)$. Consecutive diagonal terms are all of this form and may be expected to be of the same order of magnitude.

If the precipitate is formed from ions the situation is a little more complicated. Let us consider for example the case of a slightly soluble binary electrolyte. Let the diagram below represent a part of the flow sheet of the reaction:



The diagram is intended to show qualitatively the difference in energy between odd and even polymers arising from the electrostatic forces between the ions.

Let us compare at first the diagonal terms of M_{13} (read M thirteen) and M_{14} in the distribution matrix. They are $1/w_{13}$ and $1/w_{14}$. It is evident that w_{13} must be essentially greater than w_{14} because of the electrostatic forces, that is the probability for a say, negatively charged polymer to capture a positively charged monomer must be eseentially greater than the probability for a neutral polymer to capture a positively or negatively charged monomer. We therefore consider $1/w_{13}$ to be effectively zero as compared to $1/w_{14}$ and generally in the sum of diagonal terms we may simply omit the odd ones.

* read M one four

Next we compare qualitatively the significance of the second term in M_{13} and M_{14} respectively. We may write

$$M_{13} = (1 + w_{-13}/w_{14})/w_{13} + \ldots .$$

and

$$M_{14} = (1 + w_{-14}/w_{15})/w_{14} + \ldots .$$

The sum of the two second terms can be written as $(w_{-13}/w_{13} + w_{-14}/w_{15})/w_{14}$. Now the reactions (13) and (15) are of the same type, being reactions between a charged polymer and an oppositely charged monomer. Therefore w_{13} and w_{15} must be expected to be nearly equal. But w_{-13} must be essentially smaller than w_{-14} because the reaction (—13) represents the loss of a charged monomer from a neutral polymer while (—14) represents the loss of a charged monomer from a charged polymer. Therefore the second terms in M_{13} and M_{14} will differ greatly in magnitude and so that the term in M_{13} is essentially less than the term in M_{14}. Application of similar considerations to the higher terms in M_{13} and M_{14} will show that all the terms in M_{13} are essentially smaller than the corresponding terms in M_{14}. We may therefore ignore not only the diagonal terms, but the whole (horizontal) sum corresponding to odd values of i as compared to those corresponding to even values of i.

The second question is whether in $M_{14}w_{-14}/w_{15}$ is essentially greater or less than 1. It is obvious that the ratio w_{-14}/w_{15} is the ratio between the probabilities of two events, loss of a charged monomer from a charged polymer (X_{15}) and capture of a charged monomer by the same polymer (X_{15}), respectively. Without rather problematic assumptions it is, however, not possible to prophecy whether the ratio is greater or less than 1. Experiments on precipitation of barium sulphate, however, seem to indicate that the first alternative is the correct one. We may, however, with some confidence prophecy that the third term in M_{14}, $w_{-14}w_{-15}/w_{14}w_{15}w_{16}$ must be small as compared to the second. The ratio between the third and the second member is w_{-15}/w_{16} which means the ratio between the respective probabilities for loss and capture of charged monomers from the neutral polymer (X_{16}), which is probably small as compared to 1.

From these considerations it appears, therefore, that for a binary salt we may assume

$$M_i = 1/B_i(1—\alpha)^2$$

In the general case, *e.g.* for a ternary salt we are similarly lead to assume that

$$M_i = 1/B_i(1—\alpha)^\gamma$$

where γ is intergral and greater than zero. In analogy with the case discussed above we shall expect that i for lines whose M_i is not very small is an integral multiple of γ.

Denoting $(1—\alpha)^\nu B_1$ by P we thus get from (1), (2) and (3) the three equations

$$P = A \tag{I}$$

$$(1—\alpha)^\gamma \alpha = A \sum_{\nu}^{n} i/B_i \equiv AS_n \tag{II}$$

$$\dot{\alpha} = A(n + 1) \tag{III}$$

It will be remembered that B_1 contains the initial concentration of monomers a in the power ν and that similarly the B_i's contain a in the power γ. It will be seen that by the procedure indicated by the equations (1)—(2) and (I)—(II) we have included $x_1, x_2 \ldots x_{\nu-1}$ in the sum which corresponds to 1—α. This is permissible because the sums $M_2 \ldots M_{\nu-1}$ according to our assumptions concerning the nucleation are all very small as compared to M_1. It is also necessary to do so because when we measure, *e.g.* the conductivity of the solution at time zero we cannot avoid to include the small decrease in the concentration of monomers arising from instantaneous nucleation.

It is evident that a knowledge of the dependence of B_i on i is decisive for the solution of the problem. Let us assume that $B_i = i^\sigma \varkappa a^\gamma$, where σ is a fraction. We then get

$$S_n = (\int_\nu^n i^{1-\sigma} di)/\varkappa a^\gamma = (n^{2-\sigma} - \nu^{2-\sigma})/(2-\sigma)\, \varkappa a^\gamma$$

We may confidently assume that the last term in S_n is disappearingly small as compared to the first. Therefore from equations (II) and (I)

$$\varkappa a^\gamma (1-\alpha)^\gamma a \equiv Q \cdot a = P n^{2-\sigma}/(2-\sigma)$$

or

$$Q^{1/(2-\sigma)}\, \alpha^{1/(2-\sigma)} = P^{1/(2-\sigma)}\, n(1/(2-\sigma))^{1/(2-\sigma)}$$

Using (III) or rather $\dot{\alpha} = P n$ and replacing $1/(2-\sigma)$ by q we get

$$Q^q P^{1-q} \alpha^q = \dot{\alpha} q^q$$

that is

$$\frac{\dot{\alpha}}{\alpha^q Q^q P^{(1-q)}} = q^{-q}$$

As now $Q = \varkappa a^\gamma\ (1-\alpha)^\gamma$, $P = \beta a^\nu\ (1-\alpha)^\nu$ where $\varkappa$ and β are constants, integration yields

$$\int_0^a \alpha^{-q}(1-\alpha)^{-p} d\alpha = \varkappa^q \beta^{(1-q)}\, a^p q^{-q}\, t$$

where $$q = 1/(2-\sigma) \text{ and } p = \gamma q + \nu(1-q)$$

If q and p are known we have thus found the chronometric integral of the polymerisation process.

The weak (or the weakest) point in the derivation seems to be the assumption that the largest term in M_i can be represented by $1/B_i(1-\alpha)^\gamma$ where $B_i = \varkappa a^\gamma i^\sigma$. Actually the author was lead to the assumption of proportionality between B_i and i^σ by a numerical treatment by A. E. Nielsen of Turnbull's very accurate conductometric investigation of the precipitation of barium sulphate. Nielsen found by trials that the degree of conversion α according to Turnbull's result could be accurately represented by the expression

$$\int_0^\gamma \alpha^{-2/3}(1-\alpha)^{-4} d\alpha = kt$$

that is q = 2/3, p = 4. That q = 2/3 follows with some certainty from Turnbull's finding, that α when small is proportional to the cube of t. Assuming $1-\alpha \simeq 1$ integration yields $\alpha^{1/3} = 1/3\ kt$. That p = 4 came out by trials with other integral values of p. It must be added that Johnsen and O'Rourke [12] on the basis of their own experiments on the same reaction have arrived at exactly the same expression for its chronometric integral. From q = 2/3 follows $\sigma = 1/2$, and from $p = 4 = \gamma\, 2/3 + \nu/3$ it follows that $\gamma = 2$ and $\nu = 8$. For integral values of γ and ν no other values are possible. Attention may be drawn to the fact that Nielsen and the author [2] arrived at the conclusion that ν for barium sulphate was equal to 8. Obviously the expression $B_i = \varkappa a^2 \sqrt{i}$ for a binary electrolyte requires further discussion as it is by no means evident that the expression should be of this form. In the present situation the correctness of the form of the chronomal has therefore only been proved experimentally and that only in one case. The problem is being further attacked in this laboratory from the experimental and from the theoretical side. The outstanding theoretical problem seems at present to be a derivation of the dependency of B_i on i from a definite physical picture.

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The Separation of Small Amounts of Inorganic Cations by Chromatographic Methods. I. Introductory Experiments with Inactive Cations

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Separations of divalent inorganic cations are studied on ion-exchange columns, employing aqueous solutions and partition-elution by means of organic solvents. A mixture of *e. g.* Ba, Ni, Mn, Co, Cu, Zn and Bi is completely separated and collected into small fractions of elutriant.

A new three-phase ion-exchange theory is introduced in order to explain the behaviour during these elutions. It is in agreement with the experimental results and also with earlier published data.

Paper strip separations are studied in *n*-butanol- and different ketone-mixtures with acids. Complete and reproducible separations of a wide combination of ions are obtained with methyl-propyl ketone + HCl in less than 1 hour.

I. INTRODUCTION

In order to find fast methods for a complete separation of chemical elements (or different inorganic ions), produced during bombardments in a cyclotron, chromatographic methods were tested at our laboratory. Compared to old conventional separation methods they have the advantage of being clean, less laborious and most often leading to a complete resolution of a mixture of ions. The disadvantage is primarily that the methods are rather slow, more than 10 hours being needed to effect a separation. If the methods should obtain importance in the study of radioactive elements, this time had to be decreased to at least 3—4 hours. It is otherwise impossible to assay and determine the extremely small fission- and spallation-yields of isotopes with half-lives below or around one hour.

As the purpose was, first to study the spallation products of copper [1], the principal interest was directed to the study of Zn, Cu, Ni, Co, Mn and Fe.

Three different chromatographic methods enable good separations of inorganic ions:

1. paper chromatography strips,
2. chromatography on cellulose columns or some other adsorbent as *e.g.* Al,
3. ion-exchange columns.

The two latter methods enable the separation of milligram to gram quantities, paper chromatography on the contrary not more than 1 mg of each cation and usually considerably less. Paper chromatography hence necessitates an individual and almost complete separation of the target material before the chromatographic separation starts.

Cellulose columns enable excellent separations of Cu, Co, Ni and Fe[2], provided the flow rate is very small (2—3 drops a minute), and are therefore less convenient for spallation studies.

II. ION-EXCHANGE COLUMNS

A. Experimental variables

Numerous applications of ion-exchange methods to the separation of inorganic ions have been published (see Ref.[3] p. 179 and Ref.[4], p. 221), especially with the rare earth and transuranium elements, and certain rare metals as Zr, Hf, Nb, and Ta. On the contrary very little has been reported about the separation of divalent cations. Co, Ni, Fe, and Ga have been separated on an anion-exchanger with high molarity HCl-solutions [5-6], Mn and Cu partly with 1 *N* H_2SO_4[7], Cd and Zn with 0.5 *N* HCl[8] and Na, K, Mg, Ca, Li, Cd, Hg, and Be also partly with 0.7 *N* HCl.[9] An investigation [10] and an attempt [11] to separate the NH_3-complexes of Ni, Cu, Zn, and Ag has been made, but very poor separations were achieved.

Experimental variables, concerned with the separation of ion mixtures, are well established [3-4], and the most important factors to obtain optimum separations are:

1. low flow rate,
2. high temperature,
3. small particle size of the exchanger,
4. increasing length of the resin column,
5. high capacity exchanger and
6. low adsorbed ion conc.

The attainment of equilibrium is favoured by these six factors. When fast separations are wanted, as in the case of radioactive, shortlived isotopes, high temperature (70—80° C), small particle size, short columns, high capacity exchanger, low adsorbed ion conc. are needed, and the flow rate is then regulated so as to obtain the best separation.

B. Theory

Symbols:

[ ]	activity
C	concentration in mole/liter
γ	activity coefficient
g	gram oven dried (110°) ion exchanger in H^+-form
$M_{Me^{+2}}$	number of moles of the ion Me^{+2}
M_1	molecular weight of the resin (Dowex 50) in oven dried H^+-form
v	volume liquid in the column in ml

A_1 area of cross section of the mobile phase
A_s » » » » » » nonmobile phase (in g/cm)
V eluted volume of solution in ml, when the max.conc. (band) reaches the outlet of the column
p number of theoretical plates of the column.

An ion-exchange reaction of divalent cations with the resin phase in H^+-form can be written:

$$Me^{+2} + 2\ HR \rightleftharpoons MeR + 2\ H^+ \quad (1)$$

$$\frac{(MeR) \cdot (H^+)^2}{(Me^{+2}) \cdot (HR)^2} = K_0 \quad (2)$$

where K_0 is the true exchange constant.

The distribution constant K_d for the reaction, if no complex formation occurs in solution, is defined as

K_d = (conc. Me in resin phase) : (conc. Me^{+2} in liquid phase) in moles · g^{-1} : moles · ml^{-1}

$$\therefore\ K_d = \frac{(M_{Me})_R}{M_{Me^{+2}}} \cdot \frac{v}{g} = \frac{M_{MeR} \cdot 1\,000}{g \cdot C_{Me^{+2}}} \quad (3)$$

A new equilibrium constant may be defined as

$$K_j = \frac{K_0 \cdot \gamma_{Me^{+2}} \cdot \gamma^2_{HR}}{\gamma_{MeR} \cdot \gamma^2_{H^+}} = \frac{M_{MeR}}{C_{Me^{+2}}} \cdot \frac{C^2_{H^+} \cdot (M_{MeR} + M_{HR})}{M^2_{HR}} \quad (4)$$

if concentration is expressed in mole fraction in the resin phase and in mole per liter in the liquid.

When Me is present in very low conc. (in the following experiments at most 0.5 % M_{MeR} of the whole resin phase) $M_{HR} \simeq M_{MeR} + M_{HR}$ and

$$K_j = M_1/1\,000 \cdot K_d \cdot C^2_{H^+} \quad (5)$$

The plate theory [12–13] is probably the most convenient method of calculating K_d from experimental column separations. Consden [14] has defined

$$R_F = \frac{\text{movement of band}}{\text{movement of advancing front of liquid}}$$ and shown that

$$R_F = \frac{A_1}{A_1 + K_d \cdot A_s} \quad (5)$$

$$\therefore\ K_d = \frac{A_1}{A_s} \cdot \left(\frac{1}{R_F} - 1\right) \quad (6)$$

As $\frac{A_1}{A_s} = v/g$ it is simply shown that

$$K_d = \frac{V - v}{g} \quad (7)$$

Further on, it has been shown that

$$p = \frac{2V \cdot (V - v)}{W^2} \quad (8)$$

where W is the half width of the elution curve in ml at an ordinate value of 1/e of the maximum.

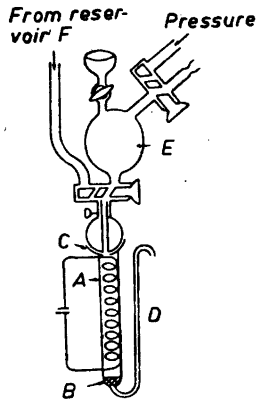

Fig. 1. Experimental column: A resin bed; B glass wool support; C joint, ball-ground; D capillary tube; E 75 ml reservoir.

C. Experimental

Apparatus: The essential apparatus, as shown in Fig. 1, is made of Pyrex glass. A helical resistance thread is wound on the outside of the columns *A* and isolated by asbestos fibre + water-glass. The winding of the thread is adapted to give a constant temperature from 20—90° throughout the whole column-length by varying the applied voltage. The columns have a fixed diameter, 0.8 cm, but different lengths and are easily exchangeable. They are filled with resin (Dowex 50) to the top.

Procedure: The columns are packed with a water-slurry of the resin and then exhaustively washed alternately with 4 *M* HCl and water, and finally with water to remove all the excess of hydrochloric acid. The divalent cation mixture is adsorbed on the top of the column from reservoir *E* (Fig. 1) in a feebly acid solution, the column washed with water to remove the anions and the required temperature obtained by applying a certain calibrated voltage to the resistance thread. The elution is simply performed by passing the desired solvent through the column with a constant flow rate either from the reservoir *E* under air pressure or from *F* under hydrostatic pressure; the effluent collected in small fractions by an automatic sample-changer and analysed for the different cations. All solutions, used in the experiments, are carefully freed from dissolved air bubbles.

Table 1.

Run	Temp. °C	Cu^{+2} added mg	Cu^{+2} found %	Flow-rate ml/min	HCl molarity	p	K_d	K_j
1	20	9.24	99.1	0.9 —0.92	0.65	9.8	46.3	3.51
2	20	9.24	100.2	0.7 —0.8	0.70	6.0	34.7	3.07
3	20	8.52		0.65—0.7	0.70		35.9	3.16
4	74	6.16	98.3	0.61—0.66	0.68	18.2	47.9	4.13
5	74	6.20	98.7	0.61—0.71	0.68	17.5	49.0	4.22
6	88	8.52	99.8	0.54—0.62	0.49		91.0	3.94
7	88	9.00	99.2	0.65—0.83	0.58	11.1	65.5	3.88
8	88	8.52		0.64—0.80	0.70		43.1	3.80
9	88	9.09		0.8 —0.9	0.73		41.3	3.96
10	88	9.09		0.51—0.57	0.73		39.2	3.75
11	88	9.21	99.8	0.45—0.63	0.73	21.7	39.7	3.79
12	88	6.24	99.7	0.63—0.80	0.80		28.6	3.30
13	88	9.00	99.7	0.69—0.71	0.86	11.9	21.0	2.79
14	88	8.52		0.54—0.6	0.92		15.8	2.40

D. Experimental results

a) *Studies of the column efficiency with* Cu^{+2}. In order to check the column properties when different experimental variables are changed, elutions were made by HCl, using a column of 12.5 cm length, filled with 3.72 g Dowex 50 (10—20 mesh). Results are collected in Table 1.

From the data in Tables 1 and 2 and the curves in Figs. 2—4 it is apparent that

1) the ion-exchange elution of Cu with HCl at different concentrations is quantitative,
2) it is advantageous to use more elevated temperatures,
3) the influence of small variations of acid conc. on the elution curve is very marked,
4) the equilibrium constant K_j is fairly constant within a large HCl-conc. range (Fig. 4). In eq. (4) it was shown that

$$K_j = K_0 \cdot \frac{\gamma_{Cu^{+2}} \cdot \gamma_{HR}^2}{\gamma_{CuR} \cdot \gamma_{H^+}^2}$$

where K_0 is the true and invariable equilibrium constant. According to Kressman [15] even the ions in the resin phase are almost completely surrounded by an ionic atmosphere, similar to that surrounding the corresponding cations in the aqueous phase, and hence their activity coefficient may be expected to vary in the same manner. The constancy of K_j is in accordance with this view, the steep decrease of K_j for HCl-conc. > 0.75 *M* being attributed to the onset of Cu-complex formation in the solution phase.

b) *The separation of Cu, Co, and Ni with 0.7 M HCl.* The best separation of these three cations is obtained with 0.7 *M* HCl. A column, dimensioned 29 × 0.8 cm, and filled with 8.70 g Dowex 50 (10—20 mesh), was used; and 3 mg each of the cations were adsorbed.

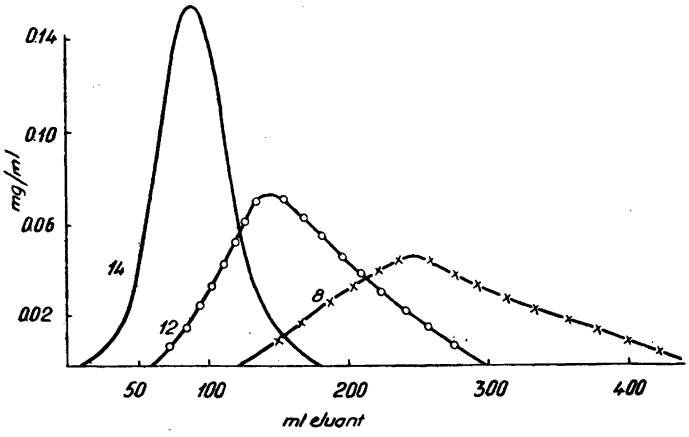

Fig. 2. Elution curve for Cu^{+2}*; column dimensions 12.5 × 0.8 cm; temperature 88°; eluant HCl in different conc. (— sample 14, ○ sample 12, and × sample 8 in Table 1).*

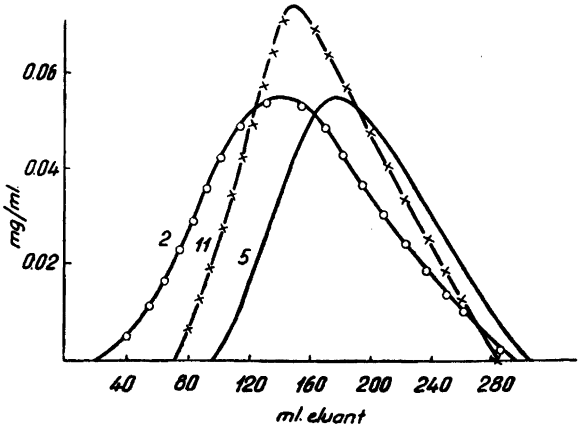

Fig. 3. Elution curves for Cu^{+2} at different temperatures; column dimensions 12.5 × 0.8 cm; eluant ~0.7 M HCl. (— sample 5, ○ sample 2 and × sample 11 in Table 1.)

The separation factor α_{AB} between two ions A and B is defined as

$$\alpha_{AB} = K_{d_B}/K_{d_A} \qquad (9)$$

Practical experiences have shown that it is only possible to effect a separation when $\alpha_{AB} > 1.20$. Results are shown in Table 2.

Table 2.

Run	Temp. °C	Flow-rate ml/min	Cu^{+2}				Ni^{+2}			Co^{+2}		
			fraction ml	K_d	K_j	α_{CuNi}	fraction ml	K_d	K_j	fraction ml	K_d	K_j
1	86	0.40—0.42	253—560	44.3	3.91	1.47	437—645	65.2	5.76	460—670	66.1	5.82
2	20	0.37—0.42	200—410	34.5	3.05	1.10	222—465	37.9	3.35	235—	38.0	3.36
3	86	0.19—0.22	275—532	42.7	3.76	1.56	436—722	66.7	5.88	460—740	69.8	6.18
4	86	0.10—0.15	340—484	43.2	3.82	1.56	486—710	67.2	5.92	552—740	70.2	6.20

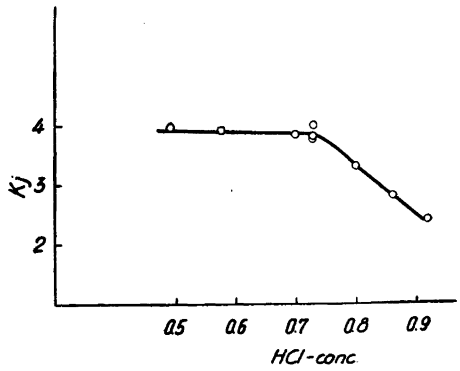

Fig. 4. Variation of Kj for Cu^{+2} with HCl-conc.; temp. 88°.

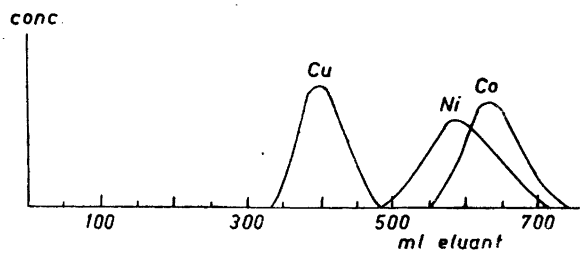

Fig. 5. Elution curves in attempt 4, Table 2. Eluant 0.7 M HCl; temp. 86°; flow rate 0.12 ml/min; column dim. 29 × 0.8 cm with Dowex 50 (10—20 mesh).

Table 2 and Fig. 5 together with a comparison to the data in Table 1 show that

1) Cu is separated from Ni and Co at 86°, though not the last two, (α_{NiCo} = 1.04 in run 4),

2) K_d and K_j are independent of column length as well as of flow rate, provided that equilibrium is attained. With longer columns and slower flow rates p increases, attaining a value of 145 for Cu in run 4, and hence facilitates the separations, as p principally determines the spreading of the elution peaks.

The procedure, tested above, is very slow. In run 4, the elution of Cu alone took more than 60 hours.

c) *Partition elution with methyl-n-propyl ketone.* To effect faster separations than in the preceding section it is necessary to increase the K_d-differences of the ions, and at the same time to maintain the high solubility in the solution. A mixture of methyl-*n*-propyl ketone and high molarity HCl was found to have these properties. Single phase mixtures are formed with HCl > 7.5 *M*, the mixture otherwise rapidly separating into two fairly pure phases. Hence the cations can rapidly be adsorbed on the top of the column from aqueous solution, and the water is directly displaced by the lighter ketone-acid mixture at the same time as the elution starts. The apparatus is constructed (Fig. 1) so as to obtain the smallest possible contact area between the ketone- and aqueous-phase in order to only slightly change the acid conc. in the ketone.

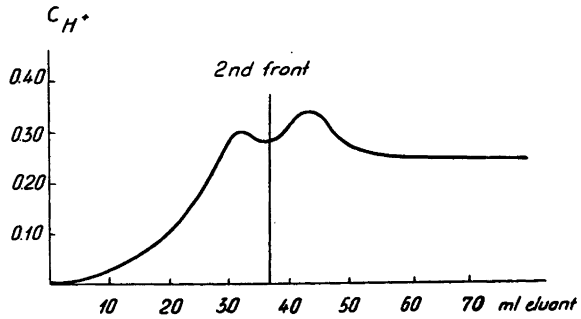

Fig. 6. C_{H^+} gradient curve; column dimensions 12.6 × 0.9 cm; 3.59 g Dowex 50 100—200 mesh; eluant methyl-iso-propylketone + 2 % conc. HCl; flow rate 0.2—0.25 ml/min.

A preliminary investigation (Fig. 6; the elution curve starting, when the first drops of ketone are passing out of the column) has revealed that a pH-gradient is developed, the form of which is about the same as in ordinary frontal analysis but with a distinct minimum at the "acid front"; that is to say where a homogeneous effluent is attained and in ordinary paper chromatography a second front developed. The "acid front" is preceded by a ketone-water emulsion with gradually rising H^+-conc. Calculations from experimental data show that a stationary H_2O-phase is held by the resin, and is only very slowly displaced by the solvent. The explanation must be that the ketone molecules, partly polymerised in the acid solution, can only gradually and with greatest difficulty penetrate into the resin phase, and hence a distribution of the HCl-molecules is obtained between a stationary H_2O-phase and a moving ketone phase. The pH in the inner solution of the resin falls, until the same H^+-normality is obtained as in the outer solution. At the same time, in the ketone phase the H^+-conc. is gradually diminishing during the passage through the column, a two-phase emulsion being formed as soon as the HCl-normality < 7.5. Hence during the elution an H^+-band is moving down the column, preceded by a ketone-water emulsion with gradually rising H^+-conc.

The regeneration of the column is easily performed by alternately passing 8 *M* HCl and H_2O. The elution procedure is the same as that already discussed in Section C. An accurate quantitative analysis was not performed. The solutions were prepared some days before the experiments, gas-bubbles otherwise being evolved in the column.

Experimental conditions: 3.52 g Dowex 50 (100—200 mesh) in H^+-form; column dimensions 12.2 × 0.9 cm; temperature 20°; 0.5 mg each of examined cations; eluant methyl-*n*-propyl ketone + varying % and molarity HCl.

Table 3 indicates calculated K_d and R_F and Figs. 7 and 8 the elution curves for two experiments.

Table 3.

Run	Flow-rate	Acid conc. *M*	Cu^{+2}		Co^{+2}		Mn^{+2}		Zn^{+2}		Cd^{+2}		Bi^{+3}	
			K_d	R_F	K_d	R_F	K_d	R_F	K_d	R_F	K_d	R_F	K_d	R_F
1	0.11—0.14	0.16	15.4	0.065	23.5	0.043			12.7	0.078				
2	0.20—0.30	0.16	15.2	0.070	24.0	0.044			12.2	0.088			7.05	0.150
3	0.09—0.12	0.18	13.4	0.080	19.8	0.054					7.70	0.126		
4	0.08—0.10	0.20	11.8				38.7							
5	0.12—0.20	0.20	11.4	0.086	18.8	0.057			8.3	0.114	7.75	0.12	5.75	0.185
6	0.12—0.14	0.24	9.6		15.7				6.2		5.6		4.20	
7	0.11—0.16	0.30	6.9	0.133	10.6	0.092	20.2	0.05	3.76	0.22				

As is shown in Figs. 7 and 8, a large mixture of divalent cations can be separated, if the eluant is successively changed and the flow rate low. In Fig. 7 the separations were complete without any contamination in the different fractions. In Fig. 8 almost complete separations were obtained except with Cd and Zn, which were impossible to separate with the eluant employed, and Cu, with 95 % collected in its own fraction but with traces in the Mn-fraction after changing the HCl-conc.

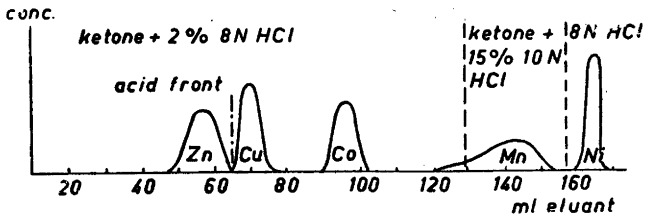

Fig. 7. Elution curves for 0.5 mg Zn, Cu, Co, Mn and Ni (as chlorides); flow rate ~ 0.12 ml/min.

Serious complications appear, when Fe-ions are present. In spite of their large solubility in the ketone they cannot be eluted in a single fraction. Fe is partly eluted earlier than Bi, but much Fe-contamination appears during the whole elution. Further, Fe renders the complete separation of the other cations, investigated, more difficult; Cu-ions especially become considerably retained. The same difficulties were observed, eluting ion-exchange columns with dilute acids as well as paper strips with ketone-acid mixtures; in the latter case, however, only with higher Fe-conc. (in paper strip scale).

Experiments have shown K_d to be independent of Cl^--conc. With the assumption of true ion-exchange in the resin phase and a distribution as Me^{+2} between ketone and aqueous solution with almost all Me^{+2} in the ketone phase, eq. (10) is obtained. K_{part} is defined as in eq. (14).

$$K_0 = M_1/1\,000 \cdot K_d \cdot K_{part} \cdot C_{H^+}^2 \cdot \frac{\gamma_{MeR} \cdot \gamma_{H^+}^2}{\gamma_{HR}^2 \cdot \gamma_{MeKet}} \qquad (10)$$

The mechanism, deduced experimentally, seems to be in agreement with this equation, but in spite of this the results in Table 3 do not satisfy the above equation, the hydrochloric acid dependence being less. Calculations, based on assumptions of complex anion formation, distribution as anions *etc.* give even worse agreements. Either the variations of the total activity coefficient product must be large and partly compensate the $C_{H^+}^2$-variations, or the assumption about ion-exchange in a solid resin phase is in error. As the experimental

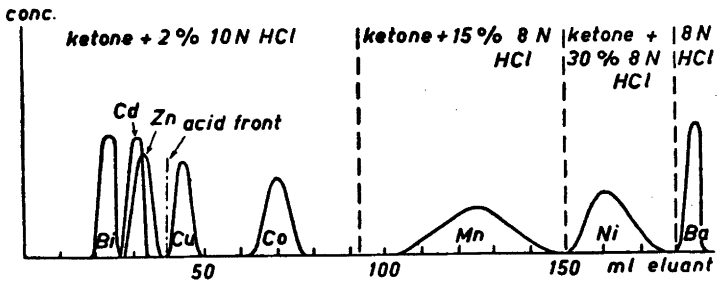

Fig. 8. Elution curves for 0.5 mg Bi, Cd, Zn, Cu, Co, Mn, Ni and Ba (as chlorides); flow rate ~ 0.16 ml/min.

data are in complete agreement with cellulose chromatography with the same solvents, and the rapid elution of Bi^{+3} is in direct contradiction to ordinary ion-exchange results, the latter seems to be the case.

A new theory. In explaining these discrepancies as well as the other observations a new ion-exchange theory has been elaborated, which is in agreement with most of the vast published data, as well as these new partition elution experiments. The whole ion-exchange process is regarded as a three-component mechanism with a solid phase, an inner solution, firmly maintained by and inseparable, even by centrifuge, from the resin, and an outer solution. The exchange with a divalent metal ion becomes, conc. in inner solution indexed i and other symbols as before.

$$Me_i^{+2} + 2HR \rightleftharpoons 2\ H_i^+ + MeR$$

$$Me^{+2} + 2\ H_i^+ \rightleftharpoons Me_i^{+2} + 2\ H^+$$

$$\frac{[H^+]_i^2 \cdot [MeR]}{[Me^{+2}]_i \cdot [HR]^2} = K_0' \tag{11}$$

$$\frac{[Me^{+2}]_i \cdot [H^+]^2}{[Me^{+2}] \cdot [H^+]_i^2} = K_0'' \tag{12}$$

K_0' and K_0'' are true equilibrium constants and $K_0' \cdot K_0'' = K_0$, the constant defined in eq. (2). A new definition of the distribution constant K_d is now introduced, as it is convenient to have all conc. per ml and not as before in g in the resin phase. If the resin volume is v_R ml

$$K_d' = \frac{(C_{Me})_R + (C_{Me^{+2}})_i}{C_{Me^{+2}}} = \frac{M_{MeR} \cdot 1\,000/v_R + (C_{Me^{+2}})_i}{C_{Me^{+2}}} \tag{13}$$

As long as $(C_{Me^{+2}})_i$ is fairly small, compared to the fraction in the resin, which is always so in aqueous solutions, a fast distribution between inner and outer solutions is obtained, and the equations, concerning only two phases, are approximately valid. In highly acid organic solvent mixtures, however, where only the acid molecules + water can rapidly penetrate into the inner solution, the acid conc. of the inner solution will become very high, the equilibrium in eq. (11) displaced so that all Me is in the inner solution, hence eq. (12) will become completely rate determining, and eq. (11) may be neglected. As the reaction is independent of Cl^--conc., the distribution in the outer solution is written

$$(Me^{+2})_{H_2O} \rightleftharpoons (Me)_{Ket} \text{ and } \frac{[Me]_{Ket}}{[Me^{+2}]} = K_{part} \tag{14}$$

where K_{part} is not a constant but a function of pH in the outer solution.

$$K_d' = \frac{(C_{Me^{+2}})_i}{C_{Me^{+2}} + (C_{Me})_{Ket}} \tag{15}$$

$$\therefore\ K_d' = \frac{K_0'' \cdot [H^+]_i^2}{[H^+]^2 \cdot (\gamma_{Me^{+2}})_i} \cdot \frac{1}{(K_{part}/\gamma_{MeKet}) + 1/\gamma_{Me^{+2}}} \tag{16}$$

With the new definition of K'_d, eq. (7) is transformed to

$$K'_d = \frac{V-v}{v_i} \tag{17}$$

where v_i is the volume of the inner solution, and depends on resin swelling and hence of $[H^+]_i$.

The distribution and the phases in eq. (15) are exactly the same as in paper strip separations, and this explains the great resemblance of the two methods. Using only 2 % HCl in the ketone, $C_{Me^{+2}}$ may be omitted in eq. (15) and

$$\therefore\ K'_d \simeq \frac{K''_0 \cdot [H^+]_i^2}{[H^+]^2} \cdot \frac{\gamma_{MeKet}}{K_{part} \cdot (\gamma_{Me^{+2}})_i} \tag{18}$$

When elution begins, the H^+-conc. is very low in the inner solution, the distribution of H^+ between inner and outer solution rapid and hence $[H^+]_i^2/[H^+]^2 \simeq 1$ for a given Me during the entire elution. Hence the second factor in eq. (18) determines the variations of K'_d with acidc onc. in the elutriant and predominantly that of K_{part}, as most probably the variations of γ are small, when the initial Me-conc. is as small as in the experiments, carried out.

It has not yet been possible to verify the deduced eq. (18), approximated as suggested above, in the experiments of Table 3, as the H^+-dependence of K_{part} is unknown, as well as the exact values of v_i. The latter was, however, found to decrease slightly with increasing C_{H^+} in the eluant, and hence in Table 3 the variations of K'_d are somewhat smaller than those of K_d as

$$K'_d = K_d \cdot g/v_i \tag{19}$$

The data of Table 3 indicate that

$$K'_d \cdot C_{H^+} = \text{constant} \tag{20}$$

which is in accordance with the view in Fig. 7 of a pH-gradient moving through the column and the different cations always travelling with the same pH.

$\therefore\ K_{part} \simeq C_{H^+} \times$ constant.

III. PAPER STRIPS SEPARATIONS

A. Experimental variables

Paper strip separations have proved to be very valuable in inorganic analysis and have been thoroughly studied at different laboratories, above all by a research team at Teddington, Pollard *et al.* in Bristol, Lacourt *et al.* in Bruxelles and Lederer in Australia (see Refs. [16,17]). Complete separations have been attained in groups of 3—5 cations and anions.

It is almost impossible to verify by mathematical calculations and previous experiments that a certain separation can be effected. Experimental testing is always necessary, since many variables influence the separation mechanism, and very small variations can lead to very great changes in separation efficiency. The most important variables are:

1) form and dimensions of the chromatographic chamber and elution mode,
2) atmosphere in the chamber,
3) elutriant, employed,
4) form, dimensions, sort and pretreatment of paper, employed,
5) concentration and acidity of initial cation mixtures,
6) place of and treatment at the point of application to the paper,
7) equilibrium during, and length of elution, and
8) temperature.

If all the variables above are held constant, reproducible results may be obtained, very careful and slow experiments, however, being necessary. The present investigation was, however, carried out in order to find rapid methods, and hence the chief object was to find optimum conditions of the above factors to enable fast and complete resolution of mixtures of ions.

B. Experimental

Apparatus. All experiments were carried out by the descending technique. The chromatograph chamber is made of glass, as strong acids are used during elution, and is closed by a glass cover, absolutely tight fitting.

Paper strips. Munktell OB filter papers are employed. The strips are cut 45 × 3.5 cm.

Technique. A small spot of the investigated cation solutions (0.005—0.010 mg each) is applied by a pipetting device to the middle of the paper, 7.5 cm from the upper edge, and dried in an air-stream. Four paper strips are placed in the chamber and partly or entirely equilibrated with the "atmosphere" from a solvent on the bed of the chamber; the desired eluting mixture is allowed to diffuse down a fixed length of the strips and finally evaporated from the strips in a hood, usually over night. A suitable reagent, sprayed by an atomizer, developes the positions of the cations.

Stock solutions. Chloride or nitrate solutions (anions in K-, Na-, or H-form) were prepared with 5 mg metal per ml and with varying acidity.

Preparation of solvents. Only pure solvents were used, all organic ones dried, redistilled and a fraction near the boiling point used. All two-phase solvent mixtures were thoroughly shaken and the two layers separated. The water layer was used as bottom solvent in the chamber.

Analytical reagents. Most sensitive spot test reagents, especially when spraying HCl-eluted strips, were found to be:

Rubeanic acid (saturated in ethanol) + NH_3: Cu^{+2}, Co^{+2}, and Ni^{+2},
Diphenylcarbazide (1 % in ethanol) + NH_3: Zn^{+2}, Tl^{+3}, Hg^{+}, Hg^{+2}, Mn^{+2}, Cr^{+3}
Dithizone (15 % in chloroform) + NH_3: Cd^{+2}, Tl^{+}, As^{+3}
Cadion (0.02 % in ethanol + 1 % 2*N* NaOH): Cd^{+2}
Dimethylaminobenzalrhodanin (0.03 % in ethanol) + NH_3: Ag^{+}, Hg^{+}, Hg^{+2}
p,p'-Tetramethyldiaminodiphenylmethane (0.5 % in 5 % citric acid): Au^{+3}
Alizarin (0.1 % in ethanol) + NH_3: Ca^{+2}, Sn^{+2}, Sn^{+4}, Sb^{+5}, Zr^{+4}, Y^{+3}, Pr^{+3}, Th^{+4}, BO_3^{-3}, Ti^{+4}
Aluminon (0.1 % in ethanol) + NH_3: Be^{+2}, Ga^{+3}, Al^{+3}, La^{+3}, Fe^{+2}, Fe^{+3}
Quinalizarin (0.01 % in ethanol) + NH_3: Mg^{+2}
a,a'-Dipyridyliodide: As^{+5}, Pd^{+2}, Sb^{+5}
Cinchoniniodide: Pd^{+2}, Pt^{+4}, Bi^{+3}
Chromotropic acid (5 % in water): Ti^{+3}
Phosphomolybdic acid (5 % in water): Sb^{+3}, Sn^{+2}
Pyrogallol (5 % in ethanol): Ce^{+3}, VO_4^{-3}, VO^{+2}, MoO_4^{-2}, WO_4^{-2}
NH_3 + *o*-Toluidine (2 % in ethanol): Ir^{+4}, CrO_4^{-2}, MnO_4^{-}, Cr^{+3} (H_2O_2)
Dipicrylamine: Cs^{+}, Rb^{+}, K^{+}
Violuric acid (0.1 *M* in water) + NH_3: Li^{+}, Na^{+}
$K_4Fe(CN)_6$: UO_2^{+2}

Measurements of R_F-values. The mid-point of the spot, where the investigated solution is applied to the paper, and the front border of the solvent front are the reference points of R_F-measurements. Unfortunately most published data only report one value of the R_F. As the spreading of the different spots can vary greatly, this indication gives rather a poor measure of the separation efficiency. Mostly two values are noted here, one for the front and the other for the rear of the spot. Where only one value is reported, the "centre of gravity" of the spot is measured.

In the following tables all R_F-values are means of several experiments.

C. Results

Most of the variables, mentioned earlier, were investigated to determine the optimum conditions of fast separations and their relative influence on R_F.

1) *Dimensions of the chamber etc.* The heights and cylindrical forms of all chambers, employed, were about the same, and the descending technique was always used, being more rapid than the ascending one. This variable is hence almost constant.

2) "*Atmosphere*". It has been shown [18] that variations of "atmospheric" conditions in the chamber can completely alter separations, previously obtained. In these experiments the "atmosphere" solvent was entirely another than the elutriant.

A comparative study of the influence on R_F as well as of the degree of separation of a cation mixture, eluted by methyl-*n*-propyl ketone + HCl directly after insertion of the paper strips in the chamber, was conducted by varying the "atmosphere" content in both ketone, HCl and water. Results are shown in Tables 4—5.

Together with Table 6 the above tables indicate the great influence of "atmospheric" conditions on the R_F. In certain cases (Co and Cu) the elution order can become reversed, merely by changing the "atmosphere" a little.

It is impossible to prevent a partial evaporation of the solvent mixture on the chamber-bed. This was clearly demonstrated, as R_F-values, obtained in "old chambers" with HCl-mixtures, diminished greatly. On the contrary it is

Table 4. R_F of different cations; elutriant methyl-n-propyl ketone + conc. HCl; "atmospheric" conditions changed; volumes in ml.

Eluant +	10 % HCl		15 % HCl				20 % HCl		30 % HCl
Atmosphere	H_2O		H_2O				H_2O		H_2O
Ions	0	30	0	3	10	30	0	30	0
Ni	0.02	0.03	0.015	0.025	0.03	0.04	0.06	0.08	0.28
Mn	0.13	0.10	0.23	0.18	0.17	0.15	0.225	0.19	
Bi^{+3}			0.48		0.44				
Co	0.60	0.37	0.67	0.56	0.50	0.44	0.60	0.52	0.86
Cu	0.65	0.74	0.67	0.64	0.62	0.62	0.66	0.63	0.79
Zn	0.75	0.82	0.81	0.78	0.75		0.84	0.81	
Cd	0.80	0.84	0.82	0.82	0.79		0.86	0.83	
Fe^{+2}				0.96	0.94		0.96	0.94	
Acid front	0.74	0.74	0.81	0.78	0.75	0.71	0.83	0.81	

Table 5.

Eluant +	5 % HCl	10 % HCl	15 % HCl				30 % HCl
Atmosphere / Ions	30 ket. + 5 conc. HCl + 8 H_2O	30 ket. + 5 conc. HCl + 8 H_2O	30 ket. + 10 H_2O	30 ket. + 4 H_2O	30 ket. + 10 8*N* HCl	30 ket. + 10 8*N* HCl + 5 H_2O	30 ket. + 10 8*N* HCl + 5 H_2O
Ni	0.01	0.02	0.03		0.04	0.03	0.10
Mn	0.11	0.16	0.08		0.26	0.12	0.29
Bi^{+3}	0.32		0.33	0.32		0.46	0.58
Co	0.41	0.60	0.30		0.69	0.46	0.74
Cu	0.50	0.74	0.47	0.42	0.65	0.58	0.74
Zn		0.82	0.54		0.75	0.68	0.85
Cd			0.59	0.55	0.86	0.76	0.86
Fe^{+2}	0.91	0.90	0.84		0.95	0.90	0.93
Acid front	0.55	0.78	0.51	0.47		0.64	

advantageous to use solvent mixtures, up to one month old, as eluants, provided these are stored in well stoppered bottles. It is true that the solution darkens, due to the formation of small amounts of certain polymerisation products, but, depending on their concentration in the "acid front" during elution, the front turns clearly visible so as to facilitate certain separations.

Both the results in Tables 4 and 5 and the observations from "old chambers" indicate the strong dependence of R_F on the % HCl in the "atmosphere". When it decreases, the R_F-values also diminish, primarily owing to the decrease of the R_F of the "acid front".

To hold the "atmospheric" variable as constant as possible in the following experiments, a freshly prepared "atmospheric" solvent was used each time with the same composition as in the eluant.

3) *Solvents employed.* In order to avoid tailing of the ions, owing to their linkage to active points of the cellulose, it has been found necessary to use strong acid mixtures in inorganic paper strip separations. Two different methods have proved to be the most efficient, either employing a complexing solvent mixture, the acid concentration being rather low so as not to prevent complex formation, or a strongly acid one-phase mixture. Both methods have been tested in this investigation.

a) Separations with one-phase mixtures of ketone + HCl: A careful investigation was carried out to find the separation efficiency, when the acid concentration was varied in different ketones, as well as the equilibrium between paper strips and "atmosphere" at the beginning of elution. Results, illustrated in Table 6, indicate that, among others, the following separations may be obtained: α) Ni, Mn, Bi, Co, Cu, Zn, Cd, and Fe, as well as Fe, Mn, Co, Zn, and Cr-Al, if the acid concentration and "atmospheric" conditions are controlled; Cr and Al can not be separated; β) Ag, Hg, Tl, and Pb, as well as Pb, Bi, Cu, and Cd, with exact control of the separation variables; γ) Au, Pd, Pt, and Ir^{+3}; δ) Ca, Mg, and Ba-Sr, if very long elution is performed; Ba and Sr are impossible to separate; ε) Li, K, Rb, and Cs; K and Rb, however, separate only partly

Table 6. R_F-values.

Ions	II	III *	III	IV *	IV	V	VI	VII
Ag^+	0—0.49	0.30—0.40	0.16—0.55	0.53—0.66	0.26—0.57	0.26—0.98	0.33—0.63	0.05—0.34
Hg^+	0; 0.71—0.92		0.73—0.88	0.79—0.89	0.84—0.91	0; 0.82—0.97	0; 0.75—0.92	0.58—0.75
Hg^{+2}	0.12—0.86	0.70—0.80	0.73—0.87	0.8		0.78—0.94	0.73—0.95	0.63—0.76
Tl^+	tail	0—0.16	0—0.19	0—0.19	0—0.19	0—0.38	0—0.19	0—0.17
Tl^{+3}				0—0.19	0—0.19			0—0.16
Pb^{+2}	0.01—0.15	0.15—0.24	0.13—0.27	0.15—0.23	0.12—0.23	0.10—0.32	0.10—0.25	0.11—0.20
Bi^{+3}	0.31—0.54	0.38—0.87	0.35—0.43	0.40	0.41—0.60	0.47—0.61	0.32—0.51	0.30—0.36
As^{+3}			0.56—0.60	0.62				0.58—0.61
Sb^{+3}	0.83—0.97	0.80—0.92	0.78—0.96	0.90	0.91—0.98	0.86—0.97	0.85—0.94	0.82—0.87
Sn^{+2}	0; 0.79—0.97	0.84—0.93 tail	0.76—0.97	0.93	0.86—0.99	0.81—0.98	0.80—0.92	0.84—0.89
As^{+5}	0.57—0.74	0.44—0.46	0.58—0.60	0.70	0.83—0.88	0.80—0.90	0.72—0.80	0.53—0.60
Sb^{+5}			0; 0.80—0.93		0; 0.88—0.95	0.89—0.94		0; 0.81—0.98
Sn^{+4}	0	0	0	0	0	0; 0.90—0.94	0	0; 0.88—0.94
Cu^{+2}	0.41—0.51	0.30—0.36	0.42—0.51	0.59—0.61	0.65—0.76	0.55—0.67	0.48—0.66	0.41—0.49
Cd^{+2}	0.76—0.89	0.45—0.52	0.65—0.78	0.75	0.82—0.93	0.80—0.95	0.82—0.92	0.69—0.77
Mn^{+2}	0.02—0.06	0.06—0.16	0.06—0.11	0.14	0.15—0.22	0.21—0.34	0.17—0.30	0.10—0.16
Co^{+2}	0.17—0.31	0.22—0.29	0.25—0.34	0.50	0.65—0.82	0.66—0.85	0.48—0.76	0.48—0.56
Ni^{+2}	0	0.04—0.09	0.03—0.08	0.02—0.04	0.01—0.06	0.02—0.07	0.01—0.05	0.02—0.07
Zn^{+2}	0.51—0.75	0.46—0.49	0.57—0.61	0.72	0.80—0.93	0.74—0.92	0.63—0.87	0.57—0.60
Fe^{+2}	0.31—0.97 tail	0.65—0.92	0.62—0.95	0.90 tail	0; 0.88—0.98	0.85—1.0 tail	0; 0.82—0.96	0; 0.72—0.94
Fe^{+3}	0; 0.82—0.91	0.92—0.97	0.71—0.76	0.96 tail	0; 0.89—0.98	0.90—1.0 tail	0; 0.83—0.95	0; 0.90—0.98
Cr^{+3}	0		0—0.06	0.03	0—0.03	0—0.09	0—0.05	0—0.05
Al^{+3}	0		0—0.06	0.03	0—0.03	0.01—0.08	0—0.04	0—0.06
Ga^{+3}	0; 0.88—0.95		0; 0.84—0.91		0; 0.88—0.97	0.91—0.97	0; 0.88—0.93	0; 0.84—0.91
Ca^{+2}	0		0.01—0.04	0.01	0.01—0.04	0.02—0.07	0—0.03	0.02—0.06
Ba^{+2}			0	0	0	0—0.02	0	0
Sr^{+2}			0—0.01	0				0—0.04
Mg^{+2}	0—0.02		0.07—0.11	0.03	0.01—0.05	0.05—0.12	0.01—0.05	0.03—0.09
Li^+	0—0.02				0.12—0.15	0.24—0.29	0.10—0.12	0.11—0.15
Na^+					0—0.06			0—0.08
K^+	0		0.05—0.06		0.03—0.05	0.08—0.12	0.02—0.05	0.05—0.07
Rb^+	0		0.05—0.06			0.10—0.12	0.03—0.06	0.09—0.11
Cs^+			0.07—0.08		0.05—0.07	0.16—0.20	0.06—0.09	0.06—0.10
Au^{+3}	0.88—0.97	0.83—0.95	0.82—0.89		0.84—0.93	0.90—0.97	0.87—0.94	0.90—0.95
Pd^{+2}	0.16—0.20 tail	0.44—0.46	0.40—0.49	0.50—0.80	0.47—0.90	0.68—0.96	0; 0.33—0.52	0.28—0.36
Pt^{+4}	0.17—0.30 0.40—0.51	0.45—0.50 0.68—0.74	0.59—0.68	0.78	0.45—0.83	0.70—0.83	0.43—0.81	0.49—0.65
Ir^{+4}			0.41—0.49		0.58—0.68	0.13—0.15	0.07—0.13	0.04—0.08 0.39—0.45

Ions	II	III *	III	IV *	IV	V	VI	VII
Be^{+2}	0—0.04		0.09—0.30		0—0.17	0.22—0.34	0—0.22	0.08—0.16
Ti^{+3}	0—0.02		0—0.07		0—0.11	0.08—0.15	0—0.08	0.04—0.09
VO^{+2}	0—0.03		0.04—0.11		0.04—0.10	0.07—0.18	0—0.11	0.05—0.11
VO_4^{-3}			0.04—0.11 tail		0.03—0.10 tail	0.07—0.18		0.05—0.10 tail
MoO_4^{-2}	0.89—0.97		0.78—0.92		0.88—0.94	0.87—0.93	0.88—0.94	0.87—0.94
WO_4^{-2}	0; tail		0	0.03	0; tail	0; tail	0; tail	0
UO_2^{+2}	0.34—0.61	0.54—0.64	0.58—0.64	0.78	0.74—0.89	0.81—0.94	0.81—0.96	0.62—0.74
Th^{+4}	0		0	0.03	0	0—0.03	0	0—0.03
Zr^{+4}	0—0.02		0—0.03		0; tail	0—0.22	0—0.12	
MnO_4^-	0.03—0.08		0.06—0.12		0.14—0.22	0.20—0.32	0.12—0.22	0.08—0.15
CrO_4^{-2}	0—0.03		0—0.05		0—0.06	0—0.13	0—0.06	0—0.12
BO_3^{-3}	0.18—0.25		0.34—0.44		0.32—0.43	0.45—0.67	0.41—0.52	0.28—0.36
Ce^{+3}	0		0.02—0.05	0.01	0—0.03	0—0.04	0—0.02	0—0.03
Y^{+3}	0		0—0.05		0—0.02	0—0.06	0—0.03	0.01—0.04
Pr^{+3}			0—0.04		0—0.03			0—0.03
La^{+3}			0—0.03		0—0.03			0—0.03
Acid-front	0.54	0.45	0.59	0.70	0.85	—	0.67	0.60

Ions	I*	I
Bi^{+3}	0—0.91	0—0.38
Cu^{+2}	0; tail	0.10—0.45
Cd^{+2}	0—0.47	0.36—0.74
Mn^{+2}		0
Co^{+2}		0—0.05
Ni^{+2}		0
Zn^{+2}	0; tail	0; 0.09—0.77
Fe^{+2}		0—0.83

The eluants in the different experiments are indicated:

I methyl-*n*-propylketone + 2 % 10*N* HCl
II » » » + 5 » » »
III » » » + 15 » 8*N* »
IV » » » + 15 » 10*N* »
V » » » + 25 » 8*N* »
VI methyl-*iso*-propyl-ketone + 15 % 10*N* HCl
VII » » butyl » + 15 » » »

*, non-equilibrium between paper and atmosphere; elution started directly after the insertion of the strips in the chamber.
Without *, equilibration during 16 hours.

from each other; ζ) Sn^{+4}, Bi^{+3}, Sb^{+3}, and As; η) W, Mo, V, and U; ∂) Ti, Th, and U; $\varkappa$) Ga from Al and Tl.

Metal ions with different valency states are most often rapidly reduced by the cellulose during elution, as is apparent from the agreement of R_F's in

Table 6 of the pairs Hg^{+}—Hg^{+2}, Fe^{+2}—Fe^{+3}, Tl^{+}—Tl^{+3}, Mn^{+2}—MnO_4^{-} and Cr^{+3}—CrO_4^{-2}. Weak tailing and hence slow reduction was observed for VO_4^{-3} and Ti^{+4}. Exceptions are Sn^{+4}—Sn^{+2}, where the higher valence ion is strongly adsorbed by the cellulose and no reduction therefore occurs; As^{+5}—As^{+3}, where an investigation has revealed that only very feeble reduction occurs in spite of the R_F's being about the same; and Ir^{+4}—Ir^{+3}, where, depending on the solvent and H^{+}-conc., Ir^{+4} may be reduced [18].

b) *n*-Butanol elutions: Only two-phase solvent mixtures were investigated. Pure *n*-butanol as well as mixtures with HCl, HNO_3, citric acid and NH_4OH were used. As previously published data are exhaustive, and R_F-values, obtained with the same eluant, mostly agree with these, no data will be mentioned but only some observations: α) As, Sb, and Sn in one of their two valence-states are almost always separated by HNO_3, citric acid and mixtures of these; β) the difficultly separable Al^{+3} and Cr^{+3} may be separated by *n*-butanol + 5% citric acid + 2 *N* HNO_3 (2 : 1 : 1), the bands, however, nearly touch; γ) a partial or complete neutralisation of the acids with NH_4OH and thereby higher pH only diminishes the R_F's, renders the separations more difficult, and increases the spreading of the spots: δ) it was found difficult to reproduce previously published R_F's of ions, where the acid concentration of the stock solutions have to be high to prevent hydrolysis (especially As, Sb, and Sn), if exactly the same initial solutions were not prepared. This is due to the feeble acidity in the organic phase of two-phase mixtures, which is hence considerably changed by the strong acid concentration in the initial spot.

4) *Filter paper.* Munktell OB was employed in all experiments. Small amounts of Cu, located in bands in the paper, necessitate prior separation of Cu. Further, the adsorbing capacity of ions is not as high, and the paper not as homogeneous as Whatman 1, but the advantage of fast diffusion was considered more important.

The form and dimension of all paper strips was the same and the solvent front diffused the same length in each experiment, but in spite of this the variable can not be regarded as held constant, as paper strips are neither absolutely homogeneous nor mutually completely alike. This caused variations in R_F from paper strip to paper strip, as the diffusion rates, especially that of the "acid front", vary considerably (up to 20 %) on account of the varying intersections of the stationary and moving phases.

5) *Initial solutions of ions.* An investigation was made into the effect of changing the H^{+}-concentration (HNO_3 and HCl) of the initial spots of Cu, Co, Ni, Fe, Mn, Zn, Ag, Hg, and VO^{+2}, thereafter eluted by ketone-HCl mixtures, but only small changes in R_F could be noted, and the tailing of Ag, Hg, Fe, and VO was not diminished. As previously mentioned, the effect of changing the initial acid concentration can be great when two-phase mixtures are employed.

Usually no difference was observed by using chlorides or nitrates in the initial solutions. When chlorides, weakly acidic or neutral, were eluted by butanol-HNO_3 mixtures, the same R_F's were found as those, previously reported by Pollard *et al.* with nitrates. Ketonic solvents were also tested and with one exception, Bi, the same R_F's were obtained.

In a mixture of Ba, Ni, Mn, Co, Cu, Zn, Cd, and Fe, eluted by ketonic solvents, large concentrations of one or two of the ions did not affect the R_F's of the other ions. No displacement effect could be observed, even when serious tailing of the more concentrated ions were obtained into the areas of the other ions. Neither did Na^+ in large concentration disturb the separations; NH_4-salts, however, even in moderate concentration completely altered the separations and produced long tails of all the ions.

The spreading of the spots of some cations after elution was measured, varying amounts initially applied to the strips in about the same area. Results are shown in Table 7.

Table 7. Eluant: methyl-n-propyl ketone + 15 % 8 N HCl; equilibration during a night before elution; acid front $R_F \simeq 0.68$.

mg applied	R_F of eluted cations					
	Ni^{+2}	Mn^{+2}	Co^{+2}	Cu^{+2}	Zn^{+2}	Fe^{+2}
0.005	0.03—0.06	0.08—0.12	0.30—0.37	0.43—0.54	0.67—0.80	0.84—0.90
0.010	0.03—0.06	0.08—0.13	0.30—0.38	0.43—0.54	0.67—0.81	0.83—0.80
0.025	0.02—0.07	0.07—0.13	0.30—0.41	0.41—0.68	0.68—0.77	0.83—0.90
0.050	0.01—0.07	0.05—0.13	0.30—0.41	0.40—0.87	0.68—0.78	0.84—0.92; 0
0.200	0.01—0.07	0.04—0.15 tail	0.26—0.41	0.39—0.87	0.69—0.80	0.70—1.00; 0 tail
0.400	0—0.07	0.04—0.16 tail	0.25—0.40		0.69—0.82	0.50—1.00; 0 tail
1.00	0—0.08	0—0.16	0.21—0.36	0.36—0.86	0.69—0.85	0—1.00
2.00	0—0.09	0—0.17	0.12—0.36		0.69—0.88	0—1.00

Table 7 indicates that only amounts < 0.025 mg of each cation are well separated. Identical experiments were performed with methyl-*iso*-propyl ketone + 15 % 10 *N* HCl. The same dependence on applied weight was obtained; the spreading, however, was a little larger.

Table 7 also shows that Ni or Mn, Co or Zn can be separated in amounts of more than 1 mg. A combined elution by ketone + 8 *N* HCl and ketone + 10 *N* HCl leads to very good separations, but unfortunately only a max. 0.025 mg Cu and Fe can be present. By applying the stock solutions in different concentration and varying the subsequent degree of drying an attempt was made to diminish the tailing and retention of Cu and Fe in higher concentration, but only small improvements were attained.

6) *Application to the paper strips.* The solutions were always applied to about the same area and at the same distance from the upper ends of the strips. Further, it is advantageous to apply in small spots and not in bands, since the long tails, produced at the outer edges of the strips, render the cutting of the paper more difficult, particularly when invisible radioactive isotopes are separated [1].

Only slight effects could be observed by varying the degree of drying after application. Hence the variable is nearly constant.

7) *Equilibrium.* The very strong influence on R_F-values, caused by different states of equilibrium between paper strips and "atmosphere" at the beginning of elution, is indicated in Table 6, particularly by a comparison between values of solvent mixture I. Apart from some exceptions, *e.g.* Ag^+, more distinct spots and better separations are attained after equilibration. Otherwise the elution is performed with different conditions at the start and at the end, since the absorbtion of "atmospheric" vapours by the cellulose is fast at first but decreases exponentially with time.

Even when investigating shortlived radioactive isotopes it is favourable always to equilibrate for at least 15 minutes.

8) *Temperature.* Temperature differences influence vapour pressures, equilibrium constants and diffusion rates and hence also R_F's. However, an investigation revealed that by working at room-temperature, held constant to within $\pm$ 3° C, R_F-differences were small, and the variable can be considered as held constant.

1—8) *Summary.* Among the investigated variables only three have a powerful influence on the R_F's, obtained: eluant + "atmosphere", equilibration and paper. The first two principally determine R_F-values and separation efficiency and may be used and applied in such a way that good separations are obtained. Differences in paper strips cannot be controlled and chiefly determine the observed variance of R_F from experiment to experiment, when the same conditions are otherwise used. Fortunately, only the absolute values of R_F vary, the separation efficiency being feebly affected.

With the technique employed, and with the ketonic solvent as elutriant the entire chromatographic separation takes less than one hour, and is hence applicable to spallation studies.

D. Discussion

The great similarity has previously been mentioned between paper strip separations and partition elutions with ion-exchange columns, eluted by the same one-phase solvent mixture. The experimental results of the two procedures make possible an interpretation of the mechanism in paper chromatography. At the application of the ion-mixture to the paper the ions are adsorbed on active points in the cellulose and in the water phase, always bound in ordinary, not especially dried paper. During the equilibration, for instance with ketone + 8 N HCl as eluant, the water phase increases, but only after several days is complete saturation attained. At the same time HCl, as well as ketone, is dissolved in the water phase. When elution starts, the eluant begins to diffuse down the paper, water is partly adsorbed by the incompletely saturated cellulose and HCl strongly adsorbed in the cellulose water phase, until the H^+-concentration becomes the same as in the elutriant (8 N). An "acid front" is developed, moving down the paper with a one-phase ketone mixture behind and a ketone-water emulsion (two-phase mixtures when $H^+ < 7.5$ M) in front of it, the latter with a rapidly increasing pH-gradient and a gradually diminishing water content, the volume of which depends on the equilibration time. When the solvent front, almost pure ketone, reaches the initial spot, a distribution of the applied ions starts to occur between the moving ketone, the station-

ary water phase, feebly acidified by the "atmosphere", and the active points in the cellulose. The significance of the latter at first is predominant, the ions being retained unless their solubilities in the pure ketone are high, but rapidly diminishes, as the acid concentration in the stationary phase increases. Pure partition between stationary acid water and ketone-water emulsion now occurs, different ions moving with a particular pH. Even behind the "acid front" partition occurs between a stationary highly acid water phase and the moving one-phase mixture.

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The Separation of Small Amounts of Inorganic Cations by Chromatographic Methods. II. An Automatic Scanner for Radio Paper Chromatograms

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The construction of a sensitive and well resolving scanner of chromatograms is described, permitting the determination of R_F-values for as little as $5 \cdot 10^{-7}$ mC Co^{60}.

The investigation of radio labelled compounds by means of paper chromatography has found application during the last ten years. Two different scanning methods have been used, either autoradiography or registering of the counts by passing a Geiger-Müller counter. The former has the disadvantage of being slow and difficult to interpret quantitatively, and resolves spots, near one another, rather badly. The second method is not as simple as the first but is fast, quantitative and gives good resolution. Different automatic scanning GM-apparatus have been described [1–4]. In a scheme of separating spallation products of copper [6], investigated at this laboratory, an apparatus was constructed, which is similar to a later published scanning [5] device in essential parts.

SCANNING INSTRUMENT

The construction and connections are illustrated in Fig. 1. The paper strips *A* are continuously passing a small slit *B* below a thin mica window of a bell-shaped GM-counter *C* (20th Century El. EW 3 H), shielded by a lead castle *D*, the lower parts of which are shown in Fig. 2. The moving paper strip is guided by a small concavity *E* with the same width, 3.5 cm, as the strips. The slit is 3.5 × 0.2 cm and its height 0.25 cm. In the cylindrical concavity *F* the GM-counter directly rests above the slit (distance window-paper strip hence 0.25 cm) or if desired with some Al- or Pb- sheets between to adsorb the β-rays.

The movement of the strips is synchronized to the movement of a writing recorder (Easterline Angus Graphic Ammeter) by a fine thread *G*, attached to the paper by a clamp *H*, resting on a Lucite stand *K*, and with its other end wound around the shaft of the drum that moves the chart of the recorder. The

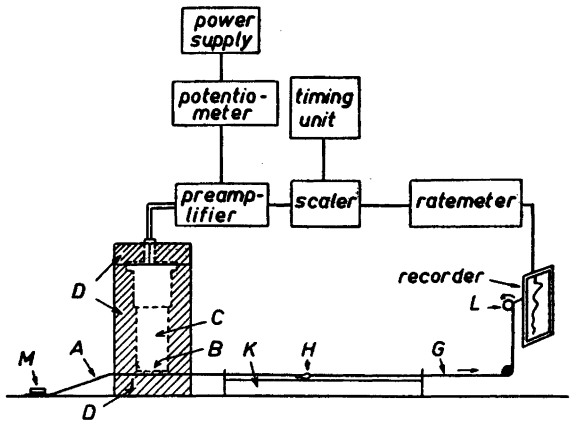

Fig. 1. Diagram of scanner and connections.

rate of the paper movement, compared to that of the chart, is regulated and shifted by small spools L of different radius and also by altering the gear connection shaft-chart in the recorder. The thread and strip are tightened by a small lead plate M.

All counting equipment is of conventional Harwell type. High voltage is supplied to the GM-counter via a potentiometer + power supply, type 200, fed into the scaler (type 1009 A) and registered both by a scale of 100 circuit + mechanical register, controlled by a timing unit (type 1003), and also by a ratemeter (type 1138 A) + recorder. The recorder is calibrated so as to write directly on the lined chart the number of counts per second, and the integrating capacities regulated to give a smooth and sensitive curve.

INVESTIGATIONS

a) *Tests of sensivity, reproducibility etc.* In order to determine the sensivity and smallest registered and well resolved amount of radioactive material, exact volumes of Co^{60} + inactive Co were applied to the strips, eluted by means hof te method, described earlier [7], counted and recorded. As the number of mC per ml of the Co-solution had been determined against a Co-standard, the

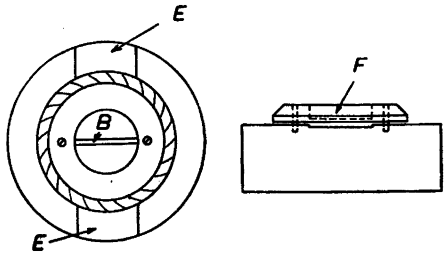

Fig. 2. Lead castle, lower part.

Table 1. Registered counts, less background, from strips, to which different λ-amounts of Co^{60} had been applied.

Number of λ	Registered impulses	
		with extra Pb-plate
1	185 ± 38 232 ± 34	67 ± 22 71 ± 26
2	450 ± 35 495 ± 36	123 ± 21 155 ± 26
3	683 ± 46	175 ± 30
4	972 ± 49	263 ± 35
5	1 180 ± 50 1 190 ± 45	321 ± 33 345 ± 29
10	2 185 ± 60 2 351 ± 54	657 ± 39 662 ± 31
20	4 607 ± 74	1 342 ± 43
25	5 968 ± 82	1 715 ± 47

smallest amount of Co^{60} to give well defined peaks with the same R_F as those, obtained by spraying the strips with rubeanic acid, could be calculated and was found to be $\sim 5 \cdot 10^{-7}$ mC (mean of 20 experiments), *e.g.* 22.5 disintegrations per second. Deducting background, only 35 impulses were then registered by the scaling circuit, but nevertheless the R_F could be determined to within ± 10 %.

11.8 % of the total disintegrations were counted by the counting assembly, *i.e.* the effective angle is ~ 40°.

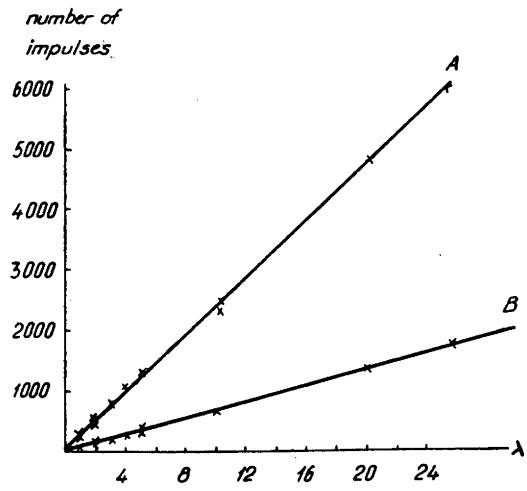

Fig. 3. Registered number of impulses of different amounts of Co^{60} (data from Table 1); A without and B with Pb-absorbator.

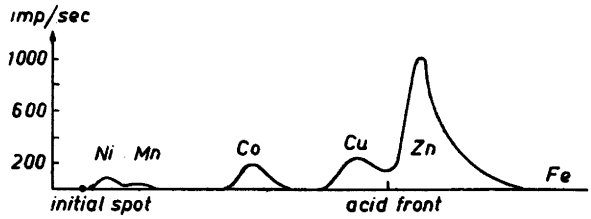

Fig. 4. Separation of Cu-spallation products with methyl-iso-propylketone + 15 % 8 N HCl.

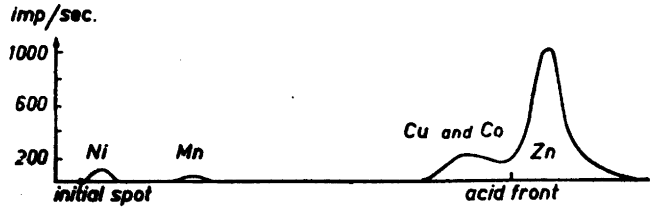

Fig. 5. Separation of Cu-spallation products with methyl-iso-propylketone + 15 % 10 N HCl.

Smooth curves could never be obtained, when very weak radioactive solutions were counted, as the integrating capacity had to be small to resolve the peaks. The width of peaks, registered from strong γ-emitting isotopes, such as Co^{60}, was larger than that of the real spots on account of γ-penetration through the thin Pb-plate near the slit, but these effects could partly be eliminated by an additional Pb-plate, placed between the mica-window and the paper, with exactly the same dimensions of the slit. The effective angle was then diminished to $\sim 12°$, the γ-boundary effects were almost screened but the sensivity also diminished.

To test the properties of the equipment, the above experiments were performed, applying Co^{60} in different areas and activities to the strips. Results are shown in Table 1 and Fig. 3, which indicate the good reproducibility and quantitative measuring properties of the apparatus, independant of the final areas of the spots.

b) *Separation efficiency.* The apparatus was used to trace the spallation products of copper [6]. The separations, obtained, and the well defined peaks are illustrated in Figs. 4—5. The Fe-activity is very weak and unregistered in the ratemeter range employed.

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The Separation of Small Amounts of Inorganic Cations by Cromatographic Methods. III. Spallation of Copper with 60 MeV Protons

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Chromatographic paper strip technique is tested to separate the spallation products of copper. Complete separation of the whole group is obtained in about 3—4 hours, and a special monitor is hence not wanted. The yields of isotopes with half-lives above 30 minutes can be determined.

Bombardment of Cu by charged particles with energies ranging from 1—400 MeV have been carried out and the yields of radioactive products, formed during the high energy process, reported [1–4]. The present investigation was performed to study the yield distribution in an intermediate range, 60 MeV, and also to test the utility of chromatographic paper strip technique [5–6] in spallation studies.

EXPERIMENTAL

a) Irradiations with 60 MeV protons were carried out in the circulating beam of the Uppsala synchro-cyclotron during 30—60 minutes with 0.1 mm thick electrolytic Cu-foils, dimensioned 2 × 16 mm, as targets.

b) Separation procedure. The Cu-foil was dissolved in hot HCl + H_2O_2, the copper precipitated as sulfide by H_2S and separated from the solution by centrifuging. All the spallation products, except almost all Cu, remained in solution, which was gently evaporated to a small volume on a steam bath, a slow warm air-stream passing through the solution. An exact volume was applied to two paper strips by means of a thoroughly calibrated micro-pipet. After 15 minutes equilibration in the chromatography chambers, the complete separation of the different cation species was attained by eluting one strip with methyl-*n*-propyl ketone + 15 % 8 *M* HCl and the other with the same ketone + 15 % 10 *M* HCl (see Figs. 4—5 in part II of this work), and their exact positions on the strips registered by special counting equipment [6]. Each ion-species, Mn and Ni from the second solvent mixture, Co, Zn, Fe, and Cu-contaminations from the first, was completely cut out from the strips, gently ashed into a small Pt-dish, the same as was used in the later radiometric analysis.

The Cu-precipitate was dissolved in concentrated HNO_3, and an exact volume pipetted into a Pt-dish. The total volume of the Cu-solution as well as the solution, containing the other ions, was determined.

Before the first spallation experiment, an investigation was made into the possible losses of both Cu and "other" isotopes during precipitation, evaporation and ashing, using both inactive and active isotope mixtures. The Cu-fraction was found to be entirely uncontaminated by the "other" ions. This was also verified in each spallation experiment as well as the Cu-losses, the latter obtained by weighing the foil before bombardment and by quantitative Cu-determination after the transfer of the aliquot to the Pt-dish. The losses of "other" isotopes were found to be (7 ± 2) %, and they principally occur during evaporation. The losses in the ashing procedure, though found to be negligible (<1 %), were always checked.

c) Counting technique. The isotopes were identified by half-life determinations and absorbtion measurements by means of a sample changer arrangement and an end-window GM-counter (Philips 8314), mounted in a lead castle with good geometry and negligible housescattering. Corrections were made for background, coincidence, backscattering, air and window absorbtion, decay during bombardment and counting efficiency of the rays.

Yields are given relative to Cu^{64}, which is arbitrarily assigned a yield 1.00.

IDENTIFIED RADIOACTIVE NUCLIDES

a) *Zn-fraction.* Zn^{62} and Zn^{65} were identified. Zn^{62} was counted in equilibrium with its daughter element Cu^{62} and is assumed to decay 10 % by β-emission and 90 % by K-capture. Zn^{65} was assumed to decay 46 % by γ-emission and was counted through sufficient Al-absorber to cut out completely the β- and X-rays, and the counting efficiency of the 1.1 MeV γ-ray was assumed to be 1.7 %. The half-life of Zn^{63} was too short to be counted by the technique, employed.

b) *Cu-fraction.* Identified activities were those of Cu^{60}, Cu^{61}, Cu^{62} and Cu^{64} with counting efficiencies, estimated to be 101, 67.8, 100.5 and 59 %.

c) *Ni-fraction.* The only observed activity was that of Ni^{57}, which was assumed to decay 50 % by β-emission with a total counting efficiency of 55 %.

d) *Co-fraction.* The radionuclides Co^{56}, Co^{57}, Co^{58}, Co^{60} and Co^{61} have been identified, and their counting efficiencies were assumed to be, respectively, 98, 100, 17.6, 103 and 100 %. Even the 9.2 hour Co^{58}-isomer was identified. The counting efficiency in the GM-counter of a 0.025 MeV γ-ray is 2.7 %, which was assumed to be the efficiency for the isotope. The yield of the short-lived Co^{62} cannot be determined with the separation technique, employed.

e) *Fe-fraction.* The activities of Fe^{55}, Fe^{59} and Fe^{60} were identified. Fe^{55} is assumed to decay 100 % by K-capture with a counting efficiency of 3.5 % and Fe^{59} 100 % by β^--decay.

In the iron decay curve a new activity was found, presumably due to the earlier reported, though uncertain, Fe^{60}-isotope, identified in fission studies at Berkeley [7]. In three bombardments its half-life was found to be 8.3, 8.1 and 8.1 hours. The β-energy was calculated to be 1.6 ± 0.2 MeV by absorbtion measurements, which also indicated that the counting efficiency of the isotope + daughter is only about (8 ± 5) %. The mass number is, however, not established, but the yield, found, supports the assumption that the isotope is Fe^{60}, and the β-energy, found, is in agreement with the energy of the daughter element Co^{60}. The β-energy of the new isotope is hence unknown. The possibility that the isotope is Fe^{52} can be eliminated, as the thresh-hold energy with deuterons for Cu → Fe^{52} has been reported to be 85 MeV[3], and the threshhold energy with protons can hardly be more than 10 MeV less. Neither the counting efficiency found, nor the β-energy corresponds to Fe^{52}.

f) *Mn-fraction.* The characteristic activity of Mn^{56} was identified as well as a very weak Mn^{54}-activity, but no Mn^{52}-activity was found. Counting efficiency of Mn^{56} was assumed to be 102 % and of Mn^{54} 6 %.

g) Neither Cr- nor V- activity was observed.

EXPERIMENTAL RESULTS

Table 1 shows the partly corrected yields in 4 bombardments together with their weighted means, and Table 2 the finally corrected yields.

Table 1. Experimental yields from Cu, irradiated with 60 MeV protons; yields are relative to Cu^{64}.

Isotope	Experimental number I		II		III		IV		Weighted mean
	$\tau_{\frac{1}{2}}$	yield	$\tau_{\frac{1}{2}}$	yield	$\tau_{\frac{1}{2}}$	yield	$\tau_{\frac{1}{2}}$	yield	
Mn^{54}								0.00023	0.00023
Mn^{56}	2.80 h	0.00362	2.59 h	0.00360	2.70 h	0.00367	2.75 h	0.00158	0.0036
Fe^{59}				0.0172	46.0 d	0.0055	45.5 d	0.0047	0.0055
Fe^{60}	8.3 h	0.000102			8.10 h	0.000188	8.1 h	0.000169	0.00075
Co^{56}						0.0088			0.009
Co^{57}		0.068		0.050		0.073		0.051	0.060
Co^{58}	9.4 h	0.004			9.15 t	0.00382	9.3 h	0.0035	0.0037
Co^{60}		0.188		0.147		0.188		0.157	0.17
Co^{61}	1.80 h	0.055	1.83 h	0.0370	1.75 h	0.0565	1.74 h	0.0473	0.052
Ni^{57}	35.9 h	0.0276	36.0 h	0.0322	35.9 h	0.0323	35.2 h	0.0176	0.032
Cu^{60}					23.2 m	0.91	23.8 m	0.83	0.87
Cu^{61}	3.3 h	2.88	3.4 h	2.52	3.43 h	2.45	3.35 h	2.61	2.6
Cu^{62}							9.6 m	2.06	2.06
Cu^{64}	12.8 h	1	12.8 h	1	12.8 h	1	12.7 h	1	1
Zn^{62}			9.15 h	0.42	9.18 h	0.50	9.26 h	0.31	0.40
Zn^{65}						0.0059		0.0030	0.0046

Table 2. Experimental yields, relative to Cu^{64} from Cu, irradiated with 60 MeV protons. × stable isotopes.

Z \ Mass number	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66
Zn_{30}											0.21		×	0.14	×
Cu_{29}									0.71	2.20	3.10	×	1	×	
Ni_{28}						$3.15 \cdot 10^{-3}$	×		×	×	×		×		
Co_{27}				0	$5 \cdot 10^{-3}$	$6.8 \cdot 10^{-2}$	0.58	×	0.137	$2.8 \cdot 10^{-2}$					
Fe_{26}	0		×		×	×	×	$3.90 \cdot 10^{-3}$	$1.18^{*} \cdot 10^{-3}$						
Mn_{25}	0		$2.66 \cdot 10^{-3}$	×	$1.99 \cdot 10^{-3}$										
Cr_{24}	×	×	×												

* uncertain.

Mass number →

DISCUSSION

The greatest advantage of using the chromatographic technique is that particularly pure samples of all spallation products can simultaneously be obtained, as indicated by the agreement of half-life determinations with earlier published data. Exact calculations of the yields are hence favoured. Unfortunately the conventional radiometric weighing procedure cannot be used, the amounts being to small to obtain reliable weights without a sensitive microbalance, and instead less accurate volumetric measurements must be made. The good reproducibility, attained in almost all bombardments as shown in Table 1, indicates, however, that the chromatographic procedure is well applicable to the study of spallation products, and on account of the small amount of work necessary, and the many yield determinations, simultaneously made in one bombardment, the method is in most aspects superiour to the conventional technique.

The proton energy in bombardment IV in Table 1 was a little less than 60 MeV, and this is also shown by the yields of Mn^{56} and Ni^{57}, which were found to be considerably smaller than in the other bombardments, and depends on a steep fall of the excitation functions of Mn^{56} and Ni^{57} immidiately below 60 MeV. Such a fall has earlier been found with deuterons just around 60 MeV[3].

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The Crystal Structure of Barium Selenopentathionate Dihydrate

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The crystal structure of barium selenopentathionate dihydrate has been determined by X-ray methods. The crystals, which have a four-molecule unit cell based on the space group D_{2h}^{16} —*Pnma*, with $a = 4.98$ Å, $b = 10.36$ Å, $c = 22.20$ Å, are isomorphous with those of barium pentathionate dihydrate.

The selenopentathionate ion consists of a divalent selenium atom to which are attached two thiosulphate groups. The resulting S—S—Se—S—S chain is unbranched and non-planar, with dihedral angles of 109°, and S—S and S—Se bond lengths of 2.13 Å and 2.17 Å, respectively. The values, 103° and 101°, respectively, were found for the S—S—Se and S—Se—S bond angles.

Some comments are made on the occurrence of rotational isomers of the pentathionate, selenopentathionate and telluropentathionate anions.

It was pointed out some time ago [1], on the basis of the finding that pentathionic, selenopentathionic and telluropentathionic acids give isomorphous salts, that the anions of these acids have analogous structures. Meanwhile, the detailed structures of two other salts of the acids, not belonging to the isomorphous series but having more favourable unit cells, have been determined by X-ray methods. These are orthorhombic barium pentathionate dihydrate [2] and ammonium telluropentathionate [3]. In order to include a salt of selenopentathionic acid, and to secure dimensional data for the selenopentathionate ion, crystals of barium selenopentathionate dihydrate have now been prepared and found to be isomorphous with those of barium pentathionate dihydrate.

EXPERIMENTAL

The salt was obtained as follows. To 20 g of crude sodium selenopentathionate trihydrate [4,5], dissolved in 25 ml of 0.2 *N* hydrochloric acid and cooled in ice, was added a solution of 25 g of barium perchlorate trihydrate in 15 ml of water. Crystals of barium selenopentathionate rapidly began to separate out. After about five minutes, the product

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was filtered off, and washed with alcohol and ether. Yield, about 17 g. For recrystallization, 5 g of the crude product was dissolved, at about 40° C, in 15 ml of 0.5 *N* hydrochloric acid. The filtered solution was warmed gently again, a few milliliters of methanol were added, and the mixture was allowed to stand at room temperature.

The product was analyzed volumetrically by oxidation with potassium bromate, and determination of the selenious acid by the Norris and Fay method, as described earlier [4] in the case of the sodium and potassium salt. (0.2380 g substance: 19.87 ml of 0.09997 *N* sodium thiosulphate. $BaSe(S_2O_3)_2 \cdot 2H_2O$ (476.6): Calc. Se 16.57. Found Se 16.47).

The crystals of barium selenopentathionate dihydrate have a pale yellowish green colour. When kept in the dark, they remain unchanged for months, whereas they slowly decompose, with liberation of selenium, when exposed to light or to X-rays. The crystals appear as flat prisms, elongated along the *a* axis and bounded by {001} and {011}. There is perfect cleavage along (001), as in the case of barium pentathionate dihydrate [2].

The unit cell dimensions were determined from oscillation and Weissenberg photographs, with an accuracy of about 0.5 % (Cu$K\alpha$ radiation, $\lambda = 1.54$ Å). For comparison, data for barium pentathionate dihydrate [2] are listed below.

	a	*b*	*c*
$BaSe(S_2O_3)_2 \cdot 2H_2O$	4.98 Å	10.36 Å	22.20 Å
$BaS(S_2O_3)_2 \cdot 2H_2O$	5.00	10.30	21.78

There are four formula units per unit cell; density, calc. 2.75, found 2.76 g/cm^3. The systematic absences, *viz.*, 0*kl* when $k + l$ is odd, *hk*0 when *h* is odd, are the same as for barium pentathionate dihydrate. It is apparent that the two salts are isomorphous, and the space group was therefore taken as D_{2h}^{16}—*Pnma*. This choise appears to be justified by the outcome of the analysis. The space group has eightfold general positions, and implies that the selenopentathionate ion possesses either a mirror plane, or a centre, of symmetry, with the barium ion also in special positions. As borne out by the analysis of the pentathionate and the present structure, the barium ion and the selenium atom lie in mirror planes. A centre of symmetry is, furthermore, incompatible with any reasonable structure for these anions.

Relative intensity measurements in the 0*kl* and *h*0*l* zones were made visually on Weissenberg photographs, using a double film technique, and after applying corrections for the Lorentz and polarization factors, the intensities were converted into relative structure amplitudes. They were ultimately put to an approximately absolute scale by comparison with calculated structure factors. 134 of the 161 0*kl* reflections and 132 of the 147 *h*0*l* reflections theoretically accessible with Cu$K\alpha$ radiation were recorded. The crystals used for the *a* and *b* axis photographs had cross-sections of 0.08 mm × 0.04 mm and 0.11 mm × 0.08 mm, respectively, and although absorption was certainly not negligible, no corrections were made. As before [2], difficulties were experienced in the cutting of the crystal for the *b* axis photographs, due to the ready cleavage. There was a slight but persistent tendency for twinning to occur, possibly with triclinic crystals [6], and this may have affected the reliability of the observed intensities.

The Patterson and Fourier summations, and the location of peaks, were made as described earlier [2], the summations being performed at 6° intervals along the *b* and *c* axes and at 12° intervals (0.166 Å) along the *a* axis.

DETERMINATION OF THE STRUCTURE

The Patterson projection along the *a* axis was worked out first. The resulting vector map was very similar to the corresponding one for barium pentathionate dihydrate [2], and is not reproduced here. As before, the map gave a very good resolution of vector peaks, and allowed approximate values for the *y* and *z* coordinates of the barium ion and the selenium and sulphur atoms to be determined and used for the calculation of signs of the 0*kl* reflections. The first Fourier map proved successful in indicating the positions of the oxygen atoms and water molecules, and the structure was refined in the usual way by successive electron density projections, leading to the final map, $\varrho(yz)$, shown in Fig. 1 and explained in Fig. 3. The *y* and *z* coordinates derived from this projection were corrected for termination-of-series errors as described by Booth[7], to give the final values of Table 1. The reliability factor, *R*, for the 0*kl* reflections changed from 0.187 to 0.181 as a result of this process.

The *x* coordinates were found chiefly from Patterson and Fourier projections along the *b* axis. The *h*0*l* Patterson map indicated that the barium ion and the four sulphur atoms lie close to the glide plane *n* normal to the *a* axis, and emphasized further the similarity of this structure with that of barium pentathionate dihydrate. Provisional *x* coordinates were estimated partly from the vector map and partly by reference to the pentathionate structure, and were employed for the calculation of signs of the *h*0*l* reflections. The first Fourier synthesis was based on 57 terms having *h*0*l* indices with *l* even and 50 terms with *l* odd. The analysis then proceeded by successive syntheses until the signs of all observed *h*0*l* reflections were thought to be established. The final electron density map, $\varrho(xz)$, is shown in Fig. 2, and a line diagram is reproduced in Fig. 4.

The oxygen atom, O_1, is better resolved in $\varrho(xz)$ than in $\varrho(yz)$, where overlapping with S_1 occurs. Trial and error calculations, however, showed that the *z* coordinate of O_1, 0.076, estimated from $\varrho(yz)$ gave a lower value for the reliability factor than the value, 0.080, derived from $\varrho(xz)$, or an intermediate value. In $\varrho(xz)$, the oxygen atom, O_2, overlaps with S_1, and the water molecule, $(H_2O)_2$, is not visible, probably due to the proximity to the selenium atom in this projection. The *x* coordinate of $(H_2O)_2$ was therefore obtained by trial and error calculations, and those of S_1, S_2, O_2 and O_3 were checked in the same way.

The final atomic coordinates, as fractions of corresponding cell edges and referring to a centre of symmetry as origin, are listed in Table 1.

Table 1. Atomic coordinates for barium selenopentathionate dihydrate.

Fourfold positions				*Eightfold positions*			
	x	*y*	*z*		*x*	*y*	*z*
Ba	0.227	0.250	−0.049	S_2	0.285	0.088	0.181
Se	0.561	0.250	0.177	S_1	0.305	0.012	0.092
$(H_2O)_1$	0.743	0.250	0.010	O_1	0.590	0.005	0.076
$(H_2O)_2$	0.320	0.250	−0.160	O_2	0.180	−0.106	0.098
				O_3	0.177	0.095	0.054

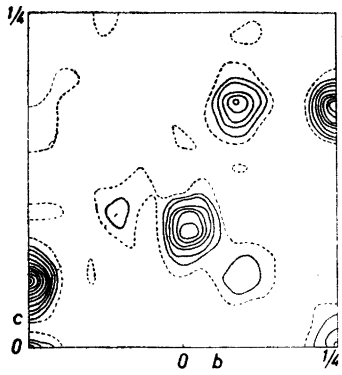

Fig. 1. Electron density projection of BaSe $(S_2O_3)_2 \cdot 2H_2O$ along the a *axis,* ϱ(yz). *One asymmetric unit is shown. Contour intervals: 10 e·Å^{-2} for the barium ion, 6 e·Å^{-2} for the selenium atom, and 4 e·Å^{-2} for the sulphur and oxygen atoms and water molecules. The 4-electron line is dashed.*

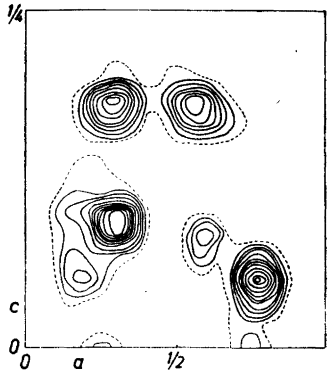

Fig. 2. Electron density projection of BaSe $(S_2O_3)_2 \cdot 2H_2O$ along the b *axis,* ϱ(xz). *One asymmetric unit is shown. The 8-electron line is dashed, and contour intervals are as in Fig. 1.*

The atomic scattering curves of the *International Tables* were employed for the calculation of structure factors, with the xenon curve in the case of the barium ion, and a temperature factor of $B = 2.0$ Å^2 for both zones. The reliability factor, $R = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$, with non-observed reflections included when $|F_{calc}|$ is larger than the smallest observable value of $|F_{obs}|$, is 0.18 for the $0kl$ reflections and 0.23 for the $h0l$ reflections. A list of observed and calculated structure factors can be supplied by the authors on request.

THE SELENOPENTATHIONATE ION

The ion is built up of an unbranched and non-planar S—S—Se—S—S chain, and possesses, by space group requirements, a mirror plane of symmetry. Distances and angles, calculated on the basis of the coordinates of Table 1, are given below. The numbering of atoms, and the outline of the ion in the two projections, are shown in Figs. 3 and 4. A prime denotes the image of an atom across the mirror plane. It is believed that the error in the Se—S and S—S bond lengths is less than 0.04 Å, and in the S—S—Se and S—Se—S bond angles less than about 3°. The errors in S—O and O—O distances and angles may be considerably greater due to the overlapping of atoms and the obscuring effect of the heavy atoms.

Table 2. Sulphur-sulphur and selenium-sulphur bond lengths and bond angles.

$S_1—S_2 = 2.13$ Å	$\angle S_1S_2Se = 103°$	S_1S_2Se/S_2SeS_2'
$S_2—Se = 2.17$	$\angle S_2SeS_2' = 101°$	(dihedral angle) = 109°

Non-bonded distances.

$S_1—S_1' = 4.93$ Å	$S_1—Se = 3.36$ Å
$S_1—S_2' = 4.59$	$S_2—S_2' = 3.36$

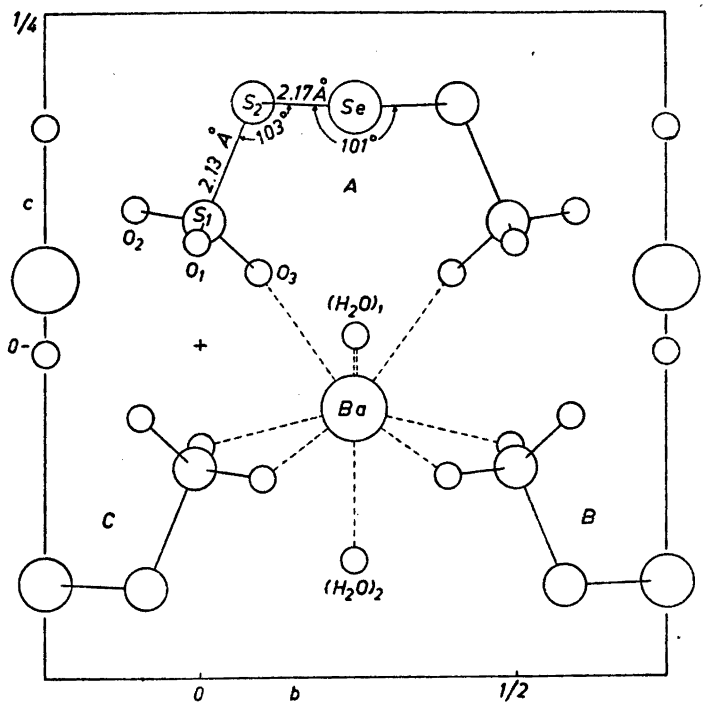

Fig. 3. Arrangement of the ions as seen along the a axis. Half a unit cell (four asymmetric units) is shown. Origin at the midpoint of the left half of the figure.

The Se—S bond is a little shorter than corresponding to the sum of the single bond radii [8], *i.e.*, 2.21 Å. The difference is within the experimental error, but similar shortenings, relative to the single bond lengths, were observed for the middle S—S bonds in the isomorphous crystals of barium pentathionate dihydrate [2], and for the Te—S bonds in ammonium telluropentathionate [3].

The S_2SeS_2' bond angle, 101°, differs slightly from the corresponding angle, $\angle S_2S_3S_2' = 106°$, in the pentathionate. It is likely that the smaller value found for the selenium bond angle is significant.

The crystal structures of two compounds containing Se—S bonds, *viz.*, selenium dibenzenesulphinate [9] and selenium dithiocyanate [10], have been described in literature earlier. The Se—S bond lengths observed are 2.20 Å and 2.21 Å, respectively, and the S—Se—S angles, 105° and 101°. As for the benzenesulphinate, the bonds from selenium are to sulphonyl sulphur and not to divalent sulphur, and the data are therefore not strictly comparable.

The oxygen atoms and the terminal sulphur atoms, together with the divalent sulphur atoms situated next to the selenium atom, constitute two thiosulphate groups. The shape of these is a distorted tetrahedral one. From the data of Table 3 it appears that the average S—O bond length is 1.40 Å. The difference between the individual values may, partly at least, be due to

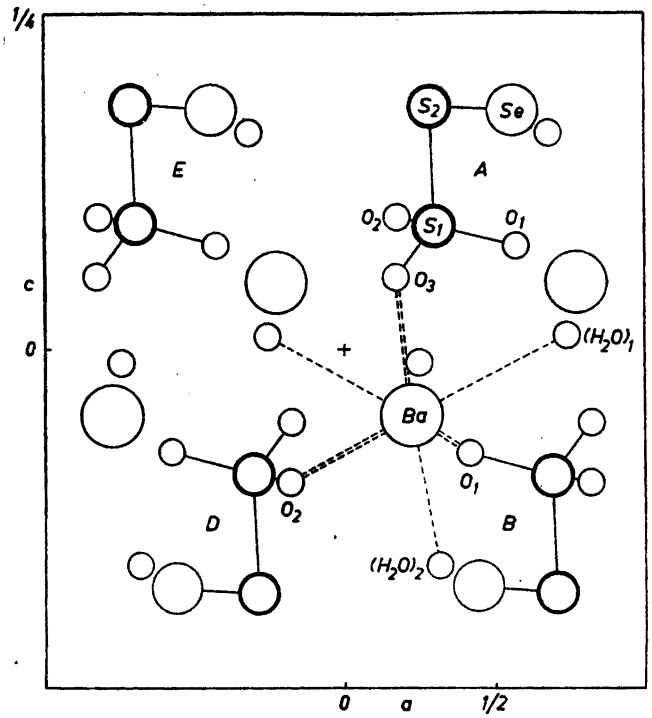

Fig. 4. Arrangement of the ions as seen along the b *axis. Four asymmetric units are shown. In this projection, two sets of sulphur atoms and sulphonate oxygen atoms overlap exactly due to the mirror plane normal to the* b *axis. The barium-sulphonate oxygen approaches, which occur once on each side of the mirror plane, are indicated by double lines (broken). Origin at the midpoint of the figure.*

experimental errors. A larger x coordinate of S_1 would have brought the S—O bond lengths closer to the average, but such a change, when tried, was found to cause a higher value of the reliability factor.

Table 3. Sulphur-oxygen bond lengths and angles.

$S_1-O_1 = 1.47$ Å	$\angle O_1S_1O_2 = 115°$	$\angle S_2S_1O_1 = 105°$
$S_1-O_2 = 1.38$	$\angle O_1S_1O_3 = 111°$	$\angle S_2S_1O_2 = 103°$
$S_1-O_3 = 1.36$	$\angle O_2S_1O_3 = 114°$	$\angle S_2S_1O_3 = 109°$

Non-bonded distances within the thiosulphate groups.

$S_2-O_1 = 2.91$ Å	$O_1-O_2 = 2.40$ Å
$S_2-O_2 = 2.78$	$O_1-O_3 = 2.32$
$S_2-O_3 = 2.87$	$O_2-O_3 = 2.30$

The non-bonded distances from the selenium atom to the oxygen atoms O_1, O_2 and O_3 are 3.39 Å, 4.50 Å and 3.70 Å, respectively. These distances are dependent on rotations about the S_1—S_2 bond. The shortest approach between the two sulphonate groups of a selenopentathionate ion is O_3—O_3' = 3.21 Å, which is the same value as found in barium pentathionate dihydrate.

THE BARIUM-OXYGEN COORDINATION

The relative arrangement of the selenopentathionate ions, the barium ions and the water molecules, as seen along the *a* and *b* axes, is shown in Figs. 3 and 4. The environment of a barium ion is, when allowance is made for minor variations in distances and angles, exactly the same as described earlier [2] for the isomorphous structure of the pentathionate. Thus, each barium ion is surrounded by nine oxygen atoms, six of which are sulphonate oxygen atoms and three are water oxygen atoms.

In Table 4, the letters *A* to *E* refer to different selenopentathionate ions, as shown in the figures. Distances listed in the left column of the table occur two times, once on each side of the mirror plane.

Table 4. Barium-oxygen distances.

$Ba-O_{3A} = 2.81$ Å	$Ba-(H_2O)_{1A} = 2.89$ Å
$Ba-O_{1B} = 2.86$	$Ba-(H_2O)_{1E} = 2.74$
$Ba-O_{2D} = 2.74$	$Ba-(H_2O)_{2A} = 2.51$

The water molecule, $(H_2O)_1$, is coordinated to two barium ions, in adjacent unit cells along the *a* axis, at distances of 2.89 Å and 2.74 Å and an angle of 124°. This water molecule probably forms hydrogen bonds to the oxygen atoms, O_1 and its image across the mirror plane in which the water molecule and the barium ions are located. The distance, $(H_2O)_1—O_1$, is 3.05 Å and the $O_1—(H_2O)_1—O_1'$ angle is 113°. It may be noted that $(H_2O)_1—O_3 = 3.39$ Å and $\angle O_3—(H_2O)_1—O_3' = 57°$, so that hydrogen bonding to O_3 and O_3' instead of to O_1 and O_1' is unlikely.

The second water molecule does apparently not form any hydrogen bonds. The distance from this water molecule to the barium ion, 2.51 Å, is very short and may be subject to maximum errors, since the *z* coordinate and particularly the *x* coordinate of $(H_2O)_2$ are probably the most uncertain ones in the structure.

As in the pentathionate structure, weak van der Waals contacts only occur across the glide plane *a*, normal to the *c* axis at $z = \frac{1}{4}$ and $-\frac{1}{4}$. The shortest approaches are Se—Se″ = 4.09 Å, S_2—Se″ = 3.74 Å and $S_2—S_2'' = 3.95$ Å, where a double-prime denotes the equivalent of an atom produced by the operation of the glide plane. The result is a layer structure which accounts for the ready cleavage of the crystals along the *c* plane. The thickness of the layers is half the *c* axis, 10.10 Å.

ROTATIONAL ISOMERISM

With the present work, the crystal structure of a salt of each of the pentathionic acids has been determined, *i.e.*, the parent acid and those derived from it by substitution of selenium and tellurium for the middle sulphur atom. In the salts, the anions are built up of unbranched and non-planar chains.

As noted earlier [11], such chains may exist in rotational-isomeric forms. The terminal sulphur atoms are rotated out of the plane of the three middle atoms, either to the same side of the plane — *cis* — or to opposite sides — *trans*.

The pentathionate and selenopentathionate ions in the isomorphous barium salts occur in the *cis* form, the ions being located in a crystallographic mirror plane. The occurrence of *cis* forms in these salts is perhaps due to the oxygen-coordinating powers of the barium ion, the anions in the *cis* form being able to act, in a sense, as bidentate chelating agents.

In the ammonium salt [3], $(NH_4)_2Te(S_2O_3)_2$, the telluropentathionate ion has a *trans* configuration, as contrasted with the pentathionate and selenopentathionate ions in the barium salts.

Unit cell and space group data are available for some other salts of these acids [1]. The following hemitrihydrates are isomorphous:

$K_2S(S_2O_3)_2 \cdot 1\frac{1}{2}H_2O$	$(NH_4)_2Se(S_2O_3)_2 \cdot 1\frac{1}{2}H_2O$	$Cs_2Te(S_2O_3)_2 \cdot 1\frac{1}{2}H_2O$
$Rb_2S(S_2O_3)_2 \cdot 1\frac{1}{2}H_2O$	$Rb_2Se(S_2O_3)_2 \cdot 1\frac{1}{2}H_2O$	$Rb_2Te(S_2O_3)_2 \cdot 1\frac{1}{2}H_2O$

The space group is D_{2h}^{14}—*Pbcn*, and there are eight molecules per unit cell. The crystal structure has not yet been worked out, and no molecular symmetry is crystallographically required, so it is not known whether the configuration is *cis* or *trans*. It is evident, however, that the three anions have the same configuration in the isomorphous salts. Therefore, either the pentathionate and selenopentathionate anions, or the telluropentathionate anion, must be able to occur in different forms in different salts.

It is perhaps to be expected, for all three anions, that *trans* forms occur in alkali salts, and *cis* forms in salts of the alkali earth metals. Preliminary experiments indicate that crystals of strontium and barium telluropentathionate may be obtained which are isomorphous with those of barium pentathionate and selenopentathionate dihydrate.

In solutions of salts of these acids, equilibrium mixtures of *cis* and *trans* forms probably occur. Inferring, however, from the structure of the anions in the crystalline salts, the nature of the cation must be expected to influence the position of the equilibria in solutions. Thus, in solutions of strontium and barium salts the relative amounts of the *cis* form should be larger than in solutions of the alkali salts.

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The Interaction between Halogen Acids and Nitro-amines

IV *. The Reactivity of Some Nitronaphthylamines

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The reaction between hydrochloric acid and some mononuclear-substituted mono- and dinitronaphthylamines has been investigated and discussed. When treated with hydrochloric acid, 2-nitro-1-naphthylamine and 1-nitro-2-naphthylamine are converted to diazonium salts while 3-nitro-2-naphthylamine remains unchanged. The *m*-nitronaphthylamines and *p*-nitronaphthylamine do not react. All three dinitro-1-naphthylamines were investigated and found to give diazonium salts.

3,4-Dinitro-1-naphthylamine was also subjected to the action of hydrobromic acid. Diazonium salts are formed but other reactions simultaneously take place. The reaction products which were isolated indicate the formation of a brominating agent obviously originating from the reaction between hydrobromic acid and the displaced nitro-group.

In connexion with this investigation 2,3- and 3,4-dinitro-1-naphthylamine have been prepared.

The present part of this series deals with the reaction between hydrochloric acid and some mononuclear-substituted mono- and dinitronaphthylamines. 3,4-Dinitro-1-naphthylamine, the most reactive of the investigated amines, was also reacted with hydrobromic acid.

PREPARATION OF THE NITRONAPHTHYLAMINES

With the exception of 2,3- and 3,4-dinitro-1-naphthylamine the compounds investigated were prepared according to methods reported in the literature.

Hodgson and Turner [1] claimed to have prepared 2,3-dinitro-1-naphthylamine by nitration of 3-nitro-1-acetnaphthalide and subsequent hydrolysis of the resulting 2,3-dinitro-1-acetnaphthalide. Several authors [2–5] seem to have been able to repeat these experiments. The present author has been unable to

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do this. Ward and Coulson [6] reinvestigated the nitration under the conditions used by Hodgson and Turner and obtained a reaction product consisting of a mixture of 3,5- and 3,8-dinitro-1-acetnaphthalide.

In the present investigation the nitration of 3-nitro-1-acetnaphthalide was carried out in glacial acetic in the presence of boron trifluoride giving in good yield a mixture of 2,3- and 3,4-dinitro-1-acetnaphthalide (ratio *ca.* 1 : 4). The isomers were separated by taking advantage of their different rates of deacylation to the corresponding amines. Owing to the mobility of the 4-nitro-group, special methods must be used for the deacylation of 3,4-dinitro-1-acetnaphthalide. Two useful methods were found, namely heating its solution in alcohol either with sodium alcoholate [7] or with boron trifluoride [8].

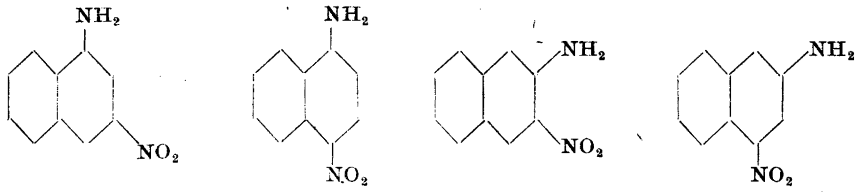
To establish the structures of the amines they were oxidized with aqueous potassium permanganate. Both isomers yielded *o*-phthalic acid which proves that they are mononuclear-substituted derivatives of naphthalene and, consequently, the structures must be 2,3- and 3,4-dinitro-1-naphthylamine. These structures were suggested already by the fact that both amines, when treated with hydrochloric acid, replace one nitro-group by chlorine with formation of diazonium salts (*cf.* below). Hitherto this reaction has been observed only for amines having a nitro-group in the *ortho* or *para* position relative to the amino-group. The structure 3,4-dinitro-1-naphthylamine is ascribed to the amine which, when treated with hydrochloric acid, yields 4-chloro-3-chloronitronaphthalene-1-diazonium chloride.

Hodgson and Turner [1] reported, for 2,3-dinitro-1-acetnaphthalide, a m.p. of 275°. The same compound (m.p. 275.5°) was obtained by Hodgson and Elliott [9] by nitration of 3-chloro-1-acetnaphthalide with nitric acid. The 2,3-dinitro-1-acetnaphthalide obtained in the present investigation melted at *ca.* 254° (decomp.) and according to a determination of the mixed melting point carried out by Ward [10] it is definitively not identical with the 2,3-dinitro-1-acetnaphthalide prepared by Hodgson and Elliott. Moreover, the corresponding amines also differ considerably. That prepared by Hodgson and Turner melted at 160—61° while that obtained by the author melted at *ca.* 233° (decomp.).

It may be mentioned that 3,4-dinitro-1-naphthylamine (but not 2,3-dinitro-1-naphthylamine) shows chromo-isomerism. Crystals separating from hot toluene or xylene solutions are orange-yellow while those from cold solutions are bright red. When heated to about 190° the red form turns orange-yellow and this colour persists on cooling.

THE REACTION BETWEEN HYDROCHLORIC ACID AND THE NITRONAPHTHYLAMINES

In the investigation of the reactivity of the nitronaphthylamines, the methods described in the previous part of this series [11] were used (*cf.* also the experimental part of the present paper). Of the amines investigated, the following remain unchanged when kept for two hours at 100° in a mixture of glacial acetic and hydrochloric acids:



The amines which give diazonium salts on similar treatment are listed in Table 1. A rough idea of the reactivity of the nitro-amines is obtained from the curves in Figs. 1—2, where the amounts of diazonium salts present in the reaction solution are plotted against the time of reaction. Only 1-nitro-2-naphthylamine and 3,4-dinitro-1-naphthylamine are reactive enough to give diazonium salts at 40° (Fig. 1); the others require a higher temperature and were, therefore, reacted at 80° (Fig. 2). Owing to the relatively low solubility of some of the nitronaphthylamine hydrochlorides the experiments had to be carried out in solutions which were more dilute than those used for the dinitro anilines. For comparison 3,4-dinitroaniline was reacted at the concentrations used for the nitronaphthylamines and the corresponding curves are shown in Figs. 1—2.

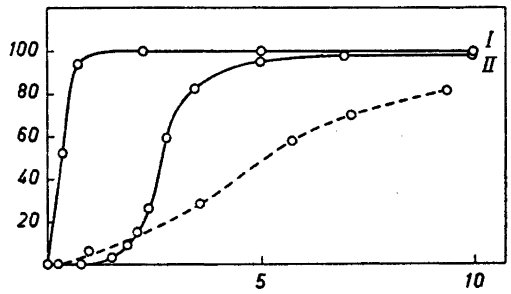


Fig. 1. Relation between yields of diazonium salts in per cent of the theoretical (ordinate) and time of reaction in hours (abscissa). Reaction temperature 40° C. I. 3,4-Dinitro-1-naphthylamine. II. 1-Nitro-2-naphthylamine. — — — 3,4-Dinitroaniline.

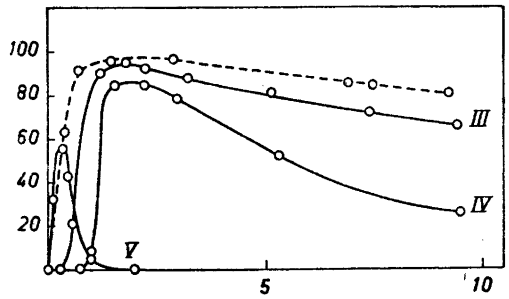
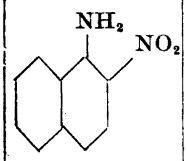
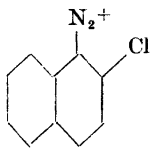
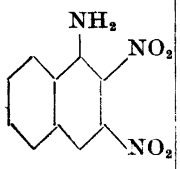
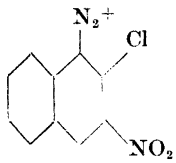
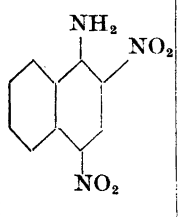
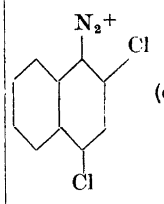
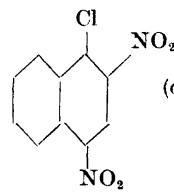
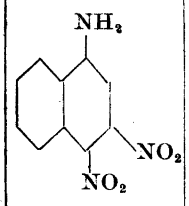
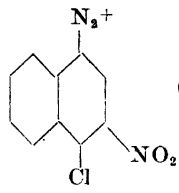
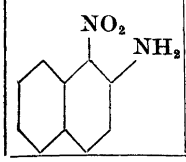
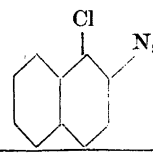


Fig. 2. Coordinates the same as in Fig. 1. Reaction temperature 80° C. III. 2-Nitro-1-naphthylamine. IV. 2,4-Dinitro-1-naphthylamine. V. 2,3-Dinitro-1-naphthylamine. — — — 3,4-Dinitroaniline.

Table 1. Compounds obtained from the nitronaphthylamines which on treatment with hydrochloric acid give diazonium salts.

Nitronaphthyl-amine	Temp. °C	Time min.	Reaction products
	80	100	 (*ca.* 95 %) *
	80	25	 (*ca.* 55 %)
	80	120	 (*ca.* 85 %);  (*ca.* 2 %)
	40	60	 (*ca.* 100 %
	40	360	 (*ca.* 100 %)

* The yields of diazonium salts correspond to the maxima of the curves in Figs. 1—2.

The products formed from the reactive nitronaphthylamines are listed in Table 1. The methods used for identification of the products appear from the experimental section but some comments will be given here.

In the cases of 2-chloro-, 2,4-dichloro- and 4-chloro-3-nitronaphthalene-1-diazonium chloride, the diazonium group was replaced by hydrogen by reduction with hypophosphorous acid; 1-chloronaphthalene-2-diazonium chloride was converted to 1-chloro-2-iodonaphthalene with potassium iodide. The

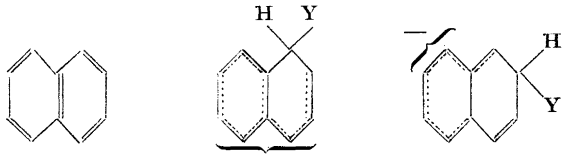
compounds were identified by determination of the mixed melting points with authentic samples. The diazonium salt formed from 2,3-dinitro-1-naphthylamine decomposes rapidly at the reaction temperature (80°) and its structure was, therefore, determined in a different way. When after three hours the presence of diazonium salt could no longer be detected the solution was worked up and a chloronitronaphthol and a small amount of the starting material were isolated. The naphthol was characterized by its methyl ether but the structure has not been rigorously proved. However, in view of the fact that, by the reaction between hydrochloric acid and nitro-amines, only nitro-groups *ortho* or *para* to the amino-group have hitherto been found to be replaced by chlorine and that, in all cases investigated, the chlorine enters the position vacated by the nitro-group, the structure of this naphthol is most probably 2-chloro-3-nitro-1-naphthol.

The chain mechanism postulated for the other nitro-amines investigated [11-12] is assumed to operate also in the case of the nitronaphthylamines.

The investigation has shown that some of the nitronaphthylamines are more reactive than the corresponding derivatives of aniline. Thus 2-nitro-1-naphthylamine and 1-nitro-2-naphthylamine react smoothly at 80° and 40°, respectively, whereas *o*-nitroaniline fails to react even at 100°. Further, 3,4-dinitro-1-naphthylamine is more reactive than 3,4-dinitroaniline. 3-Nitro-2-naphthylamine and 4-nitro-1-naphthylamine resemble *o*- and *p*-nitroaniline in remaining unchanged at 100°. 2,3-Dinitro-1-naphthylamine is less reactive than 2,3-dinitroaniline which reacts rather rapidly even at 40° (*cf.* part III[11]).

The relatively high reactivity of the 1,2-derivatives may be attributed to the greater multiplicity of a 1,2-bond of naphthalene as compared with that of a 2,3-bond (the multiplicities given by Coulson, Daudel and Robertson [13] are 1.725 and 1.603, respectively). This property of a 1,2-bond may also be responsible for the apparently great effect exerted by the 3-nitro-group in the case of 3,4-dinitro-1-naphthylamine. On the other hand, the relatively small multiplicity of the 2,3-bond may contribute to the nonreactivity of 3-nitro-2-naphthylamine and to the comparatively low reactivity of 2,3-dinitro-1-naphthylamine.

The different reactivities of the 1,2-derivatives indicate that a nitro-group in the 1-position is more easily replaced than one in the 2-position. This was to be expected from the theory presented by Dewar [14]. He has discussed the nucleophilic substitution in naphthalene and from the differences in the resonance energies of the parent compound (I) and the transition states (II, III) he concludes that substitution should take place more readily in the 1-position.



I II III

63 Cal 61 Cal 52 Cal

It should be pointed out that the velocity of diazonium salt formation is not only dependent on the replaceability of the nitro-group but also on other factors (*cf.* part III[11]). For instance, the rate of decomposition of the diazonium salt always is of importance and, as in the case of 2,3-dinitro-1-naphthylamine, the reaction products can apparently retard the process. This amine gave diazonium salt in a yield of about 55 % within 20 minutes but nevertheless small amounts of the starting material could be isolated from the reaction solution even after three hours. From this it is obvious that only an approximative comparison of the ease of replaceability of the nitro-groups may be made on the basis of the curves in Figs. 1—2.

THE REACTION BETWEEN HYDROBROMIC ACID AND 3,4-DINITRO-1-NAPHTHYLAMINE

In the replacement experiments already mentioned, hydrochloric acid was employed. In the case of 3,4-dinitro-1-naphthylamine, the most reactive of the investigated amines, the reaction with hydrobromic acid was also studied. In an experiment carried out at 80°, the presence of diazonium salt could be demonstrated even after three minutes. Large amounts of diazonium salts, however, were never present in the solution, and after three hours none could be detected. After another two hours the solution was worked up. By chromatography (Al_2O_3, benzene), fractions accounting for about 45 % of the adsorbed amount of the reaction product were isolated. The product obtained by elution of the first zone, (about 26 % of the adsorbed amount), yielded by repeated chromatography (Al_2O_3, light petroleum) three fractions, the analyses of which suggested that the main products were mono-, di- and tribromonitronaphthalene, respectively. The results are displayed in Table 2. Each fraction is named from the compound which may be the main component. The yields which are given in per cent of the total amount of the reaction product adsorbed are rather approximate.

Table 2. Compounds formed in the reaction between hydrobromic acid and 3,4-dinitro-1-naphthylamine

Fraction	M. p. °C	Yield %
Tribromonitronaphthalene	208—11	3
Dibromonitronaphthalene	115—45	8
Monobromonitronaphthalene	78—85	6
Dibromonitronaphthylamine	165—68	4
Monobromodinitronaphthylamine	239—40 (decomp.)	11
3,4-Dinitro-1-naphthylamine	199 (decomp.)	2

As indicated by the analyses and the melting points, the fractions, with the exception of 3,4-dinitro-1-naphthylamine and probably also of monobromodinitronaphthylamine, consisted of more than one compound. Nevertheless, the results give some information about the reaction.

The presence of tribromonitronaphthalene, dibromonitronaphthylamine and monobromodinitronaphthylamine indicates the formation of a brominating agent, obviously originating from the reaction between hydrobromic acid and the displaced nitro-group. This consumption of the diazotising agent inhibits the formation of the 3,4-dinitronaphthalene-1-diazonium ion which is an intermediate of importance for the total rate of the process. Therefore, the rate should be lower when hydrobromic acid is used and as a matter of fact some starting material could be recovered even after five hours (80°). For comparison, 3,4-dinitro-1-naphthylamine, when treated with hydrochloric acid at 40°, was quantitatively converted to 4-chloro-3-nitronaphthalene-1-diazonium chloride in about one hour.

The reaction mechanism outlined here is the same as that postulated for the reaction between hydrobromic acid and 2,3-dinitroaniline (*cf.* part III[11]). However, bromoderivatives indicating the formation of a brominating agent could not be isolated in that case.

The formation of a monobromonitronaphthalene is remarkable. This means that one of the nitro-groups or the amino-group has been replaced by hydrogen. For comparison, it may be mentioned that, on treatment with hydrochloric acid in the presence of *p*-chloroaniline, 4,9-dinitro-3-aminoretene is partly converted to 9-nitro-3-aminoretene [12].

As mentioned above the chromatographically isolated products accounted for only about 45 % of the reaction product. The remaining 55 %, occurring in several dark zones in the top of the column, was not further investigated.

EXPERIMENTAL *

Preparation of the nitronaphthylamines

The syntheses of *2-* and *4-nitro-1-naphthylamine* followed the method of Hodgson and Walker [15] and the compounds were purified by two crystallizations from xylene, followed by chromatography (Al_2O_3, benzene). 2-Nitro-1-naphthylamine melted at 144—45° and 4-nitro-1-naphthylamine at 196—97°.

3-Nitro-1-naphthylamine and *4-nitro-2-naphthylamine* were prepared by partial reduction of 1,3-dinitronaphthalene as described by Hodgson and Birtwell [16]. The amines were crystallized twice from 80 % ethanol and melted at 136—37° and 99—100°, respectively.

1-Nitro-2-naphthylamine. 1-Nitro-2-acetnaphthalide, prepared by the method reported in Org. Syntheses [17], was hydrolyzed as described by Schiemann and Ley [18]. The amine so obtained melted at 127—28.5°.

3-Nitro-2-naphthylamine. Verkade *et al.*, the first to prepare this compound, and Ward (*cf.* Ref.[10]) kindly placed samples at my disposal.

2,4-Dinitro-1-naphthylamine was prepared by the improved method presented by Hodgson and Birtwell [20]. After two crystallizations from glacial acetic acid it melted at 244—45°.

2,3-Dinitro-1-naphthylamine and *3,4-dinitro-1-naphthylamine.* 3-Nitro-1-acetnaphthalide (6.90 g, 0.03 mole) was suspended in glacial acetic acid (90 ml) containing boron trifluoride (9.0 g). A mixture of nitric acid (3.20 g, 0.05 mole, *d* 1.5), glacial acetic acid (30 ml), and boron trifluoride (3.0 g) was added and, while stirring, the suspension was heated to 65—70°. A clear solution was obtained in 10—15 minutes (in some experiments, where the nitration did not start at this temperature, a crystal of sodium nitrite was added). After 5 minutes the solution was cooled and poured into water (1 000 ml). The yellow precipitate formed (6.60 g) was collected, washed with water, dried and crystal-

* All melting points are corrected.

lized from glacial acetic acid (110 ml) giving a yellow well-crystalline product (5.70 g), m. p. *ca.* 235° (decomp.). The product was dissolved in hot glacial acetic acid (200 ml), the solution allowed to cool to 45—50° and the crystals which separated collected. After another crystallization from glacial acetic acid (85 ml), pure 3,4-dinitro-1-acetnaphthalide (1.60 g), m. p. *ca.* 263° (decomp.), was obtained as yellow needles. (Found: N 15.0. Calc. for $C_{12}H_9O_5N_3$ (275.2): N 15.3).

The combined filtrates were diluted with water, most of the acid neutralized, and the yellow precipitate formed (3.85 g) collected, washed with water, dried, and refluxed 40 minutes with methanol (115 ml) containing boron trifluoride (11.5 g) to deacylate 3,4-dinitro-1-acetnaphthalide. The red solution so obtained was diluted with water and the precipitated yellow product collected, washed with water, dried, and finely suspended in ether (700 ml). The suspension was refluxed and the insoluble product consisting of almost pure 2,3-dinitro-1-acetnaphthalide (1.40 g), m. p. *ca.* 250° (decomp.) was crystallized from glacial acetic acid (25 ml) giving light yellow needles (1.20 g), m. p. *ca.* 254° (decomp.). (Found: N 15.3. Calc. for $C_{12}H_9O_5N_3$ (275.2): N 15.3).

The residue obtained on evaporation of the ether consisted of 3,4-dinitro-1-naphthylamine (2.00 g), m. p. 185—87° (decomp.), which, after one crystallization from glacial acetic acid (25 ml), was obtained as reddish-brown flat needles (1.20 g), m. p. *ca.* 202° (decomp.) (the solubility of 3,4-dinitro-1-naphthylamine in ether is about 3 g/l). A sample was crystallized twice from *n*-propanol (charcoal) and formed orange-yellow needles, m. p. *ca.* 205° (decomp.). (Found: C 51.6; H 2.85; N 17.8. Calc. for $C_{10}H_7O_4N_3$ (233.2): C 51.5; H 3.03; N 18.0). From rapidly chilled toluene or xylene solutions, the amine was obtained in the form of bright red crystals. From hot solutions the orange-yellow form separated.

2,3-Dinitro-1-acetnaphthalide was hydrolyzed by dissolving the compound (1.0 g) in ethanol (80 ml), adding 50 % sulphuric acid (80 ml) and refluxing the solution for 3 hours. After dilution of the solution with water the precipitated 2,3-dinitro-1-naphthylamine (0.80 g), m. p. 225—227° (decomp.), was collected, washed with water, dried and crystallized from *n*-propanol (40 ml) giving yellow needles (0.62 g), m. p. *ca.* 233° (decomp.). (Found: C 51.3; H 2.99; N 18.2. Calc. for $C_{10}H_7O_4N_3$ (233.2): C 51.5; H 3.03; N 18.0).

3,4-Dinitro-1-acetnaphthalide was deacylated by refluxing the compound in methanol containing boron trifluoride, as described by Sihlbom [8].

The oxidation of the two amines for establishing the orientation of the nitro-groups was carried out in the following way (*cf.* Hodgson and Hathway [21]). A mixture of finely powdered dinitronaphthylamine (0.15 g), potassium permanganate (0.7 g) and water (10 ml) was refluxed for 5 hours. It was worked up as described by Hodgson and Hathway and yielded colourless crystals (0.04 g), m. p. 199°, undepressed on admixture with *o*-phthalic acid.

The structure 3,4-dinitro-1-naphthylamine was ascribed to the amine which, when treated with hydrochloric acid, yielded 4-chloro-3-nitronaphthalene-1-diazonium chloride (*cf.* below).

The reaction between hydrochloric acid and the nitronaphthylamines

Samples (0.10 g) of *3-* and *4-nitro-1-naphthylamine* and of *4-nitro-2-naphthylamine* in a mixture of glacial acetic acid (10 ml) and hydrochloric acid (2 ml, *d* 1.19) were heated to 100° for 2 hours in sealed tubes. The reaction mixtures were diluted with water, neutralized and the precipitated product collected, washed with water and dried. In each case only the starting material was obtained.

3-Nitro-2-naphthylamine (20 mg) was treated as described above. The acids were evaporated and the almost colourless needles obtained dissolved in ethanol. After the addition of some drops of ammonia and dilution with water a red crystalline product (17 mg) containing no chlorine separated. It melted at 114—15°, undepressed on admixture with 3-nitro-2-naphthylamine.

When treated with hydrochloric acid in glacial acetic acid solution, *2-nitro-1-naphthylamine, 1-nitro-2-naphthylamine, 2,4-* and *3,4-dinitro-1-naphthylamine* were quantitatively consumed and diazonium salts were formed. Similarly, *2,3-dinitro-1-naphthylamine* gave

a diazonium salt; the reaction was rapid in the beginning but soon slowed down. Even after 3 hours, unchanged dinitronaphthylamine was present in the solution.

The rate of diazonium salt formation was determined as described in the preceding paper of this series [11]. The amine (0.003 mole; in the case of the slightly less soluble 3,4-dinitro-1-naphthylamine 0.002 mole) was dissolved in glacial acetic acid (84 ml) and 12 *M* hydrochloric acid (17 ml) was added. The reaction solution was kept in a well closed flask. At suitable intervals samples were taken out and the amount of diazonium salt formed determined. 1-Nitro-2-naphthylamine and 3,4-dinitro-1-naphthylamine were allowed to react at 40° ± 0.2°. In the cases of 2-nitro-, 2,3- and 2,4-dinitro-1-naphthylamine, no diazonium salt was formed at this temperature and the reaction was, therefore, carried out at 80° ± 0.2°. In the curves in Figs. 1—2, the yields of diazonium salts are plotted against time of reaction.

The products formed when the reactive nitronaphthylamines were treated with hydrochloric acid were identified in the following way.

2-Nitro-1-naphthylamine (0.003 mole) was dissolved in glacial acetic acid (84 ml) and the solution was mixed with conc. hydrochloric acid (17 ml) and heated in a sealed flask for 2 hours. The reaction solution was then diluted with water (400 ml), extracted with ether to remove a small amount of impurities originating from that fraction of the diazonium salt which had already decomposed, mixed with 50 % hypophosphorous acid (25 ml), kept at 5—10° overnight and again extracted with ether. The latter ether solution was washed with aqueous sodium hydroxide and water, dried over Na_2SO_4 and decolourized with a little charcoal. Evaporation of the ether yielded almost colourless crystals (0.35 g, 72 %), m. p. 49—51°, which were crystallized from light petroleum giving leaflets, m. p. 55—56°, undepressed on admixture with 2-chloronaphthalene.

1-Nitro-2-naphthylamine (0.005 mole) was dissolved in glacial acetic acid (84 ml), conc. hydrochloric acid (17 ml) was added and the solution kept at 40° for 10 hours. A saturated aqueous potassium iodide solution (50 ml) was added while stirring, causing rapid decomposition of the diazonium salt. After 3 hours, water (100 ml) was added and the product precipitated was collected, washed with water, dried, and extracted with ligroin. On evaporation of the solvent a crystalline substance (1.10 g), m. p. 73—76°, was obtained. One crystallization from ethanol (10 ml) gave light orange-yellow needles (0.65 g, 45 %), m. p. 82—83.5°, undepressed on admixture with 1-chloro-2-iodonaphthalene.

2,3-Dinitro-1-naphthylamine (0.001 mole) was dissolved in glacial acetic acid (30 ml), conc. hydrochloric acid (6 ml) added and the solution kept in a closed flask at 80° for 3 hours. After dilution with water most of the acids were neutralized and the separated product (0.17 g) was collected, washed with water and dried. The product was dissolved in ether and the solution saturated with ammonia. The red precipitate formed was collected and suspended in ether. The introduction of hydrogen chloride yielded a yellow solution; the ammonium chloride was removed and the ether evaporated. An orange coloured crystalline residue (65 mg, 29 %), m. p. 154—55°, consisting of 2-chloro-3-nitro-1-naphthol was obtained. From ligroin-benzene it separated as orange coloured needles. (Found: C 53.6; H 2.69; N 6.40; Cl 16.0. Calc. for $C_{10}H_6O_3NCl$ (233.6): C 53.8; H 2.59; N 6.27; Cl 15.9).

The methyl ether. 2-Chloro-3-nitro-1-naphthol (25 mg) was dissolved in ether (15 ml) containing excess of diazomethane and the solution was kept overnight. Evaporation of the ether yielded a light-yellow product (25 mg), m. p. 75—77°. After one crystallization from ethanol light-yellow needles, m. p. 86—87° were obtained. (Found: C 55.6; H 3.57. Calc. for $C_{11}H_8O_3NCl$ (237.6): C 55.6; H 3.40).

The above ether solution, from which the ammonium naphtholate had been removed, yielded on evaporation of the ether a product which after one crystallization from benzene gave yellow needles (15 mg), m. p. *ca.* 228° (decomp.), undepressed on admixture with 2,3-dinitro-1-naphthylamine.

2,4-Dinitro-1-naphthylamine (4.0 g) was dissolved in glacial acetic acid (480 ml), conc. hydrochloric acid (100 ml) was added and the solution kept in a closed flask at 80° for 2 hours. The solution was cooled, diluted with water (2 000 ml), extracted with ether, mixed with 50 % hypophosphorous acid (100 ml), kept at 5—10° for 10 hours and again extracted with ether. Both ether solutions were washed with aqueous sodium hydroxide and water, dried over Na_2SO_4 and decolourized with a little charcoal. The ether was then evaporated.

The yellow product (0.20 g) from the first ether extract was triturated with light petroleum and crystallized from benzene giving light-yellow crystals (70 mg), m. p. 146—47°, undepressed on admixture with 1-chloro-2,4-dinitronaphthalene.

The second ether extract yielded almost colourless crystals (2.20 g, 65 %), m. p. 62—63°, undepressed on admixture with 1,3-dichloronaphthalene.

3,4-Dinitro-1-naphthylamine (0.001 mole) was dissolved in warm glacial acetic acid (42 ml) and conc. hydrochloric acid (8.5 ml) was added. The solution was kept at 40° for 1 hour, then cooled, mixed with 50 % hypophosphorous acid (20 ml), kept at room temperature for 2 hours, diluted with water (200 ml) and neutralized. The precipitate formed was collected, washed with water and dried. The product (0.15 g), m. p. 74—75°, was further purified by chromatography (Al_2O_3, benzene) giving a light-yellow product (0.12 g, 58 %), m. p. 80—81°, undepressed on admixture with 1-chloro-2-nitronaphthalene.

The reaction between hydrobromic acid and 3,4-dinitro-1-naphthylamine

3,4-Dinitro-1-naphthylamine (0.004 mole) was dissolved in hot glacial acetic acid (90 ml), 40 % hydrobromic acid (18 ml) was added and the solution then kept at 80°. The reaction was followed by coupling tests with 3-retenol. Diazonium salts were detected 2—3 minutes after mixing and were shown to be present in the solution during the next 3 hours. After 5 hours the solution was cooled and diluted with water (700 ml). The precipitate formed was collected, washed with water and dried. The product (0.94 g) was dissolved in benzene (10—20 mg of an insoluble red product containing bromine was removed) and adsorbed on a column of alumina (10 cm^2 × 25 cm). On development with benzene many zones appeared. Zones Nos. 1—3 were eluted by continuing the addition of benzene. Zones Nos. 4—5 which moved very slowly with benzene were taken out and eluted with a mixture of benzene and ethylacetate. The many dark coloured zones remaining in the top of the column were not investigated.

The first zone, very light yellow, yielded a product (0.25 g, 26 % of the adsorbed amount) consisting of at least three compounds. After trituration with warm light petroleum, almost colourless crystals (0.03 g), m. p. 208—11° were obtained. (Found: N 3.60; Br 56.4. Calc. for a *tribromonitronaphthalene*, $C_{10}H_4O_2NBr_3$ (409.9): N 3.42; Br 58.5).

The light petroleum solution was adsorbed on a column of alumina (3 cm^2 × 20 cm) which was then washed with light petroleum. Two separate, very light yellow zones were formed and successively eluted. The first eluted zone yielded a yellow-white product (0.08 g) which crystallized as needles from ethanol, m. p. 115—45°. (Found: N 4.15; Br 50.8. Calc. for a *dibromonitronaphthalene*, $C_{10}H_5O_2NBr_2$ (331.0): N 4.23; Br 48.3). The other zone gave a product (0.06 g), m. p. 78—85° which after crystallization from ethanol was obtained as yellow-white needles, m. p. 79—86°. (Found: N 5.53; Br 31.4. Calc. for a *monobromonitronaphthalene*, $C_{10}H_6O_2NBr$ (252.1): N 5.55; Br 31.7).

The second zone, yellowish brown, furnished only few mg of substance. It was not further investigated.

The third zone, yellow, yielded an amine (0.04 g), m. p. 165—68° which contained bromine. After one crystallization from ethanol it melted at 181—84°. (Found: C 35.7; H 2.00. Calc. for a *dibromonitronaphthylamine*, $C_{10}H_6O_2N_2Br_2$ (346.0): C 34.7; H 1.75).

The fourth zone, orange-yellow, also yielded an amine (0.10 g), m. p. 239—40° (decomp.). One crystallization from ethanol did not change the melting point, the amine being obtained as brown yellowish needles. (Found: N 13.1; Br 25.4. Calc. for a *monobromodinitronaphthylamine*, $C_{10}H_6O_4N_3Br$ (312.1): N 13.4; Br 25.6).

The amines isolated from zones Nos. 3—4 were readily diazotised in a mixture of glacial acetic and conc. hydrochloric acids. The diazonium salts apparently were not decomposed by the addition of water; they furnished azodyes with 3-retenol.

The fifth zone, reddish-brown, gave 3,4-dinitro-1-naphthylamine (0.02 g), m. p. *ca* 199° (decomp.), undepressed on admixture with an authentic sample.

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Short Communications

New α-Keto Acids in Green Plants.

α-Ketopimelic Acid, γ-Hydroxy-α-ketopimelic Acid, and Hydroxypyruvic Acid in *Asplenium septentrionale*, α-Ketoadipic Acid in Germinating Pea Seeds

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When investigating the mode of formation of the new amino acids α-aminopimelic acid and γ-hydroxy-α-aminopimelic acid which were recently found in this laboratory [1,2] in *Asplenium septentrionale*, we have given attention to the keto acids in this plant. Virtanen *et al.*[3] have identified keto acids in the form of their 2,4-dinitrophenylhydrazones by means of paper chromatography, and using this method they could confirm the earlier findings in this laboratory with other methods [4] of the presence of pyruvic acid, oxalacetic acid, and α-ketoglutaric acid in pea plants. If, however, a still greater number of keto acids is to be found simultaneously in the investigated material the separating capacity of this method is not great enough, especially as the hydrazones of keto acids have a tendency to form multiple spots. When investigating the keto acids of *Asplenium* we have therefore reduced the extracted dinitrophenyl hydrazones with tin in an alcoholic hydrochloric acid solution to the corresponding amino acids, which then have been determined by the paper chromatographic method using butanol-acetic acid and phenol + NH_3 as solvents. This method presupposes that the hydrazone extract is free from amino acids. Kulonen [5] and Towers and Steward [6] have recently reported that they have used a method of this kind for the determination of keto acids, the reduction being made with other agents.

Using the above-mentioned method we found α-ketoglutaric acid, pyruvic acid, glyoxylic acid, hydroxypyruvic acid, α-ketopimelic acid, and γ-hydroxy-α-ketopimelic acid in *Asplenium septentrionale*, provided that the amino acids which appeared on the paper chromatogram were formed from the corresponding hydrazones. As only very faint spots of some amino acids, and no spots of serine, α-aminopimelic acid, and γ-hydroxy-α-aminopimelic acid were formed on the chromatogram made with the extract of non-reduced hydrazones the occurrence of the above-mentioned keto acids in *Asplenium* is to be considered sufficiently proved (Figs. 1 and 2). The amount of all the keto acids is so small that their isolation was impossible with the plant material we had at our disposal (*ca.* 500 g).

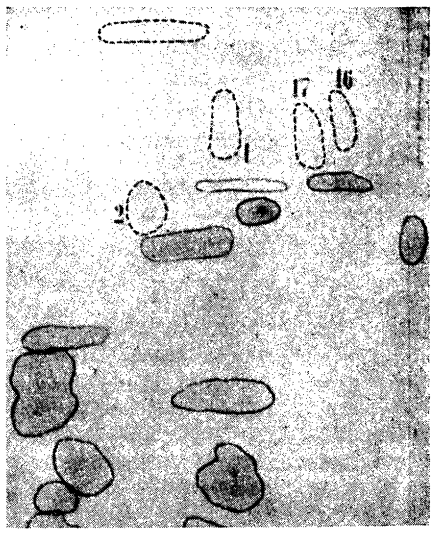

Fig. 1. Two-dimensional paper chromatogram (phenol + NH_3 and butanol-acetic acid) of the non-reduced 2,4-dinitrophenylhydrazones. Dot-lined spots = with ninhydrin produced very faint spots of 1 = gly, 2 = ala, 16 = asp, 17 = glu. Full lined spots = yellow hydrazone spots without ninhydrin treatment.

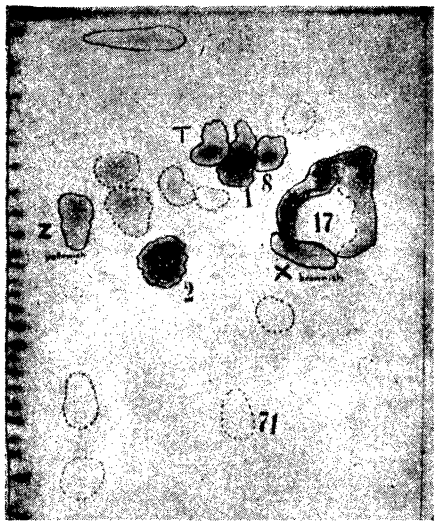

Fig. 2. Two-dimensional paper chromatogram of the reduced hydrazones. Full-lined spots = with ninhydrin produced distinct spots of 1 = gly, 2 = ala, 8 = ser, 17 = glu, X = γ-hydroxy-α-aminopimelic acid (brownish), Z = lactone of γ-hydroxy-α-aminopimelic acid (yellowish), T = unknown. Dot-lined spots = with ninhydrin produced faint spots. 71 = α-aminopimelic acid; unnumbered ninhydrin spots are unidentified.

Among the keto acids which we have found *hydroxypyruvic acid*, *α-ketopimelic acid* and *γ-hydroxy-α-ketopimelic acid* especially attract attention as they have not earlier been found in any plant as far as we know. In addition there are some ninhydrin positive unidentified spots on the chromatogram, among which spot T is particularly intense. The characterization of γ-hydroxy-α-ketopimelic acid has been possible only because the corresponding amino acid was earlier isolated from *Asplenium* [1].

In germinating pea seeds formation of *α-ketoadipic acid* could be shown. Even this acid has not earlier been found in any organism.

Our investigations suggest that α-aminoadipic acid, α-aminopimelic acid and γ-hydroxy-α-aminopimelic acid are formed *via* transamination from the corresponding keto acids. We are going to investigate these transamination reactions more closely. The formation of serine from hydroxypyruvic acid, and the formation of glycine from glyoxylic acid seems also probable.

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The Synthesis of Phenylcarbamoyl Derivatives by Lossen Rearrangement of Dibenzohydroxamic Acid

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Reports on the modification of proteins by reaction with organic *iso*cyanates (notably phenyl *iso*cyanate) have been published by numerous authors (for review, see Herriott [1]). One common feature of these experiments has been the use of an excess of the reagent. The consequence of this has probably been the production of a very complex mixture of highly substituted protein derivatives and in most cases, when the reactions were performed in aqueous solution, hydrolysis of an uncontrolled amount of the reagent has occurred.

A method by which proteins and peptides in aqueous solution could be partially substituted by reaction with phenyl *iso*cyanate under strictly controlled conditions would be highly desirable. It appeared to us that the well known Lossen rearrangement of dibenzohydroxamic acid might be useful in this respect.

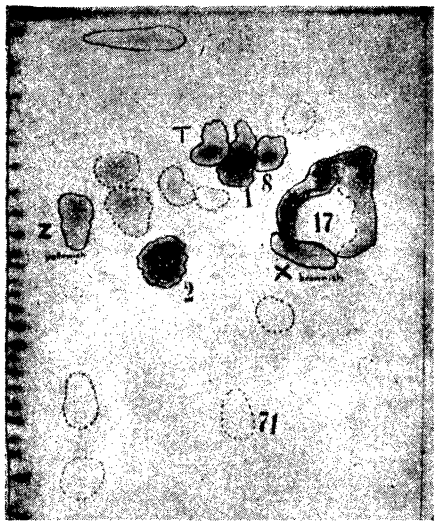

Fig. 2. Two-dimensional paper chromatogram of the reduced hydrazones. Full-lined spots = with ninhydrin produced distinct spots of 1 = gly, 2 = ala, 8 = ser, 17 = glu, X = γ-hydroxy-α-aminopimelic acid (brownish), Z = lactone of γ-hydroxy-α-aminopimelic acid (yellowish), T = unknown. Dot-lined spots = with ninhydrin produced faint spots. 71 = α-aminopimelic acid; unnumbered ninhydrin spots are unidentified.

Among the keto acids which we have found *hydroxypyruvic acid*, *α-ketopimelic acid* and *γ-hydroxy-α-ketopimelic acid* especially attract attention as they have not earlier been found in any plant as far as we know. In addition there are some ninhydrin positive unidentified spots on the chromatogram, among which spot T is particularly intense. The characterization of γ-hydroxy-α-ketopimelic acid has been possible only because the corresponding amino acid was earlier isolated from *Asplenium* [1].

In germinating pea seeds formation of *α-ketoadipic acid* could be shown. Even this acid has not earlier been found in any organism.

Our investigations suggest that α-aminoadipic acid, α-aminopimelic acid and γ-hydroxy-α-aminopimelic acid are formed *via* transamination from the corresponding keto acids. We are going to investigate these transamination reactions more closely. The formation of serine from hydroxypyruvic acid, and the formation of glycine from glyoxylic acid seems also probable.

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The Synthesis of Phenylcarbamoyl Derivatives by Lossen Rearrangement of Dibenzohydroxamic Acid

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Reports on the modification of proteins by reaction with organic *iso*cyanates (notably phenyl *iso*cyanate) have been published by numerous authors (for review, see Herriott [1]). One common feature of these experiments has been the use of an excess of the reagent. The consequence of this has probably been the production of a very complex mixture of highly substituted protein derivatives and in most cases, when the reactions were performed in aqueous solution, hydrolysis of an uncontrolled amount of the reagent has occurred.

A method by which proteins and peptides in aqueous solution could be partially substituted by reaction with phenyl *iso*cyanate under strictly controlled conditions would be highly desirable. It appeared to us that the well known Lossen rearrangement of dibenzohydroxamic acid might be useful in this respect.

This reaction, in which phenyl *iso*cyanate is produced by rearrangement of the dibenzohydroxamate anion in alkaline solution, has been thoroughly investigated by Hauser and coworkers [2,3] who found that the reaction in 0.1 *N* ammonia solution followed first order kinetics with respect to the concentration of the dibenzohydroxamate anion. We have confirmed this for potassium dibenzohydroxamate under slightly different conditions. The phenyl *iso*cyanate produced during the rearrangement was brought into reaction with an excess of β-alanine at constant pH (8.50) according to the scheme:

$$C_6H_5-N=C=O+H_3N^+-CH_2-CH_2COO^- \rightarrow C_6H_5-NH-CO-NH-CH_2-CH_2COO^-+H^+$$

The reaction was followed on a recording autotitrator [4]. Dibenzohydroxamic acid is a rather weak acid ($pK = 7.4$ in 50 % ethanol at 40° C). Accordingly the pH must be kept at (or above) 8.5 in order that the reagent be present mainly in the anionic form which undergoes rearrangement. The kinetic data were corrected for dissociation of the ammonium group of β-alanine ($pK = 10.2$) at pH 8.5. The first order rate constants were determined at 35.0° and 40.0°. Mean values of several determinations were as follows:

$k_{35°} = 2.88 \times 10^{-3}$ min^{-1} ($t_{\frac{1}{2}} = 241$ min)
$k_{40°} = 5.83 \times 10^{-3}$ min^{-1} ($t_{\frac{1}{2}} = 119$ min)

By using these rate constants it is possible, under the conditions specified above, to generate in aqueous solution almost any amount of phenyl *iso*cyanate down to about 1 micromole. The reaction is conveniently quenched after the predetermined time by addition of acid to pH 5 which causes precipitation of the insoluble dibenzohydroxamic acid.

It is well known [1] that phenyl *iso*cyanate reacts readily with amino groups of proteins at or above pH 8. However, when working with dilute solutions of these compounds, hydrolysis of the reagent might conceivably become a significant side reaction. In order to investigate this problem the model peptide, triglycine (GGG), was reacted at pH 8.5 and 40° with phenyl *iso*cyanate liberated from an excess of potassium dibenzohydroxamate. Under these conditions 85 % of the GGG is present as the free amine. The disappearance of the peptide was followed on aliquots by the ninhydrin method. The experiment (Fig. 1a) shows that the reaction between phenyl *iso*cyanate and the amnoi group is practically quantitative down to a concentration of the latter of about 15 microequivalents per 10 ml (1.5×10^{-3} equivalents per l). Below that concentration competing hydrolysis of the reagent becomes detectable. However, more than 90 % of the GGG has reacted at the time when the equimolar amount of phenyl *iso*cyanate has been liberated. Experiments at 35° gave similar results.

Craig and his coworkers [5] have observed that substitution at the hydroxyl group of tyrosine residues of peptides occurred with dinitrofluorobenzene. Since the reports

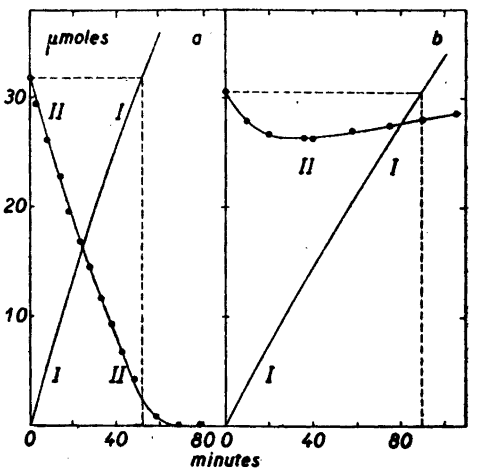

Fig. 1a — Curve I: Calculated liberation of phenyl isocyanate at pH 8.50 and 40.0° from potassium dibenzohydroxamate (121.7 micromoles) added at zero time to 10 ml of water containing 31.8 micromoles of GGG. Curve II: Disappearance of GGG.

Fig. 1b — Curve I: Calculated liberation of phenyl isocyanate at pH 8.50 and 35.0° from potassium dibenzohydroxamate (132.7 micromoles) added at zero time to 10 ml of water containing 30.4 micromoles of N-benzoyltyrosine. Curve II: Disappearance of N-benzoyltyrosine.

on the reaction of phenyl *iso*cyanate with tyrosine residues have been ambiguous [1], we decided to study the reaction with N-benzoyltyrosine under conditions similar to those given above for GGG, using the Folin method for determination of unreac-

ted N-benzoyltyrosine. Complications arise from the fact that aniline, which is produced by the competing hydrolysis of phenyl *iso*cyanate, also reduces the Folin reagent. Correction for this hydrolysis was made in a blank run containing no N-benzoyltyrosine and curve II (Fig. 1b) was obtained. Examination of the curve indicates that the phenylcarbamoyl derivative initially formed undergoes hydrolysis at the pH of the reaction (8.50). The aniline formed in the latter hydrolysis causes an apparent increase in the concentration of N-benzoyltyrosine which could not be corrected for. This probably accounts for the rise of the curve. In any case the experiment indicates that by using the present method for partial substitution of proteins, substitution at tyrosine residues would be unlikely in the presence of unreacted amino groups. Since phenyl *iso*cyanate is known to react much faster with amines than with alcohols the same would appear to hold true for the hydroxyl groups of serine and threonine residues.

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Phenylcarbamoyl Derivatives of Insulin

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Much research has in the past been devoted to the chemical modification of insulin, mainly because of its relative stability and unique physiological action. The advent of refined separation techniques has made it possible to investigate further the complexity of such protein derivatives. This was done for the first time by Harfenist and Craig [1] who were able to separate DNP-substituted insulin derivatives by countercurrent distribution. In the present communication is reported the synthesis and separation of phenylcarbamoyl derivatives of insulin.

An aqueous solution of pork insulin (60 micromoles, based on the molecular weight 5 777 for a molecule consisting of one A and one B chain) was reacted at pH 8.50 and 40° with phenyl *iso*cyanate (42 μmoles) liberated in the solution by Lossen rearrangement of potassium dibenzohydroxamate as previously described [2]. The partially substituted insulin was subjected to partition chromatography on a Hyflo Super-Cel column, using the solvent system 2-butanol – 0.01 *N* trichloroacetic acid [3]. The elution chromatogram consisted of 3 peaks (Fig. 1) of which the first appeared in the same fractions as insulin itself and obviously was the unreacted protein. According to Sanger and coworkers [4] the two-chain model of insulin contains three primary amino groups, the N-terminal α-amino groups of glycine and phenylalanine and the ε-amino group of a lysine residue. The two α-amino groups should react more rapidly under the above conditions (pH 8.5) than the latter owing to the difference in their p*K*'s (7.5 and 11.9, respectively). Consequently, by reasoning along the same lines as Craig [1], one would expect D_1 and D_2 to be mono- and di-substituted derivatives, respectively, substitution having occurred only on the α-amino groups of the insulin molecule.

The isolated components were hydrolysed in strong acid (5 *N* hydrochloric acid at 100° for 18 hours) which is known to cause cyclisation of phenylcarbamoyl derivatives of α-amino acids and peptides, thus affording the corresponding hydantoins, (p. 1724).

The hydantoins were extracted from the hydrolysis mixture with ether and determined spectrophotometrically in alkaline solution (0.1 *N* sodium hydroxyde) under which conditions they were shown to develop strong absorption peaks at 239 mμ, with a molecular extinction of 1.60 and 1.65 · 10^4 for the glycine (Gly.hyd.) and phenylalanine derivative (Phe.hyd.), respectively.

The hydantoins were identified by paper chromatography in two solvent systems (xylene-acetic acid-water, 3 : 2 : 1, and *n*-pentanol saturated with water). The spots were located on the paper by spraying with *N* sodium hydroxyde followed by photo-

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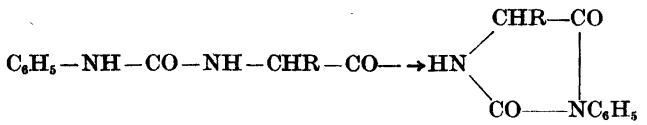


graphy in ultraviolet light. An estimate of the ratio of the two hydantoins was obtained by cutting out the spots, eluting with 0.1 *N* alkali and determining the optical density of the eluates at 239 mμ. The method is not very exact (± 10 %) owing to a rather high and varying absorption of the paper blanks. The results obtained are summarised in Table 1.

18 hours. The corresponding figure for Phe.hyd. is 90 %. This difference in stability of the two hydantoins indicates that the actual molecular ratio of D_1,Gly to D_1,Phe is even greater than 10. It will be noticed that the yield of Gly.hyd. from D_1, calculated on the basis of a minimum molecular weight for insulin of 12 000 would be > 100 %. The present experi-

Table 1.

Peak	Gly.hyd. + Phe.hyd. moles per mole of derivative		Molecular ratio Gly.hyd. : Phe.hyd.	
	theoret.	found	theoret.	found
I	0	0	—	—
D_1	1	0.7	—	10 : 1
D_2	2	0.85	1	1 : 3

D_1 is obviously a mixture of the two monosubstituted derivatives, consisting mainly (> 90 %) of the mono-derivative substituted on the N-terminal glycine (D_1,Gly). The yield obtained of Gly.hyd. (about 63 %) is consistent with the fact that Gly.hyd. is unstable under the strongly hydrolytic conditions of the cyclisation, 57 % of Gly.hyd. being recovered after

ments thus confirm the molecular weight of 6 000 found by Craig [1] and recently by Kupke and Linderstrøm-Lang [5], provided that D_1 is the monosubstituted derivative.

The molecular ratio of the two hydantoins obtained from D_2 corresponds fairly well to their respective stability during the cyclisation. The total yield of the hydantoins, however, is somewhat low compared

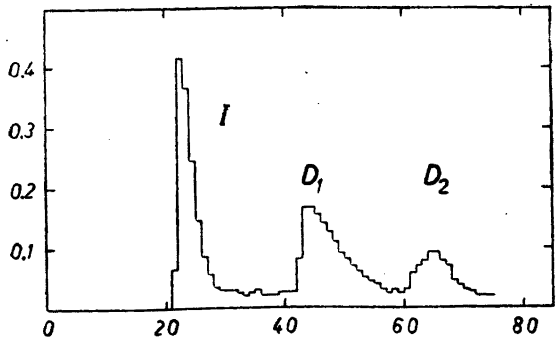
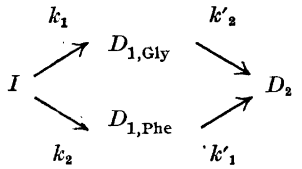


Fig. 1. Elution chromatogram of partially substituted insulin. Absc.: Number of fractions (23 ml). Ord.: Optical density at 277 mμ.

with that obtained from D_1. The possibility that D_2 is a mixture of di-derivatives must not be overlooked. It seems reasonable to suppose, however, that the derivative substituted on both N-terminal amino-groups is the major component.

The course of the substitution may be presented as follows:



where k_1 (k'_1) and k_2 (k'_2) represent the (second order) rate constants for substitution at the N-terminal glycine and phenylalanine residue, respectively. Examination of Fig. 1 shows that the amount of each component is approximately $I = D_1 = 3 \cdot D_2$ and from Table 1 is obtained $D_{1,Gly} >> D_{1,Phe}$. These facts cannot be reconciled unless it is assumed that $k'_2 > k_2$, that is: the amino group of the N-terminal phenylalanine must react faster in the mono-derivative $D_{1,Gly}$ than in insulin itself. The supposition that this »unmasking» is an intermolecular effect caused by a higher degree of dissociation of the mono-derivative into subunits as compared with insulin, is not substantiated by ultracentrifuge experiments. The possibility remains that the effect is of intramolecular origin. This would seem to require the distance between the groups involved to be rather short, or in different terms: The A and B chains of the insulin molecule would appear to be parallel rather than anti-parallel. This last conclusion is in agreement with the allocation of the cystine bridges recently published by Sanger [6]. Further details will be published in *Compt. rend. trav. lab. Carlsberg, Sér. chim.*

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Additional Note on α-Aminopimelic Acid in Green Plants

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In an earlier paper [1] we reported the detection of α-aminopimelic acid in *Asplenium septentrionale* by the paper chromatographic method. We have now been able to isolate α-aminopimelic acid from the same plant by cutting out the spot in question from some paper chromatograms before the treatment with ninhydrin (thick Munktell 20 paper was used). The pieces of paper were extracted with water, the extract evaporated to dryness, and the solid rest extracted with a mixture of abs. alcohol and ether in order to remove browncoloured impurities. The white microcrystalline substance (2 mg from 280 g fresh *Asplenium*) which on the paper chromatogram gave only one spot identical with the spot of α-aminopimelic acid, had a melting point of 204° C. Synthetic α-aminopimelic acid had a melting point of 203° C and the mixture of both 204° C. 1.4 mg of the isolated substance used 0.76 ml 0.01 *N* NaOH (0.304 mg NaOH), calculated for α-aminopimelic acid 0.80 ml (0.320 mg NaOH).

The isolated α-aminodicarboxylic acid is thus α-aminopimelic acid. Its R_F-value in phenol + NH_3 is 0.40 using Whatman 4 paper; in butanolacetic acid the R_F-value corresponds to that of γ-aminobutyric acid.

We have found free α-aminopimelic acid by the paper chromatographic method also in the seeds of a legume tree, *Ceratonia siliqua.*

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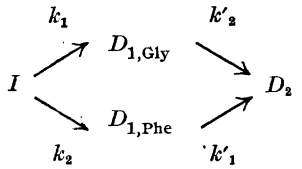

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The Relative Stability of the Alkali Metal Chelates of Dibenzoylmethane

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Brändström [1] has recently discussed the evidence bearing on the relative stability of the sodium and potassium chelates of β-ketoesters. He concludes that K^+ has a stronger tendency than Na^+ to give a chelate ring with the anion of a β-ketoester. Since none of the evidence previously available involves a direct measurement of these tendencies to form chelate rings, it seems worthwhile to consider some recent measurements in our laboratory on the tendencies of the alkali metal ions to form chelate rings with dibenzoylmethane (HCh) which is very closely related to the β-ketoesters.

By making potentiometric measurements in 75 % dioxane—25 % water instead of pure water, it becomes possible to measure differences in chelate stability which are not measurable in water [2-5]. Thus, by using tetramethylammonium hydroxide as the titrating agent (the tetramethylammonium ion is a non-chelating ion), it became possible to make measurements which permit the calculation of formation constants (as defined by Bjerrum [6]) for the alkali metal chelates of dibenzoylmethane. The results of such measurements at 30° (4×10^{-3} mole of HCh and 1×10^{-3} mole of M^+ in 100 ml solution) expressed as log K are:

Li^+ ($LiNO_3$)	5.95	K^+ (KCl)	3.67
Na^+ (NaCl)	4.18	R^+ ($RbNO_3$)	3.52
		Cs^+ (CsCl)	3.42

The order of variation with atomic number from metal to metal is the same here as for the alkali earths and other periodic groups. Thus the direct measurements obtained here show that the tendency to form the sodium chelate is definitely greater than that for the potassium chelate.

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A Remark to the Paper of W. C. Fernelius and L. G. Van Uitert on the Relative Stability of the Alkali Metal Chelates of Dibenzoylmethane

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The very interesting measurements of Fernelius and Van Uitert [1] on the relative stability of the alkali metal chelates of dibenzoylmethane which were performed in a dioxane-water mixture are in good agreement with the evidence obtained by Brändström [2-3] given in papers probably not yet accessible to Fernelius and Van Uitert. The paper of Brändström [4] cited by them deals with the behaviour in solvents of a much lower polarity (toluene and tert-butyl alcohol) where the situation may be quite different from that in a more polar medium [3] and where unfortunately no exact measurements seem possible.

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The System CaO-CaF_2

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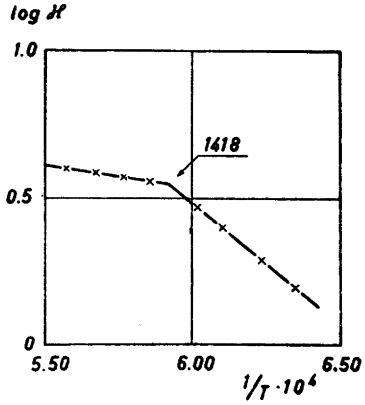
In studies on the electrical conductivity of the system $CaO - CaF_2$ it has been shown that there exists a gap of miscibility in the liquid state on the CaF_2-rich side of the diagram.

Previously this system had only superficially been studied by Eitel [1] as part of a greater work with the method of thermal analysis. In his work, he reported a value for the eutectic point. However, Eitel did not use the highest grade of purity of CaF_2 and thus found a melting point for the fluoride which was too low. The true melting point has been determined by Naylor [2] and found to coincide with the one in this work.

The experiments were carried out in a high frequency furnace and the temperature was measured with a Pt : Pt—10 % Rh thermocouple. The resistances of the melts were measured between molybdenum electrodes, the cell constant of which was determined using 0.1 *N* KCl. The determinations were made in carbon crucibles with thoroughly weighed and blended mixtures of CaO and CaF_2, A.R. grade. All experiments were carried out in a purified nitrogen atmosphere. The carbide contamination from the carbon crucibles could be shown to be negligible with



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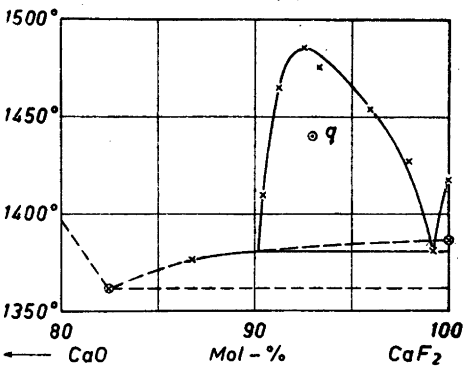

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Finally, it may be remarked that the electrical conductivity of CaF_2 at the melting point (1 418° C), $\varkappa = 3.56\ \text{ohm}^{-1}\text{cm}^{-1}$, is of the same magnitude as that of $CaCl_2$, $\varkappa = 4.17\ \text{ohm}^{-1}\text{cm}^{-1}$, extrapolated to the same temperature [3].

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The Relative Stability of the Alkali Metal Chelates of Dibenzoylmethane

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Brändström [1] has recently discussed the evidence bearing on the relative stability of the sodium and potassium chelates of β-ketoesters. He concludes that K^+ has a stronger tendency than Na^+ to give a chelate ring with the anion of a β-ketoester. Since none of the evidence previously available involves a direct measurement of these tendencies to form chelate rings, it seems worthwhile to consider some recent measurements in our laboratory on the tendencies of the alkali metal ions to form chelate rings with dibenzoylmethane (HCh) which is very closely related to the β-ketoesters.

By making potentiometric measurements in 75 % dioxane—25 % water instead of pure water, it becomes possible to measure differences in chelate stability which are not measurable in water [2-5]. Thus, by using tetramethylammonium hydroxide as the titrating agent (the tetramethylammonium ion is a non-chelating ion), it became possible to make measurements which permit the calculation of formation constants (as defined by Bjerrum [6]) for the alkali metal chelates of dibenzoylmethane. The results of such measurements at 30° (4×10^{-3} mole of HCh and 1×10^{-3} mole of M^+ in 100 ml solution) expressed as log K are:

Li^+ ($LiNO_3$)	5.95	K^+ (KCl)	3.67
Na^+ (NaCl)	4.18	R^+ ($RbNO_3$)	3.52
		Cs^+ (CsCl)	3.42

The order of variation with atomic number from metal to metal is the same here as for the alkali earths and other periodic groups. Thus the direct measurements obtained here show that the tendency to form the sodium chelate is definitely greater than that for the potassium chelate.

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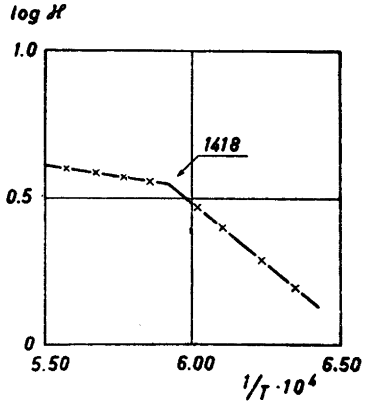

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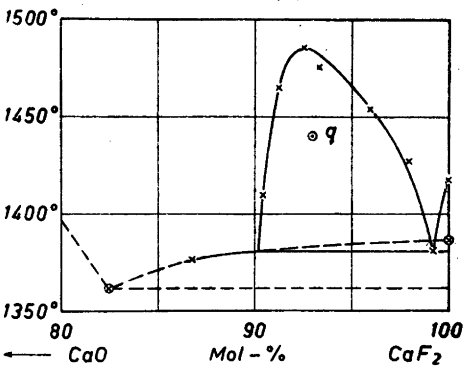

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The Chemistry of the Natural Order *Cupressales*. XIII *. The Presence of Sugiol in the Wood of *Juniperus communis* L.

JOHAN B-SON BREDENBERG and JARL GRIPENBERG

Department of Chemistry, Institute of Technology, Helsingfors, Finland

In continuation of earlier work on the family *Cupressaceae*, an investigation of the wood of the common juniper (*Juniperus communis* L.), the only species of this family endemic to Scandinavia, has been undertaken. The fractionation of the extract was carried out essentially according to Lindstedt [1]. From the fractions soluble in sodium hydroxide and from the neutral fraction a substance m.p. 290—292°, $[\alpha]_D^{21}$ + 22° ± 2° (ethanol), $[\alpha]_D^{21}$ + 15° ± 2° (acetone) was obtained in low yield (0.006 %). The substance corresponded to the formula $C_{20}H_{28}O_2$ and was difficultly soluble in the common organic solvents. It gave no colour with ferric chloride and failed to react with dinitrophenylhydrazine. The U.V. spectrum of the compound in ethanol showed a sharp maximum at 234 mμ (log ε 4.18) and a broader maximum at 286 mμ (log ε 4.12). These properties are in agreement with the properties given for sugiol (9-ketoferruginol), which has previously been isolated from the fraction soluble in 1 % sodium hydroxide of the resin from the wood of *Cryptomeria japonica* D. Don (m.p. 283—284°; $[\alpha]_D^{18}$ + 34.35° (pyridine); λ_{max} 286 mμ) [2,3] and from the neutral part of the extract of the wood of *Dacrydium cupressinum* Sol. (m.p. 295—297°; $[\alpha]_D^{20}$ + 20° (ethanol); λ_{max} 233 mμ (log ε 4.2), 285 mμ (log ε 4.1) [4,5].

A mixed melting point determination with authentic sugiol, kindly supplied by Dr. B. R. Thomas, showed no depression. The identity of the two compounds was further confirmed by comparison of the IR-spectra, which were indistinguishable.

Experimental. The finely powdered wood (6.5 kg) was extracted with ether in a continuous extractor for 24 hours. The extract was shaken successively with portions of 8 % sodium hydrogen carbonate, 10 % sodium carbonate, 0.2 % sodium hydroxide and 8 % sodium hydroxide solutions.

After acidification, chromatography of the precipitate on aluminium oxide and recrystallisation from benzene, the two last mentioned fractions gave 15 and 55 mg, respectively, of sugiol.

One third of the neutral portion was then steam distilled and from the non-volatile portion of this there was obtained, by careful chromatography, a further amount (100 mg).

The sugiol thus obtained had m.p. 290—292° (dec.) (corr.), $[\alpha]_D^{21}$ + 22° ± 2° (ethanol, *c* 0.58), + 15 ± 2° (acetone, *c* 0.52). (Found: C 79.6; H 9.4; Mol.wt. 313 (Rast). Calc. for $C_{20}H_{28}O_2$: C 79.95; H 9.4; Mol.wt. 300.4).

This work has been financially supported by a grant from *Statens Naturvetenskapliga Kommission* which is gratefully acknowledged.

The authors also wish to thank Dr. K. E. Almin, Svenska Träforskningsinstitutet, Stockholm, for determination of the IR-spectra.

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* The numbering of the three preceding papers: *Chemistry & Industry* **1952** 1267, *ibid.* **1954** 432 and *Acta Chem. Scand.* **8** (1954) 1073, should be X, XI and XII respectively.

Reduction of Hydroxylamine in the Root Nodules of Leguminous Plants

ARTTURI I. VIRTANEN, AUNE KEMPPI and EEVA-LIISA SALMENOJA

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

The formation of hydroxylamine as an intermediate has been discussed in connection with N_2-fixation. The reduction of hydroxylamine to ammonia is hereby very probable. When nitrate is reduced to ammonia hydroxylamine has been shown to be an intermediate, and Egami [1] has even proved the existence of a special hydroxylamine-reductase in nitrate reducing bacteria. From *Neurospora crassa* Zucker and Nason [2] recently isolated, and in a noticeable degree purified, the enzyme reducing hydroxylamine. This enzyme proved to be a metallo-flavoprotein.

On the other hand Colter and Quastel [3] have shown that blood hemoglobin acts as an enzyme in the dismutation of hydroxylamine to N_2 and NH_3. In the presence of cysteine or ascorbic acid hemoglobin catalyzes the reduction of hydroxylamine to ammonia. Schwyzer [4] showed in this laboratory that hemoglobin had no influence on oximes or hydroxamic acids (oxime of pyruvic acid and benzhydroxamic acid).

We have investigated the reduction of hydroxylamine and the oxime of pyruvic acid in the root nodules of leguminous plants in order to find out to what extent reduction takes place, and the role of leghemoglobin in the reduction.

Experiments were made both with water extract of root nodules and with crushed nodules. On the basis of results obtained in this laboratory N_2-fixing nodules always contain leghemoglobin, while ineffective nodules do not contain it [5]. When investigating the catalytic power of leghemoglobin + ascorbic acid in the reduction of hydroxylamine, effective root nodules of pea, clover, or soya were crushed in water, and the solution which had been clarified by centrifugation was used as agent in reduction experiments. Hydroxylamine was determined by the method developed by Csáky [6], based on Blom's principle, and ammonia by the method of Pucher. In an

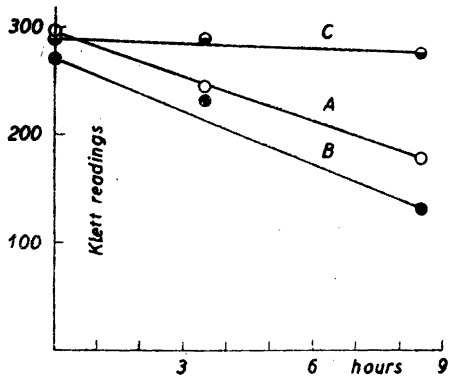

Fig. 1. Reduction of hydroxylamine. A: leghemoglobin extract from effective nodules of red clover + ascorbic acid + hydroxylamine (solution A). B: hemoglobin extract from red blood cells + ascorbic acid + hydroxylamine (solution B). C: ascorbic acid + hydroxylamine (solution C)

experiment with 3 ml of extract from 3 g of red clover nodules + 3 ml of ascorbic acid solution (= 30 mg of ascorbic acid in phosphate buffer pH 7.4) + 0.6 ml of hydroxylamine solution (10 mg $NH_2OH \cdot HCl$/ml) the loss of hydroxylamine was in 3 h at 38° C 30 %. In the control boiled nodule extract was used as agent.

In comparing the effect of leghemoglobin and blood hemoglobin on the breakdown of hydroxylamine, the content of leghemoglobin of the nodule extract was determined spectrophotometrically as pyridinehemochromogen, and a solution was prepared from red blood cells by dilution until the content of hemoglobin was the same as that of leghemoglobin in the nodule extract.

Example: From 2 g of effective root nodules of clover 5.32 ml of reddish water extract was obtained. The following solutions were prepared: 4 ml of nodule extract + 4 ml of ascorbic acid solution (= 40 mg of ascorbic acid in phosphate buffer pH 7.4) + 0.8 ml of hydroxylamine solution (10 mg $NH_2OH \cdot HCl$/ml). (Solution A)

4 ml of hemoglobin-solution of blood (1 ml red blood cells: 4 ml water, diluted to 192 ml) + 4 ml of ascorbic acid solution + 0.8 ml of hydroxylamine solution. (Solution B)

4 ml of water + 4 ml of ascorbic acid solution + 0.8 ml of hydroxylamine solution. (Solution C)

Reaction temperature t = 38° C.

The decrease of NH_2OH appears from the curves in Fig. 1. The catalytical effect of blood hemoglobin and leghemoglobin is thus practically the same.

Nodules whose red colour had changed into green (the porphin ring of leghemoglobin has opened [7]) and which thus had lost their N_2-fixing capacity [5] reduced hydroxylamine very poorly, or hardly at all. The reduction of hydroxylamine in root nodules is thus chiefly caused by leghemoglobin. This is confirmed also by the results of the experiments in which we compared the breakdown of hydroxylamine by the extract of effective nodules formed by pea bacteria, strain H 43, and ineffective nodules formed by pea bacteria, strain H VIII. In the latter nodules no leghemoglobin is to be found. Ascorbic acid was not added to the extracts. After a reaction time of 3 h the photometer readings were: in the experiment with effective nodules 83, in the experiment with ineffective nodules 152.

Formation of ammonia by breakdown of hydroxylamine was followed in some experiments. With an extract of the root nodules of soya, to which ascorbic acid had not been added, 0.28 μg of NH_2OH nitrogen disappeared during 3 h, while 0.12 μg of NH_3 nitrogen was formed, which corresponds approximately to the equation: $3\ NH_2OH = N_2 + NH_3 + 3\ H_2O$.

Experiments in which an eventual reduction of pyruvic acid oxime in root nodules was examined gave negative results. Increase of alanine as a reduction product of the oxime could not be observed in these experiments by using the paper chromatographic method. The method of determining the oxime is, however, uncertain as hydroxylamine to a great extent decomposes during hydrolysis when the solution contains organic substances like ascorbic acid.

Our findings show that an intensive breakdown of hydroxylamine takes place in N_2-fixing root nodules which contain leghemoglobin. As ascorbic acid is to be found in the nodules in relatively high concentration (2–3 times higher than in roots) [8] the reduction to ammonia is apparently practically complete in those hydroxylamine concentrations which may occur in the nodules if hydroxylamine is formed as an intermediate. In parallel experiments where the effect of red blood cells and red root nodules on the reduction of hydroxylamine in the presence of ascorbic acid was compared, the reaction velocity was found to be approximately the same. As the disappearance of hydroxylamine is very slight in ineffective and leghemoglobin-free root nodules, as well as in older ones where the leghemoglobin, because of the splitting of the porphin ring, has changed into green pigment, it is obvious that leghemoglobin is the main, or only, factor which causes the reduction of hydroxylamine in root nodules. In the case that hydroxylamine is formed as an intermediate in N_2-fixation in root nodules, leghemoglobin is thus indispensable in the reduction of hydroxylamine.

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Fixation of Molecular Nitrogen by Excised Nodules of the Alder

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The alder tree fixes molecular nitrogen at a rate sufficient to support vigorous growth on a nitrogen-poor soil. As it now is possible to demonstrate nitrogen fixation consistently with excised nodules from leguminous plants [1,2], it seemed advisable to test whether alder nodules also can fix nitrogen apart from the host plant.

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Minutes exposure to $^{15}N_2$	Atom per cent ^{15}N excess	
	Experiment 1	Experiment 2
30	0.014	0.105 0.142
60	0.064	0.153 0.114
120	0.069	0.376 0.278

Table 1. Fixation of nitrogen by excised alder nodules exposed for various periods to an atmosphere containing N_2 enriched with 45 atom per cent ^{15}N excess. Only the fraction of the nodules soluble in 3 N HCl was analyzed.

Alnus glutinosa plants were grown for 4 years in a greenhouse on a quartz sand substrate supplied with a nitrogen-free nutrient mixture [3]; N_2-fixation was very strong and the trees attained a height of 3 – 4 m. At harvest, branches of the root system were cut, sand was removed, and the root nodules were detached. The nodules immediately were placed in Warburg type respirometer vessels, and these were attached to manometers and evacuated [4]. To remove atmospheric nitrogen, the vessels were evacuated twice and filled with oxygen. After a third evacuation, 0.3 atmosphere of a mixture of 2 parts oxygen and one part $^{15}N_2$ was added, and argon then was added to atmospheric pressure. The N_2 was enriched with 45 atom per cent ^{15}N excess, and the nodules were exposed to it for 0.5 to 2 hours in the vessels which were shaken in a water bath at 24°. The nodules then were removed and ground with 3 *N* HCl in a mortar. The ground material was centrifuged, and only the soluble supernatant solution was analyzed for ^{15}N with a mass spectrometer.

Table 1 indicates the fixation of $^{15}N_2$ by excised alder nodules in 2 experiments. It is evident that excised alder nodules fix a readily detectable quantity of molecular nitrogen in their acid soluble material. We generally consider that 0.015 atom per cent ^{15}N excess indicates positive fixation, and in the most active alder nodules over 20 times this level was fixed in 2 hours.

Crushing or slicing the nodules decreased their fixation during a 2 hour exposure to $^{15}N_2$ to between a fourth and a sixth that observed with intact nodules. Fixation was very limited in tests to determine the influence on fixation of exposure of the plants to light or dark — nodules from illuminated plants had 0.014 atom per cent ^{15}N excess, whereas nodules from plants darkened for a day or for 3 days before removing their nodules for a2 hour exposure under $^{15}N_2$ had 0.006 and 0.005 atom per cent ^{15}N excess, respectively. Addition of oxalacetate, alpha-ketoglutarate, malate or sucrose stimulated fixation of $^{15}N_2$ by alder nodule slices only slightly. Experiments designed to establish the rate of fixation in whole nodules, rather than the soluble portion only, were unsuccessful, for the inert nitrogenous compounds diluted the ^{15}N of the more active soluble fraction to a level too low for accurate estimation.

Fixation of nitrogen by excised alder nodules can be demonstrated readily with ^{15}N as a tracer, but factors influencing the fixation are not yet sufficiently well under control to assure uniform results. With an improved understanding of these controlling factors, the use of excised nodules should stimulate studies of nitrogen fixation and the interesting nitrogen metabolism [5] of the relatively neglected alder plant.

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Kinetics of the Reaction between Hydrazoic Acid and Phenanthrenequinone

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It is shown that the reaction between hydrazoic acid and phenanthrenequinone consists of two separate reactions. In the first reaction hydrazoic acid and phenanthrenequinone react to give diphenamic acid and nitrogen. In the second reaction diphenamic acid takes part in a normal Schmidt reaction, giving phenanthridone, ammonia, nitrogen, and carbon dioxide.

In a previous communication [1] S. Veibel and the present author have described the stoichiometry of the reaction between phenanthrenequinone and hydrazoic acid as compared with that of the normal Schmidt reaction. The latter is, with benzoic acid as an example:

$$\varphi COOH + HN_3 = \varphi NH_2 + N_2 + CO_2$$

The former is more complicated, ammonia being formed together with phenanthridone:

$$C_{14}H_8O_2 + 2\ HN_3 + H_2O = C_{13}H_9ON + 2\ N_2 + CO_2 + NH_3$$

In this paper the comparison between the two reactions will be based upon kinetic investigations.

TECHNIQUE

Both reactions have been followed in a Brønsted shaking apparatus of the type described earlier in this journal [2]. Besides that, the second reaction was in a single run followed by titration of the ammonia formed.

The solvent was in all cases concentrated sulfuric acid (81.32—98.53 % H_2SO_4), the concentration of which was determined by titration immediately before the process was started. Owing to the small concentration of quinone and hydrazoic acid the concentration of water can be considered constant during the reaction.

An experiment showed that the spontaneous decomposition of hydrazoic acid is very slow compared to the reactions investigated, thus making any correction for pressure increment due to this reaction unnecessary.

The volume of the shaking apparatus was determined by measuring the increase in pressure, when a weighed amount of sodium azide was oxidized by ceric sulfate in excess in aqueous solution.

In a standard experiment 1.000 millimole sodium azide dissolved in 19.66 ml sulfuric acid reacts with 0. 500 millimole quinone. The pressure increase was calculated from the above mentioned data to be 22.19 cm Hg of which 15.54 cm Hg is nitrogen and 6.65 cm Hg is carbon dioxide. The calculation is based upon the following Bunsen absorption-coefficients [3] for nitrogen and carbon dioxide in 95.6 % sulfuric acid: $\alpha_{N_2} = 15.55\ 10^{-3}$; $\alpha_{CO_2} = 0.923$.

KINETICS

The reaction between benzoic acid and hydrazoic acid

Table 1 shows that the second order scheme is followed exactly to a degree of advancement of 0.65 ,with a velocity constant of $0.198 \cdot 10^{-3}$ l mole^{-1} sec^{-1}.

Table 1. The reaction between benzoic acid and acid and hydrazoic acid. 1 millimole of benzoic acid and 1 millimole of hydrazoic acid in 19.66 ml sulfuric acid (95.4 % H_2SO_4). 25.00° C.

t min	ξ	$\frac{19.66}{60}(1-\xi^{-1})-1$	$k\ 10^3$ l mole^{-1} sec^{-1}
50	0.029	0.010	0.20
100	0.057	0.020	0.20
150	0.083	0.030	0.20
200	0.109	0.040	0.20
300	0.157	0.061	0.203
400	0.194	0.079	0.198
500	0.233	0.100	0.200
1 400	0.441	0.259	0.185
1 700	0.505	0.334	0.196
2 000	0.548	0.397	0.199
3 000	0.649	0.606	0.202

mean value: $0.198 \cdot 10^{-3}$ l mole^{-1} sec^{-1}

The only available data for the influence of substituents on the velocity are those of Briggs and Lyttleton [4]. These are not very accurate and refer to a heterogenous reaction, they are therefore difficult to interpret, and it was a tempting task to determine the velocity constant for some substituted benzoic acids. It proved very difficult to obtain reproduceable results, presumably because the substituted acids decomposed in the sulfuric acid solution (dark colour). The only reliable result is the constant for *p*-methyl-benzoic acid which was found to be $0.371 \cdot 10^{-3}$ l mole^{-1} sec^{-1} in 95.4 % sulfuric acid at 25.00° C.

This indicates that if the Hammett equation [5]

$$\log k_{subst} - \log k_{unsubst} = \sigma \cdot \varrho$$

is valid, we must think of ϱ as having a comparatively great negative value.

The reaction between phenanthrenequinone and hydrazoic acid shows a sharp decrease in velocity at a degree of advancement of about one third, *cf.* Fig. 1. This indicates that the reaction cannot be described by a mechanism involving only intermediate products in quasi-stationary concentrations.

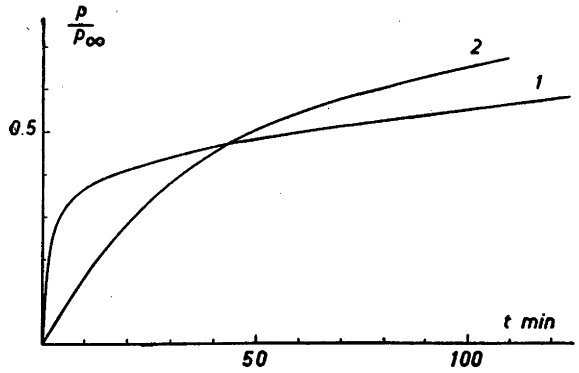


Fig. 1. Gas evolution as function of time for the reaction between hydrazoic acid and 1. phenanthrenequinone, 2. retenequinone.

Systematic guessing on chronometric integrals [6] is thus out of place, as it can be considered impossible to guess the very complicated functions which arise from mechanisms with intermediates in non-stationary concentrations.

A better way out would probably be to investigate the reaction at the very beginning and at the end. Whereas the former always will give some indication, the latter will only give interpretable results if the last measurable velocity constant is small compared to all the others.

Fig. 2 shows in arbitrary units the initial velocity as function of the concentrations of quinone and hydrazoic acid. It is evident that the first measured step in the reaction is described by:

$$-\dot{Q} = -\dot{H} = k_1 QH$$

where Q and H denote concentrations of quinone and hydrazoic acid respectively.

The following equation between pressure and time which is valid at the end of the reaction shows that the last part of the process also can be described by a second order expression.

$$\left(1 - \frac{p(t) - \pi}{p_\infty - \pi}\right)^{-1} - 1 = k_2(t - \tau)$$

π and τ are constants which have the dimension of pressure and time respectively.

To investigate the nature of the four constants (k_1, k_2, π, τ) experiments with different water contents in the sulfuric acid were carried out. Whereas π proved to be almost constant ($\sim \frac{1}{3} \cdot p_\infty$), it was found that k_1 and k_2 and τ vary greatly, but in such a manner that $k_1 \cdot \tau$ is roughly constant.

As analysis showed the gas evolved in the beginning of the reaction to be almost pure nitrogen, π was taken to be 7.77 cm Hg which is equivalent to half of the nitrogen that should be evolved according to the stoichiometry. Doing so we are forced to interpret τ, the "starting time" of the last reaction, as the

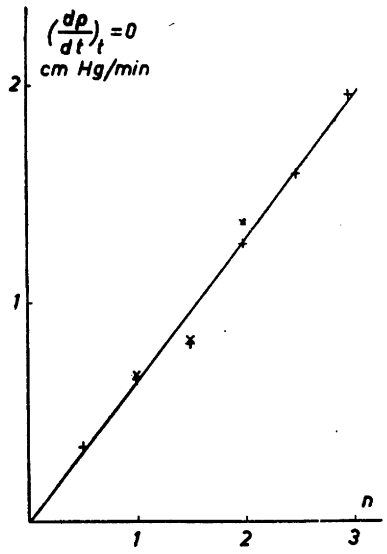


Fig. 2. Initial velocity of the reaction between hydrazoic acid and phenanthrenequinone as function of the initial concentrations.

\+ *H = 1 millimole/19.66 ml* *Q = n millimole/19.66 ml*
× *Q = 1 millimole/19.66 ml* *H = n millimole/19.66 ml*

time required to generate a compound that reacts according to this scheme. As mentioned above, $k_1\tau$ is roughly independent of the sulfuric acid concentration, indicating that all reactions occurring between the measured initial reaction and the measured last reaction are rapid as compared to these two. Table 2 shows the velocity constants as functions of the sulfuric acid concentration.

The reaction was in a single run followed by titration of the ammonia formed. It proved that the ammonia is released by a second order reaction with equivalent amounts of the reactants, and that the velocity constant equals k_2. We have thus established this scheme of reactions:

$$Q + H \rightarrow D + N_2$$
$$D + A \rightarrow P + N_2 + CO_2 + NH_3$$

where P is phenanthridone and D is some intermediate in nonstationary concentration.

Table 2. The velocity constants as functions of the sulfuric acid concentrations. 25.00° C.

Experiment No.	% H_2SO_4	k_1 l·mole^{-1}sec^{-1}	k_2 10^3 l·mole^{-1}sec^{-1}	τ sec
12	98.53	0.041	3.58	1 900
14	96.81	0.113	2.17	250
15	96.81	0.115	1.99	750
17	95.41	0.157	1.60	600
21	92.53	0.382	1.02	200
20	84.32	2.32	0.76	50

Mathematical treatment

If we by x denote the number of moles of hydrzoic acid which react according to the first reaction, and by y denote the number of moles of hydrazoic acid which reacts according to the second reaction, then we have for the reaction between equivalent amounts

$$\dot{x} = k_1(2-x-y)(1-x)$$
$$\dot{y} = k_2(2-x-y)(x-y)$$

Dividing and substituting k_1/k_2 by k we get

$$\frac{dy}{dx} = k\,\frac{x-y}{1-x} \quad \text{or} \quad y = \frac{1-kx}{1-k} - \frac{1}{1-k}\,(1-k)^k$$

and from this and the first equation we get:

$$k_1 t = \int_0^x \frac{dz}{\frac{1}{1-k}\,(1-z)^{1+k} + \frac{1-2k}{1-k}\,(1-z)^2}$$

As we have $p(t)/p_\infty = \xi\ (t) = 0.350\ x + 0.650\ y$, a numerical evaluation of this integral can give us the theoretical values of $p = p(t)$.

Owing to the fact that $k \ll 1$ this evaluation is very tedious and is omitted in favour of the following proof.

Let the reaction be carried out between 2 moles of hydrazoic acid and n moles of phenanthrenequinone. We then have

$$\dot{x} = k_1(2-x-y)(n-x)$$
$$\dot{y} = k_2(2-x-y)(x-y)$$

from which $y = \dfrac{n-kx}{1-k} - \dfrac{n^{1-k}}{1-k}\,(n-x)^k$

This equation is also valid for the values (x_∞, y_∞) reached when $t \to \infty$. For these values we further have: $x_\infty = y_\infty = 2$, as a total amount of 2 moles of hydrazoic acid is converted. For the apparent final degree of advancement $\xi^* = p_\infty/22.19$ defined as the ratio of the final pressure to the final pressure in a reaction between equivalent amounts of quinone and hydrazoic acid, we still have $\xi^* = 0.350\ x_\infty + 0.650\ y_\infty$.

Table 3. Final pressure as function of the initial concentration of quinone. Solvent: 98.53 % sulfuric acid. 25.00° C.

n = moles of quinone pro 2 moles of hydrazoic acid	p_∞	ξ^*exptl.	ξ^*theor.
1.0	22.17	0.999	1.000
1.5	18.64	0.840	0.835
2.0	16.90	0.762	0.765
2.5	16.43	0.740	0.739
3.0	16.17	0.729	0.729

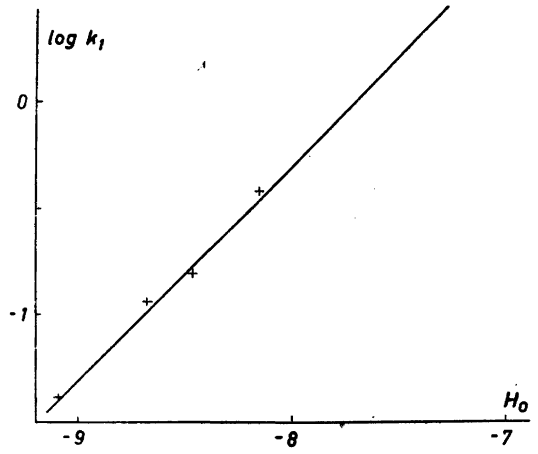


Fig. 3. log k_1 plotted against Hammetts H_0 acidity function.

By substitution we arrive at the following equation between ξ^* and n in which has been used the value $k = 0.087$ corresponding to the experiment.

$$n + 1.753 - 2.753\xi^* - n^{0.913}(n - 4.333 + 3.333\xi^*)^{0.087} = 0$$

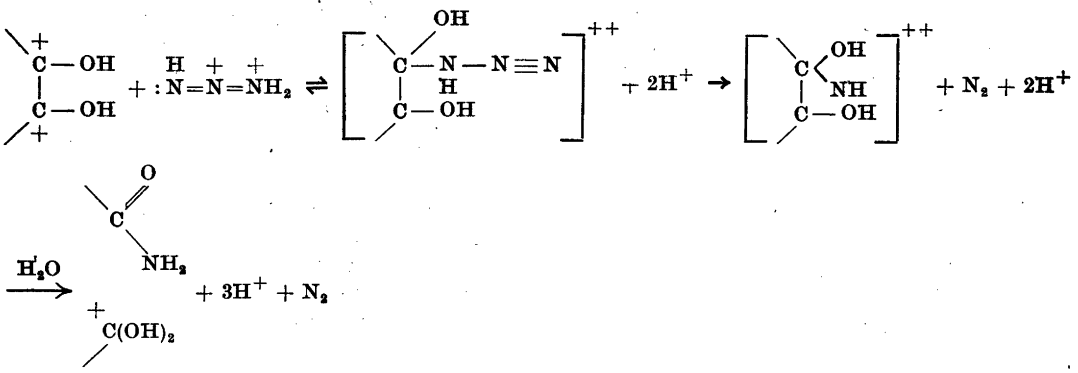
The experimental values are given in Table 3 together with the theoretical values determined by iteration. The agreement is quite satisfactory and gives good evidence for the correctness of the postulate.

Mechanism

1. Fig. 1 shows the velocity of the first reaction to be proportional to the product of the concentrations of quinone and hydrazoic acid. The velocity constant k_1 decreases very markedly, when the concentration of sulfuric acid is increased. To get an estimate of this variation we can plot log k_1 against Hammett's H_0 acidity function [7]. This is in principle incorrect, as H_0 measures the tendency of a solution to transfer a proton to a neutral base, and we know that most of the quinone and the hydrazoic acid will be found as highly protonized ions, such as: $C_{14}H_8O_2H_2^{++}$ and $H_4N_3^{+++}$. As the plot gives a straight line we assume however that in this dilute solution of water in sulfuric acid certain regularities, such as $H_0 \sim H_+ \sim H_{++}$ will be found.

In this case the slope of the line should be an integer and Fig. 2 shows it to be + 1, which means that the reaction velocity is inversely proportional to the "hydrogen ion concentration". The most reasonable explanation of this fact is that the reaction involves the ion $H_3N_3^{++}$ which is the most highly protonized form of hydrazoic acid that still possesses a lone pair.

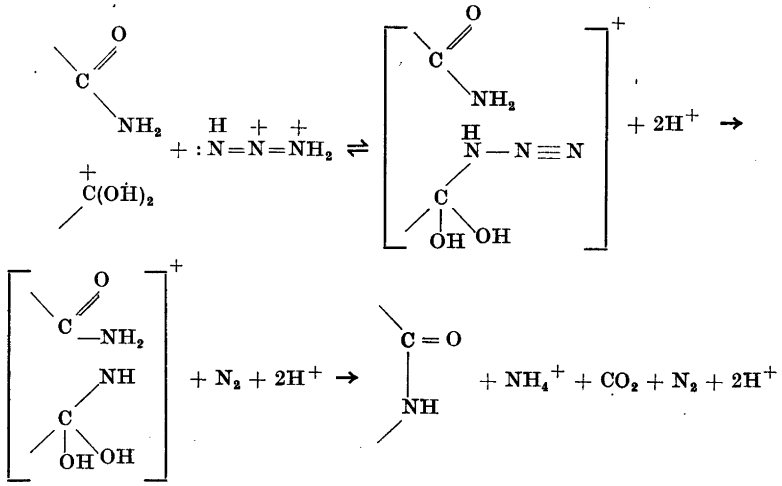
In order to prove that the last molecule in this sequence is the above-mentioned intermediate, the reaction mixture was diluted with ice and water. A white precipitate was formed, which showed no depression in melting point



when mixed with an authentic sample of diphenamic acid, prepared according to standard methods [8]. As the yield was practically quantitative, we have thus confirmed the proposed mechanism, in which we consider the second step as rate determining.

2. As the intermediate is a substituted benzoic acid, the relation to the normal Schmidt reaction has now been established.

A rough estimate of the heat of activation based upon experiments at 25.00° C and 30.00° C showed it to be 27 000 cal. for the last reaction, which is in good agreement with the heat of activation for similar reactions, such as Curtius and Lossen rearrangements [9]. The above mentioned value of ϱ is also in reasonably good agreement with values of ϱ found for these reactions [10]. The following mechanism is therefore proposed:



3. The interesting case of 1-methyl-7-*iso*propyl-phenanthrenequinone (retenequinone) demands further discussion. It reacts with hydrazoic acid to give only one compound, and not two isomer compounds as could be expected. In terms of the proposed mechanism this is explained as follows:

In the first reaction that CO group which is near to the methyl group has a very low reactivity due to steric hindrance. The intermediate product will thus be predominantly 3-methyl-4'-*iso*propyl-22'-diphenic acid(2')monamide. In this compound the carboxy group is sterically screened by a phenyl group and a methyl group, and thus ionizes to $-\overset{+}{C}=O$ instead of $-\overset{+}{C}(OH)_2$ in sulfuric acid [11].

It is however known that the former of these ions reacts more rapidly with hydrazoic acid, than does the latter [12]. It is thus seen that it is also a consequence of the mechanism that the reaction with retenequinone should be slower at the beginning of the reaction and more rapid towards the end, than is the corresponding reaction with unsubstituted phenanthrenequinone. The experiments confirm this result (Fig. 1).

The fact that k_1 and k_2 are approaching each other, render it very difficult to split up the experimental results into two reactions. But still we can find k_2/k_1 from the relation between ξ^* and n if we, in an experiment with excess of quinone, know the final pressure. From a single experiment in 95.4 % sulfuric acid was found $k = 0.98$, which confirms the previous considerations.

The authors thanks are due to Professor J. A. Christiansen and to Dr. Jørgen Koefoed for their interest in this investigation.

Financial support from *Det teknisk videnskabelige Forskningsraad* and *Carlsbergfondet* is gratefully acknowledged.

Note added in proof. Professor Veibel has informed the author that a paper related to the same research has just been published by other authors (Badger, G. M. and Seidler, J. H. *J. Chem. Soc.* **1954** 2329). These authors have studied the reaction between hydrazoic acid and crysenequinone from the point of view of its use in the preparation of phenanthridone, and have isolated the amidoacid as an intermediate product.

In the reaction only one phenanthridone is formed, and the reaction is thus analogous to that between hydrazoic acid and retenequinone. It therefore seems very probable that the two rate constants will behave in the same manner as the rate constants of the latter reaction, k_1 being smaller and k_2 greater than the corresponding rate constants of the reaction between hydrazoic acid and phenanthrenequinone.

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Studies Related to Naturally Occurring Acetylene Compounds. XVII. Four New Polyacetylenes from Garden Varieties of *Coreopsis*

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Some common garden *Coreopsis* species have furnished mixtures of new polyacetylenic compounds. Four of these have been obtained in a pure state. Dodeca-1:11-diene-3:5:7:9-tetrayne (II) dominates in the roots of all the investigated *Coreopsis* species; through the characteristic U.V.-spectrum (II) was also detected in small amounts in the thistles: *Carthamus lanatus* L., *Cnicus benedictus* L. and *Silybium Marianum* Gaertn. 1-Phenyl-*n*-hept-5-ene-1:3-diyn-7-ylacetate (V) could be isolated from all parts of the said *Coreopsis* species. The constitution and *trans* configuration of (V) was established through comparison with synthetic (V). Trideca-1:3:5:11-tetraene-7:9-diyne (IX) dominates in the oil from the leaves, whereas 1-phenyl-*n*-undeca-7:9-diene-1:3:5-triyne (XI) was the main chromophore of the oil from the flowers.

In the preceding communications of this series the occurrence of different types of acetylenic compounds in some plants belonging to the *Compositae* has been demonstrated. With the object of gathering information about the distribution of these compounds within the different tribes and subtribes of this vast family some common garden plants from different tribes lacking wild representatives in this country have been investigated. As different species of the genus *Coreopsis* have been investigated previously[1] with regard to their peculiar flower pigments, the so-called antochlor pigments, we were somewhat surprised when it turned out that this genus is rich in new members of the naturally occurring polyacetylenes.

The species investigated were the annuals *Coreopsis tinctoria* Nutt. in the garden varieties "*nigra speciosa*" "*nana radiata*" and *Coreopsis Drummondii* Torr & Gray, *C. cardaminifolia* atrosanguinea* and a small sample of the perennial *C. verticillata* L.

Since all these gardens varieties are glabrous plants we were surprised to obtain good yields of essential oils, $\sim 0.6^0/_{00}$. The essential oils from all parts of the plants showed selective absorption in ultraviolet partly with acetylenic

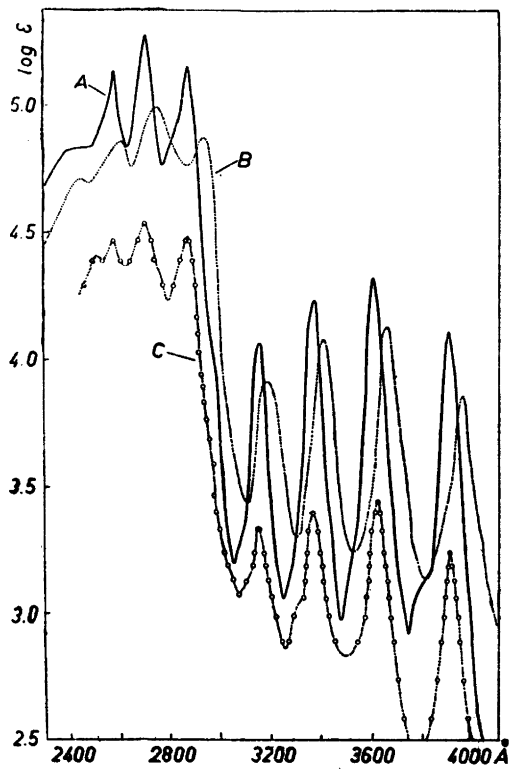


Fig. 1. Ultraviolet absorption of

A: dodeca-1:11-diene-3-5-7:9-tetrayne (II) from Coreopsis in hexane.
B: tetradeca-2:12-diene-4:6:8:10-tetrayne (I) in alcohol, E. R. H. Jones et al.
C: purest fraction of (II) from Cnicus benedictus L.

spacing between the maxima. Since the spectral curves of the oils from different parts of the plants differed rather much, the oil from the roots and flowers and the green part of the plants were worked up separately. This separation greatly facilitated the isolation of some of the new polyacetylenes, but, as will be seen from the chromatographic separation described in the experimental part, the four compounds so far isolated occur in all parts of the plants but varying in dominance.

The crude oil from the roots exhibited sharp absorption maxima in U.V.; the substance responsible for these maxima is only slightly retarded by chromatography on alumina from petroleum ether solution. The U.V. spectrum of the pure substance in hexane is given in Fig. 1, curve A, together with the spectrum of tetradeca-2 : 12-diene-4 : 6 : 8 : 10-tetrayne (I) synthesised by E. R. Jones *et al*[2].

$$H_3C-CH=CH-C\equiv C-C\equiv C-C\equiv C-C\equiv C-CH=CH-CH_3 \qquad (I)$$

$$CH_2=CH-C\equiv C-C\equiv C-C\equiv C-C\equiv C-CH=CH_2 \qquad (II)$$

We are greatly indebted to professor E. R. H. Jones who has put the full curve for (I) at our disposal. The conformity of the two curves is striking. The displacement of the maxima of I, 40—50 ÅU towards longer wave-lengths, are in all probability mostly due to the use of different solvents (alcohol with I, hexane with II).

This sharp-banded substance from *Coreopsis* roots has, when pure, an excellent crystallising power. The crystals, which are slightly yellow, are, however, very unstable. They become dark rapidly between 43 and 45°, and turn brownish-black between 45 and 48° without any further change.

By catalytical hydrogenation 10.1 moles H_2 were consumed. The perhydrocompound melted at —10.8 to —10.3°, synthetic dodecane at —10 to —9.3°, mixed m.p. —12.3 to —11.5°. Dr. W. D. Celmer, Chas. Pfizer & Cie, New York, most kindly measured the infra-red spectra of the perhydrocompound and of our synthetic dodecane which confirmed the identity.

From the conformity of the U.V.-spectra, *cf.* Fig. 1, we conclude that the *Coreopsis* hydrocarbon also must be a diene-tetrayne. Of the possible arrangements of the double and triple bonds that of II = dodeca-1 : 11-diene-3:5:7:9-tetrayne has been chosen for the following reasons. Schlubach *et al*[3]. demonstrated that the triple bond reacts much more slowly than the double bond with peracetic acid. The *Coreopsis* substance reacted very slowly with peracetic acid. The small amounts of substance available did not permit the isolation of a crystalline dioxide; but as soon as the 3 915 ÅU maximum had disappeared, the remaining chromophoric system exhibited maxima at 2 450, 2 330 and 2 235 ÅU, close to the position of the strong short-wave band group of dialkyl tetraacetylenes (2 410, 2 300 and 2 200 ÅU)[4].

Therefore we conclude that the four triple bonds are conjugated. It then remains only two constitutional possibilities, II and dodeca-1 : 3-diene-5 : 7 : 9 : 11-tetrayne. The experiences accumulated from the synthetic side during recent years[2, 3, 5] strongly suggest that a mono-alkyl tetrayne would be much too unstable to exist above 0° C.

There occurs in the infra-red spectrum of this diene-tetrayne, *cf.* Fig. 2, curve A, a definite peak at 2 130 cm^{-1} close to the position characteristic of mono-alkylated acetylenes, but infra-red data on synthetic and naturally occurring polyacetylenes of the di-alkylated type[7, 8] very often have peaks near 2 100 besides the 2 200 cm^{-1} peak. Through the courtesy of the Jackson Laboratory of the E. I. du Pont de Nemours & Co. Inc. we obtained an infra-red spectrogram of divinyl-acetylene, redrawn in Fig. 2 curve B. The agreement in the region 7—15 μ is good and indicates that the *Coreopsis* hydrocarbon has both double bonds as vinyl groups.

Thus, although the small amounts available and the extreme instability of this *Coreopsis* hydrocarbon in the crystalline state have seriously hampered our investigations, we think that (II) is fairly established as its constitutional formula*.

The extremely sharp U. V. absorption maxima of (II) facilitates the spectroscopic detection of this hydrocarbon. So far this spectrum has been found in

* *Note added in proof:* II has in the meantime been synthesised by Jones, Thomson and Whiting, *vide* this volyme p. 1944. From the data of synthetic II Jones *et al.* concluded that the *Coreopsis* hydrocarbon might be a mono-alkyl-diene-tetrayne.

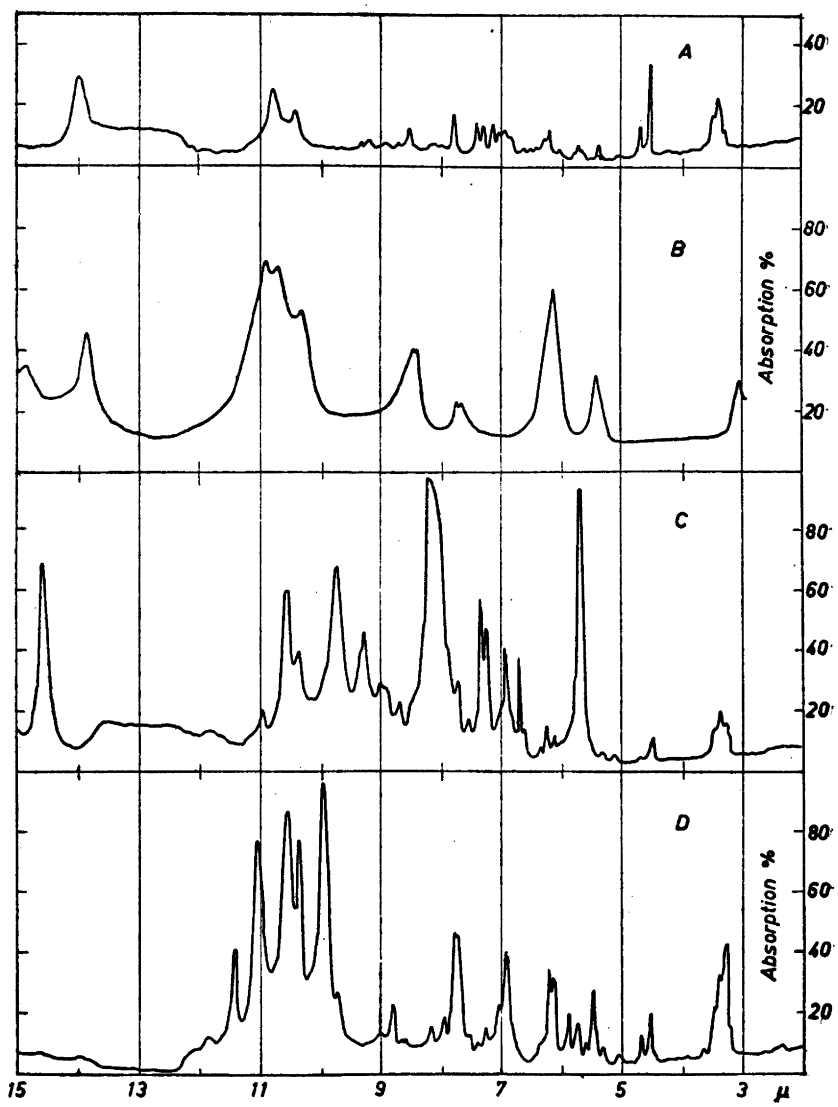


Fig. 2. Infrared absorption of

A: dodeca-1:11-diene-3:5:7:9-tetrayne (II) in CCl_4, c = 7 %, l = 0.05 mm.
B: divinyl acetylene according to du Pont, 4.25 mol % in N_2 gas, c = 400 mm, l = 10 cm.
C: 1-phenyl-hept-5-ene-1:3-diyn-7-yl acetate (V) in CCl_4, p = 13 %, l = 0.05 mm.
D: trideca-1:3:5:11-tetraene-7:9-diyne (IX) in CCl_4, c = 19.5 %, l = 0.05 mm.

5—10 % benzene in petroleum ether eluates on slightly deactivated alumina of the essential oil from the following plants: *Carthamus lanatus* L., *Cnicus benedictus* L. and *Silybum Marianum* Gaertn, all three belonging to the tribus *Cynareae*.

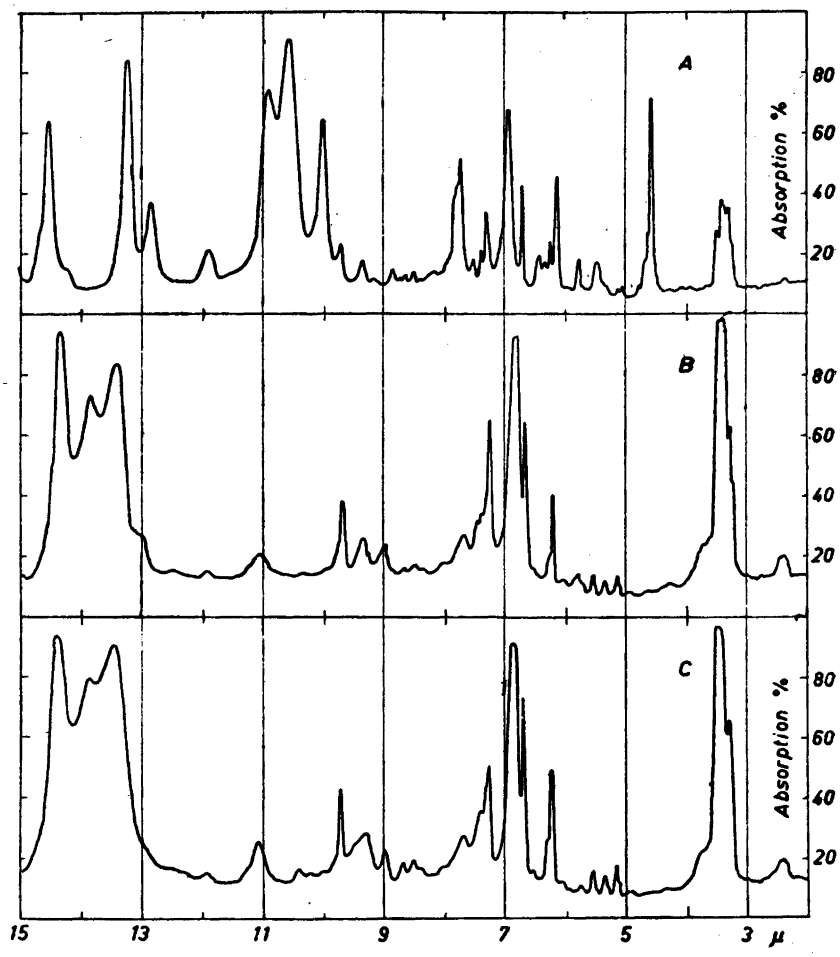


Fig. 3. Infrared absorption of
A: 1-phenyl-undeca-7:9-diene-1:3:5-triyne (XI), liq. l = 0.0125 mm.
B: 1-Phenyl-n-undecane from hydrogenation of (XI), liq. l = 0.025 mm.
C: 1-Phenyl-n-undecane synthetic, liq. l = 0.025 mm.

We intend to isolate (II) in substance from these *Cynaroideae* as soon as larger samples of the plants are available.

During the chromatographic analysis of the root oil, we have not been able to isolate any pure substance from the eluates with petroleum ether — 10 % benzene until petroleum ether — 70 % benzene. With higher amounts of benzene, sometimes only with pure benzene, there was eluted a substance, which crystallised readily as colourless plates, m.p. 43°, and analysing as $C_{15}H_{12}O_2$. The U.V.-spectrum, Fig. 4, curve A, resembled that of matricarianol[9] (IV), but the first maxima were displaced

$$CH_3-CH=CH-C\equiv C-C\equiv C-CH=CH-CH_2OH \qquad (IV)$$

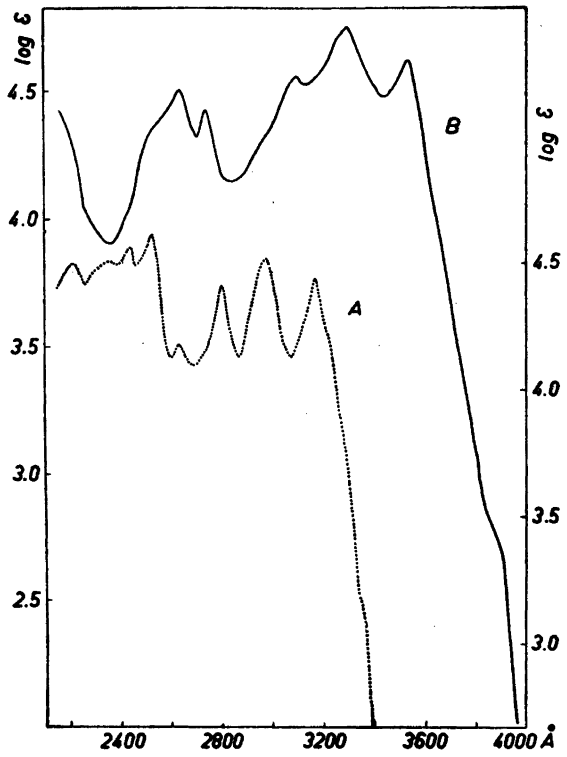
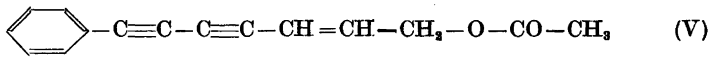


Fig. 4. Ultraviolet absorption in hexane of
A: 1-phenyl-hept-5-ene-1:3-diyn-7-yl acetate (V), scale left side.
B: trideca-1:3:5:11-tetraene-7:9-diyne (IX), scale right side.

some 50 ÅU towards longer wave-lengths. The infra-red spectrum of the 43° compound, Fig. 2, curve C, disclosed that the new compound was an ester as indicated by strong peak at 1 754 cm^{-1} and a broad and strong band at 1 225 cm^{-1}. Moreover, there occurred in the infra-red spectrum a sharp peak at 1 490 cm^{-1}, mostly characteristic of phenyl residues. In the U.V.-spectra of polyenes a phenyl group is about equivalent to 1 1/2 double bond[10], whereas in phenyl-poly-ynes it has mostly been found equivalent to about one double bond[11]. The analysis, U.V. and infra-red spectra taken together point to (V) as a reasonable constitutional formula:



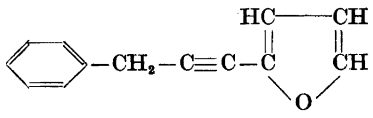
(V)

As mentioned in the following communication, (V) was synthesised by Bruun *et al.*[12] by asymmetric Glaser coupling of phenyl-acetylene with pentenynol (VII) to give the free alcohol, m.p. 72—72.5°, which, acetylated with acetic anhydride-pyridine at 0°, furnished (V), m.p. 45—46°, mixed m.p. with the

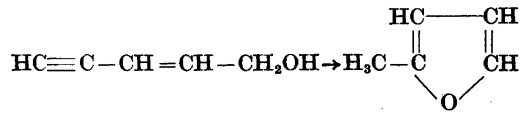
Coreopsis ester 42—43°. The identity was confirmed by saponification of the natural ester to the free alcohol, m.p. 71—72°, undepressed on admixture with the synthetic compound.

So far most of the polyacetylenic compounds isolated from the *Compositae* have occurred with *cis*-configuration at their double bonds. Since the pentenynol (VII) used in the synthesis of (V) is supposed to be the *trans*-isomer, (V) also must have the *trans* configuration.

Professor E. R. H. Jones and Dr. M. C. Whiting have most kindly pointed out the interesting formal connection between (V) and the "carlina oxide" (VI), one of the first acetylenic compounds which was isolated from an essential oil[13], *viz.* that of *Carlina acaulis* L. belonging to *Compositae*, tribus *Cynareae*, subtribus *Carlininae*. Heilbron *et al.*[14] demonstrated the ready conversion of pentenynol (VII) into α-methylfuran (VIII) in acid medium.



(VI)



(VII) (VIII)

The conversion of (V) into (VI) might have proceeded by an analogous mechanism. Several attempts carried out by the Manchester school to realise this transformation have, however, so far been without success.

The crude essential oil from the leaves and stems from the annual *Coreopsis* species showed strong selective absorption in the entire U.V.-region but with rather broad and undistinct maxima. By chromatography on alumina the main part was eluted with the petroleum ether, followed by some small fractions characterised by the presence of the sharp U.V.-maxima of (II). Immediately behind — and in part mixed up with these fractions — there followed, with 5—10 % benzene in petroleum ether, a new compound, responsible for the main U.V.-absorption of the essential oil from the leaves. This compound could be crystallised from petroleum ether at —60° as slightly yellow crystals. These crystals melted at 71—72° and showed very disagreeable properties. Polymerisation to colourless insoluble masses occurred rapidly both in the crystalline state and in solution. In contrast to (II) the 71° compound was oxidised extremely rapidly. The U.V.-spectrum of our best preparations is given in Fig. 4, curve B, the infra-red spectrum in Fig. 2, curve D. The spectra establish the presence of double and triple bonds, but give primarily very little further information.

Catalytic hydrogenation generated a hydrocarbon with the addition of 8 moles of hydrogen which by m.p. —7 to —6°, and mixed m.p. with synthetic tridecane —6.5 to —6°, gave full information about the carbon skeleton. It then remained to arrange 4 double and 2 acetylenic bonds in this normal C_{13} chain.

Valuable indications about this arrangement were provided by the reaction with maleic anhydride. Although maleic anhydride was used in a large excess the reaction product, m.p. 149—150°, had arisen through addition of only one mole of anhydride. We have not been able to purify

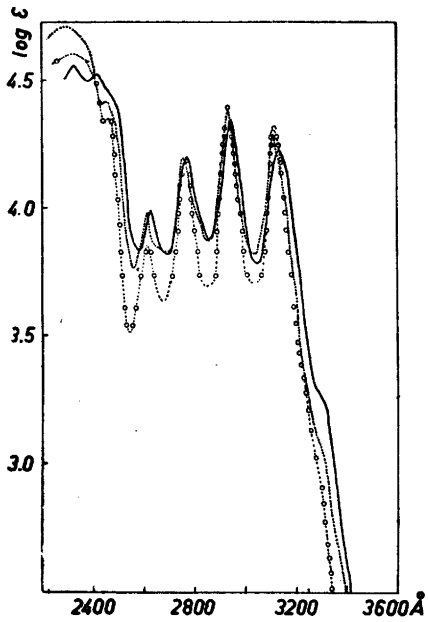
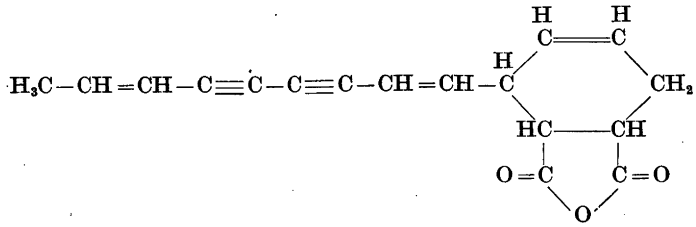


Fig. 5. Ultraviolet absorption in hexane of
——— *maleic anhydride adduct (X),*
····· *trans, trans deca-2:8-diene-4:6-diyn-1-ol (IV),*
·····○···○····· *deca-2:8-diene-4:6-diyne*

the adduct to give correct analytical data for $C_{13}H_{12} + C_4H_2O_3 = C_{17}H_{14}O_3$ but during a few preparations of this adduct we unvariably obtained the same U.V.-spectrum, reproduced in Fig. 5 together with the spectra of deca-2 : 8-diene-4 : 6-diyne and deca-2 : 8-diene-4 : 6-diyn-1-ol = matricarianol (IV). The conformity of the 3 curves establishes the maleic anhydride adduct as a derivative of a symmetrical diene-diyne-grouping with alkyl substituents at both ends. It thus follows that the maleic anhydride has been preferentially added into a butadiene end-residue. The 71° compound thus should have the constitution (IX) = trideca-1 : 3 : 5 : 11-tetraene-7 : 9-diyne with maleic anhydride adduct (X):

$$H_3C-CH=CH-C\equiv C-C\equiv C-CH=CH-CH=CH-CH=CH_2 \qquad \text{(IX)}$$



(X)

Returning to the infrared spectrum of the 71° substance, Fig. 2, curve D, some additional support of formula IX, can be obtained. Curve D has peaks at 905 and 1 005 cm^{-1} characteristic of a conjugated vinyl group[15], and at 948 and 966 cm^{-1} characteristic of conjugated and unconjugated *trans* double bonds,

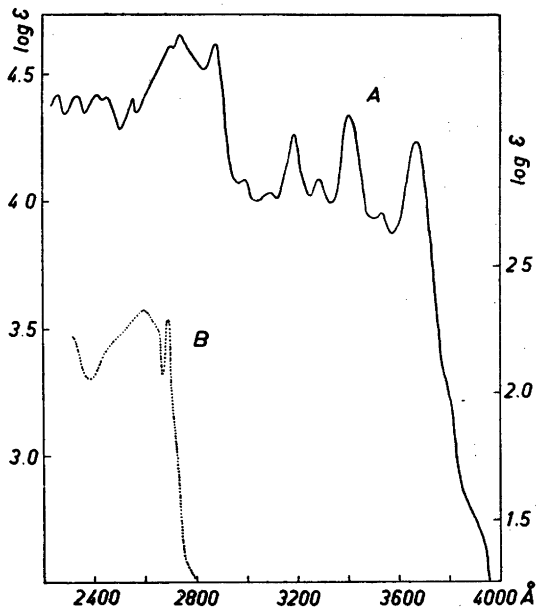


Fig. 6. Ultraviolet absorption in hexane of
A: 1-phenyl-n-undeca-7:9-diene-1:3:5-triyne (XI), scale left side.
B: 1-phenyl-n-undecane from hydrogenation of (XI), scale right side.

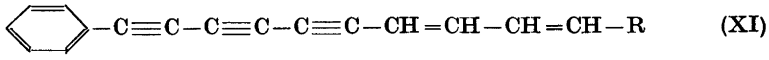
respectively. Since the solvent, CCl_4, covers most of the region where the *cis* double bond mostly absorb, a *cis* configuration is not excluded, but since the *trans* peaks are strong, most probably all double bonds are *trans*.

The crude essential oil from the flowers of the different annual *Coreopsis* species had also a strong absorption in the entire U.V.-region, but with somewhat more distinct maxima than the oil from the green part. By chromatography on alumina the main part was eluted with the petroleum ether; then followed, with 5—10 % benzene in petroleum ether, fractions which, according to the U.V.-spectra, contained small amounts of the diene-tetrayne (II) together with a new substance responsible for the main U.V.-absorption of the flower oils. The separation of this hydrocarbon was somewhat tedious as described in the experimental part. Fig. 6, curve A, gives the U.V.-absorption curve for the main component in hexane. The intersected weak maxima might indicate a mixture of compounds. It should be added, however, that these weak maxima did occur with the same intensities relative to the main maxima in all fractions. After the position of the first maximum at 3 670 ÅU it has been named the "3 670-hydrocarbon". This hydrocarbon is liquid at room temperature, but can be crystallised from petroleum ether at —70°.

Since it is difficult to get rid of the solvent at low temperatures, no m.p. can be given for the "3 670-hydrocarbon".

The infra-red spectrum, Fig. 3 curve A, indicated, by maxima at 1 600, 1 490, 1 445, 917 and 687 cm^{-1}, that the hydrocarbon contained a phenyl nucleus. This was confirmed by the infra-red spectrum of the product of catalytical hydrogenation ($Pd/BaSO_4$, Fig. 3, curve B) which, by maxima at 1 610, 1 500, 1 460 and 695 cm^{-1} was characterised as a mono-alkylbenzene. The conclusion was further confirmed by the U.V.-spectrum of this hydrogenation product, Fig. 6 curve B, which agrees perfectly with those of the toluene group[16]. The hydrogenation product melted at —8 to —6,5°, which comes closest to the data given in literature for 1-phenyl-*n*-undecane, m.p. —5°[17], —10°[18], whereas 1-phenyl-*n*-decane has m.p. —14,4° and 1-phenyl-*n*-dodecane has m.p. + 3°. We have synthesised 1-phenyl-*n*-undecane in different ways, but invariably we have found a m.p. at about —15° for the synthetic phenylundecane. The infra-red spectrum of synthetic 1-phenyl-*n*-undecane, Fig. 3, curve C, agreed completely with that of the hydrogenation product. The American Petroleum Institute has no references to the preparation of their standards or to their purification methods, nor do they give any reason for the rather remarkable difference between the published data, nor is any sample of the standards available. The situation is rather unsatisfactory, but as a preliminary working hypothesis we have supposed that the hydrogenation product of the "3 670-hydrocarbon" is 1-phenyl-*n*-undecane; the possibility of polymorphism cannot be excluded.

Additional information about the structure of the "3 670-hydrocarbon" was rendered by the reaction with maleic anhydride. The reaction was very slow, seemed to some extent to be speeded up by illumination of the "3 670-hydrocarbon" before reaction. The adduct crystallised in small spherical aggregates, which decomposed at 146°. The amount of reaction product was not sufficient for analysis but the U.V.-spectrum was very informative, *cf.* Fig. 7, curve A, where the spectrum of 1-phenyl-octa-1 : 3 : 5-triyne, the synthesis of which is described in the following communication[12], is given for comparison, curve B. The agreement of the two curves leaves no doubt that the adduct is a derivative of phenyl-triacetylene. Then the "3 670-hydrocarbon" must have the constitutional formula XI



(XI)

Indications are that R is CH_3. A substitution on the diene system seems very unlikely since the infra-red spectrum, Fig. 3 curve A, has strong peaks at 944 cm^{-1}, characteristic of R—CH = CH—R^1. A branching on the diene system should give a hydrogenation product with a significantly lower m.p. The methyl peak in curves A and B, Fig. 3, is scarcely strong enough to account for more than one methyl group, *cf.* synthetic 1-phenyl-*n*-undecane, Fig. 3 curve C.

We are convinced that still more polyacetylenic compounds could be isolated from the essential oil of *Coreopsis* if chromatographic separation could be performed with somewhat larger amounts of oil. So far some preliminary investigations of other species of the genus *Coreopsis* indicate that further polyacetylenes dominate in other species.

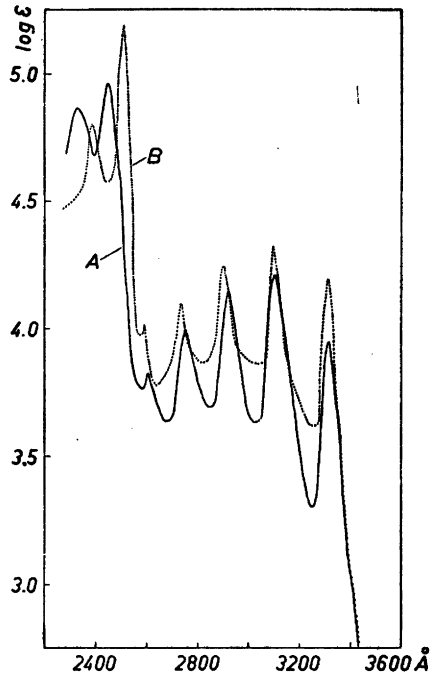


Fig. 7. Ultraviolet absorption in hexane of
A: maleic anhydride adduct of (XI).
B: 1-phenyl-octa-1:3:5-triyne.

EXPERIMENTAL PART

The garden varieties of the following *Coreopsis*-species were cultivated at Norges Tekniske Högskole during the season 1952 on seed samples supplied by Norsk Frö A/S, Trondheim, the numbers in brackets refer to the catalogue of that firm: *Coreopsis cardaminifolia * atrosanguinea* (3215); *C. Drummondii* (3218); *C. tinctoria* (3221); *C. tinctoria "nana radiata"* (3226); *C. tinctoria "nigra speciosa"* (3229). The material of the perennial *C. verticillata* L. was provided by Mr. R. Persson, Trondheim. As soon as some plants flowered a qualitative investigation was carried out on essential oils prepared from the roots, the stem + the leaves, and from the flowering part of the plants. It was found necessary to carry out the steam distillation with exclusion of light and in a closed system under nitrogen in order to keep decomposition and oxidation at a minimum. For the chromatographical investigation the oils from the different garden varieties were combined according to the agreement of the U.V.-spectra. As adsorbent has been used a slightly deactivated alumina[19].

Root oils

The U.V.-spectra of all the essential oils from the roots of the different varieties and that of *C. verticillata* L. agreed very well and were dominated by the sharp peaks belonging to the dienetetrayne (II). By the chromatography an appreciable part, mostly about 1/3, went in the first petroleum ether washings. This fraction was a colourless liquid with selective absorption below 2 600 ÅU only, and might consist of terpene hydrocarbons.

The last washings with petroleum ether and the first eluates with 5 % benzene, which together accounted for only 3 % of the oil, contained all the diene-tetrayne (II). These fractions from the different varieties were combined and rechromatographed until no further change in the U.V.-absorption curve. From petroleum ether these fractions deposited slightly yellow crystals when stored at $-15°$ for some hours. The crystals were

washed with petroleum ether at −15° and dried by evacuation at the oil pump at room temperature. In a sealed m.p. tube the crystals became discoloured from 43° and turned black at about 47° with no further change until 200°.

U.V.-spectrum in hexane, Fig. 1, curve A.

ε_{max}	13 400	20 900	17 200	11 650	143 000	189 000	143 000	
λ_{max}	3 905	3 610	3 370	3 155	2 875	2 710	2 580	ÅU
$\Delta\nu_{max}.10^{-12}$		62.8	59.2	60.7				

Infra-red spectrum Fig. 2, curve A.

Catalytical hydrogenation. 4.44 mg was hydrogenated in alcoholic solution with 31.3 mg catalyst. Consumed (21° C, 740 mm) 7.58 ml, calculated for $C_{12}H_6$, 4/≡, 2/= 7.52 ml. The hydrogenation product was isolated in the usual way and distilled at 0.3 mm, 40° air bath temperature. The distilled hydrocarbon crystallised readily below −15° and melted sharply between −10.8 and −10.3°. For comparison *n*-dodecane was prepared by catalytical hydrogenation of pure lauryl bromide with $Pd/CaCO_3$-catalyst and melted at −10 to −9.3°, given in literature −9.6°[21]. Mixed m.p. of equal amounts of the hydrogenation product and synthetic dodecane −12.3 to −11.5°.

Reaction with peracetic acid: 6.6 mg of (II) was dissolved in the calculated amount of 10 % peracetic acid and left at room temperature for 2 days. A U.V.-spectrum revealed that practically no reaction had taken place. 2.6 mg of crystalline (II) was then dissolved in 2 ml of 10 % peracetic acid. After 24 hours at room temperature the characteristic spectrum of (II) had been displaced by new sharp maxima at 2 450, 2 330, 2 235 ÅU. Dialkyl-tetraynes[4]: 2 410, 2 300, 2 200 ÅU.

With 10 % benzene in petroleum ether until 70 % benzene in petroleum ether some fractions were eluted, which together amounted to about 10 % of the oil, and showed undistinct and variable U.V.-spectra. Since each fraction was small and the spectra agreed with corresponding fractions from flower and leave oils they have not been investigated any further.

When the percentage of benzene was raised above 70 %, about 30 % of the starting material was eluted which readily deposited small amounts of colourless plates from petroleum ether in the cold. Since the total eluates showed only a broad step out in U.V., the mother liquor should contain a mixture of chromophores mainly with short-waved selective absorption. After some recrystallisations the colourless plates melted constantly at 43°. (Analysis: *Micro-analytische afdeling, Laboratorium v. Org. Scheikunde der Universiteit v. Amsterdam.* Found: C 79.8; H 5.6. Calc. for $C_{15}H_{12}O_2$ (224.2): C 80.3; H 5.39).

Ultraviolet absorption spectrum in hexane; *cf.* Fig. 4.

ε_{max}	27 600	32 400	25 700	15 200	40 800	37 200	32 000	
λ_{max}	3 175	2 980	2 810	2 630	2 530	2 450	2 220	ÅU
$\Delta\nu_{max}.10^{-12}$		61.8	60.9	73.1				

Infra-red spectrum in carbon tetrachloride, *cf.* Fig. 2 curve C.
A 1:1 mixture with synthetic (V), m. p. 45−46°[12] melted at 42−43°.

Saponification: Some mg of not quite pure 43°-ester was saponified at room temperature under nitrogen with aqueous alkali with addition of acetone until the solution was homogeneous. After 1 hour the saponification mixture was worked up. The chromophore was obtained in the unsaponifiable part which in the crude state was a red oil. This oil was distilled at 0.001 mm and 90° air bath temperature and the distillate recrystallised twice from petroleum ether; colourless crystals, m.p. 71−72°, synthetic 1-phenylhept-5-ene-1:3-diyn-7-ol 72−72.5° mixed m. p. 71−72°.

The amount of essential oil from the *green part* of the plants amounted to some grammes from each of the garden varieties and therefore the chromatographic separation was carried out with each single variety. The results of the different runs were in very good agreement.

About 3/4 of the oil went in the first washings with petroleum ether and consisted of a colourless oil with U.V.-maximum at 2 280 ÅU (natural farnesene).

With 10 % benzene in petroleum ether the main chromophore of the oil from the green parts was eluted. These crude fractions amounted to some 3 % of the starting material and mostly left a crystalline residue by evaporation *in vacuo*. Recrystallisation from petroleum ether afforded yellow needles, m. p. 71—72°.

U.V.-absorption spectrum in hexane, Fig. 4.

ε max	42 200	57 200	36 000	26 700	32 000	
λ max	3 535	3 300	3 100	2 470	2 640	ÅU
$\Delta\nu$ max.10^{-12}		60.4	57.6			

Infra-red spectrum in carbon tetrachloride solution, Fig. 2, curve D. At an air bath temperature of 68—70° this hydrocarbon can be distilled slowly at 0.0001 mm; if the temperature exceeds 75° decomposition takes place.

Catalytical hydrogenation: 54.2 mg was hydrogenated in alcoholic solution with $Pd/BaSO_4$-catalyst. $C_{13}H_{12}$, 2/≡, 4/= calc. 19°, 750 mm 64.0 ml H_2, observed 65 ml H_2.

The hydrogenation product was isolated in the usual way and distilled at 0.3 mm and 50° air bath temperature. The distillate crystallised readily in the refrigerator and then melted at −7 to −6° C.

For comparison *n*-tridecane was synthesised from *n*-tridecan-4-ol synthesised by Grignard reaction of *n*-propyl bromide with *n*-decylaldehyde. The *n*-tridecan-4-ol was recrystallised from dilute alcohol in the refrigerator and distilled at 0.3 mm. n_D^{20} obs = 1.48325, given[22] is 1.483. The *n*-tridecan-4-ol was dehydrated with $KHSO_4 + K_2SO_4$ at 170° for 5 hours and the olefin isolated in the usual way and distilled over a little sodium metal. 1.14 g of tridecene was hydrogenated with $Pd/BaSO_4$ catalyst in alcohol solution, $C_{13}H_{26}$, 1/= calc. 144 ml at t = 21° and 754 mm H_2, consumed 135 ml. The hydrogenation product, *n*-tridecane was isolated in the usual way and distilled. The distillate melted at −6 to −5.8°, given in literature[21] −5.3°. Mixed m. p., synthetic *n*-tridecane with the hydrogenation product from the 71° hydrocarbon from *Coreopsis*, at −6.5 to −6°. For comparison a mixed m. p. determination was carried out also between synthetic dodecane, m. p. −10 to −9.3°, and the hydrogenation product from *Coreopsis*, m. p. −7 to −6; a 1:1 mixture melted unsharply at −13 to −11.5° C, *i. e.* with a definite depression of the lower m. p.

Reaction with maleic anhydride: 120 mg of the 71°—substance was dissolved in a few ml benzene and a great excess (~ 2 g) of maleic anhydride was added and the solution sealed up under pure nitrogen and heated for 1 hour at 60—65° C. The reaction mixture was evaporated, the solvent at 15 mm, the excess maleic anhydride at 0.0001 mm, air bath at 40° C. The solid residue was dissolved in benzene and after addition of petroleum ether left in the refrigerator. After 24 hours the solutions had deposited colourless needles, m. p. 146—148°. Recrystallisation from benzene-petroleum ether 1:1 raised the m. p. to 149—150° C. Found: C 75.2, 75.8; H 5.1, 5.5. Calc. for $C_{13}H_{12} + C_4H_2O_3 = C_{17}H_{14}O_3$: C 76.6; H 5.3. Calc. for $C_{12}H_{10} + C_4H_2O_3 = C_{16}H_{12}O_3$: C 76.1; H 4.8. The C-values thus indicated $C_{12}H_{10}$ which had been excluded by the catalytical hydrogenation. The adduct decomposed readily and for this reason was difficult to analyse.

U.V.-spectrum in hexane, Fig. 5, curve A.

ε max	16 800	22 200	16 000	9 900	33 600	36 000	
λ max	3 135	2 940	2 770	2 630	2 420	2 330	ÅU
$\Delta\nu$ max.10^{-12}		63.5	62.6	57.7			

The chromatographic column was eluted further with increasing concentrations of benzene in petroleum ether. The fractions eluted were small and with undistinct spectra. Benzene eluted strongly coloured yellow fractions which from petroleum ether deposited the colourless plates of (V) m. p. 42.5 undepressed by (V) from the root oil.

Flower oils

By the chromatography of the essential oil from the flowers the main part — about 2/3 — also was eluted with the first petroleum ether fractions and, like the corresponding fractions from the leaves, seemed to consist of terpene hydrocarbons. With a few per cent benzene in petroleum ether some fractions were eluted, totalling about 10 % of the starting material, which according to spectroscopical U.V.-evidence contained mixtures of the diene-tetrayne (II) and the "3 670-hydrocarbon". We tried to separate this mixture by distillation at 0.0001 mm, but the distillate obtained at a bath temperature of 50° was small and, according to the U.V.-spectrum, seemed to contain the same mixture of the two hydrocarbons. The residue decomposed.

The best method appeared to be rechromatography on deactivated alumina with large amounts of petroleum ether followed by a mixture of 1 % benzene in petroleum ether. The fractions devoid of the 3 915 peak characteristic of (II) were redistilled, boiling range 50—60° at 0.0001 mm.

Ultraviolet absorption in hexane, Fig. 6, curve A.

ε_{max}	17 100	(8 800)	21 600	(12 000)	18 400	(10 800)	12 400	41 200	45 200	
λ_{max}	3 670	(3 525)	3 405	(3 285)	3 186	(3 095)	2 990	2 875	2 735	ÅU
$\Delta\nu_{max}.10^{-12}$		63.7		60.5		61.7				
			62.1		56.1					

Infra-red absorption, liq., Fig. 3, curve A.

Catalytical hydrogenation: 183 mg of freshly distilled "3 670-hydrocarbon" was checked spectrographically in hexane solution, the hexane solution concentrated and added to freshly prepared $Pd/BaSO_4$ catalyst in alcohol. The consumption of H_2, 8.5 moles, was somewhat in excess of the amount calculated for $C_{17}H_{12}$, 3/≡, 2/=.

The hydrogenation product was isolated in the usual way and distilled at 0.0001 and 35° air bath temperature, m. p. −10 to −9°. It could be crystallised from petroleum ether at −70°. The recrystallised hydrocarbon melted at −8 to −6.5° C. Infra-red spectrum, Fig. 3, curve B. U.V.-spectrum, Fig. 6, curve B.

Maleic anhydride adduct. About 10 mg of "3 670-hydrocarbon" was diluted with pure benzene and the benzene solution saturated with maleic anhydride, sealed up in a glas tube under pure nitrogen and heated to 40°. The bath temperature was raised slowly to 70° during 4 hours. The solvent was evaporated and the large excess of maleic anhydride sublimed off at 40° and 0.001 mm. The residue was dissolved in petroleum ether. In the refrigerator this solution deposited small spherical clusters with decomposition point 146°.

U.V.-spectrum, Fig. 7, curve A.

ε_{max}	9 060	16 500	13 700	9 620	6 570	91 700	72 800	
λ_{max}	3 317	3 105	2 920	2 750	2 605	2 450	2 327	ÅU

During chromatography of the oil from the flowers the "3 670 fractions" were immediately followed by fractions containing the trideca-1:3:5:11-tetraene-7:9-diyne (IX), m. p. and U.V.-spectra. With 50 to 70 % benzene in petroleum ether there followed some liquid, orange-red fractions amounting to 1—2 % of the starting material with very undistinct spectra in both visible and U.V.-regions. We have not been able to separate this fraction into pure compounds.

Pure benzene eluated about 1 % of the starting material as semisolid coloured fractions, which by crystallisation from petroleum ether afforded the colourless leaflets of (V) m. p. 43°.

Coreopsis verticillata L. A few flowering plants of this species were bought from Mr. R. Persson, Trondheim, to obtain a preliminary insight into a perennial *Coreopsis* species. The flowers (29 g) gave 1 ‰ of essential oil with only very weak and undistinctive absorption in U. V.

The leaves and stems (215 g) gave 0.4 ‰ of essential oil with rather strong selective absorption in U. V., maxima at 3 100, 2 963 and 2 830 ÅU. The positions of these maxima as well as the general trend of the U.V.-absorption curve were in excellent agreement with those of cosmene = 2:6-dimethyloçta-1:3:5:7-tetraene[15]. The concentration of cosmene in the essential oil of *C. verticillata* amounted to 17 %.

The roots (120 g) gave 0.4 ‰ of essential oil with a U.V.-spectrum composed of the very characteristic maxima of II and those of cosmene. The calculated content of dodeca-1:11-diene-3:5:7:9-tetrayne II was very small, 1.6 %, whereas cosmene amounted to 20 % of this essential oil.

Carthamus lanatus L. We are indebted to professor dr. Rolf Nordhagen, director of the Botanical Garden at the University of Oslo, for small samples of the investigated thistles. Mr. F. E. Eckblad, assistant at the University Botanical Garden, had the kindness to dry the plants at ordinary temperature and in absence of light. The sample of *C. lanatus* L. was so small that a division of the plants in the main parts had to be abandoned. The essential oil of the whole plant had a broad and complex absorption in U.V. By chromatography on alumina the petroleum ether washings brought out a small fraction (1.4 mg) with the very distinct maxima of dodeca-1:11-diene-3:5:7:9-tetrayne (II). The calculated content of (II) in this small fraction was —3 %. With about 20 % benzene in petroleum ether there was eluted a chromophore with an extremely complex U.V.-spectrum resembling some phenanthrene-spectra. Our hydrocarbon which possesses first maxima at 3 550, 3 335 and 3 180 ÅU, has been found in small amounts in some other plants of the Compositae.

Cnicus benedictus L. The starting material was a few dried plants from the University Botanical Garden in Oslo, *cf.* above. The material was practically devoid of roots and was steam distilled without separation. The essential oil had a strong absorption in the entire U.V.-region with several small peaks. Chromatography on alumina disclosed that this oil consisted of a very complicated mixture. With 2 % benzene in the petroleum ether there was eluted a fraction (12.9 mg) with the very distinct spectrum of (II), calculated concentration 0.4 % 20 % benzene in petroleum ether eluted the same hydrocarbon, maxima at 3 550, 3 335 and 3 180 as with *Carthamus*. None of the fractions contained enough substance to permit preparative work.

Silybium Marianum Gaertn., starting material a few dried plants from the University Botanical Garden of Oslo *cf.* above. The small amounts of roots were extracted with acetone, the small quantity of extract (5.7 mg) showed only a step out below 3 000 ÅU.

The rest of the plants were distilled with steam, to give an oil which had a broad and rather undistinct absorption in U. V. By chromatography on alumina the oil was split up into a number of fractions, which according to the U.V.-spectra mostly still should be rather complex mixtures. The fraction eluted with 10 % benzene, however, had an U.V.-absorption conform with the standard curve for II; the content of (II) in this fraction amounted to only 1 ‰.

Grants from *Norges Almenvitenskapelige Forskningsråd* which made it possible to carry out these investigations are gratefully acknowledged. We wish also to express our gratitude to *Norsk Hydro-Elektrisk Kvælstofaktieselskab, Oslo*, who in the past 10 years has kept our laboratory with nitrogen devoid of any traces of oxygen. The importance of these gifts for experimental investigations of natural compounds of the unstability of the *Coreopsis*-components treated above can scarcely be overestimated.

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Studies Related to Naturally Occurring Acetylene Compounds. XVIII. The Synthesis of Some Phenylacetylenes Related to *Compositae* Compounds

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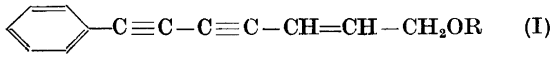
1-Phenyl-hept-5-ene-1:3-diyn-7-yl acetate (I), methyl 1-phenyl-hept-5-ene-1:3-diyn-7-oate (II) and 1-phenyl-octa-1:3:5-triyne (VII) were synthesised according to known procedures. (I) turned out to be identical with one of the acetylenic components of the essential oils of some *Coreopsis* species. (VII) showed an U.V. absorption spectrum so close to those of ene-triynes that qualitative spectroscopical separations of these chromophores are obliterated.

Some of the acetylenic compounds which occur in *Compositae* plants are present in very small amounts and so it is sometimes difficult to obtain sufficient amounts of the pure compounds for degradational experiments to establish their structure. There has in recent years accumulated a rather large body of material concerning the ultra-violet as well as the infra-red spectra of synthetic and naturally occurring acetylenic compounds which sometimes makes it possible to predict a very restricted number of possible structures from the spectroscopical data.

J. S. Sörensen and N. A. Sörensen[1] in their work on the polyacetylenes of some *Coreopsis* species isolated small amounts of a substance, m.p. 43°, which they assumed to be 1-phenyl-hept-5-ene-1 : 3-diyn-7-yl acetate (I, R = $COCH_3$)



(I)

Since this compound should be readily available by a Glaser coupling[2] of phenylacetylene with the pent-2-en-4-yn-l-ol of Heilbron *et al*[3]. this synthesis was supposed to be the easiest way to get a confirmation of the structure and at the same time to establish the unknown configuration at the double bond. As described in the experimental part the synthesis of (I, R = $COCH_3$) through the corresponding free alcohol (I, R = H) was successful. The m.p. of synthetic

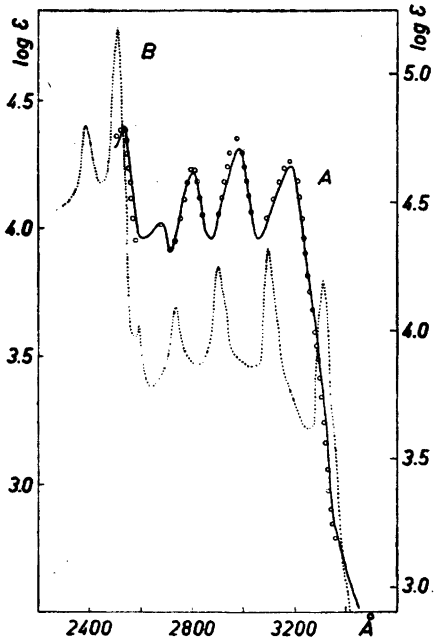


Fig. 1. Ultraviolet absorption in hexane of
A: O O O O *1-phenyl-hept-5-ene-1:3-diyne-7-ol (I), synthetic, scale left side.*
A: ——— *1-phenyl-hept-5-ene-1:3-diyne-7-ol (I), from Coreopsis, scale left side.*
B: ····· *1-phenyl-octa-1:3:5-triyne, scale right side.*

(I, R = $COCH_3$) was found at 45—46°, mixed m.p. with the ester from *Coreopsis* 42—45°. As is seen from Fig. 1 the U.V. spectra were identical. The identity was further confirmed by mixed m.p. determination with the saponification product of the *Coreopsis* ester.

The methyl ester of the acid corresponding to the alcohol (I, R = H) was also prepared by an asymmetric Glaser coupling. This ester (II) may be regarded as a phenyl analogue of the matricaria ester (III)


$-C\equiv C-C\equiv C-CH=CH-COOCH_3$ (II)

$H_3C-CH=CH-C\equiv C-C\equiv C-CH=CH-COOCH_3$ (III)

As will be seen from Fig. 2, the similarity of the two U.V. absorption curves is rather striking. The agreement of the maxima in the long-wave band group is good; curiously enough (II) has the most pronounced fine structure. In the short-wave band group (II) has maxima at about 50 ÅU longer wavelengths than (III).

Sörensen *et al.* in the cited paper[1] transferred a liquid hydrocarbon, known to contain a monosubstituted benzene ring, into a maleic anhydride adduct with a beautiful fine structure in the UV spectrum, with maxima occurring in positions close to those of ene-triynes, represented by one of the compounds of the oil from the root of *Artemisia vulgaris*[4] (Table 1).

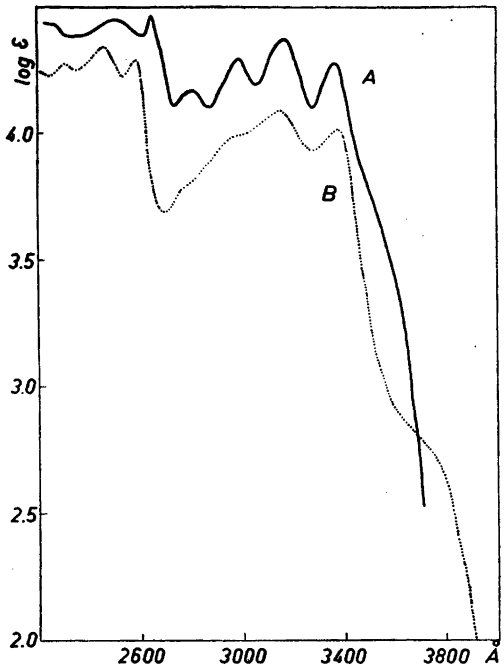


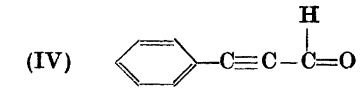
Fig. 2. Ultraviolet absorption in hexane of A: methyl 1-phenyl-hept-5-ene-1:3-diyn-7-oate (II). B: methyl deca-2:8-diene-4:6-diyn-1-oate (III).

Table 1.

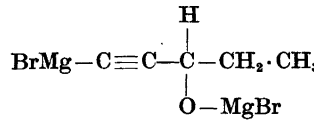
Found in adduct	3 317	3 105	2 920	2 750	2 605	2 450	2 327
Found in $R-(C\equiv C)_3-CH=CH-R'$	3 286	3 081	2 890	2 725	2 577	2 425	2 310
Found in $Ph-(C\equiv C)_3-CH_2-CH_3$	3 315	3 100	2 905	2 735	2 595	2 510	2 387

In this connection it was desired to obtain information about the UV spectrum of phenyltriynes of the type $Ph-(C\equiv C)_3-R$ with R = alkyl. Since amyldiacetylene was at hand from another synthesis[5], we first tried a Glaser coupling with this substance and phenylacetylene. From the reaction mixture fractions could be isolated with UV maxima in the expected position as far as regards the weak long-wave band group. The strong short-wave band group was, however, mixed up with very sharp peaks corresponding to diamyl tetra-acetylene[5], and our efforts to separate this mixture have been without success.

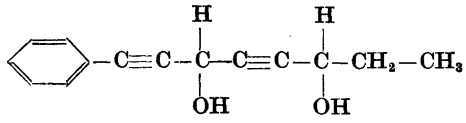
1-Phenyl-octa-1 : 3 : 5-triyne (VII) was then synthesised according to reactions worked out in Manchester for analogous reactions[6]. Phenylpropargyl aldehyde (IV) was reacted with the Grignard complex of pent-1-yn-3-ol (V) to 1-phenyl-octa-1 : 4-diyne-3 : 6-diol (VI). The dichloride of (VI) was dehydrohalogenated with potassium ethoxide according to Bohlmann[7].



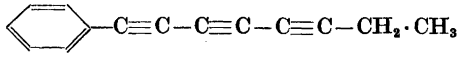
(IV)



(V)



(VI)



(VII)

(VII) was purified by chromatography on alumina and carefully distilled at 0.0005 mm and 60°. (VII) decomposed readily on superheating and elementary analyses were quite unsatisfactory. The constitution of the synthesised hydrocarbon, therefore, was proved by catalytical hydrogenation to 1-phenyl-*n*-octane, identical in the infra-red spectrum with authentic material.

The UV spectrum of (VII) is given in Fig. 1, curve B. The maxima come very close — as can be seen from the compilation of the data in Table 1 — to those of ene-triynes. In diacetylenes the phenyl group is spectroscopically somewhat more active than one double bond; in the tri-yne series the difference is too small for qualitative spectroscopical examinations.

EXPERIMENTAL

1-Phenyl-hept-5-ene-1:3-diyn-7-ol. Air was passed through a mixture of phenylacetylene (5 g), pent-2-en-4-yn-1-ol (4.1 g) and a solution of ammonium chloride (112.4 g), copper(I) chloride (72.5 g) and iron(III) chloride (5.9 g) in water (280 ml) for 6 ½ hours at room temperature. The reaction product was isolated with ether. The residue left after the ether had been removed was extracted with three times 100 ml of petroleum b.r. 40/70°. The material which had been extracted by the petroleum was chromatographed on de-alkalised alumina[8]. Diphenyl-diacetylene was removed by petroleum and petroleum-benzene mixtures. Elution with ether furnished an oil (0.7 g), which from petroleum afforded long, thin, colourless needles of 1-phenyl-hept-5-ene-1:3-diyne-7-ol, m.p. 71—72° (0.3 g). Light absorption maxima: 3 180, 2 980, 2 810 and 2 530 Å, ε 18 000, 24 000, 18 000 and 24 000, respectively. (Found: C 86.05; H 5.55. $C_{13}H_{10}O$ requires C 85.65; H 5.55). A mixture with the saponified compound from *Coreopsis*[1] (m. p. 71—72°) melted at 71—72°, all three samples taken at the same time. The substance was acetylated in pyridine with a large excess of acetic anhydride with standing in the refrigerator for 2 days and then 3 hours at room temperature. The *acetate* was isolated with ether and crystallised from petroleum, m. p. 45—46°, colourless plates, light abs.max. 3 185, 2 975, 2 810, 2 660, 2 535, 2 435, 2 390 and 2 320 Å, ε 15 000, 19 000, 15 500, 10 000, 31 500, 42 500, 43 000, 43 000, respectively. (Found: C 80.25; H 5.35. $C_{15}H_{12}O_2$ requires C 80.35; H 5.4.) A mixture with the *Coreopsis* compound[1] (m. p. 40—43°) melted at 42—45°, all three taken at the same time.

Methyl 1-phenyl-hept-5-ene-1:3-diyn-7-oate. The ester was prepared by oxidative coupling of phenylacetylene (8.0 g, impure) with methyl pentenynoate[3] (3.75 g) by passing air for 6 hours through a solution of ammonium chloride (76 g), copper(I) chloride (49 g) and iron(III) chloride (4.0 g) in water (190 ml). The reaction product was isolated with ether and chromatographed on de-alkalised[8] alumina. The fractions eluted with petroleum ether-benzene (1:1) and benzene were combined and crystallised from petro-

leum ether. The deposited yellow crystals, m. p. 42—43°, were rechromatographed carefully to remove the last traces of diphenyl diacetylene. The purest material was crystallised twice from petroleum ether. The methyl 1-phenyl-hept-5-ene-1:3-diyn-1-oate formed nearly colourless, hard crystals, m. p. 44—45° (Found: C 80.15; H 4.8. $C_{14}H_{10}O_2$ (210.2) requires C 80.0; H 4.8). Light abs. max. in hexane: 3 365, 3 150, 2 980, 2 800, 2 640, and 2 500 ÅU, ε 18 500, 22 000, 19 500, 14 000, 28 500 and 28 000, respectively.

Phenylpropargyl aldehyde. This aldehyde was prepared according to[9], with the exception that the cyclic ethylene acetal of α-bromocinnamic aldehyde was used as an intermediate instead of the diethyl acetal. It was prepared in the following way (compare[10]. We are indebted to B. Löken, Assistant Director of Development, Syntex S/A, for the details of the procedure): α-Bromocinnamic aldehyde (105 g) and ethylene glycol (25.4 g) in toluene (150 ml) were refluxed in a water determination apparatus with addition of *p*-toluenesulphonic acid (100 mg) until the theoretical amount of water (7.4 ml) had been collected. The *cyclic acetal* boiled at 168—172° at 16 mm (Found: C 74.6; H 6.1. $C_{11}H_{11}O_2Br$ (256.2) requires C 74.4; H 6.1).

1-Phenyl-octa-1:4-diyne-3:6-diol. Ethyl magnesium bromide was prepared in the customary manner from 8 g of magnesium in 100 ml of tetrahydrofuran. To the filtered solution of the Grignard compound pent-1-yn-3-ol (15 g)[11] in tetrahydrofuran (30 ml) was added drop-wise with stirring and cooling during 1 hour. The solution was refluxed for another hour. Freshly distilled phenylpropargyl aldehyde (15 g) was added to the refluxed solution, drop-wise, with stirring and cooling during one hour, heated for another hour and decomposed, whilst still warm, with ice-water and diluted sulphuric acid. The tetrahydrofuran and water layers were separated and the water solution extracted with ether (200 ml). The combined ether and tetrahydrofuran solutions were dried over sodium sulphate. Removal of the solvents, finally in a vacuum, left a viscous, orange liquid (20 g), b. p. 100° at 0.005 mm.

1-Phenyl-3:6-dichlorocta-1:4-diyne. The diol (above) (17 g) was dissolved in pyridine (15 ml) and thionyl chloride (15 ml) was added drop-wise with stirring and cooling to keep the temperature below —15°. The stirring was continued for 4 hours at room temperature, and then the reaction mixture was decomposed by addition of water. Organic material was extracted with 3 × 100 ml ether. The combined ether extracts were washed with 100 ml of 2 *N* hydrochloric acid and then with a 5 % sodium carbonate solution until neutral reaction. The ether solution was dried with magnesium sulphate. The crude reaction product (12 g) was not purified but used straight away for the following reaction.

1-phenyl-octa-1:3:5-triyne. Potassium ethoxide (from 4 g of potassium) in ethanol (100 ml) was cooled to —15 to —20° and the crude dichloride above (12 g) was added drop-wise during ½ hour (nitrogen) with stirring. Stirring was continued for 1 ½ hours, when water was added and the reaction product extracted with ether. The ether solution was dried with magnesium sulphate. The extracted material was purified by repeated chromatography on alumina. The pure *1-phenyl-octa-1:3:5-triyne* distilled at 60° and 0.0005 mm as a faintly yellow liquid (1.3 g). Light abs. max. 3 315, 3 100, 2 905, 2 735, 2 595, 2 510 and 2 385 Å, ε 16 000, 21 000, 18 000, 13 000, 10 000, 155 000, 63 000, respectively. On hydrogenation in ethanol with a palladium-on-barium sulphate catalyst 5.9 moles of hydrogen were consumed. The hydrogenation product had n_D^{20} 1.4850 (given for *n*-octylbenzene n_D^{20} 1.4851). Infra-red spectra of the hydrogenation product and of synthetic *n*-octylbenzene were identical in the 2—15 μ range.

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Studies Related to Naturally Occurring Acetylene Compounds. XIX. The Isolation of 1-Acetoxy-*n*-Trideca-2:10:12-triene-4:6:8-triyne from *Carlina vulgaris* L.

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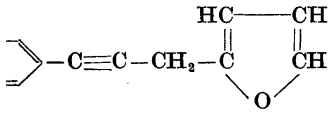
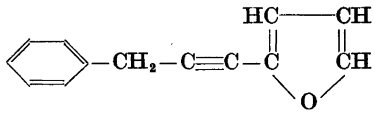
1-Acetoxy-*n*-trideca-2:10:12-triene-4:6:8-triyne (V) has been isolated from common Carlinethistle, *Carlina vulgaris* L.* *eu-vulgaris* Holmboe. (V) is the main chromophore of the lipids of all parts of the thistle. (V) bears interesting relations to the "carlina oxide" (II) isolated from *C. acaulis* L. and to 1-phenyl-*n*-hept-5-ene-1:3-diyn-7-ol (VI) recently isolated as the acetate from some species of the genus *Coreopsis*.

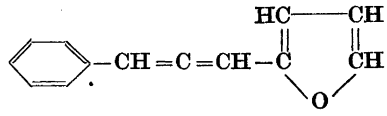
More than 50 years ago F. W. Semmler[1] isolated the so-called "carlina oxide" from the essential oil of *Carlina acaulis* L. The degradational studies which Semmler himself carried out[2] left only three constitutional possibilities (I)-(III).



(I)



(II)



(III)

Of these three possibilities Semmler — lead by an irrational aversion against the occurrence of acetylenic compounds in nature — chose (III), and it remained for A. St. Pfau *et al.* in 1935[3] to prove that "carlina oxide" has the constitution (II).

In this laboratory the essential oils from some two hundred plants belonging to the family *Compositae* have been investigated with regard to their content of acetylenic compounds. The old "carlina oxide" we have, however, so far never met with. It has to be added that none of the plants investigated by us were in a botanical sense close relatives to *C. acaulis* L. which belongs to the tribe *Cynareae*, subtribe *Carlininae*. A recent preliminary investigation of the

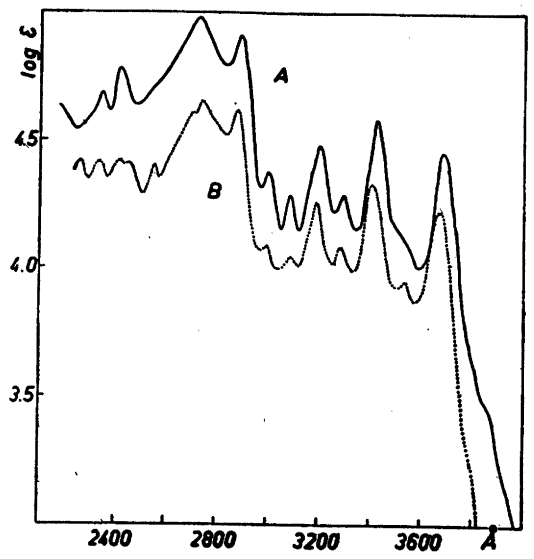
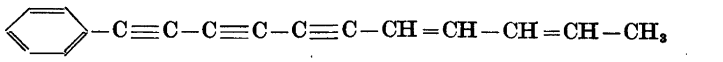


Fig. 1. Ultraviolet absorption in hexane of
A: 1-acetoxy-n-trideca-2:10:12-triene-4:6:8-triyne (V) from Carlina.
B: 1-phenyl-n-undeca-7:9-diene-1:3:5-triyne (IV) from Coreopsis.

essential oils from some members of the tribe *Cynareae*[4] revealed that they contained some conjugated polyenynes, but we found no indication of the presence of "carlina oxide". *Carlina acaulis* L. does not occur in Norway, where the only members of the genus *Carlina* are two subspecies of *C. vulgaris* L., *viz.*, *eu-vulgaris* Holmboe and *longifolia* (Rchb.) Neum. which occur in restricted areas of Southern Norway[5]. The starting material for our chemical investigations were seed samples of the subspecies *eu-vulgaris* Holmboe, most kindly collected by Dr. Hugo Sjörs in the surroundings of Uppsala in Sweden. The seed sample gave about 100 of this small prickly thistle. Qualitative investigations disclosed that acetone extraction at room temperature and steamed distilled essential oils gave the same U.V.-spectra, and that there were only slight differences between the spectra from the different parts of the thistle. All samples had strong selective absorption in the entire U.V.-region, with absorption maxima very close to that of 1-phenyl-*n*-undeca-7 : 9-diene-1 : 3 : 5-triyne (IV) isolated recently[4] from some species of the genus *Coreopsis*.



(IV)

By chromatographic analysis the essential oil from *Carlina vulgaris* was separated into a lot of fractions. Only the fractions containing the main chromophore have so far been worked up in detail. The main chromophore is retained on the alumina column until pure benzene or benzene-ethyl ether mixtures are used for elution. (IV) as a hydrocarbon is eluted already with

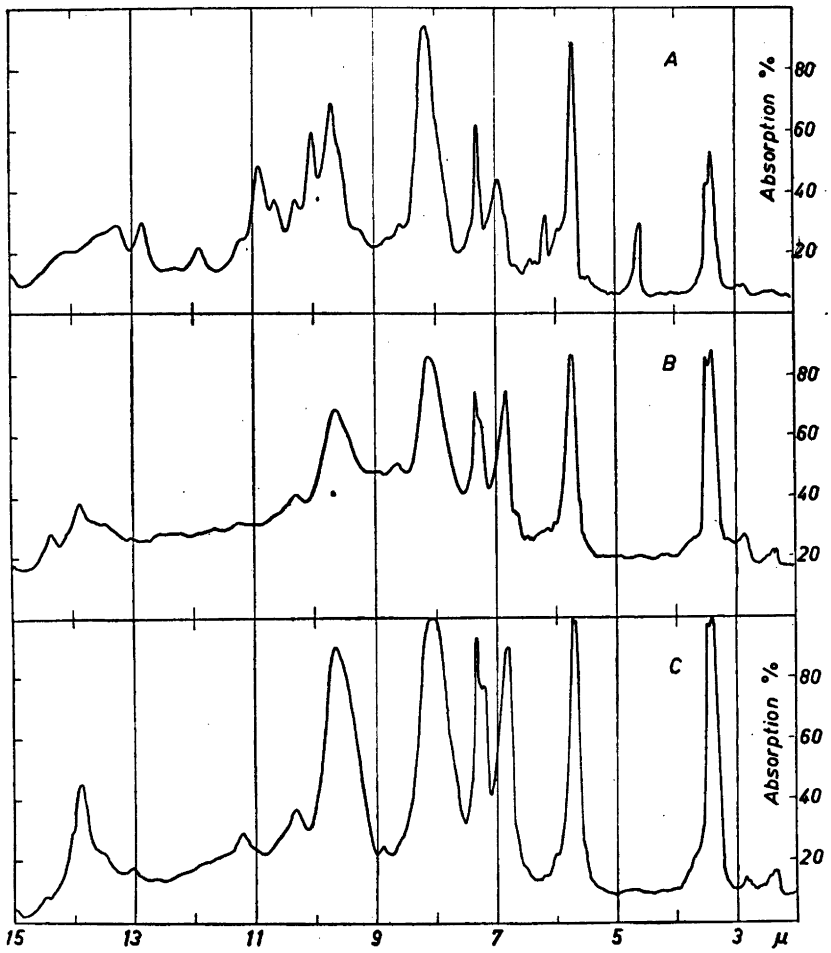


Fig. 2. Infrared absorption of

A: 1-acetoxy-n-trideca-2:10:12-triene-4:6:8-triyne (V) liq., demontable cell, thickness ca. 0.01 mm.
B: 1-acetoxy-n-tridecane from hydrogenation of (V), liq., thickness ca. 0.01 mm.
C: 1-acetoxy-n-tridecane synthetic, liq., thickness ca. 0.025 mm.

2—5 % benzene in petroleum ether and so it was obvious that the *Carlina* chromophore contained some more polar groups. By a combination of partial distillation and rechromatography the main chromophore was purified to constant spectral properties. The pure compound was a slightly yellow liquid, which solidified at —15°. We have found no solvent suited for recrystallisation. Our best fractions melted somewhat above —15°. The U.V.-spectrum in hexane is given in Fig. 1 together with that of (IV). The conformity is rather striking. The infra-red spectrum, Fig. 2, curve A, besides confirming the

acetylenic nature of the substance revealed two important facts, firstly that the *Carlina* chromophore was an acetate, sharp peak at 1 750 cm^{-1} strong band at 1 225 cm^{-1}; secondly there was no indication of a phenyl residue as in (IV).

The perhydro compound, prepared by catalytical hydrogenation, had an infra-red spectrum, Fig. 2, curve B, which resembled very much that of *n*-decyl-acetate which we had obtained by hydrogenation of the matricarianyl acetate from some *Aster* and *Grindelia* species[6]. Thus obviously the hydrogenated compound is an aliphatic acetate.

This perhydro ester was saponified. The unsaponifiable part turned out to be *n*-tridecan-1-ol as shown by mixed m.p. with authentic material.

The *Carlina* chromophore reacted slowly with maleic anhydride. Although the amount of adduct obtained was too small for analysis, its U.V.-spectrum furnished valuable information. The sharp U.V.-maxima of the adduct agreed with that of a ketone isolated from *Artemisia vulgaris*[7], which has an isolated carbonyl function and a conjugated ene-triyne system of the type

$$R-C\equiv C-C\equiv C-C\equiv C-CH=CH-R''$$

In the *Carlina* chromophore the diene system which have reacted with maleic anhydride must take part in the end residue R to account for the striking conformity of the U.V. spectrum of (IV) with the *Carlina* chromophore. Therefore the acetate group must be present in R''. Since the infra-red spectrum informs us that the acetate ester group is not a vinyl ester, R'' must be $-CH_2-O-CO-CH_3$. The *Carlina* chromophore then is given the structure (V) of 1-acetoxy-*n*-trideca-2 : 10 : 12-triene-4 : 6 : 8-triyne.

$$CH_2=CH-CH=CH-C\equiv C-C\equiv C-C\equiv C-CH=CH-CH_2-O-CO-CH_3 \qquad (V)$$

Professor E. R. H. Jones and Dr. M. C. Whiting have, as mentioned in the XVIIth-communication[4] of this series, pointed out the analogy between the prototropic rearrangement of pentenynol to α-methyl furan[8] and an isomerisation of 1-phenylhepta-5-ene-1 : 3-diyn-7-ol, found as the acetate (VI) in some *Coreopsis* species, to the "carlina oxide" of *C. acaulis*



$$-C\equiv C-C\equiv C-CH=CH-CH_2-O-CO-CH_3 \qquad (VI)$$

The alcohol corresponding to (V), the main chromophore of the essential oil of *C. vulgaris*, the alcohol corresponding to (VI) and the "carlina oxide" all have the formula $C_{13}H_{10}O$. That the furan ring in (II) has arisen through some rearrangement similar to the prototropic rearrangement of acetylenic compounds to furan-derivatives as demonstrated by I. M. Heilbron *et al*[8]. is very likely; the constitution (V) ascribed to the *C. vulgaris* chromophore raises the interesting question wether the phenyl residue in (II) and (VI) is formed by curling up of the dienyne tail of (V).

EXPERIMENTAL

The starting material consisted in part of acetone extracts obtained at room temperature and of steam distilled oils of the roots, the stem + leaves, and of the flower heads of the small thistle. All crude oils were chromatographed in petroleum ether solution on slightly deactivated alumina followed by elution with benzene-petroleum ether mixtures of per centage 10, 25, 50 and 100 benzene, ethyl ether and methanol.

Only the fractions eluted with 50 % benzene contained substances with long-wave selective absorption in the U.V. The first of these fractions showed absorption bands at 3 320, 3 140 and 2 580 ÅU. This chromophore accompanied also the main chromophore in the benzene and benzene-ether eluates. By the distillation of these fractions at 0.0001 mm the substance with a first max. at 3 320 distilled somewhat earlier, air bath temperature $< 70°$, than the main chromophore, which may be distilled at this pressure with air bath temperature at about 80°.

The distillates were rechromatographed on deactivated alumina, and then crystallised at −15°. U.V.-spectrum, Fig. 1.

ε_{max}	28 900	39 000	19 500	30 600	19 500	24 000	81 400	95 600	66 400	49 000
λ_{max}	3 685	3 420	3 300	3 195	3 085	3 000	2 890	2 725	2 415	2 345 ÅU
$\Delta\nu_{max}10^{-12}$	63.1		61.7		61.1					
			63.3							

Infra-red spectrum, Fig. 2 curve A.

Catalytical hydrogenation: 10.5 mg substance was hydrogenated in alcoholic solution with a $Pd/BaSO_4$-catalyst. Consumed, $t = 21°$, $p = 755$ mm, 10.9 ml H_2: calculated for $C_{15}H_{12}O_2$, 3/≡, 3/= 10.5 ml. The hydrogenation product was isolated in the usual way and distilled at the oil pump. Infra-red spectrum Fig. 2 curve B, synthetic 1-acetoxy-*n*-tridecane Fig. 2 curve C.

The hydrogenation product was saponified with alcoholic potassium hydroxide and the unsaponifiable part isolated. After distillation at 3 mm/air bath temperature 100° the product melted at 17.5−18.5°, crystallised from hexane m. p. 24°, recrystallised, m. p. 26.5−26.7°.

For comparison *n*-tridecan-1-ol was synthesised by the Grignard reaction of lauryl bromide with trioxymethylene according to Ou[9]. Synthetic *n*-tridecan-1-ol m. p. 28°, literature[10] 30.5°. Mixed m. p. of the unsaponifiable matter from the hydrogenation of the *Carlina* chromophore with synthetic *n*-tridecan-1-ol 27.5−28°. For comparison a mixed m. p. determination was carried out on a mixture about 1:3 of synthetic *n*-dodecanol and *n*-tridecanol, found unsharp melting range 23−26°.

Maleic anhydride adduct. 3 mg of pure *Carlina* chromophore was dissolved in pure benzene and a large excess of freshly sublimed maleic anhydride dissolved in benzene was added, and the solution left at room temperature under nitrogen for 2 hours. A sample showed that the U.V.-spectrum had not changed. The solution was then sealed up in a glass tube under pure nitrogen and heated at 75° for 1 ½ hours. A U.V.-spectrogram revealed that still no reaction had taken place.

A solution of about 2 mg of pure *Carlina* chromophore in benzene was illuminated for 1 ½ hours with a mercury lamp. Again the U.V.-spectrum of a sample revealed that the chromophore had not changed. To the irradiated solution a saturated solution of maleic anhydride in benzene was added the solution sealed up under pure nitrogen and heated at 60° for 3 hours. The solvent and the excess of maleic anhydride were removed at 40° and 0.0001 mm. The residue was rather insoluble in petroleum ether; it was dissolved in a little ethyl ether and diluted with spectral hexane. U.V.-maxima

maleic anhydride adduct:	3 320	3 108	2 905	2 740	—	2 450	ÅU
$R-C\equiv C-C\equiv C-C\equiv C-CH=CH-R''$:	3 286	3 086	2 890	2 725	2 577	2 425	»

We wish to thank Chem. Eng. Dagny Holme and Aili Torvund for valuable collaboration. Grants from *Norges Almenvitenskapelige Forskningsråd*, which made it possible to carry out these investigations, are gratefully acknowledged.

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Studies Related to Naturally Occurring Acetylene Compounds. XX. A Preliminary Communication on Some Polyacetylenic Pigments from *Compositae* Plants

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A reinvestigation of the essential oil from some species *Helipterum* rendered a complicated mixture of polyacetylenic compounds. Three of these, with first maximum at 3 985, 4 005 and 4 100 ÅU, respectively, were obtained spectroscopically pure. Of these, the first polyacetylenic pigments found in Nature, only the 4 100-pigment could be isolated in substance. The spectral properties of the 4 100-pigment point definitely to an ene-pentayne structure (V), the extreme instability corresponds well to that described for synthetic relatives. The very characteristic spectrum of (V) has so far been found in a dozen *Compositae* plants belonging to the tribes *Inuleae* og *Heliantheae*. Acetylenic pigments thus seem to be rather common in nature. The small concentrations in which they have been found and their instability may explain why they have not been detected before.

In the XIIth communication of this series[1] Skatteböl and Sörensen synthesised methyl deca-2 : 4-diynoate-1 (I) with the object of obtaining the

$$CH_3-(CH_2)_4-C\equiv C-C\equiv C-COOCH_3 \qquad (I)$$

$$R-C\equiv C-C\equiv C'-CH=CH-R' \qquad (II)$$

U.V.-spectrum for comparison with an unknown chromophore with first U.V.-maxima at 2 862 and 2 650 ÅU found in essential oils from some species belonging to the genus *Helipterum*. The observed maxima of this *Helipterum* chromophore occurred at somewhat shorter wave-lengths than those of enediynes (II). (I) turned out to have maxima at somewhat longer wave-lengths than (II), and so we had to return to an investigation of new samples of essential oils from *Helipterum*. In the meantime the problem had considerably altered since it on a spectroscopical investigation of a small sample of the essential oil from *Gnaphalium luteo-album* L. root was observed that this oil had

strong maxima in the same positions 2 865 and 2 650 ÅU as the *Helipterum* oils, but besides these strong maxima the *Gnaphalium* oil had very weak but extremely sharp maxima at 4 100, 3 780, 3 290 and 3 075 ÅU, and when this oil was investigated chromatographically it turned out that the weak long-waved band group belonged to the same substance as the 2 865 and 2 650 ÅU maxima. The same turned out to be the case also for *Helipterum* oils when new samples became available.

The *Gnaphalium luteo-album* had been drawn from a seed sample most kindly put at our disposal by Dr. K. Bisvas, superintendent of the Indian Botanic Garden, Calcutta, who on the request of professor Dr. Ove Arbo-Höeg had sent us a small collection of seeds of Indian *Compositae*. This collection consisted partly of seeds from some widely distributed tropical weeds. The selection of these samples was for our purposes the most lucky one, as all of them turned out to be rich in polyacetylenic compounds and some of them contained the chromophore with first maximum at 4 100 ÅU. As described in the experimental part this chromophore — the first polyacetylenic pigment observed in Nature — has so far been spectroscopically indicated in the root of the following plants:

	Tribe and subtribe according to Hofmann[2]	
Blumea lacera D. C.	*Inuleae*	*Plucheinae*
Blumea lacinata D. C.	»	»
Gnaphalium luteo-album L.	»	*Gnaphalinea*
Gnaphalium silvaticum L.	»	»
Helipterum Manglesii F. V. Muell.	»	»
Helipterum roseum Benth.	»	»
Helipterum Sandfordii	»	»
Pulicaria vulgaris Gaertn.	»	*Inulinae*
Xanthium strumarium L.	*Heliantheae*	*Ambrosinae*
Xanthium, unknown sp.	»	»
Sanvitalia procumbens Lam.	»	*Zinninae*
Synedrella nudiflora Gaertn.	»	*Coreopsidinae*

Thus this pigment seems to have a rather wide occurrence. The amounts present in essential oils or acetone extracts are mostly so small that only a few of the strongest U.V.-absorption maxima stand out on the general step out towards shorter wave-lengths generally met in crude oils.

Acetone extracts of the root of *Helipterum Manglesii* F. v. Muell. and *Helipterum roseum* Benth. have given the highest amounts of the 4 100-pigment and have permitted a preliminary study of the structure. These extracts contained a complex mixture of substances with selective absorption in U.V. So far we think that only 3 of them have been obtained spectroscopically pure; only the 4 100-pigment was present in sufficient amount to be isolated in substance.

When these *Helipterum Manglesii & H. roseum* root extracts were chromatographed on slightly deactivated alumina[3], 1—2 % benzene in petroleum ether eluted a slightly yellow substance with the U.V.-spectrum reproduced in Fig. 1 curve B, first maximum 4 005 ÅU. With the first washings with 5 % benzene in the petroleum ether there followed mixtures of this 4 005 chromo-

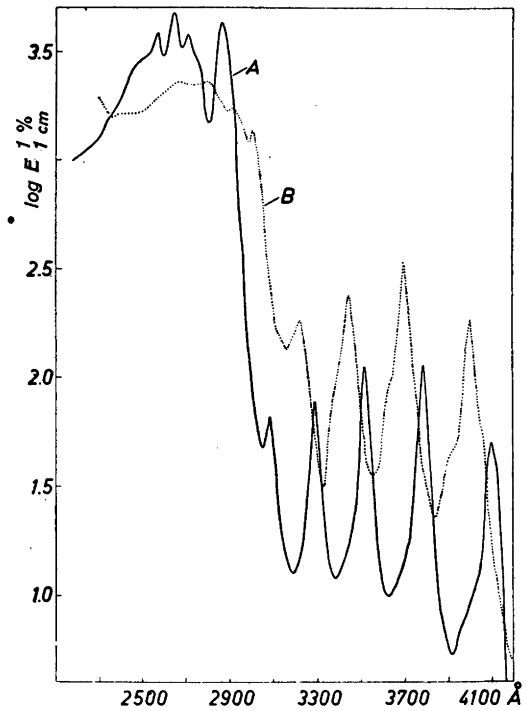

Fig. 1. Ultraviolet absorption in hexane of
A: 4 100-pigment from H e l i p t e r u m, B: 4 005-pigment from H e l i p t e r u m.

phore with the 4 100-pigment. With the later washings with 5 % benzene in petroleum the 4 100-pigment may sometimes be obtained quite pure. When evaporated *in vacuo* at room temperature this solution gives a residue of yellow elongated prisms, which turn black and insoluble at room temperature in some minutes. When stored at —15° they acquire a bronze-coloured surface in a few minutes and when redissolved a dark pattern of the surface of the crystals are left as an insoluble residue on the wall.

The U.V.-spectrum of the crystalline 4 100-pigment is given in Fig. 1, curve A. As the extreme instability described above prevents all exact operations on a balance the extinction coefficient is rather uncertain.

The U.V.-spectrum of the 4 100-pigment has the same general shape as those of the symmetrical diene-polyenes synthesised by E. R. H. Jones *et al.*[4], *cf.* our XVIIth communication[5] where the spectrum of tetradeca-2 : 12-diene-4 : 6 : 8 : 10-tetrayne (III) is reproduced together with the spectrum of dodeca-

$$H_3C-CH=CH-C\equiv C-C\equiv C-C\equiv C-C\equiv C-C\equiv C-CH=CH-CH_3 \qquad \text{(III)}$$

$$H_2C=CH-C\equiv C-C\equiv C-C\equiv C-C\equiv C-C\equiv C-CH=CH_2 \qquad \text{(IV)}$$

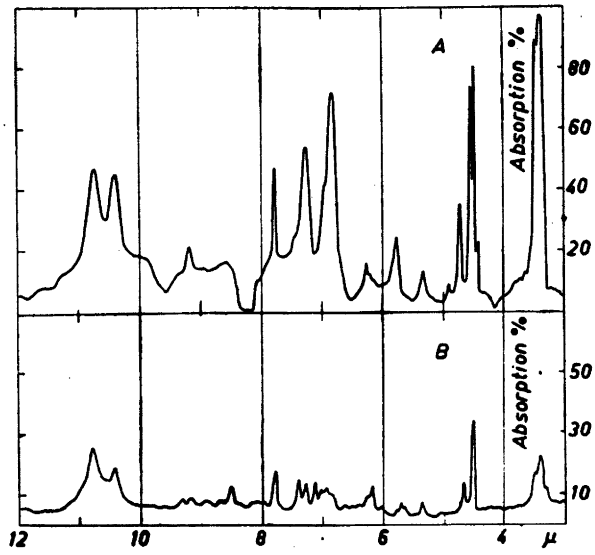

Fig. 2. Infrared absorption of

A: 4 100-pigment in $CHCl_3$, c = ca. 18 %, l = 0.05 mm, solution contaminated with spectral hexane.
B: dodeca-1:11-diene-3:5:7:9-tetrayne in CCl_4, c = 7 %, l = 0.05 mm.

1 : 11-diene-3 : 5 : 7 : 9-tetrayne (IV) isolated from some *Compositae* plants*. It is remarkable that 3 of the maxima in the strong short-waved group, *viz.* 2 863, 2 705 and 2 570 ÅU occur in nearly identical positions in the spectrum of the 4 100-pigment and in the spectra of (III) and (IV). The long-waved band group of the *Helipterum*-pigment, however, is displaced some 120 ÅU towards longer wave-lengths relative to (III), some 185 ÅU relative to (IV). Besides this displacement of the long-waved band group at longer wave-lengths the most interesting feature is that the difference in intensity of the two band groups is about 1.0 in log ε in the spectra of (III) and (IV) whereas it amounts to no less than 1.5 in log ε in that of the 4 100-pigment.

From the numerous spectra of synthetic polyene-ynes[4,6,7] and poly-ynes we know that the difference between the intensity of the two band groups are the greater the more we approach the pure poly-ynes, where the difference in dialkylpoly-ynes is ~3.0.

That the 4 100-pigment still contains at least one double bond is shown by the infra-red spectrum reproduced in Fig. 2, curve A, together with that of IV, curve B; both have the vinyl band in the position of vinylacetylenes, at 933, 962 and 1 870 cm^{-1}. The intensity and complexity of the acetylenic band group about 2 200 cm^{-1} is the strongest so far met with in the infra-red spectra of naturally occurring acetylenes.

* *Cf.* foot-note page 1743.

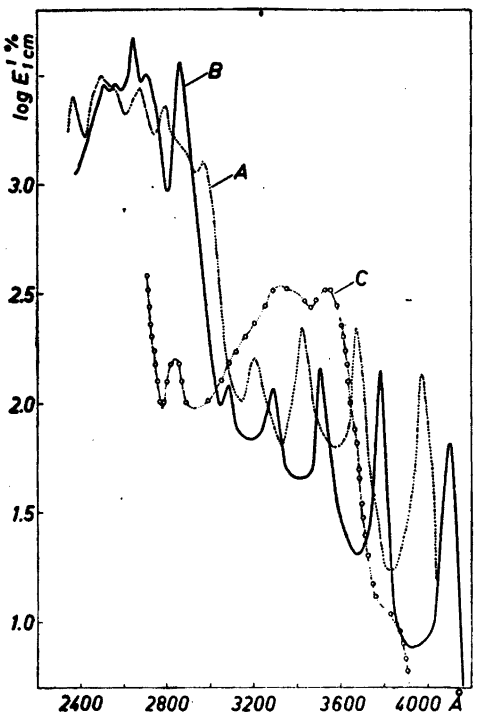

Fig. 3. Ultraviolet absorption in hexane of

A: 3 985-pigment from Helipterum. B: 4 100-pigment from Xanthium. C: polyene fraction from Helipterum.

So far no vinyl-penta-acetylene is known. The U.V.-spectrum of that type must be intermediate between that of dialkyl-hexaynes and the divinyl-tetrayne (IV):

	$R-C\equiv C-(C\equiv C)_4-C\equiv C-R$[6,7]	4 300	3 925	3 605	3 385	ÅU
	$CH_2=CH-(C\equiv C)_4-CH=CH_2$[5]	3 905	3 610	3 370	3 160	»
Mean	$R-C\equiv C-(C\equiv C)_4-CH=CH_2$	4 100	3 768	3 488	3 273	»
Observed	*Helipterum* chromophore:	4 100	3 780	3 515	3 285	3 080 »

The agreement is, as will be seen, very satisfactory as the data is taken from measurements in different solvents.

Thus the 4 100-chromophore should be an enepentayne. As to the nature of the residue R the amounts available have not permitted quite conclusive experiments. The catalytical hydrogenation of a small amount of the crystalline compound furnished a perhydrocompound of m.p. —14°, in amounts insufficient for recrystallisation. R = H, which should be excluded because of the instability of monoalkyl-poly-ynes, corresponds to the perhydro compound dodecane

m.p. —9,6°. R = CH_3 corresponds to tridecane m.p. —5,3°. The constitutional formula of the 4 100-chromophore thus might be given as (V)

$$CH_2=CH-C\equiv C-C\equiv C-C\equiv C-C\equiv C-C\equiv C-R \qquad (V)$$

where R may be CH_3, (V) is the hexa-dehydro-derivative of the tetraenediyne (VI) isolated recently from some *Coreopsis* species:

$$CH_2=CH-CH=CH-CH=CH-C\equiv C-C\equiv C-CH=CH-CH_3 \qquad (VI)$$

When the percentage of benzene in the petroleum ether was raised to about 20, a complex mixture of compounds was eluted. One of the components has a rather broad maximum in U.V. at 4 300 ÅU, we have so far not been able to obtain the substance responsible for this maximum in a pure state. We have, however, succeeded in obtaining one component of this mixture, with regard to the U.V.-spectrum very similar to the 4 005-chromophore in a spectroscopically pure state, *cf.* Fig. 3, curve A. The difference in the position of the maxima of the two compounds are only some 20 ÅU. The amounts of the two chromophores with first max. at a litle above and below 4 000 ÅU was too small to permit further investigations.

EXPERIMENTAL

Helipterum. The different garden varieties of *Helipterum, viz., H. Manglesii* (Nos. 4012, 4014 and 4016) *H. roseum* (No. 4020) *H. Sandfordii* (No. 4025) (seeds from A/S Norsk Frö) were cultivated at Norges Tekniske Högskole during the summers 1952 and 1953.

	Essential oil Mean value ‰	U.V.-absorption
Flowers	0.05	no selective abs., step out below 3 500 ÅU
Leaves and stems	0.05	no selective abs., almost step out below 3 500 ÅU

The roots were repeatedly extracted with acetone at room temperature, the lipids transferred to petroleum ether and this solution chromatographed on alumina. The petroleum ether washings contained only substances with selective absorption below 2 600 ÅU. The washings with 1 % benzene eluted the 4 005-chromophore; increasing the amount of benzene to 5 % brought out mixtures of the 4 005 and the 4 100-chromophore. The tail of the 5 % benzene washings contained the pure 4 100-pigment. Since the subsequent 20 % benzene eluates turned out to be very complex mixtures, the column was emptied with ethyl ether, the solvents removed in a vacuum and the chromatography started the next day from 20 % benzene onwards. After small amounts of the 4 100-chromophore there followed a complex orange-red mixture of substances. The orange colour belonged to a substance with first maximum at 4 380 ÅU which, however, could never be obtained pure. A polyenic chromophore with rather broad and undistinct maxima at 3 545 and 3 320 ÅU, *cf.* Fig. 3, curve C, was eluted somewhat easier than the 4 300-pigment; in the later fractions the 3 985-chromophore dominated. This last substance, with the striking spectral conformity with the 4 005-pigment, could be separated completely by rechromatography from the 4 300-pigment, *cf.* Fig. 3, curve A.

All the fractions dominated by the 4 100-pigment were combined and rechromatographed, which only increased somewhat the depth of the minima in the longwaved band group. When the solvent was removed from these fractions, the residue crystallised immediately as yellow prisms of extreme instability. It was possible neither to obtain a m. p. determination nor to carry out ordinary weighings for spectral investigations as the 4 100-pigment turned into a black insoluble mass at room temperature in a few minutes.

The extinction coefficient of the U.V.-curve given in Fig. 1, curve A, is probably somewhat in error, since the removal of the solvent in a vacuum, in order to avoid decomposition, has not been carried out long enough to garantee complete absence of solvent. The concentration of the infra-red spectrum, Fig. 2, curve A, is only approximative. The total substance used for the infra-red spectrum was afterwards used for the catalytical hydrogenation and the concentration is based on the weight of the perhydrocompound which, of course, was completely stable.

As long as the 4 100-pigment was handled in dilute solutions decomposition seemed to be rather slow. Since 2:6-dimethyl-octatetraene = cosmene had polymerised extensively in the infra-red cells in the course of the about 20 minutes necessary for a 2—15 μ run, we were rather surprised that the 4 100-pigment did not show any sign of decomposition during the course of the spectral measurements, as judged from a rerun of some of the first peaks.

This phenomenon: extreme instability as crystalline solid — fair stability in dilute solutions, has been described earlier in connection with synthetic poly-acetylenes and was most striking in the work of Celmer and Solomons[9] on the antibiotic mycomycin and its isomerisation product iso-mycomycin from *Norcardia acidophilus*.

It is an interesting question wether this extreme instability has some connection with the fact that this 4 100-pigment so far has been found in the different plants only in very small amounts, whereas the thermally rather stable diacetylenic compounds of the type matricaria ester, lachnophyllum ester and the esters of matricarianol often occur in remarkably high concentrations, 0.3—1 % of the fresh plant.

Gnaphalium luteo-album *L.*; seed sample Indian Botanic Garden, Sibpur, Calcutta, cultivated at Norges Tekniske Högskole 1953.

	g	Ess. oil ‰	U.V.-absorption
Flowers	103	0.14	Step out below 3 800 ÅU, no selective abs.
Leaves & stem	250	0.27	» » » 3 400 » , » » »
Roots	8.5	11.7	(extract) maxima at 4 100, 3 790, 3 516, 2 860, 2 645

Chromatography on slightly deactivated alumina gave in the petroleum ether washings only material without selective absorption. The chromophore went in the 20 % benzene-petroleum ether eluates and was then conform with that of the 4 100-pigment. 50 % benzene and ethyl ether eluted further fractions with only broad and undistinct spectra.

Gnaphalium silvaticum *L.*, collected at Lian near Trondheim, 1950.

	g	Ess. oil ‰	
Flowers, leaves and stems	1 082	0.041	step out below 3 400 ÅU, no selective abs.
Roots	277	0.29	maxima at 2 860, 2 650 ÅU

Blumea lacera *D. C.*, seed sample Indian Botanic Garden, Calcutta, cultivated in a greenhouse at Norges Tekniske Högskole 1953.

	g	Ess. oil ‰	U.V.-absorption
Flowers	49	1.1	semisolid substance, step out below 3 500 ÅU
Leaves & stems	1 680	2.2	liquid, step out below 3 500 ÅU
Roots	890	2.2	(extract) 4 100-chromophore on general step out at shorter wave-lengths

The root extract was chromatographed on alumina. The fractions eluted with petroleum ether were devoid of selective absorption. 20 % benzene in petroleum ether eluted a fraction (362 mg) with the pure spectrum of the 4 100-pigment, calculated content of (V) was 3 %. This fraction deposited yellow rods from petroleum ether solution. Since at that time the extreme instability of this pigment was unknown, the main part was lost by decomposition.

The eluates with 60 % benzene until pure benzene eluted another chromophore with very distinct peaks:

λ_{max}	3 485	3 250	3 055	2 872	2 675	2 560 ÅU
$\Delta\nu_{max}.10^{-13}$		62.3	58.9	62.6		

The spacing between the maxima reveals that this chromophore is acetylenic. In fact the maxima observed come very close to those of the symmetrical dienetriyne class, first exemplified in nature by the "Centaur X"-hydrocarbons of N. Löfgren[10, 11, 12]; observed for:

$$R-CH=CH-C\equiv C-C\equiv C-C\equiv C-CH=CH-R'' \qquad \text{(VII)}$$

λ_{max}	3 485	3 260	3 064	2 895	2 694	2 592 — — — ÅU

The "Centaur X" hydrocarbons are eluted with petroleum ether alone or with a few % benzene; obviously the chromophore from *B. lacera* must contain some polar group. The amounts of substance was too small for further investigations.

Blumea lacinata, *D. C.*, seed sample Indian Botanic Garden, Calcutta, cultivated in a greenhouse at Norges Tekniske Högskole 1953.

	g	Ess.oil ‰	
Flowers	95	1.7	step out below 3 400 ÅU, no selective abs.
Leaves & stems	900	0.4	» » » 3 400 » , » » »
Roots	360	1.4	maxima at 3 780, 3 520, 3 300, 3 100, 2 860, 2 650 ÅU

The essential oil from the root was chromatographed as above. The 20 % benzene eluate gave a very clear spectrum of (V).

maxima: 4 110, 3 785, 3 520, 3 295, 3 090, 2 865, 2 715, 2 650 ÅU

The eluates with higher percentages of benzene and with ethyl ether gave broad and undistinct spectra.

Pulicaria vulgaris *Gaertn.*, seed sample University Garden, Aarhus, and Agricultural College, Budapest, cultivated at Norges Tekniske Högskole 1952.

The essential oil of *Pulicaria vulgaris* Gaertn. was investigated by one of us, Dagny Holme, in her graduate work 1951; in the oil from the roots and the leaves some sharp peaks were then observed at 3 778, 3 505, 3 287, 2 865 and 2 645 ÅU standing out on a general step out towards shorter wave-lengths. The concentration of the 4 100-pigment was so small that the first maximum was overlooked. The 1952-material was chromatographed in the way found convenient with *Helipterum* oils. The petroleum ether washings absorbed only below 2 600 ÅU. Eluates with 10 % benzene showed maxima at 4 103, 3 780, 3 520, 3 290 ÅU and a striking conformity with the curve for the crystalline 4 100-pigment.

Eluates with 20 % benzene showed broad and only rather weak absorption in the region of 3 300 ÅU. The more strongly adsorbed substances have so far not been investigated.

Sanvitalia procumbens *Lam;* seed sample Olsens Enke No. 4 484, cultivated at Norges Tekniske Högskole 1952.

	g	Ess. oil ‰	
Flowers	47.5	0.46	step out below 3 500 ÅU, no selective abs.
Leaves + stems	179	0.21	» » » 4 000 » , » » »

The essential oil from the roots, 70.8 mg = 0.44 ‰, was chromatographed on a small column of deactivated alumina. The petroleum ether washings were devoid of selective absorption in U.V., the eluate with 50 % benzene contained the 4 100-pigment, seemingly

without presence of other chromophores, observed maxima: 4 100, 3 780, 3 520, 3 190 and 2 865 ÅU.

The column was then eluted with ethyl ether; this fraction had only a broad absorption band at 3 300 ÅU.

X a n t h i u m *unknown sp.* Seed sample Abbot Co., cultivated at Norges Tekniske Högskole 1952. The plants did not develop flowers and thus a botanical determination was not possible.

	g	Ess. oil ‰	
Leaves + stem	14 000	0.04	maxima at 3 670, 3 408, 3 100 ÅU
Roots	1 380	0.56	maximum at 2 860 ÅU

The crude essential oil from the roots had a rather sharp peak in U.V. at 2 870 ÅU. The oil was chromatographed from petroleum ether followed with 50 % benzene-petroleum ether. The first 1/4 of the petroleum ether washings contained rather large amounts of colourless hydrocarbons without selective absorption in U.V. (terpenes?). The first washings with 50 % benzene contained the characteristic peaks of the 4 100-pigment. The solvent was evaporated and the fraction rechromatographed using elution with 8, 16, 25 and 50 % benzene in petroleum ether. The 25 % benzene eluates had a fair U.V.-spectrum of the 4 100-pigment. On evaporation of the solvent 1 mg of yellow crystals was left which immediately was dissolved in spectral hexane, U.V.-spectrum Fig. 3, curve B.

The essential oil from the leaves + stems were also chromatographed on alumina. The 10 % benzene in petroleum ether eluate had U.V. absorption close to that of 1-phenyl-*n*-undeca-7:9-diene-1:3:5-triyne,

$$C_6H_5-C\equiv C-C\equiv C-C\equiv C-CH=CH-CH=CH-CH_3 \qquad \text{(VIII)},$$

isolated recently from some *Coreopsis* species. Rechromatography brought the spectrum very close to that of (VIII). As the amount of pure substance isolated was very small only an infra-red spectrum could be taken, which was in good agreement with that of (VIII). Thus either (VIII) or a close relative of (VIII) must be present in *Xanthium* oils.

X a n t h i u m s t r u m a r i u m *L.* Seed sample Indian Botanic Garden, Calcutta. A dozen plants of this rather tiny species were cultivated in a greenhouse at Norges Tekniske Högskole 1953. The male and female flowers were worked up separately, but neither of these oils showed any selective absorption in U.V. The essential oil from leaves and stems (16 ‰) had no selective abs.

The essential oil from the roots was chromatographed on alumina as above using 10 and 20 % benzene in petroleum ether as eluents.

The 20 % eluate contained the characteristic peaks of the 4 100 chromophore.

S y n e d r e l l a n u d i f l o r a *Gaertn.* Seed sample Indian Botanic Garden. Cultivated in a greenhouse at Norges Tekniske Högskole 1953. This weed has very small flowers which were collected through the entire flowering season in order to obtain somewhat more material. The oil extracted with acetone showed, however, no selective absorption in U.V.

	g	Ess. oil ‰	
Leaves and stems	564	0.4	no selective abs. in U.V.
Roots	88	4.3	maximum at 2 860 ÅU

The essential oil from the roots (0.38 g) was chromatographed on alumina. The petroleum ether washings were followed with 20 % benzene in petroleum ether, which eluted a fraction with U.V.-spectrum conform with that of the 4 100-pigment.

Observed maxima: 4 110, 3 805, 3 515, 3 285 ÅU

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Versuche zur Darstellung optisch aktiver bororganischer Verbindungen. I. Über Ester der Arylborsäuren

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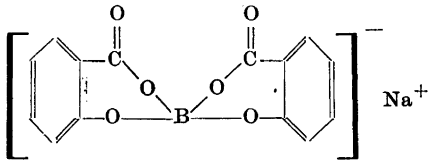
Es wird über die Darstellung und Eigenschaften der Arylborsäureester berichtet. Diese Substanzen sind alle farblose, feuchtigkeitsempfindliche Flüssigkeiten. Eine Berechnung der Atomrefraktion des Bors ist durchgeführt worden. Bei der Umsetzung von Zinkdiphenyl mit Bortrifluoridätherat entsteht Phenylbordifluorid. Die Absicht ist, über die genannten Verbindungen optisch aktive bororganische Verbindungen darzustellen.

Bortriphenyl lagert in ätherischer Lösung Phenyllithium an und bildet das stabile Tetraphenylborlithium-Salz [1]:

$$(C_6H_5)_3B + C_6H_5Li \rightarrow [(C_6H_5)_4B]^- Li^+$$

Es liegt dann die Frage nahe, ob es möglich wäre, eine Tetraarylborverbindung mit vier verschiedenen an Bor gebundenen Resten darzustellen. Eine Verbindung dieser Art enthält ein asymmetrisches Boratom und könnte demgemäss optische Aktivität zeigen. Böeseken und Muelenhoff [2] spalteten schon 1924 das Natriumdisalicylborat in seine Antipoden auf. In diesem Salze fehlen jedoch die B-C-Bindungen.



Die symmetrischen Bortrialkyle oder Bortriaryle werden durch Grignardierung von Bortrifluorid in ätherischer Lösung dargestellt [3–5].

$$3\ RMgX + BF_3 \rightarrow R_3B + 3\ XMgF\ (R = Ar \text{ oder } Alk)$$

Asymmetrische Bortrialkyle oder -aryle vom Typus $R_1R_2R_3B$ sind noch nicht bekannt.

Bei der Umsetzung von Bortrifluorid in ätherischer Lösung mit Phenylmagnesiumbromid bildet sich nach Krause und Nitsche [4] Phenylbordifluorid,

das jedoch nicht isoliert werden konnte. Auch unsere Versuche, nach der Methode von Krause das Phenylbordifluorid darzustellen, sind gescheitert *. Wegen der grossen Reaktivität der Grignard-Verbindungen und des Bortrifluorids ist es schwierig, die Reaktion auf der Stufe des Monophenylbordifluorids zu halten. Auch bei Überschuss von Bortrifluorid bilden sich Diphenylborfluorid und Triphenylbor. Die zinkorganischen Verbindungen sind weniger reaktiv als die Grignard-Verbindungen, und wir haben deshalb die Reaktion von Zinkdiphenyl mit Bortrifluoridätherat untersucht. Noch weniger reaktiv sind die quecksilberorganischen Verbindungen. Bei der Umsetzung von Quecksilberdiphenyl mit Bortrifluoridätherat bei 130—40° konnte kein Phenylbordifluorid nachgewiesen werden.

Zinkdiphenyl [6] reagiert unter Wärmeentwicklung mit Bortrifluorid, wobei sich Phenylbordifluorid als eine farblose, feuchtigkeitsempfindliche Flüssigkeit bildet. Nur die eine der beiden Phenylgruppen des Zinkdiphenyls lässt sich zur Reaktion bringen.

$$BF_3 + (C_6H_5)_2Zn \rightarrow C_6H_5ZnF + C_6H_5BF_2$$

Phenylbordifluorid bildet mit Aminen feste kristalline Addukte.

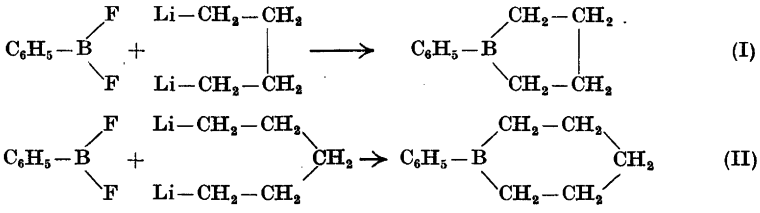
$$C_6H_5BF_2 + NH_2R \rightarrow C_6H_5BF_2 \cdot NH_2R$$

Lässt man nun eine andere Grignard-Verbindung auf das Phenylbordifluorid einwirken, so könnte man erwarten, dass sich das Diarylborfluorid bilden sollte:

$$RBF_2 + R'MgX \rightarrow RR'BF + MgXF$$

Es erwies sich als schwierig, die Reaktion auf der Stufe der Diarylborverbindung zu halten. Auch bei —60° bildet sich zum grossen Teil die Triarylborverbindung. Beim Aufarbeiten konnte ein Teil der Ausgangssubstanz zurückgewonnen werden. Das Diarylborfluorid wurde unrein und in schlechter Ausbeute gewonnen. Man muss also eine Monoarylborverbindung finden, die reaktionsträger als das Difluorid ist. Es ist zu erwarten, dass die Arylborsäuredialkylester bessere Ausbeuten von Diarylborverbindungen ergeben werden. Die Trialkylborate liefern z.B. bessere Ausbeuten von Arylborsäure als das Borfluorid [7,8].

Nach West und Rochow[17] lassen sich 1,4-Dilithiumbutan und 1,5-Dilithiumpentan in guter Ausbeute darstellen. Bei der Umsetzung von Phenylbordifluorid mit den Dilithiumverbindungen tritt Ringschluss ein. Es bilden sich Phenyl-cyklotetramethylenborin (I) und Phenyl-cyklopentamethylenborin (II).



(I)

(II)

* Nach einer Privatmitteilung von Herrn Prof. Dr. J. Goubeau, Stuttgart, ist es ihm nach der Methode von Krause und Nitsche, allerdings in etwas abgewandelter Form, gelungen, die Substanz in ungefähr 20 %-iger Ausbeute herzustellen.

Der Weg, den wir angetreten haben, um eine optisch aktive bororganische Verbindung darzustellen, wird über folgende Zwischenstufen gehen:

a. $B(OR)_3 + ArMgX \rightarrow ArB(OR)_2 + XMgOR \xrightarrow{\text{Hydrolyse}} ArB(OH)_2$
b. $ArB(OH)_2 + 2\ ROH \rightarrow ArB(OR)_2 + 2\ H_2O$
c. $ArB(OR)_2 + Ar'MgX \rightarrow ArAr'B(OR) + XMgOR \xrightarrow{\text{Hydrolyse}} ArAr'BOH$
d. $ArAr'BOH + ROH \rightarrow ArAr'BOR + H_2O$
e. $ArAr'BOR + Ar''MgX \rightarrow ArAr'Ar''B + XMgOR$
f. $ArAr'Ar''B + Ar'''Li \rightarrow [ArAr'Ar''Ar'''B]^- Li^+$

Die Hydrolysen und Umesterungen b. und d. sind notwendig. Es ist nicht möglich, die Mono- oder Diarylborsäureester aus dem Reaktionsgemisch herauszudestillieren, denn sie werden von den Magnesiumsalzen infolge Komplexbildung festgehalten.

Die Arylborsäuren wurden zuerst von Michaelis und Mitarbeitern[9] durch Einwirkung von Bortrichlorid oder Bortribromid auf Diarylquecksilber dargestellt.

$$BCl_3 + HgAr_2 \rightarrow (I)\ ArBCl_2 + ArHgCl \xrightarrow{H_2O} (II)\ ArB(OH)_2$$

(I) wird leicht von Wasser hydrolysiert, wobei (II) entsteht. Äthylalkohol ergibt Arylborsäurediäthylester, der auch leicht hydrolysierbar ist.

$$ArBCl_2 + 2\ C_2H_5OH \rightarrow ArB(OC_2H_5)_2 + 2\ HCl$$
$$ArB(OC_2H_5)_2 + 2\ H_2O \rightleftharpoons ArB(OH)_2 + 2\ C_2H_5OH$$

Es handelt sich hier um ein Gleichgewicht zwischen Ester und Säure, das zur Seite der Säure stark verschoben ist. Eine Lösung von Arylborsäure in Propanol oder Butanol lässt sich vom Wasser durch azeotrope Destillation befreien. Bei der Darstellung des Methylesters wurde das Wasser in einem Soxhlet-Apparat mit wasserfreiem Kupfersulfat extrahiert.

Die Arylborsäureester bilden farblose, leicht bewegliche Flüssigkeiten. Anfangs wurden die Destillationen unter Stickstoff ausgeführt. Es zeigte sich jedoch, dass man sie ebenso gut an der Luft ausführen kann.

In wässriger Lösung wird nach Ainley und Challenger [14] die Dioxyborgruppe der Phenylborsäure durch Halogen abgespalten, und es bilden sich dabei Halogenbenzol und Borsäure. Wir fanden jedoch, dass die reinen Ester von Brom und Jod nicht angegriffen werden. Die Hydrolysierbarkeit nimmt mit wachsender Grösse des Alkylrestes ab, was wahrscheinlich auf eine sterische Hinderung zurückzuführen ist. In Tabelle 1 sind die physikalischen Daten zusammengestellt.

In Kolumne 8 ist die Molekularrefraktion berechnet. Die Unterschiede zwischen den verschiedenen isomeren Tolylborsäureestern sind nicht grösser als die, die man bei sonstigen isomeren Benzolderivaten gefunden hat. Der Unterschied zwischen zwei homologen Estern beträgt im Durchschnitt 9,36. Eisenlohr [10] gibt für 2 CH_2 den Wert 9,24 an.

In Kolumne 9 findet man die Atomrefraktion des Bors wieder. Je grösser die Zahl der Substituenten am Benzolkern ist, um so grösser wird der Beitrag des Phenyls zur Molekularrefraktion, d. h. um so stärker tritt der ungesättigte

Tabelle 1.

Säure	Ester	Molekulargewicht	Siedepunkt °C	Siedepunkt Torr	Brechungsindices n_D^{25}	Dichte d_4^{25}	Molekularrefraktion	Atomrefraktion des Bors	Ausbeute %	Analysen Ber. Bor	Analysen Gef. Bor
Phenylborsäure	Dimethyl	150,0	74— 5	11	1,4926	1,0024	43,46	3,63	83	7,21	7,04
»	Diäthyl	178,1	90— 1	11	1,4760	0,9480	52,99	3,93	77	6,08	6,16
»	Dipropyl	206,1	112— 3	10	1,4738	0,9297	62,28	3,98	83	5,25	5,20
»	Dibutyl	234,1	139—40	10	1,4718	0,9166	71,49	3,96	96	4,62	4,65
p-Tolylborsäure	Dimethyl	164,0	89—90	10	1,4948	0,9860	48,49	3,82	71	6,60	6,64
»	Diäthyl	192,1	106— 7	12	1,4793	0,9389	58,05	4,15	67	5,63	5,48
»	Dipropyl	220,1	128— 9	11	1,4770	0,9225	67,42	4,28	85	4,92	4,91
»	Dibutyl	248,2	159—60	13	1,4758	0,9127	76,67	4,30	92	4,36	4,26
o-Tolylborsäure	Dimethyl	164,0	74— 5	9	1,4870	0,9798	48,14	3,47	86	6,60	6,50
»	Diäthyl	192,1	91— 2	10	1,4737	0,9351	57,70	3,80	74	5,63	5,76
»	Dipropyl	220,1	116— 7	9	1,4721	0,9206	66,96	3,82	66	4,92	4,94
»	Dibutyl	248,2	139—40	9	1,4706	0,9099	76,19	3,82	93	4,36	4,39
m-Tolylborsäure	Dimethyl	164,0	85— 6	10	1,4926	0,9846	48,37	3,70	79	6,60	6,59
»	Diäthyl	192,1	101— 2	11	1,4796	0,9388	58,09	4,19	57*	5,63	5,72
»	Dipropyl	220,1	128— 9	11	1,4757	0,9226	67,25	4,11	96	4,92	4,93
»	Dibutyl	248,2	146— 7	8	1,4744	0,9126	76,49	4,12	90	4,36	4,45

* 36 % wurde als Säure zurückgewonnen.

Charakter der Doppelbindungen des Benzols in Erscheinung. Auch verschiedene Substituenten haben verschieden grosse Einflüsse. Es ist deshalb schwierig zu schätzen, wie gross der Einfluss der Dialkoxyborgruppe auf die Refraktion des Phenyls ist. Die Refraktion des Phenyls haben wir aus Toluol berechnet, das wie die Phenylborsäureester ein monosubstituiertes Benzol ist. (Refraktion des Phenyls = Refraktion des Toluols minus Refraktion des Methyls = 25,34.) In ähnlicher Weise haben wir die Refraktion des Tolyls aus Xylol berechnet[11]. (Refraktion des Tolyls = Refraktion des Xylols minus Refraktion des Methyls = 30,18.) Der Mittelwert der Molekularrefraktion der drei Isomeren ist bei der Berechnung verwendet worden. In Tabelle 2 ist die Atomrefraktion des Bors für einige Verbindungsklassen zusammengestellt. Man findet, dass die Atomrefraktion des Bors in folgender Reihe zunimmt:

$$\text{Trialkylborat} \leq \text{Bortrihalogenid} < \text{Bortrialkyl} < \text{Arylborsäureester} < \text{Diarylborsäureester}.$$

Bisher sind keine bei Zimmertemperatur flüssigen Triarylborverbindungen bekannt. Über die Diarylborsäureester wird in der nächsten Arbeit dieser Serie berichtet. Zum Vergleich sind aber hier einige Messungen veröffentlicht (Tabelle 2).

Tabelle 2.

Verbindung oder Verbindungsklasse	Atomrefraktion des Bors	Literatur
Trialkylborat	3,07 [a]	Arbuzov u. Vinogradova [12 b]
Bortrichlorid	3,07	Ghira [13 b]
Bortribromid	3,26	» [b]
Bortrialkyl	3,66 [a]	Krause u. Nitsche [3]
Monoarylborsäureester	3,94 [a]	Diese Arbeit
Diarylborsäureester	5,27 [a]	Torssell [c]

[a] Mittelwert.
[b] Vom Verfasser auf Grund der Literaturangaben berechnet.
[c] Wird demnächst in *Acta Chem. Scand.* erscheinen.

Die Arylborsäuren reagieren mit Quecksilberdichlorid unter Bildung von Arylquecksilberchlorid, Borsäure und Chlorwasserstoff.

$$ArB(OH)_2 + HgCl_2 + H_2O \rightarrow ArHgCl + H_3BO_3 + HCl$$

Nach Wittig und Mitarbeitern [1] ist diese Reaktion für die quantitative Analyse verwendbar. Der Arylborsäureester wird in Wasser hydrolysiert und mit wässriger Quecksilberdichlorid-Lösung versetzt. Festes Natriumchlorid wird zugegeben, um die Hydrolyse der Quecksilberionen zurückzudrängen. Die Salzsäure wird mit 0,1 *N* Natriumhydroxyd und Methylrot titriert. Nach Zusatz von Mannit und ein paar Tropfen Phenolphtalein-Lösung wird die Borsäure in derselben Probe titrimetrisch bestimmt. Es zeigt sich dabei, dass das Verhältnis Salzsäure zu Borsäure gleich 1 : 1 ist. Über Einzelheiten siehe "Experimenteller Teil".

EXPERIMENTELLER TEIL

Phenylborsäure

Diese Säure wurde hauptsächlich nach der Methode von Bean und Johnson [7] dargestellt. Wir fanden es vorteilhafter, das Reaktionsprodukt mit Petroläther (50–60°) zu extrahieren, wobei die öligen Bestandteile gelöst wurden. Die Phenylborsäure, die in Petroläther schwerlöslich ist, blieb zurück. Unten ist die von uns verwendete Aufarbeitungsmethode beschrieben worden.

Nach den Vorschriften von Bean und Johnson wurde Tributylborat [16] mit Phenylmagnesiumbromid umgesetzt. Das Reaktionsgemisch wurde hydrolysiert und die ätherische Schicht von der wässrigen getrennt. Der Äther wurde auf dem Wasserbad abdestilliert. Nachdem man die Lösung im Destillierkolben mit Kaliumhydroxyd deutlich alkalisch gemacht hatte, wurde das Butanol im Vakuum abgetrieben. Die zurückgebliebene Lösung wurde mit Schwefelsäure schwach angesäuert, wobei die Phenylborsäure zum grössten Teil ausfiel. Nachdem der Kristallbrei über Nacht im Kühlschrank gestanden war, wurde er abfiltriert, mit etwas kaltem Wasser gewaschen und dann durch Pressen möglichst vom Wasser befreit. Die an der Luft getrocknete Substanz wurde in einen Kolben mit Rückflusskühler übergeführt und mit Petroläther (etwa 200 ml pro 100 g Säure) eine Viertelstunde auf dem Wasserbad gekocht. Nach Filtrieren wurde die Extraktion noch einmal wiederholt. Man wusch mit etwas Petroläther nach. Aus den vereinigten Filtraten liess sich nach Stehen etwas ausgefallene Phenylborsäure zurückgewinnen. Die Phenylborsäure wurde aus Wasser umkristallisiert. F. 214–16°. Ausbeute 60 %.

Analog wurden die *o*-, *m*- und *p*-Tolylborsäuren dargestellt. Die Ausbeuten waren 47 %, 49 % und 51 %.

Phenylborsäuredimethylester

Eine Lösung von 5 g Phenylborsäure in 25 ml Methanol und 60 ml Chloroform wurde in einem Soxhlet-Apparat auf dem Wasserbad über Nacht gekocht. Der kondensierte Dampf wurde mit geglühtem Kupfersulfat in der Extraktionshülse entwässert. Die Lösung wurde vor Luftfeuchtigkeit mit einem Chlorcalcium-Rohr geschützt. Nach dem Abdestillieren des Lösungsmittels wurde der Ester im Vakuum überdestilliert. Im Kolben hinterblieb eine geringe Menge nicht umgesetzter Phenylborsäure. Nach erneuter Destillation unter Stickstoff über eine kleine Kolonne wurde der Ester als eine farblose, leicht bewegliche Flüssigkeit analysenrein gewonnen. Kp. 74—5°/11 Torr; $d_4^{25} = 1{,}0024$; $n_D^{25} = 1{,}4926$. Sie hydrolysiert sich schnell an feuchter Luft, und es ist deshalb bei der Herstellung darauf zu achten, dass sie vor Feuchtigkeit sorgfältig geschützt wird. Der Ester ist sonst an der Luft stabil. Er wurde in zugeschmolzenen Ampullen aufbewahrt. $C_8H_{11}O_2B$ (150,0): Ber. B 7,21; Gef. B 7,04. Ausbeute 83 %.

Die Methylester der verschiedenen Tolylborsäuren wurden analog dargestellt.

Umsetzungen von Phenylborsäuredimethylester mit Halogenen

Umsetzung mit Brom. 2,4 g Phenylborsäuredimethylester wurden mit 2,5 g Brom und 3 ml wasserfreiem Benzol 3 Stunden bei etwa 80° erwärmt. Man konnte keine Reaktion beobachten. Bei der nachfolgenden Destillation wurde der Ester grössenteils zurückgewonnen.

Umsetzung mit Jod. 1,1 g Phenylborsäuredimethylester wurden mit 1,9 g Jod 3 Stunden bei 120—30° unter Stickstoff erwärmt. Der Methylester wurde grössenteils zurückgewonnen.

Phenylborsäurediäthylester

In einem kleinen Kolben wurden zu 5 g Phenylborsäure 25 ml Äthanol und 30 ml Benzol gegeben. Die Lösung wurde vorsichtig über eine Kolonne destilliert, wobei zuerst das ternäre Azeotrop Wasser-Äthanol-Benzol überging. Dann kam das binäre Azeotrop Äthanol-Benzol und zuletzt der Überschuss von Äthanol. Im Kolben hinterblieb der Phenylborsäurediäthylester, der im Vakuum überdestilliert wurde. Eine erneute Destillation ergab ein für eine Analyse genügend reines Produkt. Phenylborsäurediäthylester bildet eine farblose, feuchtigkeitsempfindliche Flüssigkeit vom Kp. 90—1°/11 Torr. $n_D^{25} = 1{,}4760$; $d_4^{25} = 0{,}9480$. Sie ist an trockener Luft stabil. Zum Aufbewahren wurde die Substanz in Ampullen gefüllt. $C_{10}H_{15}O_2B$ (178,1): Ber. B 6,08; Gef. B 6,16. Ausbeute 77 %.

Die Tolylborsäurediäthylester wurden analog dargestellt.

Phenylborsäuredipropylester

Eine Lösung von 5 g Phenylborsäure in 20 ml Propanol wurde über eine Kolonne destilliert. Das konstant siedende Gemisch Wasser-Propanol ging zuerst über, dann der Überschuss von Propanol. Der Rest wurde im Vakuum destilliert, wobei der Phenylborsäuredipropylester als eine farblose Flüssigkeit bei 112—3°/10 Torr überging. Der Propylester ist wie die anderen Ester feuchtigkeitsempfindlich. $n_D^{25} = 1{,}4738$; $d_4^{25} = 0{,}9297$. $C_{12}H_{19}O_2B$ (206,1) Ber. B 5,25; Gef. B 5,20. Ausbeute 83 %.

In ähnlicher Weise lassen sich die *Dibutylester* herstellen. 4—5 ml Butanol pro g Arylborsäure wurden verwendet.

Phenylbordifluorid

In einem Mörser wurden Ampullen mit 18 g Zinkdiphenyl [6] rasch zertrümmert und dann sofort in einen Claisenkolben von 100 ml Inhalt unter Stickstoff eingeführt. Durch einen Tropftrichter wurden langsam 20 g frisch destilliertes Bortrifluoridätherat hinzugegeben, wobei sich die Masse erwärmte. Nach dem Zusatz wurde das Gemisch auf dem Wasserbad vorsichtig auf 90° erwärmt, und man liess es bei dieser Temperatur eine Stunde stehen. Das gebildete Phenylbordifluorid wurde aus dem Reaktionskolben abdestilliert,

bis das innere Thermometer ungefähr 105° zeigte. Nach wiederholten Destillationen über eine Kolonne wurden 7,0 g Phenylbordifluorid vom Kp. 82—5°/730 Torr gewonnen. Ausbeute 68 % (oder 34 %, wenn angenommen wird, dass die beiden Phenyle des Zinks reagieren).

Bei schneller Destillation traten weisse Dämpfe von Phenylbordifluorid auf, die schwierig zur vollständigen Kondensation zu bringen waren. Es erwies sich auch als schwierig, den Siedepunkt konstant zu halten, was wahrscheinlich darauf beruht, dass das Phenylbordifluorid immer etwas Bortrifluoridätherat enthielt. Phenylbordifluorid bildet eine farblose, an der Luft stark rauchende Flüssigkeit. Sie ist sehr feuchtigkeitsempfindlich, weshalb sie bei der Destillation sorgfältig vor Feuchtigkeit geschützt werden muss. Unter Stickstoff oder trockener Luft ist sie für längere Zeit haltbar.

Umsetzung von Phenylbordifluorid mit Anilin. Zu 1,52 g Phenylbordifluorid in 10 ml Benzol wurden 1,10 g Anilin in 5 ml Benzol unter Stickstoff und Feuchtigkeitsausschluss gegeben. Es fiel sofort ein weisser Niederschlag vom Anilinaddukt heraus. Rohschmp. 176°. Ein Teil wurde aus einer Mischung von abs. Benzol und etwas Tetrahydrofuran umkristallisiert. F. 178—9°. Ausbeute von Rohprodukt 92 %. Leicht löslich in Tetrahydrofuran, schwerlöslich in Äther und Benzol. ($C_6H_5BF_2 \cdot NH_2C_6H_5$ (219,1): Ber. C 65,77; H 5,53. Gef. C 65,35; H 5,79).

Umsetzung von Phenylbordifluorid mit Methylamin. Beim Zusammengeben von Phenylbordifluorid in wenig Benzol und einer gesättigten, benzolischen Lösung von Methylamin fiel das weisse Methylaminaddukt aus. Farblose, blätterförmige Kristalle vom F. 98—101°. (Aus wenig Benzol umkristallisiert.) ($C_6H_5BF_2 \cdot NH_2CH_3$ (157,0): Ber. C 53,52; H 6,42, Gef. C 53,01; H 6,59).

Phenyl-cyklotetramethylenborin

Tetramethylendilithium wurde nach West und Rochow[17] aus Lithium und Tetramethylendibromid hergestellt und die Lösung in eine Vorratsbürette übergeführt. Die Lösung scheint einige Tage haltbar zu sein.

Zu 7,5 g Phenylbordifluorid in 20 ml absol. Äther wurden in einem Kolben unter Stickstoff und Kühlung mit Eiswasser 160 ml 0,38 *M* Tetramethylendilithium-Lösung langsam hinzugetropft. Die Lösung wurde gut geschüttelt. Ein weisser Niederschlag von Lithiumfluorid bildete sich allmählich. Man liess die Lösung nach dem Zusatz eine Stunde stehen und destillierte dann den Äther unter Stickstoff bei Atmosphärendruck ab. Der Rest wurde im Vakuum destilliert. Die Hauptfraktion ging bei 80—100°/11 Torr über. Erneute Destillation über eine Kolonne ergab eine farblose Flüssigkeit vom Kp. 85—87°/11 Torr. Ausbeute 3,8 g. Sie entzündet sich von selbst an der Luft. ($C_{10}H_{13}B$ (144,0): Ber. C 83,39; H 9,09; B 7,52. Gef. C 81,93; H 9,02; B 7,60).

In ähnlicher Weise wurde das *Phenyl-cyklopentamethylenborin* aus Phenylbordifluorid und Pentamethylendilithium dargestellt. Es bildet eine an der Luft selbstentzündliche farblose Flüssigkeit vom Kp. 105—107°/11 Torr. ($C_{11}H_{15}B$ (158,1): Ber. B 6,85; Gef. B 6,97).

Phenyl-di-*o*-tolylborin

In einem Claisenkolben wurden 7,4 g Phenylbordifluorid in 10 ml Äther tropfenweise mit einer *o*-Tolylmagnesiumbromid-Lösung unter Stickstoff bei —60° im Mol-Verhältnis 1:1 versetzt. Es trat ein weisser Niederschlag auf, und ein Öl bildete sich am Boden des Reaktionsgefässes. Man liess die Lösung über Nacht in der Kältemischung stehen und langsam Zimmertemperatur erreichen. Der Äther wurde abdestilliert. Bei der Vakuumdestillation wurden folgende Fraktionen erhalten:

I	60—90°/12	Torr	~ 0,2	ml
II	90—140	»	~ 0,3	»
III	140—190	»	~ 0,5	»
IV	190—210	»	~ 3,0	»

} Phenyl-*o*-tolylborfluorid?

Fraktion IV bildete eine farblose viskose Flüssigkeit, die bei erneuter Destillation zum grössten Teil bei 203—205°/12 Torr überging. Sie begann nach einigen Stunden zu erstarren. Ausbeute 3,5 g. ($C_{20}H_{19}B$ (270,2): Ber. C 88, 90; H 7,09. Gef. C 87, 75; H 7,18).

Wegen der geringen Ausbeute wurden die anderen Fraktionen nicht weiter bearbeitet.

Messungen von Brechungsindices und Dichten

Die Brechungsindices wurden für die D-Linie mit einem Abberefraktometer bei 25° ± 0,1 gemessen. Eine schnelle Ablesung beiseitigte die Hydrolysengefahr.

Die Dichtemessungen wurden mit einem Ostwaldpyknometer[15] unter sorgfältigem Feuchtigkeitsausschluss ausgeführt.

Analytisches

Eine Ampulle, 50—100 mg Arylborsäureester enthaltend, wird in einem E-Kolben unter 20 ml Wasser zertrümmert. Ausgefallene Arylborsäure wird durch Erwärmen in Lösung gebracht. Zusatz von etwas Methanol erhöht die Löslichkeit der Arylborsäure erheblich. 10 ml bei Zimmertemperatur gesättigter Quecksilberdichlorid-Lösung und 4—5 g festes Natriumchlorid werden zugegeben. Die Arylquecksilberchloridverbindung fällt als ein weisser Niederschlag aus. Man lässt die Lösung etwa 10 Min. stehen. Sie wird mit ein paar Tropfen Methylrotindikator versetzt und dann (bei Zimmertemperatur) mit 0,1 *N* Natriumhydroxyd bis zum gelben Umschlag titriert. Die verbrauchte Lauge entspricht der gebildeten Salzsäure.

Derselben Probe werden Mannit und einige Tropfen Phenolphthalein-Indikator zugesetzt. Man titriert dann weiter bis zum hellroten Umschlag. Die Farbe darf bei weiterem Zusatz von Mannit nicht verschwinden. Die verbrauchte Lauge entspricht der gebildeten Borsäure.

Es ist nicht notwendig, die Titration in zwei Stufen auszuführen. Die Gesamtsäuremenge lässt sich durch Titration mit Lauge in Anwesenheit von Mannit und Phenolphthalein als Indikator bestimmen. Dabei ist zu bemerken, dass die Borsäure nur der Hälfte der verbrauchten Laugenmenge entspricht. Bei den Diarylborsäuren oder Triarylborverbindungen ist das Verhältnis Borsäure zu Gesamtsäure gleich 1:3 bzw. 1:4.

Herrn Prof. Dr. G. Wittig spreche ich hiermit für sein Entgegenkommen, mir in seinem Laboratorium einen Arbeitsplatz zur Verfügung gestellt zu haben, meinen ergebensten Dank aus. Durch meine dortige Arbeit erhielt ich viele wertvolle Anregungen. Für einen Reisebeitrag bin ich dem *Statens Naturvetenskapliga Forskningsråd*, Stockholm, zu Dank verpflichtet.

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The Crystal Structure of Selenium Diselenocyanate

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The crystal structure of selenium diselenocyanate has been determined by X-ray methods, from Patterson and Fourier projections along the *a*, *b* and *c* axes. The crystals are orthorhombic bipyramidal, with $a = 10.07$ Å, $b = 13.35$ Å, $c = 4.48$ Å. The space group is D_{2h}^{16}-*Pnma*, and there are four molecules per unit cell.

The molecule consists of an unbranched and non-planar N≡C—Se—Se—Se—C≡N chain, with a Se—Se bond length of 2.33 ± 0.03 Å and a Se—Se—Se bond angle of 101 ± 2°. The following, less reliable values were found for dimensions involving carbon and nitrogen atoms: Se—C = 1.83 Å, C—N = 1.05 Å, ∠ Se—Se—C = 95°, ∠ Se—C—N = 164°, and the dihedral angle, SeSeSe/SeSeC = 94°. The middle selenium atom is located in a crystallographic mirror plane of symmetry, and the molecule thus occurs in a *cis* form in the crystals.

The sixth-group elements, sulphur and selenium, do both form many-membered chains and rings. Beside the S_8 rings of orthorhombic and monoclinic sulphur, and the very long chains of plastic and fibrous sulphur, there are polysulphides and polythionic compounds with six sulphur atoms or more in the molecule. Selenium occurs as Se_8 rings in the monoclinic forms, and as infinite spirals in the hexagonal state, and polyselenide anions up to and including hexaselenide appear to exist.

When, however, the selenium chains are terminated by other atoms, and thus are parts, only, of larger molecules, the chain-forming capacity of selenium becomes inferior to that of sulphur. The largest number of selenium atoms in the chains, in any such compound isolated hitherto, appears to be three. Even so, the representatives known are relatively few. The simplest, and one of the more stable, is selenium diselenocyanate [1-5], also called [3-5] cyanogen triselenide.

This compound, $Se(SeCN)_2$, was first prepared by Verneuil [1] in 1886, by treatment of potassium selenocyanate with oxidizing agents. The substance occurs, together with selenium dicyanide, as a rearrangement product of selenocyanogen [2], and the same products result from reactions of diselenium

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dibromide with silver cyanide [2] and of bromine with lead selenocyanate [3]. The molecular weight of selenium diselenocyanate in boiling benzene and chloroform corresponds to a single formula unit [4], and the electric moment, in benzene at 25° C, is 3.98 Debye units [5].

A preliminary note on the crystal structure of selenium diselenocyanate has been published [6]. Unit cell and space group data have earlier been reported for triselenium dibenzene- and di-*p*-toluenesulphinate [7], but no detailed structure determination is available for these nor for any other triselenium derivative.

CRYSTAL DATA

The compound was prepared from potassium selenocyanate and nitrogen dioxide as described by Muthmann and Schröder [4], and recrystallized from benzene. The substance consisted of orange-yellow prisms, which were elongated in the direction of the *c* axis. The faces {010} were most prominently developed, with {110} and {111} also appearing. Recrystallization from chloroform gave thin plates, having a more yellow appearance but giving the same X-ray photographs as the crystals obtained from benzene.

The crystals are stable when kept in the dark, but decompose slowly, with liberation of selenium, when exposed to light or to X-rays. The slight deterioration did not, however, appear to influence the quality of the X-ray photographs.

Oscillation and Weissenberg photographs, using Cu$K\alpha$ radiation, $\lambda = 1.52_2$ Å, lead to the axial lengths: $a = 10.07 \pm 0.03$ Å, $b = 13.35 \pm 0.04$ Å, $c = 4.48 \pm 0.02$ Å. There are four molecules per unit cell; density, calc. 3.18, found 3.12 g/cm^3. On the basis of the systematic absences, $0kl$ when $k + l$ is odd, $hk0$ when h is odd, the centrosymmetric space group, D_{2h}^{16}-*Pnma*, was provisionally adopted, and this choise appears to be justified by the agreement ultimately obtained between observed and calculated structure factors. The space group implies that the selenium diselenocyanate molecule possesses either a mirror plane, or a centre, of symmetry.

Intensity measurements were made visually on zero layer Weissenberg photographs, using a double film technique. The crystals used for the *a*, *b* and *c* axis photographs had cross-sections of 0.05 × 0.06 mm, 0.08 × 0.09 mm, and 0.05 × 0.09 mm, respectively. 176 reflections out of a theoretically accessible 210 were measured in the principal zones, $0kl$, $h0l$, amd $hk0$. The intensities were corrected for the Lorentz and polarisation factors, but not for absorption. They were converted to relative structure factors, and later put to an approximately absolute scale by comparison with the calculated values.

The Patterson and Fourier summations were made with Beevers-Lipson strips, at intervals of 6° along the *a* and *b* axes and 12° (0.149 Å) along the *c* axis.

ANALYSIS OF THE STRUCTURE

The positions of the selenium atoms were determined from Patterson projections along the *a*, *b* and *c* axes, and the complete structure was subsequently worked out by means of Fourier projections along the same axes. The selenium

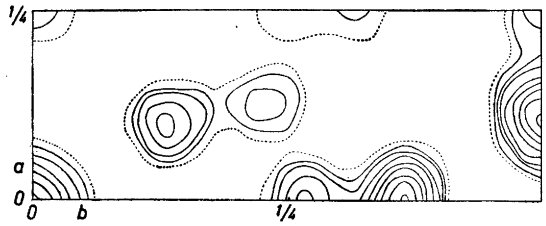

Fig. 1. Patterson projection along the c *axis. Contours at arbitrary but equal intervals, except at the origin peak, where the intervals are three times as large.*

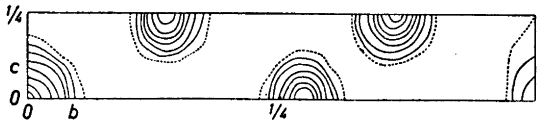

Fig. 2. Patterson projection along the a *axis. Contours as in Fig. 1.*

atoms, which account for 80 % of the electrons in the unit cell, were found to determine the signs of all observed structure factors in the three zones, except for a single one, 2,10,0.

The asymmetric parts of the Patterson projections are shown in Figs. 1 to 3. The number, heights and positions of vector peaks are in accordance with the requirements of the space group D_{2h}^{16}—*Pnma*, with one selenium atom situated in the mirror plane.

The following x and y coordinates were derived from the Patterson projection along the c axis: Se_1, $x = 0.536$, $y = 0.250$; Se_2, $x = 0.438$, $y = 0.116$. These values were used for the calculation of signs of the $hk0$ reflections for the first Fourier synthesis. The resulting map showed peaks corresponding to the carbon and nitrogen atoms, and the projection was refined by successive approximations in the usual way. The final electron density map, $\varrho(xy)$, is shown in Fig. 4.

The approximate z coordinates, 0.50 for Se_1 and 0.25 for Se_2, were derived from the Patterson projections along the a and b axes, and were employed, together with the x or y coordinates from $\varrho(xy)$, as a basis for the first Fourier projections along these axes. The refinement process, by Fourier methods as above, led to the electron density maps, $\varrho(yz)$ and $\varrho(xz)$, shown in Figs. 5 and 6.

Two sets of x, y and z coordinates were obtained from the three projections. The values finally adopted are listed in Table 1. The x and y coordinates are those derived from $\varrho(xy)$, since this projection is based on a larger number of

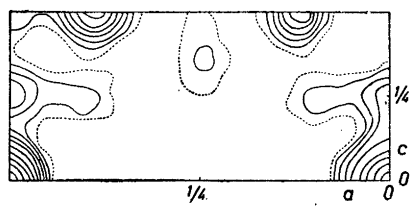

Fig. 3. Patterson projection along the b *axis. Contours as in Figs. 1 and 2.*

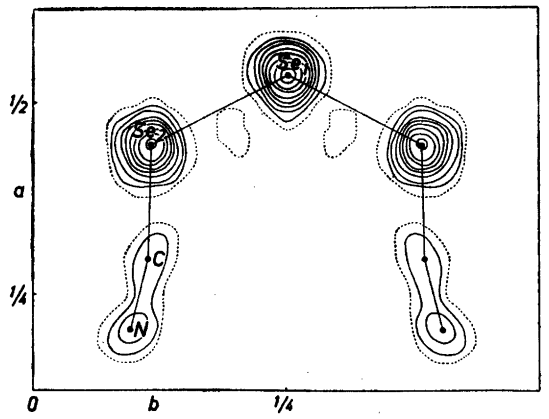

Fig. 4. Electron density projection of Se(SeCN)$_2$ along the c *axis,* ϱ(xy). *The area shown (from* x = ⅛ *to* ⅝*) is selected so as to cover one complete molecule. The 4-electron line is dashed. Contour intervals: 6 e.Å^{-2} for the selenium atoms and 2 e.Å^{-2} for the carbon and nitrogen atoms. In this and the two following figures, the positions corresponding to the final atomic coordinates are marked with dots, and lines are drawn to show the outline of a molecule.*

observed reflections (84) than the *a* axis (47) and *b* axis (45) projections. As for the *z* coordinates, overlapping occurs for Se_1 in $\varrho(xz)$ and for Se_2 in $\varrho(yz)$, but not *vice versa*. The carbon and nitrogen atoms are well resolved in $\varrho(xy)$ and $\varrho(xz)$ but not in $\varrho(yz)$, and the *z* coordinates chosen for these atoms are those derived from $\varrho(xz)$. Thus, all the coordinates of Table 1 have been determined from resolved peaks in electron density maps.

Table 1. Atomic coordinates, as fractions of corresponding cell edges. Origin at a centre of symmetry.

	x	*y*	*z*
Se_1	0.540	0.250	0.492
Se_2	0.442	0.115	0.249
C	0.295	0.112	0.488
N	0.203	0.095	0.586

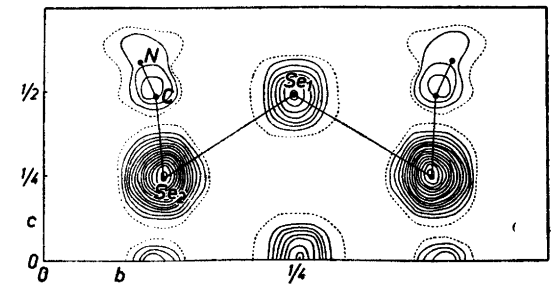

Fig. 5. Electron density projection of Se(SeCN)$_2$ along the a *axis,* ϱ(yz). *The Se_2 atoms are overlapped by other Se_2 atoms belonging to different molecules. The 6-electron line is dashed, and the contour intervals are 8 e.Å^{-2} for the selenium atoms and 2 e.Å^{-2} for the carbon and nitrogen atoms.*

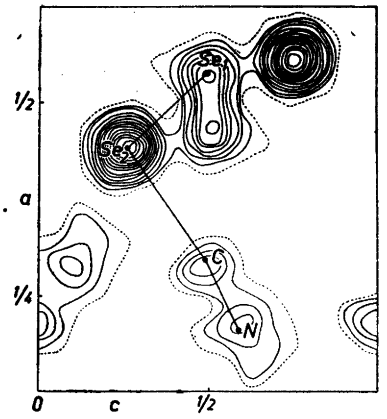

Fig. 6. Electron density projection of Se (SeCN)$_2$ along the b *axis,* ϱ(xz). *As in Fig. 4, the drawing covers the* a *axis from* $\frac{1}{8}$ *to* $\frac{5}{8}$. *The two selenocyanate groups of a molecule overlap exactly because of the mirror plane normal to the projection axis, and* Se_1 *atoms, from different molecules, overlap partly across a centre of symmetry. The 8-electron line is dashed, and contour intervals are 8 e.Å^{-2} for the selenium atoms and 3 e.Å^{-2} for the carbon and nitrogen atoms.*

In Table 3, observed and calculated values of structure factors are compared for all *hk*0, 0*kl* and *h*0*l* reflections within the range of Cu$K\alpha$ radiation. The calculated values are based on the coordinates of Table 1, and the atomic scattering curves of the *International Tables*, with a temperature factor of $B = 3.0$ Å^2 for all three zones. The realiability factor, $R = \Sigma \,||F_{obs}| - |F_{calc}|| / \Sigma |F_{obs}|$, with non-observed reflections included in the difference only when $|F_{calc}|$ is greater than the lowest observable value of $|F_{obs}|$, is 0.15, 0.12 and 0.13, respectively, for the *hk*0, 0*kl* and *h*0*l* reflections.

THE SELENIUM DISELENOCYANATE MOLECULE

The analysis has shown that the molecule is built up of an unbranched and non-planar chain, with three divalent selenium atoms in the middle and the cyano groups at the ends. The dimensions, calculated on the basis of the

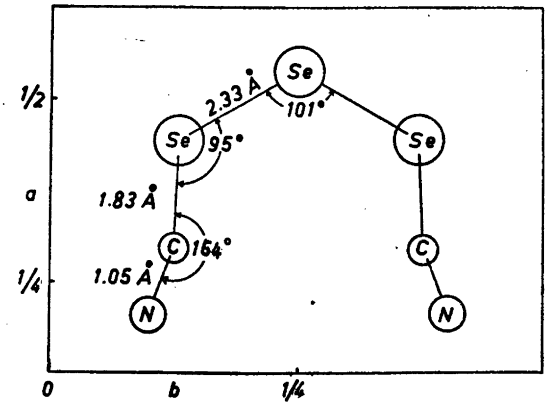

Fig. 7. The molecule as seen along the c *axis, showing bond distances and angles. The figure covers the same area as Fig. 4.*

coordinates of Table 1, are given in Table 2. The Se—Se bond length and Se—Se—Se bond angle are probably correct to within 0.03 Å and 2°. In view of the large contributions of the heavy selenium atoms to the observed X-ray intensities, but little reliance can be placed in the coordinates found for the carbon and nitrogen atoms. The coordinates may be in error by 0.1 Å or more, with corresponding errors in distances and angles involving these atoms. Due to the low and almost equal scattering power of the carbon and nitrogen atoms, the present analysis is incapable of deciding whether the structure of the selenocyanate groups is Se—C—N or Se—N—C. However, chemical evidence, and the close similarity with the thiocyanate group, leave little doubt that the former structure is the correct one.

Table 2. Bond lengths and bond angles.

Se—Se	= 2.33 Å	∠ Se—Se—Se	= 101°
Se—C	= 1.83	∠ Se—Se—C	= 95°
C—N	= 1.05	∠ Se—C—N	= 164°

Shortest non-bonded distances within the molecule.

Se—Se	= 3.60 Å	Se—C	= 3.08 Å
C—C	= 3.68	Se—N	= 2.85

The length, 2.33 Å, found for the Se—Se bond, compares with the current value [8], 2.34 Å, for the Se—Se single bond distance. Recent determinations have given the values 2.34 ± 0.02 Å in α-monoclinic [9] and β-monoclinic [10] selenium, 2.29 ± 0.01 Å in diphenyl diselenide [11], and 2.33_5 ± 0.032 Å in perfluoromethyl diselenide [12]. In hexagonal selenium [8 p. 79] as well as in α- and β-monoclinic selenium [9,10] the Se—Se—Se bond angle is 105°, as compared with the present value of 101°. These are the only data available for the bond angle of divalent selenium when bonded to two other selenium atoms.

The selenium diselenocyanate molecule possesses a mirror plane of symmetry, which passes through the middle selenium atom. The cyano groups are rotated out of the plane of the selenium atoms, to the same side of the plane, and the molecule thus occurs in a *cis* form [13] in the crystals. The dihedral angle between the Se—Se—Se and Se—Se—C planes is 94°. It is interesting to note that the configuration of the triselenium group, including the directions of the bonds extending form the external selenium atoms, is the same as that of a triselenium group in the eight-membered rings of the element. In the crystals of triselenium di-*p*-toluenesulphinate [7, 13], a twofold axis of molecular symmetry is present, and the external valencies of the triselenium group are there extending to opposite sides of the plane of the selenium atoms.

This appears to be the first structure determination of a compound containing the selenocyanate group. The analogous thiocyanate group has always been found to be linear, except in the mercury tetrathiocyanate-copper diethylenediamine complex [14] where the weighted mean value, ∠ S—C—N = 163° 12′, was reported. The deviation from linearity in the present structure, ∠ Se—C—N = 164°, may, partly at least, be due to experimental errors. The bond lengths within the group, Se—C = 1.83 Å and C—N = 1.05 Å, are to be compared

Table 3. Observed and calculated values of structure factors for selenium diselenocyanate.

k	F_{obs}	F_{calc}
	$hk0$ zone	
	$0k0$	
2	58	− 67
4	135	−156
6	161	−182
8	189	+194
10	46	+ 29
12	42	− 17
14	100	− 81
16	40	+ 38
	$2k0$	
0	163	+214
1	50	+ 45
2	93	− 95
3	134	+202
4	9	− 16
5	100	−108
6	95	− 86
7	58	− 54
8	126	+126
9	< 12	− 1
10	23	− 16
11	84	+ 83
12	< 13	− 9
13	20	− 16
14	57	− 48
15	24	− 25
16	40	+ 35
17	13	− 16
	$4k0$	
0	98	+ 92
1	59	+ 46
2	49	− 39
3	195	+216
4	24	+ 11
5	119	−110
6	62	− 50
7	69	− 59
8	53	+ 42
9	32	− 31
10	13	− 10
11	100	+ 93
12	21	+ 12
13	18	− 19
14	27	− 20
15	20	− 15
16	11	+ 10
	$6k0$	
0	63	− 67
1	< 12	− 5
2	24	+ 12
3	139	+139
4	70	+ 65
5	102	−103
6	< 10	+ 15
7	31	− 28
8	35	− 38
9	34	− 16
10	25	− 23
11	76	+ 86
12	31	+ 24
13	24	− 24
14	11	+ 15
15	9	− 17
	$8k0$	
0	116	−123
1	39	− 29
2	< 13	+ 4
3	58	+ 54
4	73	+ 70
5	45	− 39
6	46	+ 49
7	20	+ 20
8	58	− 68
9	32	− 26
10	28	− 26
11	30	+ 26
12	18	+ 19
13	12	− 15
	$10k0$	
0	61	− 66
1	38	− 36
2	18	+ 23
3	< 13	− 3
4	15	+ 15
5	12	− 4
6	28	+ 29
7	30	+ 33
8	37	− 47
9	20	− 16
	$12k0$	
0	31	− 42
1	25	− 28
2	13	+ 15
3	16	− 29
4	< 4	0
5	< 2	+ 12
6	16	+ 25

k	F_{obs}	F_{calc}
	$0kl$ zone	
	$0k0$	
2	59	− 67
4	138	−156
6	157	−182
8	183	+194
10	43	+ 29
12	29	− 17
14	94	− 81
16	40	+ 38
	$0k1$	
1	130	+147
3	131	+155
5	68	− 77
7	123	−126
9	34	+ 32
11	93	+ 80
13	< 15	0
15	48	− 47
17	8	− 7
	$0k2$	
0	66	− 55
2	105	− 95
4	182	+193
6	35	− 29
8	39	− 30
10	91	− 76
12	82	+ 75
14	< 12	+ 7
	$0k3$	
1	83	− 75
3	108	−113
5	58	+ 49
7	84	+ 79
9	< 15	− 3
11	62	− 59
13	< 11	− 3
15	16	+ 27
	$0k4$	
0	105	+118
2	36	− 28
4	38	− 37
6	56	− 52
8	69	+ 77
10	< 12	+ 5
12	< 9	− 12
14	27	− 39

k	F_{obs}	F_{calc}
	$0k5$	
1	34	+ 38
3	33	+ 32
5	15	− 15
7	29	− 39
	$h0l$ zone	
h	$h00$	
2	151	+214
4	103	+ 92
6	66	− 67
8	125	−123
10	71	− 66
12	41	− 42
	$h01$	
1	101	+107
2	137	+152
3	85	+ 73
4	142	+149
5	44	+ 26
6	114	+107
7	< 16	− 9
8	21	+ 13
9	24	− 22
10	35	− 29
11	30	− 23
12	28	− 35

h	F_{obs}	F_{calc}
	$h02$	
0	59	− 55
1	27	− 23
2	70	− 73
3	< 14	+ 4
4	41	+ 29
5	< 16	− 8
6	80	+ 64
7	< 18	− 9
8	63	+ 49
9	< 18	+ 3
10	34	+ 32
11	< 14	− 2
12	8	− 10
	$h03$	
1	64	+ 61
2	72	− 68
3	32	+ 30
4	109	−114
5	35	+ 22
6	64	− 58
7	< 18	− 14
8	23	− 22
9	< 15	− 13
10	18	+ 19
11	9	− 15

h	F_{obs}	F_{calc}
	$h04$	
0	110	+118
1	< 18	− 6
2	89	+ 85
3	< 18	0
4	34	+ 26
5	< 18	− 4
6	28	− 29
7	< 15	− 6
8	37	− 50
9	< 11	− 1
	$h05$	
1	30	+ 22
2	27	+ 30
3	18	+ 8
4	32	+ 34
5	16	+ 14
6	18	+ 27

with the standard bond lengths [8], Se—C = 1.94 Å, Se=C = 1.74 Å, C=N = 1.27 Å, and C≡N = 1.15 Å. The rather large uncertainties in the carbon and nitrogen coordinates do hardly permit definite conclusions to be drawn concerning the multiplicity of the Se—C and C—N bonds.

The shortest intermolecular distances in the crystal are 3.04 Å and 3.24 Å and occur between selenium (Se_2) and nitrogen atoms. The figure, 3.04 Å, is rather low, considering the van der Waals radius of nitrogen [15], 1.4—1.5 Å, and of selenium [9,10], 1.75—1.80 Å. The closest approach between selenium atoms of different molecules is 3.98 Å.

The crystal structure of an isomorphous compound, *viz.*, selenium dithiocyanate, $Se(SCN)_2$, has been published recently [16]. The Se—S distance, 2.21 Å, corresponds to a single bond, as does the Se—Se distance in the present structure, and the selenium bond angle is the same, 101°. A van der Waals approach of 2.98 Å occurs between selenium and nitrogen atoms.

It appears possible that the relatively unstable crystals of sulphur dithiocyanate [17], $S(SCN)_2$, may be isomorphous with those of selenium dithiocyanate and diselenocyanate.

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An X-ray Diffraction Study to Determine the Effect of the Method of Preparation upon the Crystal Structure of TiO_2

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The existence of different polymorphic forms of hydrolytically prepared TiO_2 is reviewed briefly. A quantitative X-ray diffraction technique by means of a recording spectrometer for determination of rutile and anatase in mixtures of the two is described.

Precipitation, drying, and calcination of TiO_2 samples were carried out under varying conditions. Both sulfate and chloride solutions of titanium were used, and the products were examined by X-ray diffraction. It was observed that the initial partially dehydrated product always was amorphous, even after drying at 110° C in practically all cases. At elevated temperatures the first crystalline form of samples originating from sulfate solutions always was anatase. Direct boiling of chloride solutions produced rutile as initial crystalline product, other methods of hydrolysis resulted either in anatase or in mixtures of the two. All samples from both sulfate and chloride solutions were converted into rutile at last between 700 and 920° C, the rate of conversion being somewhat depending on the origin of the sample. No brookite was found at any step.

As is well known, titanium can be precipitated from aqueous solutions where it exists as sulfate, chloride, or nitrate either by alkaline or acid hydrolysis, the latter being usually effected by boiling. It has been observed by different workers that the appearance, physical and chemical behaviour of the precipitate largely depend on the previous treatment. The present work deals with the correlation between the crystal form of the hydrolyzed product (TiO_2) and the method of hydrolytic precipitation.

This problem has been studied by Parravano[1], among others, and he has given the following schema which outlines the different methods and resulting products.

According to Schossberger[2] sulfate solutions of titanium always give products which show the crystal form of anatase. This can be converted at temperatures between 600° and 1 000° C into rutile. Instead of that, chloride solutions produce rutile directly; if sulfuric acid is added, an increasing amount

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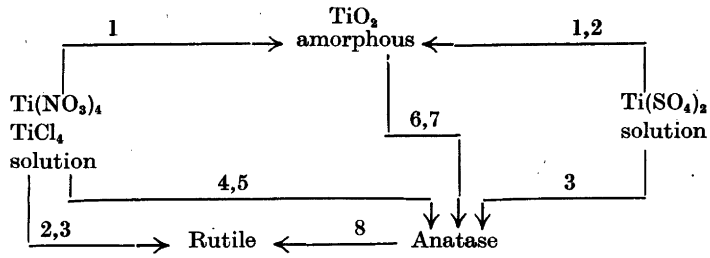

1. Precipitation (alkaline hydrolysis)
2. Hydrolysis at room temperature
3. Hydrolysis by boiling
4. Hydrolysis in the presence of SO_4^{2-} or PO_4^{3-} ions
5. Hydrolysis in the presence of excess Cl^- ions
6. Dehydration
7. Moderate heating
8. Igniting

of anatase is formed. If titanium oxyhydrate was precipitated with ammonia, redissolved in HCl and precipitated again, anatase was formed. If aged in the mother liquor for a longer time and then dried at 300°, the resulting product showed a rutile crystal form. Schossberger also states that no brookite was found in any product.

Weiser *et al.*[3] obtained amorphous or anatase type products by using ammonia precipitation from both sulfate, chloride, and nitrate solutions of titanium. If the hydrolysis was carried out by boiling, the precipitate from sulfate solutions was found to be anatase, and that from chloride or nitrate solutions rutile, if no excess Cl^- or NO_3^- ions were present in the latter case. Otherwise the resulting product was anatase also in this case. These workers explain that the rate of transformation of the more soluble and less stable anatase modification into the less soluble and more stable rutile modification is influenced by two opposite factors, *viz.* retardation of the transformation by an adsorbed layer of ions on the anatase which reduces its rate of solution, and acceleration of the transformation by an ionic environment in which anatase is more soluble. Anatase formed by hydrolysis of titanium chloride or nitrate solutions is transformed fairly rapidly into rutile at 100°. The change is slowed down by the presence of alkali chloride or nitrate, speeded up in the presence of excess hydrochloric or nitric acid. Further they state that anatase formed by the hydrolysis of titanium sulfate solutions is not transferable into rutile in any reasonable time even in strong sulfuric acid solution.

It is of interest to mention an inconsistency concerning this matter which can be found in the latest monograph about titanium, edited by Skinner *et al.*[4]: "Alkaline hydrolysis of $TiCl_4$ yields a precipitate which on drying shows the anatase structure. If hydrolysis of $Ti(SO_4)_2$ or of $TiCl_4$ in presence of the sulfate ion is carried out, the oxide formed on drying is rutile." — As can be easily seen, the whole matter has been reversed; in addition to that, the statement is confusing and oversimplified. The same book claims that Lashchenko

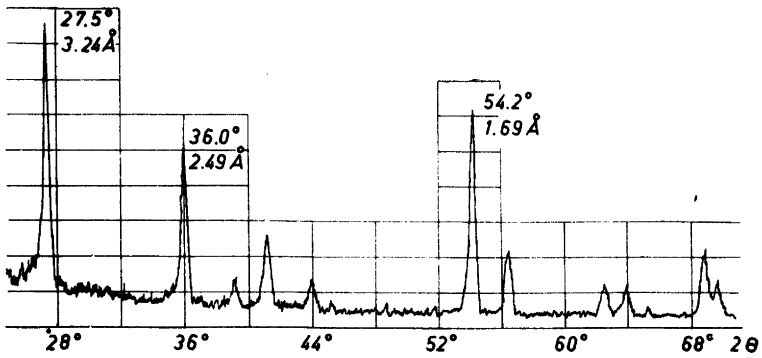

Fig. 1. A typical rutile X-ray diffraction pattern obtained by a Geiger-counter recording spectrometer using Cu Ka-radiation and Ni filter. The curve is shown between the 2 Θ-values 25° and 70°. The 2 Θ-angles and corresponding values of the d-spacings are given for the principal diffraction lines of rutile.

and Kompanski[5] have prepared brookite by dehydrating metatitanic acid. Unfortunately, we were not able to check this matter in the original paper, but the *Chem. Abstracts* reference, used also by the editors of the monograph, did not know anything about a possible brookite formation.

Inuzuka[6] reports the formation of brookite on glass surface dipped into aqueous solution of $TiCl_4$ containing HCl, dried at 100° C and heated at 300—400° C for an hour.

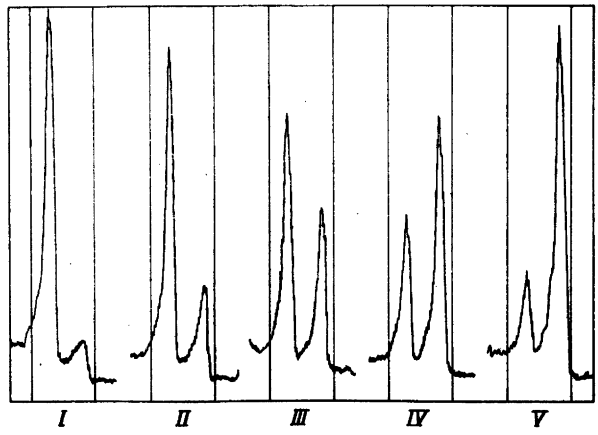

Fig. 2. X-ray diffraction records obtained from different standard mixtures. I. 12.5 % rutile, 87.5 % anatase; II. 25 % r., 75 % a.; III. 50 % r., 50 % a., IV. 75 % r., 25 % a., and V. 87.5 % rutile. 12.5 % anatase. Only the main peaks are shown, 3.51 Å for anatase (left peak), and 3.24 Å for rutile (right peak).

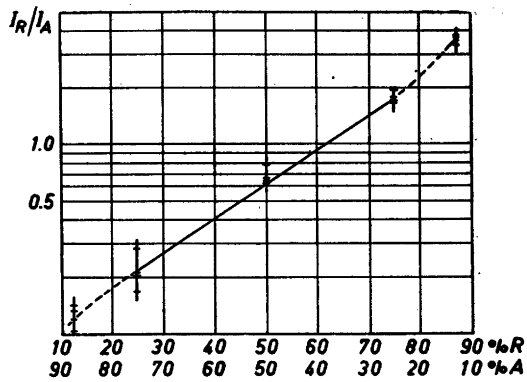

Fig. 3. Working curve for quantitative determinations of rutile and anatase in mixtures of the two. I_R intensity (height) of the rutile peak at 3.24 Å, I_A intensity of the anatase peak at 3.51 Å.

Various methods of "seeding" sulfate solutions for producing rutile directly exist in patent literature only, and they seem to lack any broader scientific basis. This author has not been able to effect a direct rutile hydrolysis from sulfate solutions by any of the numerous recommended seeding agents he tried; the moderately heated product was always anatase and changed into rutile by further ignition.

EXPERIMENTAL

In this work several methods of preparation of titanium dioxide were employed, and the calcination was carried out stepwise thus allowing the checking of the crystal form produced at the respective temperature. Ammonia precipitation was used for both chloride and sulfate solution; sodium hydroxide precipitation for chloride solution, and hydrolysis at boiling temperature for both solutions. One experiment was made using dilution technique. Table 1 contains additional information about these experiments.

X-ray technique

A North American Philips' Geiger-counter X-ray diffraction spectrometer with a strip-chart recorder was used for the anatase-rutile determinations. Cu $K\alpha$-radiation, 30 kV, 6 mA, with a nickel filter was employed. The sample was packed into a rectangular cavity in a plastic slide, and a flat and smooth surface was formed with a spatula. The plastic slide was placed in position on the spectrometer, the goniometer arm was set to $2\ \Theta = 72°$, if the whole characteristic diffraction pattern of rutile and/or anatase was desired. For the strongest peaks only, the goniometer arm was set to 32°. In both cases, the apparatus was run at a speed of 2° per minute to about 23°. A typical rutile diffraction pattern is shown in Fig. 1.

According to Klug[7] the degree of precision and reproducibility in a quantitative analysis of powder mixtures with the Geiger-counter spectrometer is dependent upon different factors: instrument stability and reproducibility, crystallite size of the powder, mounting of the specimen, mixing of the internal standard with the sample, *etc.* Absorption effects are present which usually prevent one from directly comparing line intensities of a component in a mixture with the pattern of the pure component prepared under identical conditions. This is always the case if the mixture contains components with

Table 1. Results from different hydrolysis experiments

Method of precipitation	Crystal form found at different drying or calcination temperatures						
	Room temp. Dehydrated with alcohol	110° C, 2 hrs	300° C, 1 h	500° C, 1 h	700° C, 1 h	800° C, 1 h	920° C, 1 h
Alkaline precipitation. 10 % aqueous solution of $TiCl_4$ added to a 1 : 1 diluted NH_4OH at room t.	amorphous	amorphous	anatase fairly well developed	anatase	57 % anatase 43 % rutile	rutile with traces of anatase	rutile
Alkaline precipitation. 10% aqueous acid solution of $Ti(SO_4)_2$ added to a 1 : 1 diluted NH_4OH at room t.	amorphous	amorphous	anatase poorly developed	anatase	anatase, with traces of rutile	rutile with traces of anatase	rutile
Alkaline precipitation. 10 % aqueous HCl-acid solution of $TiCl_4$ added to a 20 % NaOH solution. Room temperature.	amorphous	amorphous	72 % rutile 28 % anatase	rutile with traces of anatase	rutile	—	—
Hydrolysis by boiling a 10 % aqueous HCl-acid solution of $TiCl_4$.	amorphous	amorphous, traces of rutile	rutile poorly developed	rutile	—	—	—
Hydrolysis by adding a 10 % aqueous HCl-acid solution of $TiCl_4$ to a large volume of boiling water.	amorphous	—	68 % rutile 32 % anatase	rutile	—	—	—
Hydrolysis by boiling a 10 % aqueous acid solution of $Ti(SO_4)_2$.	amorphous	amorphous, traces of anatase	anatase, poorly developed	anatase	anatase	—	rutile

different X-ray absorption. Mixtures of two polymorphic components, however, like anatase and rutile, usually can be analyzed directly, the intensity of the line being proportional to the concentration.

In this work standard mixtures of pure rutile and pure anatase were prepared, by milling, containing 12.5, 25, 50, 75 and 87.5 % of rutile, the rest being anatase in each case. No internal standard was added. The principal peaks of the diffraction curve representing d-spacings of 3.51 Å for anatase and of 3.24 Å for rutile are shown in Fig. 2 for all these five cases.

A working curve, presented in Fig. 3, was prepared by measuring the height of the peak from the base line using arbitrary units, here millimeters. The relation I_R/I_A (I_R intensity (height) of the rutile peak, I_A intensity of the anatase peak, respectively) was plotted against concentration on semilogarithmic paper. The curve with its evidently straight middle portion was drawn on the paper based on four different I_R/I_A values, representing four different runs, the specimen being remounted in each case. These values have also been marked on the paper. The analysis of an unknown mixture is carried out in the same way: after making a run, the peak heights are measured, I_R/I_A calculated, and the working curve gives the sought composition. As can be seen in Fig. 2, the principal peaks of these polymorphic forms are very favorably located, rather close, but not so close that they would interfere with each another. Hence, the X-ray operation itself takes only about 5 minutes.

RESULTS AND DISCUSSION

As can be seen in Table 1, an alkaline precipitation with NH_4OH gave anatase initially from both sulfate and chloride solutions. The crystal structure, however, was developed faster in the latter than in the former case with the increasing temperature, and the change into rutile took place earlier. If alkaline precipitation was carried out with NaOH, the main part of the precipitate dried at 300° C consisted of rutile, and a complete change into rutile was effected at 700° C. Hydrolysis by boiling a rather concentrated solution of $TiCl_4$ produced rutile directly after the amorphous state without any anatase formation. If dilution technique was used, anatase also was present at lower heating temperatures. Normal acid hydrolysis of a sulfate solution produced first anatase (after the amorphous state), a complete conversion into rutile took place between 700° and 920° C. In all cases the product partly dehydrated with alcohol and dried at room temperature was completely amorphous, and even at 110° only a very weak crystallization could be observed in a couple of cases.

The main tendency of the anatase-rutile polymorphism observed in these experiments agrees with that presented by Parravano and Schossberger (*cf.* p. 1796). It has been shown, however, that an amorphous phase always exists before any crystallization takes place, even in the case of direct rutile formation. Other deviations from this schema were also observed, such as the difference between products if the alkaline hydrolysis was carried out with ammonia or sodium hydroxide, or the existence of mixtures of both crystal forms at different calcination temperatures.

As far as the third polymorphic form of TiO_2, brookite, is concerned, all specimens prepared during this work and also during another of the same nature, described elsewhere[8], representing a great variety of methods of hydrolysis and calcination temperatures were checked carefully for the characteristic X-ray diffraction lines of this particular substance. No traces of brookite could be found in any case, however.

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Studies on Local Anesthetics IX[1-8]

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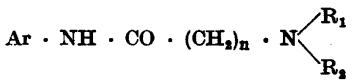
Many N-(aminoacyl)-amines, mainly of the type

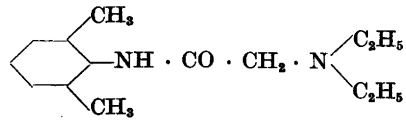


$n = 1$ or 2, R_1 = H or alkyl, R_2 = alkyl

have been synthesized by Löfgren *et al.*[1-8]. The compounds have been investigated pharmacologically and especially as to their local anesthetic action. Also, in many cases, physico-chemical properties (distribution coefficients oleyl alcohol/water [5], molar refractions [5], absorption spectra in the ultraviolet [5], ionization constants [5], and dipole moments [9]) have been determined and their relation to structure and anesthetic activity studied.

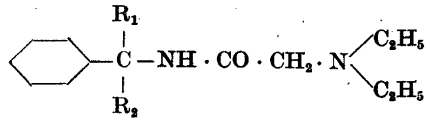
The interfacial tension/time curve, as obtained when anesthetics are distributed through an interface paraffin oil/water at which an ergosterol film is adsorbed has been studied by Seelich [10] (general anesthetics) and by Tammelin and Löfgren [11] (local anesthetics of xylocaine type). Mainly on the basis of these experiments a new theory for the mechanism of anesthesia has been presented (Tammelin and Löfgren [11], Löfgren [5]).

One of the compounds synthesized earlier [1] is xylocaine



which has outstanding local anesthetic and physico-chemical properties as compared with the other closely related compounds [1,5,9]. Xylocaine is now used extensively in all kinds of clinical local anesthesia.

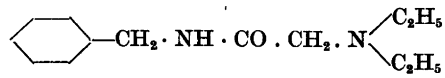
In the present work three benzylamides of the formula



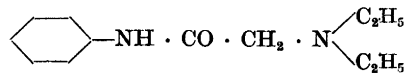
I: $R_1 = H$, $R_2 = COOC_2H_5$
II: $R_1 = CH_3$, $R_2 = COOC_2H_5$
III: $R_1 = H$, $R_2 = CH_2COOC_2H_5$

have been synthesized and their local anesthetic action studied.

Earlier [1] the benzylamide

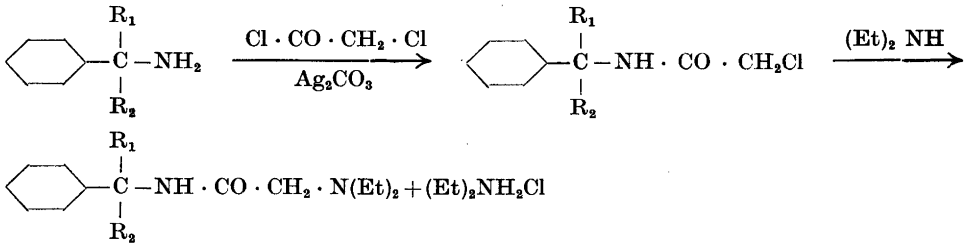


was investigated. The compound was demonstrated to have a shorter duration than xylocaine or about the same effect as the corresponding anilide (test performed on the tongue):



Whereas the anilide was found to be almost free from an irritating effect, the benzylamide was shown to be a strong irritant.

The compounds were synthesized according to the following scheme:



The chloroacetylation step was carried out in dry ether, silver carbonate being used to catch the hydrogen chloride produced.

In the earlier works by Löfgren *et al.* (*cf.* above) the reaction between acyl halides and amines was usually carried out according to the aqueous acetate buffer method described by Löfgren[1, 5]. In the present work the chloroacetylation of water-sensitive esters was made in the absence of water and with *silver carbonate* as a hydrogen chloride acceptor. It is known from the literature that silver carbonate has been used as a hydrogen halide acceptor in some reactions but as far as we know the salt has never been used in the reaction between acyl halides and amines.

After removal of the silver chloride and the ether, the remaining crude chloroacetyl derivative was heated with diethylamine in ethanol solution to give the desired product.

The three compounds were tested for their local anesthetic action on the rabbit cornea. For this purpose 2 % hydrochloride solutions of pH 6 were

prepared. In this test, compound I was inactive whereas the other two were active. The duration of II and III was a fourth and a fifth, respectively, of the value of xylocaine. As compared with xylocaine the latency times of II and III were much longer. — The toxicities of the three compounds were found to be low. Thus, when injected subcutaneously in white mice, the LD_{50} values for I, II, and III are 2.2, 0.78, and 2.2 g/kg, respectively. The corresponding value for xylocaine is 0.39 g/kg.

The three compounds, especially II, have some structural similarities with analgesic agents (compounds with a morphine-like action) *. Therefore by the toxicity determinations, special attention was paid to the occurrence of the Straub phenomenon (erection of tail). This effect is typical of analgesic agents. However, some compounds are known which give a weak Straub reaction but are devoid of analgesic action (Heinekamp [13]). All three compounds gave a positive but weak Straub test, compound II having the most pronounced effect. Because of the weak effects a more detailed evaluation of a possible analgesic action did not seem worth while.

The compounds were also tested for their spasmolytic and histaminolytic power. No appreciable effects were found.

EXPERIMENTAL **

Ethyl N-(diethylaminoacetyl)-α-aminophenylacetate (I). α-Aminophenylacetic acid was prepared as described by Steiger [14]. To synthesize the ethyl ester, we followed the directions given by Kossel [15]. It was obtained as a colourless oil; b. p. 129—131°/9 mm; yield 61 %.

In a 150-ml Erlenmeyer flask 5.30 g (0.0296 mole) of ethyl α-aminophenylacetate were dissolved in 10 ml of absolute ether. The solution was cooled to 5° and 3.34 g (0.0296 mole) of chloroacetyl chloride diluted with 5 ml of absolute ether were added drop by drop under vigorous stirring. During this process the temperature was allowed to rise slowly to about 20°. To the mixture, now protected against the light, 4.11 g (0.0149 mole) of silver carbonate, freshly prepared and dried, were added in portions during half an hour and under continued stirring which was carried on for another four hours. After this time the evolution of carbon dioxide had ceased, and the silver chloride was filtered off. On removing the ether by distillation, colourless needles separated. A very small amount was recrystallized from di-*n*-butyl ether; m. p. 68—69°.

The crude chloroacetyl derivative was dissolved in 60 ml of absolute ethanol, and 5.85 g (0.0800 mole) of diethylamine were added. The mixture was kept at 70° for six hours in a sealed bottle. The alcohol and the excess of diethylamine were then driven off under reduced pressure and at a bath temperature of 40°. After dilution with absolute ether, diethylammonium chloride separated and was filtered off. The solvent was driven off and on fractional distillation of the residue, a colourless oil was collected; b. p. 160—162°/0.8 mm; $n_D^{20} = 1.5015$; yield 4.95 g (0.0169 mole), *i. e.* 57 %, calculation based on ethyl α-aminophenylacetate. (Found: C 65.8; H 8.33; Equiv.w. 288 ***. Calc. for $C_{16}H_{24}N_2O_3$ (292.4): C 65.7; H 8.27; Equiv.w. 292).

Perchlorate. Colourless needles from absolute ethanol; m. p. 179—181°. (Found: $HClO_4$ 25.8 ****. Calc. for $C_{16}H_{25}ClN_2O_7$ (392.8): $HClO_4$ 25.6).

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* For a survey of the relationships between structure and analgesic action see for instance Eddy [12].

** All melting points and boiling points are uncorrected.

*** Titration of the base in 30 % ethanol with 0.1 *N* HCl; mixed indicator methylene blue-methyl red.

**** Analysis by precipitating the potassium perchlorate — see Löfgren [1].

Ethyl N-(diethylaminoacetyl)-α-amino-α-phenylpropionate (II). α-Amino-α-phenylpropionic acid was obtained in accordance with the description given by Steiger [16]. The esterification with ethanol was carried out as described by Mc Kenzie and Myles [17]. The fraction with b. p. 134—136°/14 mm was collected; yield 49 %.

From this ester the final base was obtained by chloroacetylation and subsequent treatment with diethylamine as described in the preceding preparation. The base was obtained as a colourless oil; b. p. 137—139°/0.1 mm; $n_D^{20} = 1.5017$; yield 58 %, calculation based on ethyl α-amino-α-phenylpropionate. (Found: Equiv.w. 303 *. Calc. for $C_{17}H_{26}N_2O_3$ (306.4): Equiv.w. 306).

Ethyl N-(diethylaminoacetyl)-β-amino-β-phenylpropionate (III). β-Amino-β-phenylpropionic acid was prepared as described by Steiger [18] and was esterified according to Mc Kenzie and Richardson [19]. On distillation a colourless oil was collected; b. p. 133—134°/9 mm; $n_D^{20} = 1.5112$; yield 52 %.

From this ester the final base was obtained by chloroacetylation and subsequent treatment with diethylamine as described under the preparation of compound I. The product was collected as a colourless oil; b. p. 150—151°/0.08 mm; solidifying p. 5°; yield 45 %, calculation based on ethyl β-amino-β-phenylpropionate. (Found: Equiv.w. 308 *. Calc. for $C_{17}H_{26}N_2O_3$ (306.4): Equiv.w. 306).

SUMMARY

Ethyl N-(diethylaminoacetyl)-α-aminophenylacetate, ethyl N-(diethylaminoacetyl)-α-amino-α-phenylpropionate and ethyl N-(diethylaminoacetyl)-β-amino-β-phenylpropionate have been synthesized and tested for their local anesthetic, spasmolytic and histaminolytic power.

We are indebted to A.-B. Astra, Södertälje, Sweden, for financial support and to Dr. S. Wiedling of the Biological Department of Astra for performing the pharmacological tests. Our sincere thanks are also due to Professor A. Girardet of the University of Lausanne who read through the manuscript.

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* The same analytical method as described under compound I.

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Studies on Local Anesthetics X[1–9]

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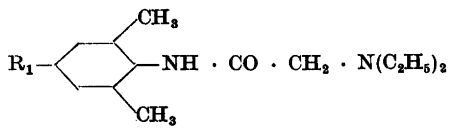
Xylocaine, *i.e.* ω-diethylamino-2,6-dimethylacetanilide, and a fairly large number of compounds related to it have been synthesized by Löfgren *et al.*[1–9] The compounds have been tested pharmacologically, especially for their local anesthetic action. On certain of the compounds, some physico-chemical measurements have been carried out, and relations of physico-chemical properties to structure and anesthetic activity studied [5,10,11].

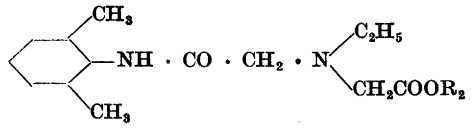
In the present investigation six new compounds have been synthesized and their local anesthetic action studied.

The structure of three of these compounds (I, II, III) is represented by the formula



where R_1 = HO (I), CH_3O (II), or C_3H_7COO (III).

The three other compounds (IV, V, VI) belong to another series



where R_2 = C_2H_5 (IV), *n*-C_3H_7 (V), or *n*-C_4H_9 (VI).

Compounds I, II, and III were prepared by chloroacetylation of the actual aromatic amine, using Löfgren's acetate buffer method [1,5], and subsequent treatment of the chloroacetyl derivative with diethylamine.

To obtain compound II, ω-diethylamino-4-methoxy-2,6-dimethylacetanilide, we first tried to methylate I, ω-diethylamino-4-hydroxy-2,6-dimethylacetanilide, with diazomethane; but this reaction failed. Nor did we succeed to methylate ω-chloro-4-hydroxy-2,6-dimethylacetanilide with the aid of the same reagent. Owing to the risk of side reactions methylation with dimethyl sulphate was not tried. (The tertiary base may give a quaternary compound; the chloro derivative may be sensitive to alkali.)

For the chloroacetylation of 4-hydroxy-2,6-dimethylaniline the above mentioned acetate method was modified because of the slight solubility of the

aminophenol in glacial acetic acid. The chloroacetylation was made by adding first chloroacetyl chloride to a water solution of the hydrochloride and then, without any delay, an aqueous solution of sodium acetate, this mixture being at once shaken vigorously . — The aminophenol, 4-hydroxy-2,6-dimethylaniline, was obtained by nitrosating 3,5-dimethylphenol followed by reduction with ammonium sulphide. This aminophenol has been previously prepared by treating 2,6-dimethylphenylhydroxylamine with diluted sulphuric acid [12], and by reduction of 4'-nitro-4-hydroxy-3,5-dimethylazobenzene [13]. — An efficient synthesis for 4-methoxy-2,6-dimethylaniline — the starting amine for preparing compound III — has been devised by Saunders and Watson [14]. They coupled 3,5-dimethylphenol with benzenediazonium chloride and methylated the resulting azo compound with dimethyl sulphate. The methoxyazo compound was then reduced under high pressure with hydrogen and Raney nickel. We followed their method, but for the reductive cleavage we used sodium hydrosulphite instead of hydrogen and Raney nickel.

Compounds IV, V, and VI were obtained by reaction between ω-ethylamino-2,6-dimethylacetanilide and the actual alkyl bromoacetate. (*Cf.* Löfgren and Widmark [4] who previously used the same method for preparation of ω-(N-methyl-carbethoxymethylamino)-2-methylacetanilide).

The compounds were tested for their local anesthetic potency on the rabbit cornea and compared with xylocaine, the method of Wiedling [15] being followed. As a measure of the potency, the *relative activity* according to Wiedling is used, *i.e.* an activity which will be found by dividing the concentration of a xylocaine standard solution by the concentration of the actual compound at which the duration of anesthesia is the same as that of the standard solution.

By this method 0.25 ml of the test solution is instilled into the rabbit's conjunctival sac, so that the cornea is covered, and allowed to remain for 30 seconds. Simultaneously the same volume of 2 % xylocaine hydrochloride solution of the same pH value is applied to the animal's other conjunctival sac. The time elapsing from the application of the local anesthetic solution to the onset of anesthesia is termed the *latent period*. The latent period and *duration* of complete anesthesia are established by the use of a rigid mechanical irritant, *i. e.* a graphite point. The pressure is adjusted so that a depression of the cornea is just elicted.

The 2 % xylocaine · HCl (0.0738 *M*) is said to have unit activity. A concentration of the actual compound at which the duration is the same as that of the xylocaine standard is selected. By dividing the molarity of the standard (= 0.0738) by the selected molarity of the test solution we thus obtain a value which we term the *relative activity* of the compound *.

As a rule, the test solutions are applied to the cornea at a pH of about 6. Sometimes, however, it may happen that one or other of the compounds within a series must be measured at a lower pH value (sometimes down to pH 4) since otherwise the compound can not be brought into solution. The duration at this lower pH value may differ slightly from that which would have been obtained at the higher pH value **; but since the stand-

* This way of calculating the relative activity differs from that of Wiedling who takes the quotient between the percentages instead of dividing the molarities.

** Thus for instance the 2 % hydrochloride solution of xylocaine gives durations from about 13 minutes down to about 10 minutes if the pH value is varied from 7 to 4. (The figures for the duration are given as average values from many experiments.) When, however, *minimal effective concentrations* are measured to evaluate local anesthetics, the influence of pH is very great (*cf.* Trevan and Boock [16], Löfgren [5]).

ard solution of xylocaine is measured at the same lower pH value, the calculated relative activity will be found to be consistent with the values of the other compounds.

In the first series of compounds (*cf.* the general formula given on p. 1806), I and III gave no anesthesia when tested as 2 % solutions on the rabbit cornea (solutions of the hydrochlorides, containing 2 % of the bases; pH = 6.0). They may therefore be described as inactive. Compound II gave a slight relative activity, the value being estimated to 0—0.5.

In a recent paper of 1951, Büchi *et al.*[17] report on a compound, ω-diethylamino-4-*n*-butoxy-2,6-dimethylacetanilide, which differs from our compound II in having a *n*-butoxy group instead of a methoxy group. They found the compound to be much more active than xylocaine on the rabbit cornea. We synthesized this butoxy compound following the directions given by Büchi *et al.* who describe the compound as being an oil at room temperature. On the contrary we found the compound to be a solid (m. p. 37—38°, *cf.* the synthetic part compound II:a). When tested on the rabbit cornea, the compound gave a relative activity of 18 (xylocaine used as a standard, *cf.* above). At the same time it was observed that the compound is a pronounced irritant, causing conjunctivitis and corneitis. As mentioned above, Büchi *et al.* also performed their measurements on the rabbit cornea but they have not reported any irritating property of the compound *. We also tested the butoxy compound in injection anesthesia on man, comparing it with xylocaine in subcutaneous wheals and in block anesthesia of the fingers.

For the tests in subcutaneous wheals, 1.5 % hydrochloride solutions of both compounds were compared. Each solution had a physiological NaCl content and an epinephrine concentration of 1:80 000; the pH value of the solutions was 4.5. On each of six persons, 1 ml of the two solutions was injected subcutaneously (dorsal side of the *brachium*). During injections, the compound of Büchi *et al.* caused marked pain. The average duration of the compound was 7.2 hr whereas xylocaine gave a corresponding value of 7.3 hr. The compound of Büchi *et al.* injured strongly the tissues in the centre of the infiltrated area and owing to this damage the centre was anesthesized for a long period, in some cases up to two weeks ("irreversibel" anesthesia).

For the experiments in block anesthesia of the fingers, a 1 % xylocaine · HCl solution was compared with a 1.5 % solution of the hydrochloride of the butoxy compound. (Both solutions had the same pH value, NaCl content, and epinephrine concentration as the solutions used for the subcutaneous wheals.) In each of four persons, 3 ml of the xylocaine solution were injected at the base of a third finger and the same operation was made with the butoxy compound on the other third finger. The xylocaine solution gave an average duration of 5.7 hr whereas the corresponding value of the solution of the butoxy compound was 5.6 hr. The latent period of the xylocaine solution was 3.8 minutes; the corresponding period for the other solution was 9 minutes. The extent of anesthesia obtained with the xylocaine solution was greater than that of the solution of the butoxy compound. Further it was observed that the compound of Büchi *et al.* injured the tissues, so that sore swellings persisted for several days.

In a later paper, Büchi[18] reports: "Die Verbindung übertrifft das Pantocain als Oberflächenanästheticum und das Novocain und das Xylocain als Infiltrationsanalgeticum".** He says nothing about the irritating properties of the compound. — To judge from our experiments on man, the compound of Büchi *et al.* is not superior to xylocaine in infiltration anesthesia. Furthermore, our experiments show definitely that the compound is useless as a clinical anesthetic.

Toxicity measurements, as carried out by subcutaneous injections in white mice, gave the same LD_{50} value of I and II, *viz.* 0.7 g/kg. The LD_{50} value of III was found to be 0.9 g/kg. The corresponding value for xylocaine is 0.34 g/kg.

* In their paper, Büchi *et al.* have graduated most of their compounds according to a certain scale of irritant power.

** In what way these measurements were carried out is not stated. — In the same paper, Büchi has misinterpreted Löfgren's discussions[5] on the relationsships between structure and local anesthetic action.

Compounds IV, V, and VI, belonging to the other group of compounds, gave relative activities 0.5 (approx.), 2.3, and 2.7, respectively. Compound VI showed a slight irritation of the cornea. The toxicity of the compounds was found to be low, the LD_{50} values (white mouse) for all three compounds being > 2 g/kg.

Compound V which has an activity 2.3 times that of xylocaine on the rabbit cornea was also tested subcutaneously on man and compared with xylocaine In this experiment the compound was found to be strongly inferior to xylocaine.

For the test on man, a 0.5 % hydrochloride solution with a physiological NaCl content and with an epinephrine concentration of 1:50 000, was prepared (pH of the solution = 5). Of this solution, four subcutaneous injections, each of one ml, were made on the volar side of the forearm. During injections strong pain was observed. The average duration of the anesthesia was 12 minutes. A solution of xylocaine · HCl as weak as 0.2 % tested under the same conditions, gave a duration of at least four hours.

The experiments with compound V show that a compound may have a higher activity than xylocaine on the rabbit cornea but a lower activity than xylocaine in subcutaneous anesthesia on man (*cf.* also the measurements on the compound of Büchi *et al.* as described above). We have often observed this discrepancy previously between the two methods *.

All six compounds were tested for their spasmolytic and histaminolytic power. No appreciable effects were found.

DESCRIPTION OF SYNTHESES **

ω-Diethylamino-4-hydroxy-2,6-dimethylacetanilide (I). 4-Hydroxy-2,6-dimethylaniline was obtained by nitrosating 3,5-dimethylphenol and subsequent reduction of the nitroso derivative with ammonium sulphide. As regards the details, the directions given by Kremers *et al.*[19] for the preparation of aminothymol were followed. The compound was recrystallized from water; colourless crystals melting at 179—182° (decomp.), in agreement with the recorded melting points [12,18]; yield 22 %, calculation based on xylenol.

For the preparation of ω-chloro-4-hydroxy-2,6-dimethylacetanilide, the following method was used: 35 g (0.26 mole) of 4-hydroxy-2,6-dimethylaniline were dissolved in 21 ml of 12.2 *N* hydrochloric acid and 120 ml of water. The solution was cooled to 17°. An acetate solution was prepared by dissolving 74 g of AcONa · $3H_2O$ in 120 ml of water, and cooled to 2°. To the solution of the aminophenol · HCl were first added 32 g (0.28 mole) of chloroacetyl chloride and then, without any delay, the acetate solution. The mixture was then immediately agitated by vigorous shaking which was carried on for five minutes, and was thereafter cooled in an ice-bath. The precipitate was filtered off, washed with ice-cold 1 *N* hydrochloric acid, then with water of the same temperature and finally dried in a desiccator over silica gel. The yield of the crude product was 38 g (0.18 mole), *i. e.* 69 %. The substance was purified by recrystallization from glacial acetic acid and colourless crystals were obtained.

From the chloro derivative, ω-diethylamino-4-hydroxy-2,6-dimethylacetanilide was prepared in the following manner. In a flask fitted with a reflux condenser, a mixture of 26 g (0.12 mole) of the purified 4-hydroxy-2,6-dimethylacetanilide, 20 g (0.27 mole) of diethylamine, and 200 ml of benzene was boiled for five hours. The precipitated diethylammonium chloride was filtered off from the hot solution by suction and then washed with a small quantity of hot benzene. The filtrate and the washings were combined. As the temperature dropped, the ω-diethylamino-4-hydroxy-2,6-dimethylacetanilide

* *Cf.* for instance Löfgren and Takman [7].

** All melting points and boiling points are uncorrected unless otherwise stated.

crystallized from the solution. The product was filtered from the cold solution by suction, dried, and washed with ice-cold water. The substance was then dried in a vacuum desiccator over silica gel and finally recrystallized from benzene with the addition of some methyl ethyl ketone. Colourless needles of m. p. 181—182° (corr.) were obtained.

Anal. Calcd. for $C_{14}H_{22}N_2O_2$ (250.3): C 67.2, H 8.86. Found: C 67.2, H 8.88.

Hydrochloride. When recrystallized from *n*-propanol with the addition of some methanol, the salt was obtained as colourless laths. It melted at 260—266° (corr.) under decomp.

Anal. Calcd. for $C_{14}H_{23}ClN_2O_2$ (286.8): C 58.6, H 8.08, N 9.77. Found: C 58.4, H 8.06, N 9.96.

ω-Diehylamino-4-methoxy-2,6-dimethylacetanilide (II). 4-Methoxy-2,6-dimethylazobenzene was prepared according to Saunders and Watson [14]. From this compound, 4-methoxy-2,6-dimethylaniline was obtained by reduction with sodium hydrosulphite in the following way: in a flask fitted with a reflux condenser, 45.0 g (0.185 mole) of 4-methoxy-2,6-dimethylazobenzene were dissolved in 900 ml of 50 % ethanol by heating to boiling, and then 120 g (0.55 mole) of sodium hydrosulphite were added portion by portion under continued boiling. When the red colour had disappeared most of the alcohol was removed by distillation under reduced pressure. The residue was made slightly alkaline and then extracted with ether. The ethereal solution was dried (Na_2SO_4), the ether driven off, and the residue was fractionated by distillation under reduced pressure. The 4-methoxy-2,6-dimethylaniline was collected as an almost colourless oil at 130—135°/15 mm. The oil soon solidified and recrystallization from light petroleum gave crystals melting at 42°, in agreement with the recorded values of the melting point [14,20]. (Owing to an accident we are unable to state the yield.)

The 4-methoxy-2,6-dimethylaniline was chloroacetylated, using Löfgren's acetate buffer method [1,5]. The yield of the crude ω-chloro-4-methoxy-2,6-dimethylacetanilide was 88 %. On recrystallization from toluene, colourless crystals were obtained; m. p. 179—180°.

Anal. Calcd. for $C_{11}H_{14}ClNO_2$ (227.7): C 58.0, H 6.20. Found: C 58.1, H 6.13.

The chloro derivative was then treated with diethylamine in benzene solution as described in the reaction between ω-chloro-4-hydroxy-2,6-dimethylacetanilide and diethylamine (see under the preparation of compound I). After having boiled the reaction mixture for four hours, the precipitated diethylammonium chloride was filtered off and washed with some hot benzene. The filtrate and the washings were combined and the benzene solution was extracted exhaustively with 3 *N* HCl. The aqueous solution was made alkaline and the base taken up in ether. The ethereal solution was dried (Na_2SO_4), the solvent removed, and the residue then fractionated by distillation under reduced pressure. The ω-diethylamino-4-methoxy-2,6-dimethylacetanilide was collected as a colourless oil boiling at 144—145°/0.02 mm; $n_D^{20} = 1.5257$; yield 57 %.

Anal. Calcd. for $C_{15}H_{24}N_2O_2$ (264.4): C 68.1, H 9.15. Found: C 68.6, H 9.13.

*ω-Diethylamino-4-*n*-butoxy-2,6-dimethylacetanilide* (II:a). This compound has earlier been prepared by Büchi *et al.*[17] (synthesis of 4-*n*-butoxy-2,6-dimethylaniline, chloroacetylation of this amine, and subsequent treatment of the chloro derivative with diethylamine). Büchi *et al.* describe the compound as being an oil at ordinary room temperature. We synthesized this compound following the directions given by Büchi *et al.* and found the compound to be a solid which could be recrystallized from petroleum ether.

The 4-*n*-butoxy-2,6-dimethylaniline, as obtained by us, had the same b. p. as that stated by Büchi *et al.* We also prepared the hydrochloride of this amine *; colourless small crystals from absolute ethanol with the addition of some absolute ether; m. p. 208—210° (decomp.).

Anal. Calcd. for $C_{12}H_{19}NO$ (229.75): C 62.7, H 8.77, N 6.10. Found: C 62.4, H 8.72, N 5.92.

For ω-chloro-4-*n*-butoxy-2,6-dimethylacetanilide we obtained the same m. p. (120—121°) as that given by Büchi *et al.*

The ω-diethylamino-4-*n*-butoxy-2,6-dimethylacetanilide (II:a) was collected as an oil boiling at 166—167°/0.03 mm (Büchi *et al.* state 158°/0.1 mm). The oil soon solidified and after recrystallization from light petroleum the compound showed a m. p. of 37—38°; $n_D^{20} = 1.5137$ (determined on the *supercooled* liquid compound).

* Not prepared by Büchi *et al.*

Anal. Calcd. for $C_{18}H_{30}N_2O_2$ (306.4): C 70.6, H 9.87, Equiv.w. 306. Found: C 70.4, H 9.80, Equiv.w. 305 *.

ω-Diethylamino-4-butyroxy-2,6-dimethylacetanilide hydrochloride (III). In a flask, fitted with a calcium chloride tube, a solution of 4.0 g (0.016 mole) of ω-diethylamino-4-hydroxy-2,6-dimethylacetanilide (compound I) in 50 ml of dry dioxan was mixed with 1.7 g (0.016 mole) of *n*-butyryl chloride under vigorous shaking. The mixture was allowed to stand at room temperature over-night. 150 ml of absolute ether were then added and after half an hour the hydrochloride of ω-diethylamino-4-butyroxy-2,6-dimethylacetanilide was filtered off; colourless crystals of m. p. 103—104°; yield 3.5 g (0.010 mole), *i. e.* 63 %.

Anal. Calcd. for $C_{18}H_{29}ClN_2O_2$ (356.9): C 60.6, H 8.19. Found: C 60.3, H 8.11.

ω-(N-Ethyl-carbethoxymethylamino)-2,6-dimethylacetanilide (IV). ω-Ethylamino-2,6-dimethylacetanilide was prepared as is described by Löfgren and Widmark [4]. To a solution of 10.5 g (0.051 mole) of this compound in 50 ml of dry benzene were added 4.3 g (0.026 mole) of ethyl bromoacetate under vigorous stirring. The mixture was then heated for 48 hours at 50° in a sealed bottle. The separated hydrobromide of ω-ethylamino-2,6-dimethylacetanilide was filtered off and washed with some hot benzene. From the combined filtrate and washings the solvent was driven off. The residue, a viscous oil, on distillation gave an almost colourless oil; b. p. 180—181°/0.5 mm; $n_D^{20} = 1.5162$; yield 4.0 g (0.014 mole), *i. e.* 54 %.

Anal. Calcd. for $C_{16}H_{24}N_2O_3$ (292.4): C 65.7, H 8.27. Found: C 66.0, H 8.42.

*ω-(N-Ethyl-carbo-*n*-propoxymethylamino)-2,6-dimethylacetanilide* (V). This compound was synthesized by the same method as described above for the preparation of compound IV, with the only difference that *n*-propyl bromoacetate was used instead of ethyl bromoacetate. — A viscous oil was obtained; b. p. 155—156°/0.05 mm; $n_D^{20} = 1.5109$; yield 94 %.

Anal. Calcd. for $C_{17}H_{26}N_2O_3$ (306.4): C 66.6, H 8.55, Equiv.w. 306. Found: C 66.8 H 8.52, Equiv.w. 307 **.

Hydrochloride. Colourless small hygroscopic crystals from dioxan; m. p. 151—152°

Anal. Calcd. for $C_{17}H_{27}ClN_2O_3$ (342.9): Cl 10.34. Found: Cl 10.31 (Mohr).

*ω-(N-Ethyl-carbo-*n*-butoxymethylamino)-2,6-dimethylacetanilide* (VI). The compound was synthesized from *n*-butyl bromoacetate and ω-ethylamino-2,6-dimethylacetanilide by the method described above for the preparation of compound IV. — A colourless viscous oil was obtained; b. p. 151—152°/0.025 mm; $n_D^{20} = 1.5081$; yield 83 %.

Anal. Calcd. for $C_{18}H_{28}N_2O_3$ (320.4): C 67.5, H 8.81, Equiv.w. 320. Found: C 67.4, H 8.90, Equiv.w. 321 ***.

Hydrochloride. Colourless small crystals from dioxan; m. p. 133—134°.

Anal. Calcd. for $C_{18}H_{29}ClN_2O_3$ (356.9): Cl 9.94. Found: Cl 10.01 (Mohr).

SUMMARY

Six new compounds, related to xylocaine, have been synthesized and tested for their local anesthetic, spasmolytic, and histaminolytic action.

We are indebted to AB Astra, Södertälje, Sweden, for financial support and to Dr. S. Wiedling of the Biological Department of Astra for performing the pharmacological tests. Our sincere thanks are also due to Professor A. Girardet of the University of Lausanne who read through the manuscript.

* Titration of the base in 30 % ethanol with 0.1 *N* HCl; mixed indicator methylene blue-methyl red.

** Titration with perchloric acid in glacial acetic acid; indicator crystal violet.

*** The same analytical method as described under compound V.

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Purification of Bacitracin Polypeptides by Charcoal Chromatography and Zone Electrophoresis

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Experimental conditions for the fractionation of polypeptides by charcoal chromatography are discussed. By combining charcoal chromatography and zone electrophoresis in cellulose columns an efficient fractionation of a commercial crude bacitracin preparation was achieved. Peptides, nucleotide(s) and amino acids were resolved as different fractions. Two new peptides similar to bacitracin A, called bacitracin H and I, were isolated. The ultraviolet absorption curves of the peptides are compared with those of fractions reported in the literature which were obtained by counter-current distribution. A description of a simple apparatus for zone electrophoresis in vertical columns is also given.

Efficient purification of bacitracins, antibiotic peptides obtained from *Bacillus licheniformis* has been achieved by Craig and Newton and their collaborators[1-4]. Both groups of investigators use only counter-current extraction. In spite of the inherent flexibility of this method, a complete resolution of such complicated mixtures often requires the application of complementary procedures which depend on different physical or chemical properties. Since the mixture is composed of closely related peptides the advantage of using independent methods is evident. UV-absorption curves and other data of characterization, published by the above-mentioned authors was considered to make bacitracins particularly favorable objects for comparative studies.

In a previous report, a bacitracin preparation (purified by counter-current extraction) was shown to contain considerable amounts of impurities[5]. The method used (carrier displacement analysis on charcoal) was, however, inconvenient for purification of crude polypeptide mixtures where the peptide to be purified is not the main component. Moreover, under the conditions used, the yield of activity and material was not entirely satisfactory. As was recently shown in the cases of insulin and ACTH-polypeptides, severe losses may be avoided by the use of properly pretreated charcoal[6].

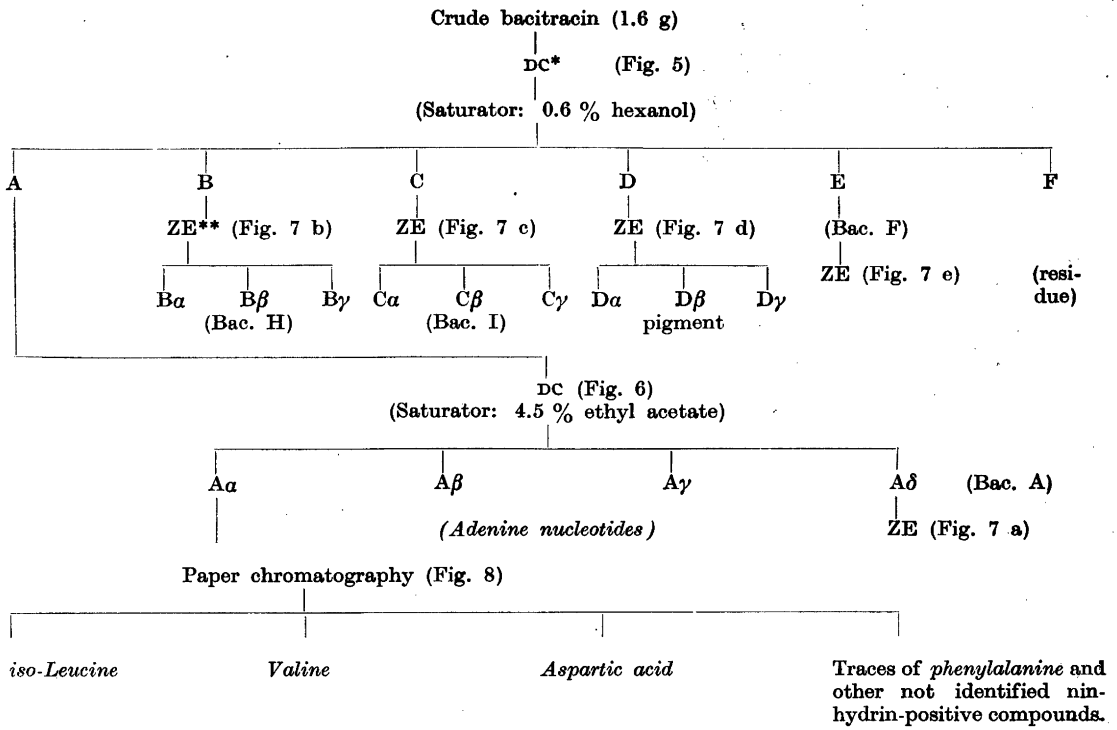
The pretreatment of the charcoal consists of deactivation of the most active adsorption centers by means of a saturator as was suggested by Tiselius and

co-workers [7,8]. The saturator may be incorporated in the eluting or displacing solvents (flowing saturator). Pretreated charcoal should be used, for otherwise extensive irreversible adsorption may occur. *Poor recovery always results from strong adsorption*, even if a very strong displacer is used to effect the desorption. On the other hand, if charcoal is pretreated with a large quantity of a strong saturator, adsorption to a significant extent may not occur. Somewhere between these extremes, a concentration range of the saturator can presumably be found where the peptide will be only weakly adsorbed. Within, or above this concentration range, chromatography must be performed to secure a high yield of the peptide in question. Thus, suitable working conditions can be found if the recovery of the peptide is determined in a series of displacement analysis experiments using varying saturator concentrations and keeping other factors constant. When this is done it is found, as could be anticipated, that the recovery increases as the concentration of the saturator increases. At a certain concentration of the pretreating agent the peptide appears in the eluate with only slight retention or without any adsorption at all occurring. The saturator concentration at which this takes place has been called the critical saturation point of the peptide. This concentration is dependent upon a number of factors which may all be easily controlled.

In view of these facts the first full scale chromatogram should be performed with charcoal at a saturation close to the critical saturation point of the component having the strongest adsorbability. The latter can be recovered by displacement, while the other components will be found in the eluate. By repeating this procedure an appropriate number of times using charcoal at decreasing saturations, a not too complex mixture may be resolved.

Desorption can be effected either by displacement (as assumed in the preceding discussion) or by gradient elution. Displacement analysis has the advantage of being easily reproducible and thus more suitable for group separations. The proper conditions can be determined with a few milligrams and applied on a gram scale, as is shown in this paper. According to Drake it is usually not possible to attain better separation by gradient elution [9]. Displacement analysis is therefore preferable in preparative charcoal chromatography.

Instead of attempting a complete separation of a complex mixture such as the crude bacitracin using charcoal chromatography alone it appears to be more efficient to use this procedure only for a group separation. When necessary, other methods can be explored for further resolution. In this work zone electrophoresis was chosen as a supplementary procedure because the electrophoretical separation presumably depends on entirely different properties of the material to be resolved. Unfortunately, only a narrow pH range of buffers could be employed, for bacitracin A is unstable and bacitracin F has a low solubility at higher pH values. Acetate buffer of pH 4.5 was used throughout this investigation. Zone electrophoresis of amounts less than 1/2 gram may easily be performed in throughs or in columns. Vertical columns were preferred because better defined zones yielding more satisfactory separations could be obtained. The peptide material was easily freed from buffer salts by charcoal chromatography.



* DC = Displacement chromatography on charcoal.
** ZE = Zone electrophoresis in acetate buffer (pH 4.5; μ 0.05)

The investigation was started with a frontal analysis. This gave useful information about the complexity of the crude bacitracin sample. The fractionation on a preparative scale was then made by adsorption analysis on charcoal and zone electrophoresis, as summarized in the fractionation scheme above.

EXPERIMENTAL TECHNIQUE

Charcoal chromatography

Apparatus. Sectioned columns were used. These were of the type developed by Hagdahl[10] but plastic-lined as described by Li and the author[6]. It is desirable to avoid contact of the solvent with metals, for traces of certain metal ions inactivate bacitracin [4].

Solvents. All solvents were made 0.1 *N* with acetic acid, because bacitracin A is reasonably stable in slightly acid solutions. Hexanol or ethyl acetate were found to be excellent saturators and the latter was also used as a displacer. These substances are easily removed before lyophilization by extraction with pentane or peroxide-free ether.

Adsorbents. Carbo Active (Merck, Darmstadt) was used in all experiments. Prior to use it was washed with 20 % acetic acid. The charcoal was mixed with three times its weight of Hyflo Supercel or Celite. The columns were packed with this mixture when unsaturated charcoal or charcoal saturated with a solution of ethyl acetate was to be used. Saturation of the charcoal with hexanol could be carried out on the column, but

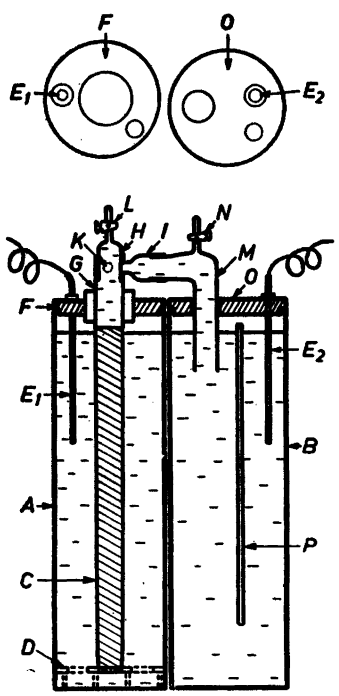


Fig. 1. Schematical drawing of the zone-electrophoresis assembly with the two cylinder lids. Explanation of the letters in the text.

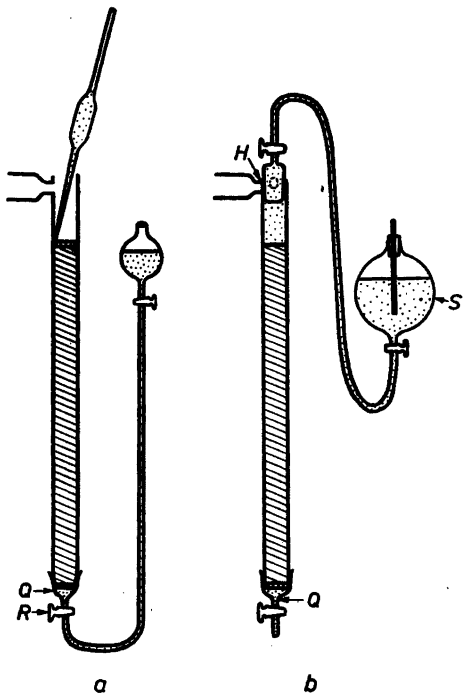


Fig. 2. a. Arrangement for application of the solution to be analyzed. b. Arrangement for displacement of the zones after the run.

in order to save time a combination of batchwise and column procedures was preferred. The charcoal-filter aid mixture (200 g) was suspended in 1 000 ml 0.6 % hexanol solution and stirred for 1/2 hour. The suspension was allowed to settle and the supernatant decanted and discarded. This treatment was repeated twice. To ensure satisfactory saturation the column of packed charcoal was always washed with the saturator solvent (at least three times the dead volume) before use. When ethyl acetate was used as a saturator, the column of untreated charcoal was saturated by washing with ethyl acetate solution until the ester appeared in the eluate.

Oxidation has never been found to occur on strongly pretreated charcoal.

Zone electrophoresis

Apparatus. A column (*C* in Fig. 1), similar to that described by Haglund and Tiselius [12] and Flodin and Porath [13], was used. It is made of glass — or quartz, if UV-absorption is to be recorded before elution. The solution is slowly forced into the column by about 5 cm water pressure (Fig. 2 a). The rinsing with buffer is also done cautiously. The zone is removed from the upper surface by lowering the level in the container. The flow should not exceed 10 ml per hour. When the zone is in the desired position the stop-cock *R* is closed and the column filled with buffer solution to the ground joint at the top. A connect-

ing tube M, bent as a knee, and a top piece H with a narrow tip and a stopcock L, are attached to the column. The ground joint of the top piece has an opening K, which is turned to coincide with the opening at the side arm I. The stop-cock L is opened and M turned upwards. Buffer solution is poured into M until the level reaches L. The stop-cock L is now closed H turned, M emptied and turned downwards. The adapter Q at the lower end of the electrophoresis column can then be detached without allowing the buffer solution to flow through the column. The column and the connecting tube can now be placed in the electrode vessels.

Two glass cylinders A and B, each containing 7—8 liters of buffer solution, serve as electrode vessels (Fig. 1). Platinum wires twisted around rods of plastic are used as electrodes. The column C is placed in the center of A. The lower end of the column is supported by a plastic disc of the same diameter as that of the cylinder A. The disc, which rests on 3 cm high rods of the same material, has a large hole in the center. This fits to the lower end of the column while other large holes allow passage of the current. The upper end of C is kept in position by a stopper G in the center of a plastic lid F on the cylinder A. The lid O of the cylinder B has an opening for the tube M. Electrode rods E_1 and E_2 are inserted in the lids which also have openings for the gases formed by the electrode reactions. The electrode vessel B also has a loose-fitting dividing wall of plastic P, to prevent electrode products from reaching M.

Liquid connection is simply effected by suction at the tip above N, subsequent closing of N when M is filled, and turning K to open position. A siphon is placed between the two electrode vessels through the holes for gas outlet in the lids F and O. When the buffer solutions in the two vessles are at the same level the siphon is removed and the electrodes connected to a power supply.

When a run is finished, K is closed and the column and connecting tube M are lifted off the electrode vessels. The tube M is removed and the adapter Q attached to the column. This is placed above a fraction collector and the flow rate is controlled by a siphon and a Mariotte flask S as shown in Fig. 2 b.

The apparatus has the advantage of being easily manipulated. The large volume of buffer solution makes it possible to perform runs of long duration or several short runs without changing the buffer.

No extra cooling device is necessary especially not if the run is performed in a cold room.

General analytical methods

The material distribution was determined by the ninhydrin reaction and UV-absorption. The former was performed according to the description of Moore and Stein [14], the latter by aid of a Beckman quartz-spectrophotometer model DU. The entire samples or suitable aliquots were taken for analysis.

PRELIMINARY EXPERIMENTS

Starting material. The main experiments and most of the other experiments were performed with a preparation (Bacitracin 50R 5 986), kindly supplied by Merck and Co., Inc., Rahway, N.J., U.S.A. It originated from Commercial Solvents Corporation (Lot No. B. 50 070 605). At the time of this investigation the activity was found to be about 35 units/mg. The dry preparation was faintly yellowish while concentrated solutions were of a dark-brown color.

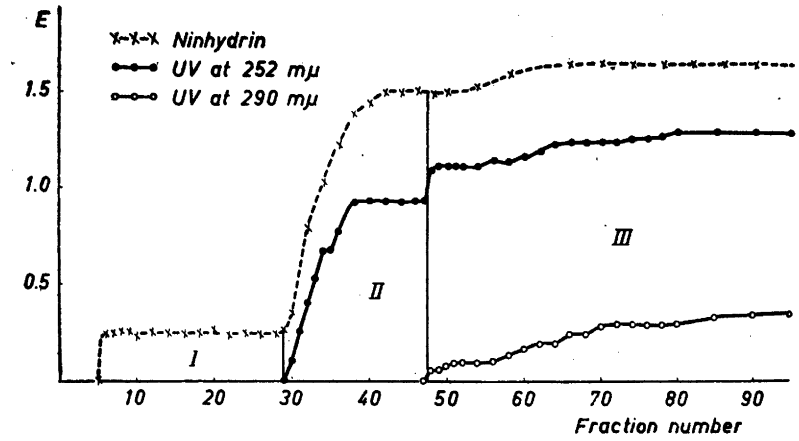


Fig. 3. Frontal analysis of crude bacitracin.

Crude bacitracin shows a very strong absorption in the ultraviolet region below 300 mμ. Craig and Newton and their collaborators have found one (or possibly several) of the bacitracin polypeptides, bacitracin F, to have an absorption maximum at 290 mμ, while the most active component, bacitracin A, exhibits a slight maximum at about 252 mμ.

Frontal analysis of crude bacitracin. For orientation, a frontal analysis was carried out. The column consisted of three sections, 3.1, 1.6, and 0.8 ml, filled with a suspension of a mixture of charcoal and filter aid (proportions 1:3) in 0.1 *N* acetic acid. A solution of 0.15 % crude bacitracin in 0.1 *N* acetic acid was pressed into the column at a rate of 6 ml per hour. Fractions of 1.7 ml were collected and measured spectrophotometrically at 252 and 290 mμ after dilution to double the volume. For ninhydrin determinations 0.5 ml of each fraction was taken.

The diagram (Fig. 3) reveals a variety of compounds of very different absorption and adsorption characteristics. Three regions can be distinguished. Region I, consisting of the least adsorbable material, gives a strong ninhydrin color but no significant absorption at the wavelength measured. No antibiotic activity with the *Corynebacterium xerosis* test was found in this region. Paper chromatography of the material in this fraction gave diagrams identical with those of Aα (see Fig. 8 and p. 1821). It consists chiefly of amino acids. In Region II a rise in the absorption at 252 mμ appears in fraction 30. That the additional material emerging in this region is heterogeneous can be seen from the change in the intensity of the ninhydrin color/UV-absorption ratio of fractions 30 to 42. The first step of the 252 mμ-curve obtained in several experiments, points to the same conclusion. The biological activity roughly parallels the UV-curve. In tube No. 48 another rise in the 252 mμ curve occurs and at the same time material absorbing at 290 mμ emerges from the column. All the curves show a small increase in absorption in Region III. This strongly indicates the appearance of several other compounds. The breakthrough of a yellow pigment is also observed in this region.

The results of the frontal analysis suggest excellent possibilities for an efficient fractionation by charcoal chromatography.

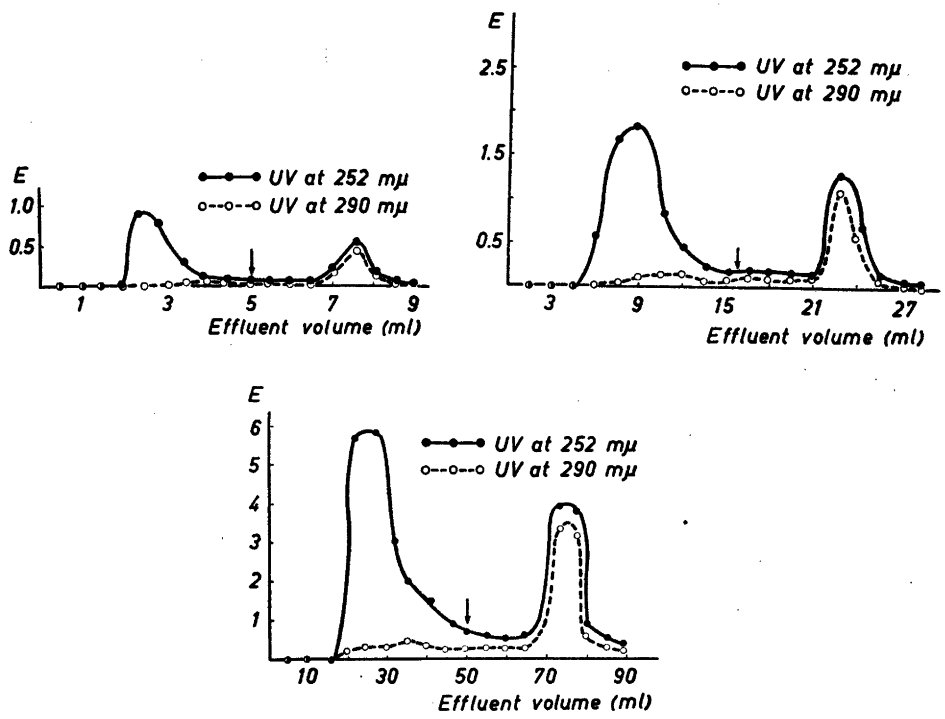


Fig. 4. Displacement chromatograms of crude bacitracin on pretreated charcoal columns. Saturated ethyl-acetate was introduced at the top of the column at effluent volumes indicated by the arrows. a. A 5 mg sample applied to a column of 1.6 ml volume. b. A 25 mg sample applied to a 4.7 ml column. c. A 125 mg sample applied to a 18.8 ml column.

Displacement analysis of crude bacitracin. Frontal analysis of crude material revealed that the yellow pigment and the material absorbing strongly at 290 mμ were the components most strongly adsorbed on charcoal. Thus, the charcoal must be pretreated with a saturator strong enough to prevent extensive irreversible adsorption of material absorbing at 290 mμ. Preliminary displacement analysis experiments were carried out to determine suitable working conditions.

In the first series the column volume (1.6 ml), the charge (5 mg) and the flow rate (2 ml per hour) were most the same, while the concentration of the flowing saturator (*n*-hexanol in 0.1 *N* acetic acid) was varied. The hexanol-acetic acid solvent saturated with ethyl acetate was used as a displacer. A 0.6 % hexanol solution was chosen for further investigation, because in this case a complete recovery within experimental error, was achieved. Fractionation was also satisfactory (Fig. 4 a).

In a new series, the charge was raised to 25 mg and the flow rate to 5 ml per hour. The amount of adsorbent was varied. The use of a 4.7 ml column volume resulted in about 95 % recovery of material absorbing at 290 mμ (Fig. 4 b).

Satisfactory fractionation and recovery were obtained when 125 mg of material were placed on a column of 18.8 ml volume (Fig. 4 c). In this case as in all large scale experiments, the flow rate was 20 ml per hour. Approximately the same charge/column volume ratio was used when gram quantities were fractionated.

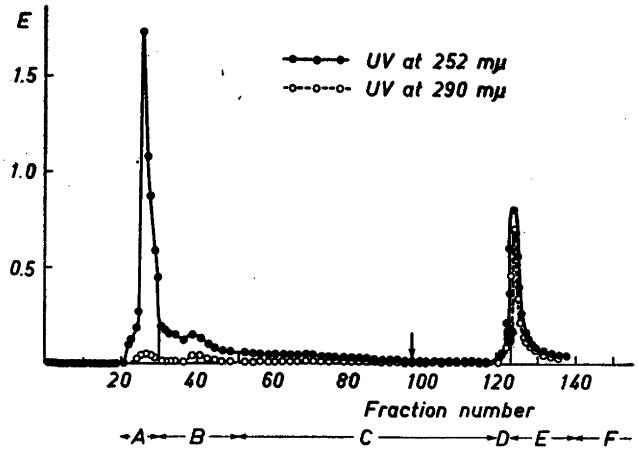


Fig. 5. Diagram showing the fractionation of 1.6 g crude bacitracin on a column made of seven 31.4 ml sections. The small fractions were pooled to the larger Fractions A—F as indicated. The arrow has the same meaning as in Fig. 4.

PREPARATIVE FRACTIONATIONS

Charcoal chromatography

Starting material: See "Preliminary Experiments".

Displacement chromatography of crude bacitracin. A column of seven 31.4 ml sections filled with charcoal saturated with 0.6 % hexanol in 0.1 *N* acetic acid was loaded with 1.6 g crude bacitracin dissolved in 4 ml of the same solvent. The flow rate was adjusted to 20 ml per hour by applying a pressure of about 3 kg per cm^2. Fractions were collected in a time-controlled fraction collector (10 min. per fraction). When 400 ml solvent had passed through the column the elution was stopped and the displacement was started, keeping the flow at the same rate as before. The distribution of material with respect to UV-absorption at 252 and 290 mμ was determined and the small fractions were pooled into six large fractions, as indicated in the diagram (Fig. 5). The solutions were extracted with peroxide-free ether to remove hexanol and ethyl acetate. After evaporation of the excess ether the solutions were lyophilized.

All the freeze-dried samples were non-hygroscopic powders. Fraction D, which contained the pigment, was yellowish; the others were white. Fraction A possessed the bulk of the activity, while Fraction F was least potent. The material was distributed as follows: Fraction A, 741 mg; B, 112 mg; C, 129 mg; D, 117 mg; E, 325 mg; F, 36 mg. Thus 92 % was recovered on a weight basis.

Displacement chromatography of Fraction A. Some of the material of Fraction A is strongly adsorbed on untreated charcoal. Since the material of Fraction A could pass the column in the experiment described above without being adsorbed a suitable saturator concentration of hexanol could presumably be found. However, ethyl acetate was used instead, because much experience had been gained from other preliminary experiments using this substance as a saturator. Acceptable yields and fractionations were obtained with 4—4.5 % ethyl acetate. Large scale experiments were performed as follows.

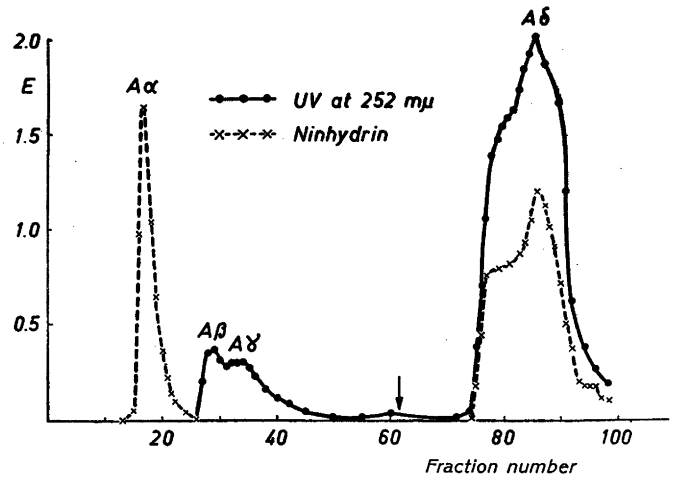


Fig. 6. Displacement analysis diagram of 512 mg of Fraction A (see Fig. 5) obtained from a chromatographic run on charcoal pretreated with 4.5 % ethyl-acetate. A column of four 31.4 ml sections was used. Displacement was started when 300 ml of eluent had passed the column.

A solution of Fraction A containing 0.6 % hexanol was freed from the latter by extraction with pentane and evaporation of the excess hydrocarbon *in vacuo*. The hexanol-free solution so obtained (15 ml), containing 512 mg of solid, was pressed into a column of four 31.4 ml sections containing the adsorbent in equilibrium with 4.5 % ethyl acetate in 0.1 *N* acetic acid. Elution was effected with 4.5 % ethyl acetate. When 300 ml had passed the column the displacement with saturated ethyl acetate was started. Fractions of 5 ml volume were collected. The material containing fractions were pooled, the ethyl acetate removed and the solutions lyophilized.

The elution peak Aα (Fig. 6) contained 13 mg of ninhydrin reactive material which showed very slight absorption at 252 mμ. The material in this fraction is, of course, the same as that in the first step of the frontal analysis chromatogram (Region I). From Aβ and Aγ only about 2 mg were recovered, in spite of the strong absorption. From Aδ 442 mg were recovered.

Zone electrophoresis of various fractions

Fraction Aδ. The column (45 × 3.5 cm) was filled with a suspension of cellulose powder in sodium acetate buffer of pH 4.5 and μ 0.05. The packing was made with a hydrostatic pressure of about two meters of water [15]. Forty-five milligrams of material were dissolved in 1 ml of buffer solution and pressed into the column [13]. The zone was first displaced to 8 cm below the surface of the column using 50 ml of buffer solution. A current of 25 mA was passed through the column for 55 hours (400 V between the electrodes). After disconnecting the tube, the buffer containing the peptide material was displaced from the column at a rate of 25 ml per hour and collected in 5 ml portions.

Under these conditions (Fig. 7 a) Fraction A δ appears to be electrophoretically homogeneous. Paper electrophoresis at pH 5.3 and 6.0 give a single spot.

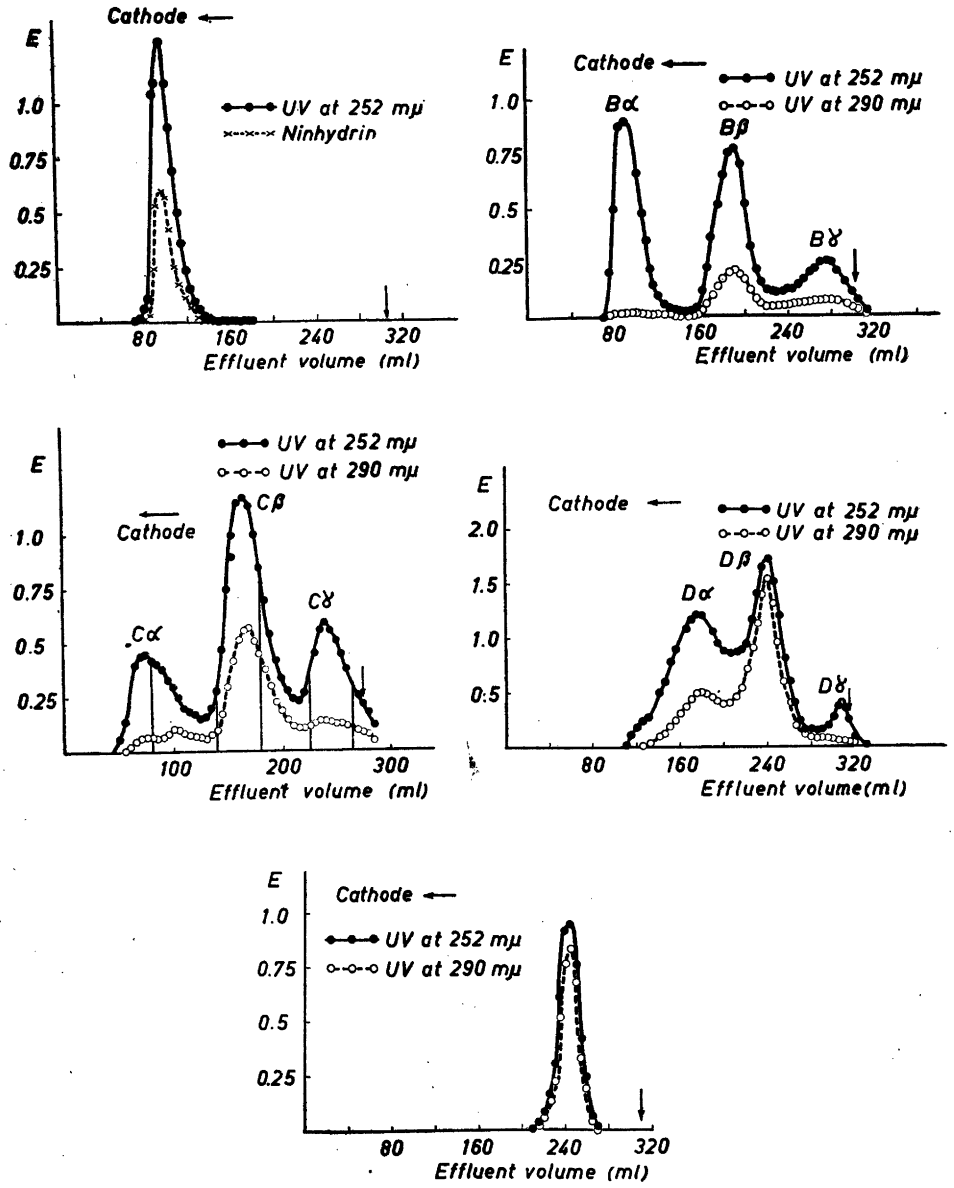


Fig. 7. Zone electrophoresis of fractions obtained from charcoal chromatograms (see Figs. 5 and 6). a. Fraction Aδ. b. Fraction B. c. Fraction C. d. Fraction D. e. Fraction E. The arrows indicate the volume in the column between the filter plate and the starting position of the original zone. All runs were carried out with acetate buffer (pH 4.5; μ 0.05). For details see text.

Other experiments, performed in a similar way, are compiled in Table 1.

Table 1.

Fraction	Amount applied (mg)	Column (length × diameter) (cm)	Distance of starting zone from the upper surface (cm)	Current (mA)	Duration (hours)	Diagram in Fig.
B	50	45 × 3.5	6	28	60	7 b
C	80	40 × 3.5	5	20	70	7 c
D	50	45 × 3.5	8	28	60	7 d
E	50	45 × 3.5	6	28	60	7 e

DISCUSSION

The completely inactive Fraction Aα (Fig. 5) was further analysed by paper chromatography. Hydrolysis changes the chromatogram only slightly with respect to ninhydrin-positive substances (Fig. 8). The strongest spot appears in the same region as leucine and *iso*leucine while a weaker spot occupies the position of valine. Besides these amino acids, aspartic acid and traces of phenylalanine, as well as a number of other unidentified ninhydrin-positive compounds are present. The large quantity of *iso*leucine is noteworthy for *iso*leucine has been reported as a DNP-derivative in the hydrolysate of dinitrofluorobenzene-treated bacitracin A[16]. All commercial bacitracin samples

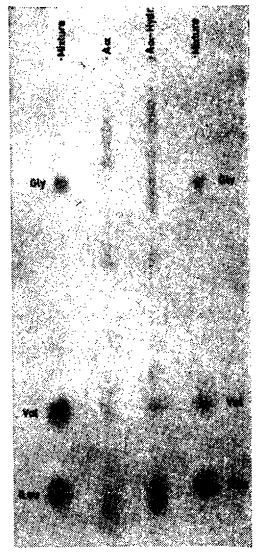


Fig. 8. Paper chromatograms of unhydrolyzed and hydrolyzed fraction Aa, and mixtures of glycine, valine and i s o leucine. Solvent system: butanol-acetic acid-water (4 : 1 : 5).

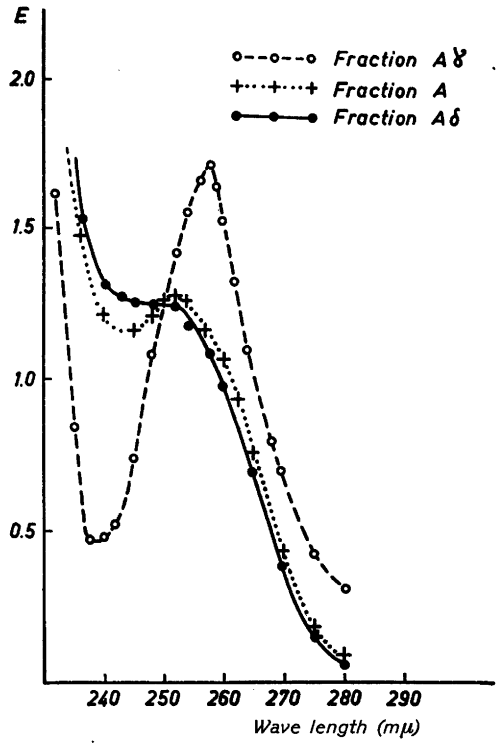


Fig. 9. Absorption curves of Fraction Aγ (adenine-nucleotide?). Fraction Aδ (bacitracin A) and Fraction A (bacitracin A contaminated by Aβ and Aγ). Ordinate: Optical density for 0.1 % solutions in dilute acetic acid (pH 3.0).

analyzed by the present author were contaminated with the same amino acids, although in different amounts. These amino acids have not been accounted for in fractionations, reported in literature, where counter-current distribution was used[1-4]. The yield of Aα material in a number of crude bacitracin samples varies between 1 and 3 %. The small amount of DNP-*iso*-leucine found in bacitracin A purified with counter-current distribution may possibly originate not from bacitracin A itself but from small amounts of contaminating free *iso*leucine [2]. Under the conditions used here zone electrophoresis does not remove *iso*leucine from bacitracin A (Fraction A). Since bacitracin A is fairly unstable, it might be thought that some chemical reaction which releases free *iso*leucine occurs on the charcoal. Rechromatographing Fraction Aδ on a charcoal column under the same conditions used for the purification proved that bacitracin A does not change its properties on the column. Only a single displacement peak was obtained and paper chromatography definitively proved the absence of any free amino acid. The difficulties in the separation are probably to be explained by an association of the free amino acids with the peptides.

Adsorption chromatography appears to be a powerful method of splitting such complexes.

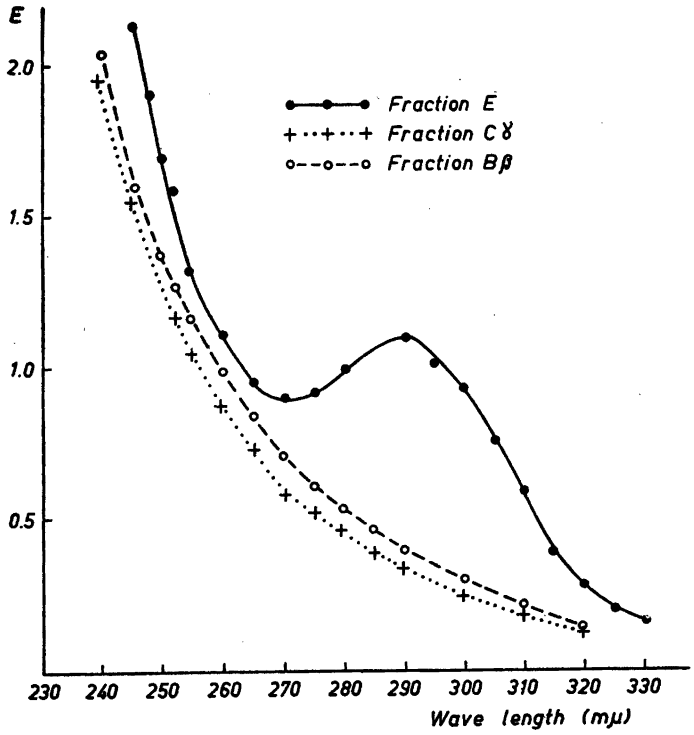


Fig. 10. Absorption curves of Fraction Bβ (bacitracin H) and Fraction Cγ (bacitracin I) and Fraction E (bacitracin F). Ordinate: Optical density for 0.1 % solutions in dilute acetic acid (pH 3.0).

The fractions Aβ and Aγ show ultraviolet absorption curves in solutions of different pH which are similar to adenine and adenine nucleotides (Fig. 9). Due to lack of material a closer examination of these fractions could not be undertaken. Chromatography of Aβ and Aγ after heating to 120° C with 6 *N* hydrochloric acid in sealed tubes for 20 hours did not reveal any significant amount of ninhydrin-positive substances. This indicates that adenine, if present, is in a bound state. Fraction Aγ mixed with Aδ in certain proportions give absorption curves similar to those of bacitracins C and G.[4] It is therefore questionable whether the latter compounds are pure bacitracin peptides.

The form of the peak Aδ might give the impression of heterogeneity. However, the material in different parts of the peak gives identical absorption spectra (that of Aδ in Fig. 9). The antibacterial activity is also uniformly distributed. Most likely the asymmetry is caused by non-equilibrium conditions during the displacement. The high potency (76 u/mg) and chemical properties of fraction A proves that it consists of bacitracin A. The absorption curve of Fraction A is very similar to the bacitracin B curve of Craig *et al.* and has a slight maximum at 252 mμ. After removing the Aβ and Aγ substances the curve becomes similar to that of bacitracin A. The differences in absorption

curves of the bacitracins A and B found by Craig and his collaborators[1] may be explained by traces of contaminating adenine-compounds.

The Fractions B, C, and D contain a number of different components, two of which, Bβ and Cγ, have been more closely examined. With the exception of Bα, the other fractions are certainly not pure. Bα consists of bacitracin A. Fractions Bβ and Cγ consist of peptides structurally related to bacitracin A as will be shown in a forthcoming paper. The absorption curves (Fig. 10) differ from those of the reported bacitracins. Since these fractions cannot be further purified by these methods the peptides in Fractions Bβ and Cγ will be referred to as bacitracin H and bacitracin I, respectively.

Fraction E seems to be electrophoretically homogeneous. The substance in this fraction has an absorption curve similar to or identical with that reported for bacitracin F.

From this investigation, it appears that fractionation of bacitracin polypeptide mixtures by zone electrophoresis and charcoal chromatography are capable of considerable further purification than is readily effected using counter-current distribution alone. It should be borne in mind that extremely small amounts of purine material would considerably change the absorption curve in the range 250—260 mμ. The bacitracins reported in the literature may, therefore, be pure enough for further chemical investigations.

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Added in proof: Recent studies by the present author on the structure of bacitracin A and F as obtained by the procedures described here are in agreement with the amino acid sequence published by Craig, Hausmann and Weisiger (*J. Am. Chem. Soc.* **76** (1954) 2839) and that found by Lockhart and Abraham (personal communication). There is thus no reason to believe that the compounds here isolated are different from those described by these authors.

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ACTA CHEMICA SCANDINAVICA 8 (1954) 1827—1829

Constitution of Resin Phenols and their Biogenetic Relations. XVIII *. Conversion of α-Conidendrin to Galbulin

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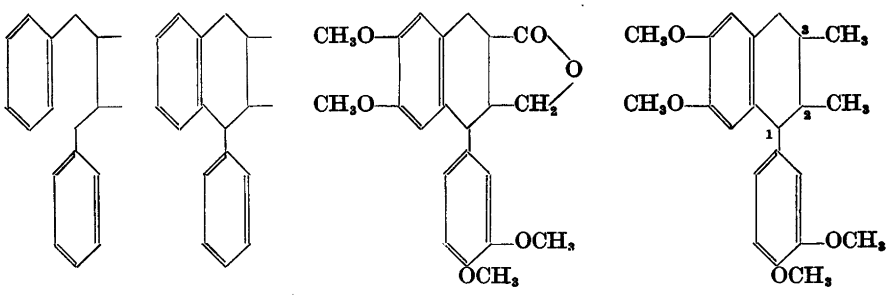
The dimethylethers of α- and β-conidendrin (III) have been converted to the corresponding fully reduced compounds (IV) which differ according to the configuration at carbon atom 3. The reduction product from the α-isomer is identical with galbulin — a new lignan isolated from *Himantandra baccata* Bail.

The lignans are dimerides of phenylpropanes characterized by the closely related carbon skeletons (I) or (II).



I II III IV; V All rings aromatic

The nonaromatic portion of the molecules frequently contains hydroxyl, ether, aldehyde or carboxylic (lactone) groups. The structure elucidation of the lignans has mainly been accomplished by means of oxidative degradations. Several interesting stereochemical relations between the various lignans have also been demonstrated. It is sometimes a matter of some difficulty to ascertain

*) Part XVII *Acta Chem. Scand.* 8 (1954) 806.

whether a natural product belongs to the lignan series or not. A simple method would be the reductive conversion to fully reduced compounds of the *d*- and *l*-dihydroguaiaretic acid and meso-dihydroguaiaretic acid types or to the corresponding cyclised analogues. It would therefore be of value to possess a series of such compounds for reference purposes.

In the present paper the reduction of the α- and β-conidendrin dimethylethers to compounds of the structure (IV) is reported. The work was initiated due to the paper chromatographic observation that guaiacum resin apparently contains a series of unknown compounds some of which may well be lignans. The facile conversion of guaiaretic acid dimethylether into dehydroguaiaretic acid dimethylether[1—2] and the recent isolation in these laboratories of dehydroguaiaretic acid from the products obtained by dehydrogenation of *iso*eugenol[3] would make the possible occurrence of phenylnaphthaline or phenyltetraline derivatives in guaiacum resin an attractive hypothesis.

The dimethylethers of α- and β-conidendrin (III) were reduced with lithium aluminium hydride to the previously-described, corresponding diols[4—5]. After esterification with *p*-toluenesulphonyl chloride the diol esters were subjected to another reduction with the same reagent[6] which resulted in formation of the desired compounds (IV). Since no optical inversion is likely to occur during these processes the fully reduced compounds retain the configuration of the original lactones — α- and β-conidendrin dimethylether — and hence differ only in respect to the configuration at carbon atom 3 (IV). Their structure was confirmed by catalytic dehydrogenation to dehydroguaiaretic acid dimethylether (V).

When this work was in progress we learned by the good services of Professor Birch (Sydney, Australia) that Hughes and Ritchie had isolated four new lignans from *Himantandra* species, one of which, galbulin, possesses the structure (IV). Their work has now been published[7]. Our compound from α-conidendrin was found to be identical with galbulin by mixed melting point and optical rotation. This constitutes a confirmation of the structure proposed by these authors and moreover connects galbulin stereochemically with α-conidendrin.

Quite recently we learned from Dr. Schrecker, National Cancer Institute, Bethesda, USA, that he has also converted α-conidendrin to galbulin[8].

EXPERIMENTAL *

p-Toluenesulphonates. 1. The diol (2 g) from α-conidendrin dimethylether was dissolved in dry pyridine (30 ml) and *p*-toluene-sulphonylchloride (6 g) added in one lot at a temperature of 5—10° and the mixture then allowed to attain room temperature slowly. Two hours after mixing, water (1.5 ml) was added dropwise with cooling and half an hour later the mixture was poured on to ice and hydrochloric acid (conc. 35 ml). The precipitated ester was collected, washed and dried in a vacuum desiccator, yield 2.8 g. Crystallization from ethanol gave a product melting at 158.5—159.5°, $[\alpha]_D^{20}$ +4.1° (*c* 1.95 in chloroform). (Found: OCH_3 17.2. Calc. for $C_{36}H_{40}O_{10}S_2$ (696.8): OCH_3 17.8).

2. The diol (2 g) from β-conidendrin dimethylether was treated similarly to the α-product above. Yield of dried crude β-ester 2.9 g. After several crystallizations from

*) All melting points uncorrected.

methanol it melted at 117.5—118.5°, $[\alpha]_D^{20}$ +53.8°. (*c* 1.80 in chloroform). (Found: OCH_3 17.5. Calc. for $C_{36}H_{40}O_{10}S_2$ (696.8): OCH_3 17.8).

Reduction of the p-toluenesulphonates. 1. A solution of the α-tosylester (1 g) in absolute benzene (20 ml) was added to a slurry of lithium aluminium hydride (1 g) in absolute ether (20 ml) and the mixture boiled for 3 h. Excess of the hydride was destroyed with ethyl-acetate, hydrochloric acid added and the product extracted with ether. The extract was then washed with sodium carbonate solution and water, dried over sodium sulphate and evaporated. The residue (0.45 g) was crystallized from methanol. M. p. 129.5—130.5°, $[\alpha]_D^{20}$ −7.6° (*c* 2.6 in chloroform). For analysis the substance was distilled *in vacuo*. (Found: C 73.94; H 8.01; OCH_3 34.12; C—CH_3 5.15. Calc. for $C_{23}H_{28}O_4$ (356.4): C 74.13; H 7.92; OCH_3 34.8; C—CH_3 8.4).

2. The β-tosylester treated as above gave after crystallization from methanol a product melting at 85—85.5°, $[\alpha]_D^{20}$ +45° (*c* 2.0 in chloroform). Distilled for analysis. (Found: C 73.80; H 8.01; OCH_3 34.0. Calc. for $C_{23}H_{28}O_4$ (356.4): C 74.13; H 7.92; OCH_3 34.8).

Dehydrogenation experiments. 1. The fully reduced α-compound (IV) (100 mg) was heated under reflux with palladized charcoal (10 %, 25 mg) in diethylene glycol (1 ml) for 4 h. The catalyst was then filtered off by suction and the colorless solution poured into water. After washing with water the precipitate (90 mg) was crystallized from glacial acetic acid and melted then at 176—177° undepressed by mixing with an authentic sample of dehydroguaiaretic acid dimethylether.

2. Using the same procedure the β-isomer of (IV) was also converted to dehydroguaiaretic acid dimethylether.

The author is indebted to *Statens Tekniska Forskningsråd* for financial support, to Dr. Hughes for determination of mixed melting point with galbulin and to Professor Erdtman for his interest in this work.

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ACTA CHEMICA SCANDINAVICA 8 (1954) 1830—1834

Preparation of Some Organic Silicon Hydrides

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The preparation of the following nine silanes from the corresponding ethoxysilanes is described: di- and tripropylsilane, di- and tributylsilane, mono-, di-, and triamylsilane, monohexylsilane and diphenylsilane. Reductions were carried out with lithium aluminium hydride either directly or after treatment with aluminium chloride. Densities, refractive indices, and molecular refractivities are given.

As in this laboratory a number of alkylethoxysilanes were available, it was desired to use them for preparation of the corresponding alkylsilanes by substituting the ethoxygroups with hydrogen. An attempt to reduce tri-*n*-propylethoxysilane with lithium aluminium hydride dissolved in diethyl ether, was not successful. If, however, lithium aluminium hydride and the ethoxysilane were mixed without a solvent and heated to about 100° for two hours, tri-*n*-propylsilane was obtained in 75—80 % yield. Reductions were also carried out in dibutyl ether at the same temperature, but the yields were not appreciably improved. Thus, tri-*n*-amylethoxysilane was reduced both without solvent and in dibutyl ether. The yield was 78 and 80 % respectively.

The methods just described were found to be unsatisfactory for the preparation of *n*-amylsilane, because polymerisation occurred, decreasing the yield to about 30 %.

Voronkov, Dolgov, and Dimitrieva[1] have reported that trialkylhalogensilanes can be prepared by distillation of disiloxanes or octaethyltrisiloxane from aluminium halides. I have tried a similar but modified method, where mono-, di-, and trichlorosilanes probably are intermediates. The ethoxysilane was mixed with aluminium chloride without any solvent present. When the reaction which then occurred was complete, the reaction mixture was dissolved in diethyl ether. This solution was decanted from precipitate, if any, and then introduced dropwise into a stirred slurry of lithium aluminium hydride in diethyl ether. Spontaneous reaction occurred giving the desired alkyl silanes. By this method it was possible to prepare *n*-amyl- and *n*-hexylsilane in yields of 65—70 %.

It seems not to be necessary to use more than one third of a mole of aluminium chloride per ethoxy group. Tri-*n*-butylsilane was prepared from alumi-

nium chloride and tri-*n*-butylethoxysilane in the molar ratios 1 : 1 and 1 : 2.7. In both cases the yield was about 70 %.

Except for the case that three ethoxygroups, bonded to silicon, are reduced, this method has no advantage over the direct reduction first described.

When alkyltriethoxysilanes were reduced by this method small amounts of gas were formed. They were presumed to be silane. This can easily be explained as a rearrangement of intermediate alkyltrichlorosilane in the presence of aluminium chloride[2], giving small amounts of dialkyldichlorosilane and silicon tetrachloride, the latter of which on reduction gives silane, *e.g.*

$$2\ C_6H_{13}SiCl_3 \xrightarrow{AlCl_3} (C_6H_{13})_2SiCl_2 + SiCl_4$$

$$SiCl_4 + LiAlH_4 \longrightarrow SiH_4 + LiCl + AlCl_3$$

In order to suppress this reaction prolonged heating with aluminium chloride should be avoided. Regarding the risk of spontaneous ignition, it is recommended to carry out the reductions in an atmosphere of nitrogen.

The reaction products were isolated in two different ways.

1. The reaction mixture was rapidly filtered and the solvent was carefully removed by distillation. After cooling at room temperature and standing for some hours, two layers were formed. Sometimes the lower layer solidified on prolonged standing. The upper layer, which contained the silane, was distilled. In some cases, particularly when the silanes could be distilled at moderate temperatures, separations of the layers were not achieved, but the silicon hydrides were distilled directly from the mixture, sometimes at reduced pressure.

The silanes isolated in this way were washed first with 5 *N* hydrochloric acid, then twice with water, dried with calcium chloride and finally redistilled.

2. After filtration the reaction mixture was hydrolysed by dropwise addition to an excess of ice-cooled 5 *N* hydrochloric acid in a three-necked flask fitted with reflux condenser, dropping funnel and mechanical stirrer. When hydrolysis was completed the silane was separated, washed twice with water, dried with calcium chloride and distilled. This method of isolation must be used for diphenylsilane (see the experimental part). For the alkylsilanes method 1 is preferable.

When studying the substitution of ethoxy groups by propyl groups in methylethoxysilanes with the aid of propylmagnesium chloride[3], a reduction, similar to those described here, was once observed. Thus methyltriethoxysilane and *n*-propylmagnesium chloride on heating to 150° yielded some methyldi-*n*-propylsilane together with the expected methyltri-*n*-propylsilane and methyldi-*n*-propylethoxysilane.

EXPERIMENTAL

Silicon analyses, refractive indices, densities and molecular refractivities are given in Table 1. The molecular refractivities found refer to the Lorenz-Lorentz formula. For the alkyl groups the bond refractivities given by Smith[8], and for the silicon-hydrogen bonds the value 3.20 given by Warrick[9] were used to obtain the calculated molecular values.

Table 1. Physical constants and analyses.

Substance	B.p. °C	Pressure mm	n_D^{20}	d^{20}	MR_D found	MR_D calc.	% Si found	% Si calc.
$(n-C_3H_7)_2SiH_2$	111[a]	760	1.4111	0.7205	40.08	40.09		
$(n-C_3H_7)_3SiH$	171[b]	760	1.4284	0.7601	53.63	53.73		
$(n-C_4H_9)_2SiH_2$	160[c]	760	1.4241	0.7458	49.38	49.36		
$(n-C_4H_9)_3SiH$	104[d]	12	1.4380	0.7793	67.50	67.63		
$n-C_5H_{11}SiH_3$	86	748	1.4042	0.7019	35.62	35.71	27.6	27.5
$(n-C_5H_{11})_2SiH_2$	81	12	1.4324	0.7636	58.62	58.64	16.3	16.3
$(n-C_5H_{11})_3SiH$	147	17	1.4436	0.7894	81.54	81.54	11.4	11.6
$n-C_6H_{13}SiH_3$	112	760	1.4131	0.7189	40.35	40.35	24.3	24.2
$(C_6H_5)_2SiH_2$	125[e]	11	1.5802	1.0027	61.19	61.26	15.0	15.2

Boiling points reported in literature: [a] 111° [4,5]; [b] 170° [6]; [c] 160° [4]; [d] 86—87° at 2.5 mm[7]; [e] see text. The amylsilanes and hexylsilane do not appear to have been published before.

A. Reduction of ethoxy groups with lithium aluminium hydride without solvent

Tri-n-amylsilane. 28.6 g (0.1 mole) of tri-*n*-amylethoxysilane and 1.9 g (0.05 mole) of lithium aluminium hydride were mixed in a 100 ml round bottomed flask. A slow spontaneous reaction began and the temperature rose to 40°. The mixture was heated for two hours to about 100°. After cooling, the reaction mixture was extracted with anhydrous ether and filtered in a dry atmosphere. The ether was withdrawn, and distillation yielded 19.0 g (78 %) tri-*n*-amylsilane boiling at 144—146° (14 mm).

Tri-n-propylsilane was prepared in the same way from 10.0 g (0.05 mole) tri-*n*-propylethoxysilane and 1.0 g (0.026 mole) lithium aluminium hydride. The yield was 6.4 g (81 %) tri-*n*-propylsilane b.p. 59° (13 mm).

Diphenylsilane was prepared from 12.8 g (0.047 mole) diphenyldiethoxysilane and 2.4 g (0.063 mole) lithium aluminium hydride. Isolation was achieved by hydrolysis with 75 ml hydrochloric acid according to method 2 described above. Distillation yielded 5.8 g (67 %) diphenylsilane boiling at 124—126° (11 mm). West and Rochow[4] have reported the boiling point 97° at 13 mm for diphenylsilane which does not seem to be correct.

In another experiment with the same amounts of starting materials the ether was distilled off after filtration as described for triamylsilane. A solid was left behind which did not distil, and no diphenylsilane could be isolated by distillation. When, however, the solid was dissolved with 5 *N* hydrochloric acid while cooling with ice the diphenylsilane was liberated and two phases were formed, which were separated. The upper one weighed 7.1 g (0.039 mole) and contained almost exclusively diphenylsilane. The lower aqueous phase was analysed for metals and was found to contain no lithium, but 1.19 g (0.044 g atoms) of aluminium, determined as alumina. Thus the solid compound contained about one gram atom of aluminium per gram atom of silicon. (The fact that the silicon aluminium compound on hydrolysis with hydrochloric acid gave only a silicon dihydride and an aluminium salt might indicate that the crystalline compound contained aluminium silicon bonds.) Diphenylsilane was also prepared from diphenyldichlorosilane by reduction with lithium aluminium hydride according to Benkeser, Landesman and Foster[10]. The diphenylsilane obtained according to this method distilled at 126—127° (13 mm).

B. Reduction of ethoxy groups with lithium aluminium hydride in dibutyl ether

Tri-n-amylsilane. 28.6 g (0.1 mole) of tri-*n*-amylethoxysilane was introduced dropwise into a stirred slurry of 1.2 g (0.03 mole) of lithium aluminium hydride in 25 ml of dibutylether. It was heated for two hours at 100°. 21 g (87 %) of a substance boiling at 146—153°

(15 mm) was isolated. This product and that prepared without solvent were redistilled together and a fraction was collected at 147° (17 mm) and analysed. (See Table 1.)

Di-n-amylsilane was prepared in the same way from 26.0 g (0.1 mole) of di-*n*-amyldiethoxysilane and 4.2 g (0.11 mole) of lithium aluminium hydride. The yield was 64 %, boiling at 81—82° (12 mm).

n-Amylsilane. 23.4 g (0.1 mole) of *n*-amyltriethoxysilane and 6.3 g (0.165 mole) of lithium aluminium hydride yielded 3.1 g (30 %) *n*-amylsilane, boiling at 85—87° (748 mm).

n-Amylsilane was also prepared from *n*-amyltrichlorosilane by reduction with lithium aluminium hydride.

n-Amyltrichlorosilane. *n*-Amylmagnesium chloride, prepared from 7.3 g (0.3 g.at.) of magnesium and 32 g (0.3 mole) of *n*-amyl chloride in 100 ml anhydrous ether was added during one hour to 42.5 g (0.25 mole) silicon tetrachloride dissolved in 50 ml ether. Thereafter the reaction mixture was refluxed for two hours. The precipitate of magnesium salts was removed by filtration and washed with ether. Distillation of the filtrate yielded 30.5 g (60 %) *n*-amyltrichlorosilane boiling at 60.5° at 15 mm. Whitmore, Sommer, Di Giorgio, Strong, Van Strien, Baily, Hall, Pietrusza, and Kerr[11] obtained a yield of 52 % using amyl bromide, instead of the chloride, and silicon tetrachloride in the molar ratio 1:1.

n-Amylsilane. 27.2 g (0.13 mole) of *n*-amyltrichlorosilane dissolved in 25 ml anhydrous dibutyl ether was added during 30 minutes to a slurry of 4.2 g (0.11 mole) lithium aluminium hydride in 25 ml dibutylether. By direct distillation from the reaction mixture 11.6 g of *n*-amylsilane were collected at 84—86° (760 mm).

C. Preparation with the aid of aluminium chloride

Tri-n-butylsilane. To 12.2 g (0.05 mole) of tri-*n*-butylethoxysilane was added 2.5 g (0.019 mole) of aluminium chloride in one portion. There was a spontaneous slight evolution of heat. The mixture was kept at 80° for a further 10 minutes. After cooling, anhydrous diethyl ether was added whereby a white precipitate was formed (about 2 g). The ether solution was added dropwise to a stirred slurry of 0.6 g (0.016 mole) of lithium aluminium hydride in diethyl ether. After filtration 7.5 g (75 %) of tri-*n*-butylsilane boiling at 104—106° (12 mm) was isolated by distillation.

In an additional experiment equimolecular amounts of tributylethoxysilane and aluminium chloride were used, but the yield (70 %) was not improved. No precipitate was formed when the solution was diluted with ether before reduction.

Di-n-butylsilane. 27.5 g (0.12 mole) of di-*n*-butyldiethoxysilane was stirred in a three-necked flask fitted with reflux condenser, and 12.0 g (0.09 mole) of aluminium chloride was added in portions. The reaction was much more intense than for the trialkyl derivative. After heating for 10 minutes to 80°, 25 ml of ether was added, and the decanted solution introduced dropwise into a slurry of 2.5 g (0.066 mole) of lithium aluminium hydride. 11.8 g (69 %) of di-*n*-butylsilane boiling at 52—53° (15 mm) was isolated.

Di-n-propylsilane was prepared as given above for dibutylsilane from 20.4 g (0.1 mole) of di-*n*-propyldiethoxysilane, 10.6 g (0.08 mole) of aluminium chloride, and 2.2 g (0.06 mole) of lithium aluminium hydride. The yield was 72 %, b.p. 110—111° (1 atm.).

n-Amylsilane. To 12.7 g (0.054 mole) of *n*-amyltriethoxysilane was added in portions 7.0 g (0.052 mole) of aluminium chloride while cooling with water. After dilution with ether reduction was carried out as described above with 1.6 g (0.042 mole) of lithium aluminium hydride. Evolution of small amounts of gas was observed during the reduction. (See below for *n*-hexylsilane.) The yield boiling at 86° (760 mm) was 63 %.

n-Hexylsilane was prepared as just described from 24.8 g (0.10 mole) of *n*-hexyltriethoxysilane, 20.0 g (0.15 mole) of aluminium chloride, and 4.4 g (0.115 mole) of lithium aluminium hydride. The yield was 7.5 g (66 %) of *n*-hexylsilane boiling at 112—113° (761 mm).

The reduction was accompanied by the evolution of a small amount of gas. Part of the gas was collected and ignited. The gas burned giving a white smoke of silicon dioxide. Another sample of gas was absorbed in acetone and tested with 1 % silver nitrate solution. A dark precipitate indicated the presence of reducing groups (Si—H bonds).

Methyldi-n-propylsilane. *n*-Propylmagnesium chloride was prepared from 4 gram atoms of magnesium in 400 ml of anhydrous ether and 4 moles of *n*-propyl chloride in 200 ml of ether. One mole methyltriethoxysilane in 100 ml of ether was added. After refluxing for

two hours the ether was withdrawn and the reaction mixture was kept at 150° for 12 hours. The ether was again added and the excess of propylmagnesium chloride was decomposed with two moles of anhydrous ethyl alcohol. After filtration and washing with anhydrous ether the filtrate was fractionated. At 126—127° 11 g of methyldi-*n*-propylsilane was collected. (Found: H 13.8. Calc. for $C_7H_{18}Si$ 13.9). (Furthermore methyldi-*n*-propylethoxysilane and methyltri-*n*-propylsilane were isolated.)

I am indebted to Professor Erik Larsson for his interest in this work.

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A New Method of Determining Small Osmotic Pressures

I. Principle and Apparatus

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The principles of an interferometric method of determining small pressure differences are discussed. An accuracy of 1—2 μ in the measurements can be obtained. An extensive description of the apparatus is given. It has been used for dynamic osmometry. Some experimental data from measurements on a polystyrene sample are reported.

In many physico-chemical methods the ability to measure small pressure differences is of great importance. This is particularly true in determinations of the osmotic pressure of solutions of macromolecules because all extrapolation procedures will become more certain and very large molecular weights can be determined more accurately. Further, very small changes in the difference of level between the menisci can be detected, thus making a dynamic method more reliable. As a rule, the menisci are observed in capillaries by means of cathetometers giving an accuracy of about 0.01 mm. Svedberg and Jullander[1,2] introduced the osmotic balance which was modified and improved by Enoksson[3,4] and Masson and Melville[5]. By this method of measurement it was possible to observe differences in menisci levels as small as 0.001 mm.

We have devised an interferometric method giving about the same accuracy as the osmotic balance. As the apparatus has proven to be simple and convenient in operation, a detailed description of it might be of interest. It has been in constant use for about two years and has been mainly used for the determination of osmotic pressures dynamically.

The basic idea behind the method is the interferometric determination of a difference in optical path. The principle has long been known and has been used by many scientists in various fields of research. In manometry the interferometric methods have been applied in different ways to obtain high accuracy. For example, a barometer of very high precision using the reflection of light beams from the mercury surfaces has been recently described[6]. In our case the organic solvents used for the solutions of polymers are also most conveniently used as the manometric liquid.

In designing the apparatus the chief aim was to obtain the greatest simplicity in construction and operation. It was thus considered expedient to make the optical arrangement as simple as possible and to try to reduce the number of adjustments to a minimum. This was accomplished by building the apparatus in such a way that the two light beams passed through the manometric liquid *.

If the two light beams of wavelength λ pass through two layers of length h with a difference in refractive index $\triangle n$, the basic formula is

$$h \triangle n = N_\lambda \lambda \tag{1}$$

where N_λ is the number of fringes of wavelength λ corresponding to the change $h \triangle n$ in optical path. Of course any one of the variables in (1) can be calculated if the other three are known. Since in our case h (see M in Fig. 1) denotes the difference in level between two menisci of the same liquid and $\triangle n = n$—1 ** where n is the refractive index of this liquid, (1) becomes

$$h (n - 1) = N_\lambda \lambda \tag{2}$$

Here n, N_λ and λ will be known and consequently h computed. For the sake of simplicity white light is used. Essentially no difficulties arose from this.

One of the main problems was to find the most suitable diameter of the tubes where the light has to pass menisci, *i.e.* in M (Fig. 1), which will be called the manometer. Because of the curvature of the menisci it is advantageous to work with large diameters. The use of large diameters also has the great advantage that errors due to surface tension are reduced. From another point of view, it is not desirable to have a large distance between the two light beams since in our simple Rayleigh system the fringes will then be too close to each other. Furthermore, small changes of the levels due to flow of liquid through the membrane correspond to a fairly large volume of liquid in wide tubes. This results in a change of the concentration of the solution in the osmotic cell which is usually made to hold a small volume. We have found that in our system a diameter smaller than 20 mm is hardly possible. For practical reasons this necessitates a distance of 25 mm between the light beams. However, very clear and distinct fringes were obtained with most liquids, and it was possible to determine N_λ reproducibly (eq. 1) with an accuracy of one fringe[7]. This means that for most liquids, where $1.3 < n < 1.5$, the error will be 1—2 μ. A change in level of 0.1 mm corresponds to about 0.03 ml of liquid, this being less than 0.5 % of the volume of the cell (7 ml). As will be seen later[8], it was not necessary to wait for such an amount to pass through the membrane before a final pressure could be determined. Furthermore, it may be observed that M is completely symmetrical and that the absolute positions of the menisci is of no importance. It was easy to thermostat the cell and manometer within a fairly wide temperature range. M is of course closed to prevent errors due to evaporation.

* Work with light beams reflected at the menisci is in progress at the Institute for other purposes where higher accuracy is required.

** More exactly, $\triangle n = n - n_1$, n_1 denoting the refractive index of the mixture of saturated vapour and air. $n_1 = 1$, however, is a very good approximation.

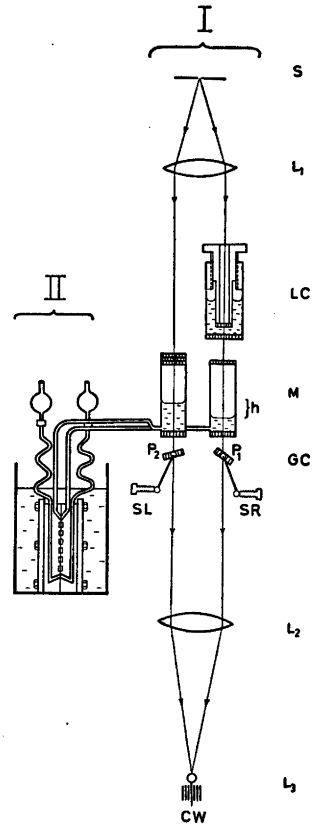

Fig. 1. Sketch showing the principle features of the apparatus.

A detailed drawing of the apparatus is shown in Fig. 1. The instrument can be divided into two main parts: (I) the manometer (interferometer) and (II) the osmotic cell. (I) is built on the stand of an upright drilling machine giving a rigid and stable support to the different units. These consist of the slit S, the lenses L_1, L_2, and L_3, the glass compensator GC, the liquid compensator LC, and the manometer M. In the ocular L_3, a cylindrical lense, the fringes are observed on a cross-wire CW. Since for practical reasons the optical system is made vertical, a mirror (not drawn in Fig. 1) is placed between L_2 and L_3 reflecting the light beams upwards in order to facilitate observation. It has been found necessary to use mirror, lenses and glass plates of good quality. L_1 is a "Rodenstock-Extra-Rapid-Aplanat" 1 : 7.7 ($f = 400$ mm) and L_2 is a "Hilger quartz-glass-quartz triple objective" 1 : 3.5 ($f = 1\,000$ mm). As concerns the glass plates the manufacturer gives the following guarantees: curvature $> 1\,000$ m, the angle between the two surfaces $< \pm 5''$.

Fig. 2 shows the glass compensator unit GC. It is entirely of conventional type and its description is merely given for the sake of completeness. The two compensating glass plates P_1 and P_2 (see also Fig. 1) are placed on holders in a

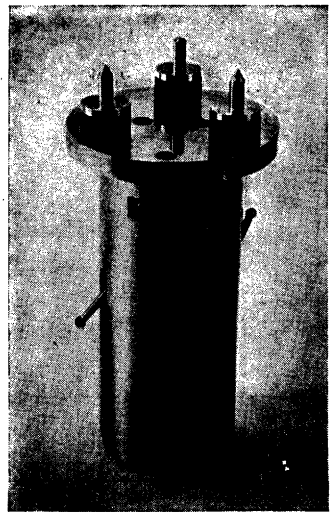

Fig. 2. The glass compensator (GC in Fig. 1).

brass tube to the upper end of which is attached a disc with a three point support for M. To the other end of the tube the lense L_2 is screwed. The angle of inclination of the glass plates can be varied by metal arms pressed by means of springs against micrometer screws SR and SL (Fig. 1). The readings are made on these micrometers. N_λ is obtained from a calibration of the readings in terms of the number of fringes of known wavelength. We used $\lambda_D = 589.3$ mμ. This calibration is made once and for all. The whole mechanical unit is fitted into the holder of the drilling machine table where also the micrometer screws SR and SL are fastened.

For various reasons[7] (white light is used, dispersion correction, extension of the range of measurements) it is convenient to use a liquid compensator LC (Fig. 1) in combination with GC. It is essentially constructed as a micrometer screw fitted into a tube containing the liquid used as a solvent for the polymer under investigation. The geometrical path of the light beam through the liquid can be varied from 0 to 25 mm. The pitch of the screw (all metal parts in this unit are of stainless steel) was not accurate enough to be used for the localization of the fringes but it could be used in whole turns (*i.e.* every 0.5 mm) without any distorsion of the image. The micrometer screw was calibrated by means of a microcomparator. It was of course necessary to use glass plates in the system and these are pressed against the bottoms of the tubes by means of nuts. Thin polythene films were used as packings. The liquid in LC was thermostated by water pumped through a jacket around the outer tube.

A detailed drawing of the manometer M is shown in Fig. 3. The cylindrical stainless steel block (1) contains the two manometer limbs (2) with a distance of 25 mm between their centres. They are closed at the bottom by a glass plate (4) tightened in a manner similar to that in LC (*i.e.* a metal plate (5) a nut (6), and a polythene washer). Two precision bore (20 mm) glass tubes are inserted into the manometer limbs with very good fitting. They are easily removed for occasional washing.

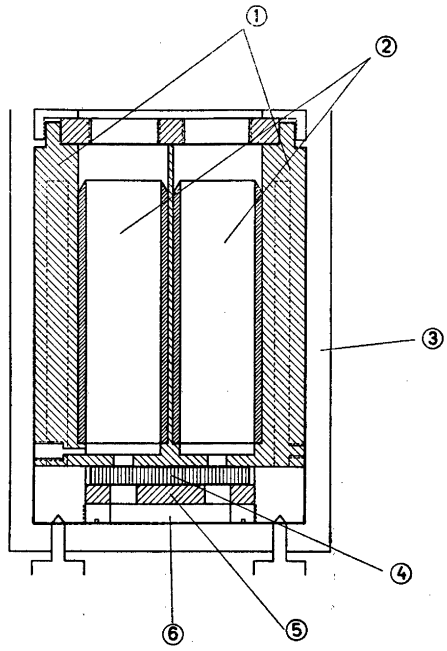

Fig. 3. Sketch of the manometer (M in Fig. 1).

On top of the block there are holders for glass plates. The latter have two functions: they prevent evaporation, and are of such thickness as to compensate exactly for the glass in LC. Tiny grooves under the glass plates ensure atmospheric pressure above the liquid in (2). M is provided with canals through which thermostated water is circulated and is covered with insulating material (3) having holes for the passage of the light beams.

The osmometer cell (II) (Fig. 1) is of a somewhat modified Fuoss-Mead pattern[9]. It is machined from silver blocks (containing about 17 % copper) which are clamped together by a frame of stainless steel. The canals for filling each half of the cell are drilled in the block following a design indicated by Sirianni *et al.*[10] but arrangements for closing them have been excluded as they are unnecessary (*cf.* Gilbert *et al.*[11]). One canal from each half of the cell is connected by a stainless steel cannula to the manometer M and the other two are provided with cannulas bent in the shape of spirals ending with short glass capillaries which are connected through standard tapers to the bottoms of 50 ml round flasks. The design is very practical for many reasons. When the filled cell is already free of air bubbles, and in use, the excess of solvent is easily removed to such an extent that the menisci are to be found in the capillaries. When a solution or fresh solvent is to be brought into the cell the steel spirals are pressed together with the result that the menisci rise into the flasks. In this way the cell can be easily kept free of air bubbles during long periods. The cell is filled initially without the inclusion of air bubbles by rapidly sucking a large amount of liquid through each of its halves. Another important point in this particular case is that a final adjustment of the positions of the



Fig. 4. The complete apparatus.

menisci in M can be very sensitively managed by means of the spirals. These permit the attainment of level differences of up to 50 mm. All joints between the cannulas, the manometer M, and the osmotic cell are tightened by means of nuts and teflon packings. The glass capillaries are cemented on to the metal by means of lead oxide and glycerol. The silver blocks have been made as flat as possible in a grinding machine and as silver is somewhat resiliant the strong force which can be applied by means of the steel frame is enough to prevent leakage at the membranes. No problems of leakage have arisen.

The thermostat is filled with water and contains two electric heaters, one of which is continuously in operation while the other is regulated by the contact thermometer and a relay. The stirrer is made as a double pump circulating the water also through the manometer M and the liquid compensator LC. The osmotic cell is completely immersed in a small bath mounted inside the main thermostat. The temperature fluctuations in the inner bath are smaller than $\pm$ 0.005°.

Fig. 4 shows the complete apparatus.

As mentioned above the apparatus has been mainly used for dynamic osmometry. In a subsequent paper[8] the technique and results will be extensively discussed. In this paper a typical result of such measurements will be shown where the method of extrapolation is the one used by Gee[12], Montonna and Jilk[13] and others (*e.g.* Refs. 2 and 3). The rate of flow of solvent through

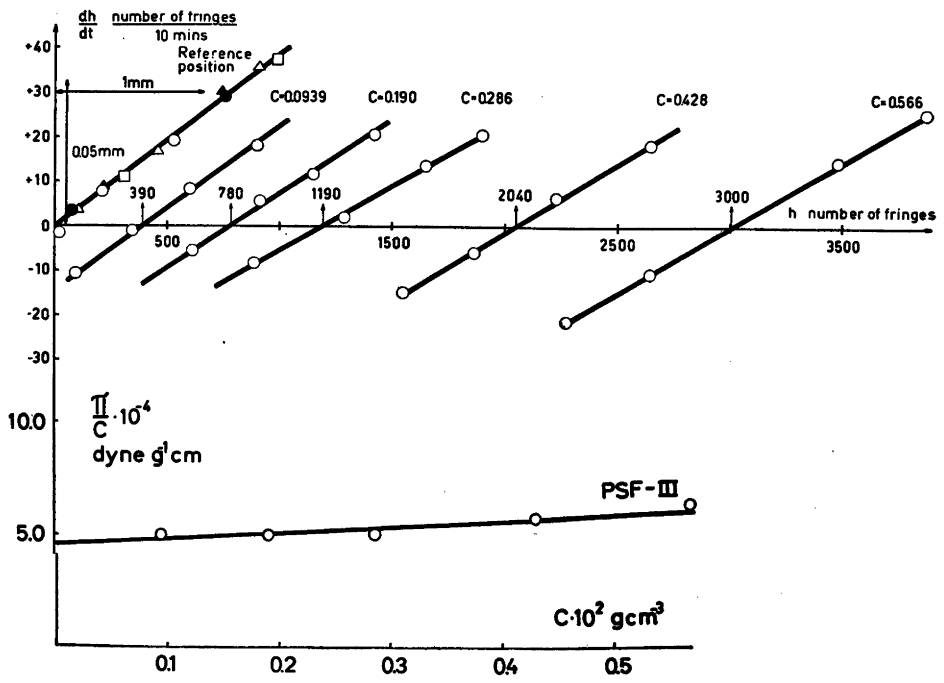

Fig. 5. Typical rate curves for a polystyrene fraction (Ref. 14) in methyl ethyl ketone and the corresponding $\frac{\pi}{c}$ *—c relationship.*

the membrane, $\frac{dh}{dt}$ at a certain level difference h between the menisci, is determined and plotted against h. A straight line is obtained, the intercept of which on the h-axis gives the value of h where $\frac{dh}{dt} = 0$, *i.e.* the equilibrium value. Fig. 5 shows the results of some measurements on a polystyrene fraction in methyl ethyl ketone for different concentrations using an Ultracellafilter membrane. From this figure some idea of the sensitivity of the rate determination can be obtained. The accuracy of the extrapolation is generally about 0.01—0.02 mm. The corresponding $\frac{\pi}{c}$ —c plot is drawn in the same figure. The molecular weight of the fraction, denoted PSF—III, an international polystyrene fraction, has been found to be 540 000. A survey of measurements on this sample can be found in "Report on Molecular Weight Measurements of Standard Polystyrene Samples"[14] (the fraction is labelled III).

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A New Method of Determining Small Osmotic Pressures

II. Measurements and Results

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Measurements with an interference osmometer are reported. A dynamic technique has been consistently used. A detailed description of the sequence of operations is given. Different polymer — solvent systems have been examined; diagrams are shown and the time for and the accuracy of the measurements are discussed.

In an earlier paper[1] an interferometric method of measuring small differences in liquid levels with an accuracy of 1—2 μ was described. For some time now osmotic measurements on different polymer — solvent systems have been performed in this apparatus. The largest source of error in osmometry, however, seems to be the membranes, the influence of which on the measurements is very little understood. In spite of this, osmometry is an indispensable tool of high polymer chemistry and it is of great importance to develop methods which can facilitate work in this field.

Although this type of apparatus can be used for any kind of osmometry, it is particularly well suited for dynamic measurements. The sensitivity of the interferometric system makes it possible to accurately determine the rate of flow through the membrane even when working near to the equilibrium position. The cell (of Fuoss-Mead pattern) is of the "dynamic" type and the apparatus is on the whole convenient and quick in operation. It has therefore been considered to be of interest to devise a dynamic technique which would provide rapid measurements of satisfactory accuracy.

The chief advantage of a dynamic method in comparison with a static one is the relative rapidity with which measurements can be carried out. Problems in connection with osmometry with special emphasis on dynamic osmometry have been extensively discussed in a series of papers by Staverman[2,3]. Among the dynamic methods in use the one introduced by Gee[4] and Montonna and Jilk[5] has been found to be most convenient for this apparatus.

Suppose that during the time dt a volume of liquid dV passes through the membrane. If π is the equilibrium position, *i.e.* the difference between the

menisci at osmotic equilibrium, and h denotes an arbitrary difference in menisci levels, $\frac{dV}{dt}$ is assumed to be proportional to the difference $(h—\pi)$ and we obtain

$$\frac{dV}{dt} = k_1 (h—\pi) \tag{1}$$

But as the tubes in the manometer are cylindrical

$$dV = k_2 dh \tag{2}$$

Thus

$$\frac{dh}{dt} = K (h—\pi) \tag{3}$$

where

$$K = \frac{k_1}{k_2} \tag{4}$$

Now if $\frac{dh}{dt}$ is determined at different positions h and plotted against h, a straight line is obtained from which the value of h at $\frac{dh}{dt} = 0$ can be found, *i.e.* $h = \pi$. The factors governing a possible deviation of this "dynamic" value from the "thermodynamic" value are the properties of the membrane (porosity, elasticity and thermodynamic stability), the mode of measuring and the accuracy of the rate determination. Together with other experimental errors — with the exception of factors giving rise to systematic deviations — this may appear as a dispersion of the points in the $\frac{\pi}{c}$—c plot.

As pointed out by Staverman[2,3] it is very important not to have any diffusion of polymer through the membrane even if a "quick" dynamic method is applied. Although a fairly porous membrane has been used in the work reported here none or extremely small diffusion of solute has been detected. This was achieved by checking the constancy of the equilibrium position over a long time (several days) for one solution of each fraction examined. The usual control of the solvent side after the measurements by pouring an excess of precipitant into the contents of the cell was also made. In no case did turbidity arise.

For the following description of the sequence of operations the reader is referred to Fig. 1 in the preceding paper[1].

The membrane was conditioned from one solvent to another in the usual manner by the successive increase of the amount of new solvent in mixtures of new and former solvent. In cases where the two solvents were not miscible in all proportions a suitable intermediary solvent was used. Throughout this work it was found that, for the type of membrane used (Ultracella-filter), this procedure of conditioning must be extended over a period of at least one week. When treated in this way the membranes showed a small and satisfactorily reproducible "cell constant" (see below).

After the initial filling of the cell any air bubbles remaining were removed by rapidly sucking a large amount of degassed solvent through both halves of the cell. A quantity of liquid was then sucked off such that the menisci were situated in the glass capillaries which were held by means of the springs in such a position as to give a suitable level in M.

The reference position was now determined with solvent in both halves of the cell. Using LC (in steps of 0.5 mm) and SR (with fixed SL) the fringes were located. The levels were then adjusted to positions desired, usually in intervals of 0.5 mm, by pipetting calculated amounts of solvent into the flasks (at II) without introducing air bubbles into the cell. For each level difference h the rate of flow $\frac{dh}{dt}$ was determined from the movement of the interference fringes during a suitable time, usually 10 mins. Generally, only two readings on SR were made, one at the time $t = 0$ and one at $t = 10$. The rate values could of course be smoothed out by taking readings at closer time intervals (*e.g.* every minute) but this is fairly tiring and does not improve the interpolation accuracy essentially. The readings on LC and SR give h and $\frac{dh}{dt}$ respectively:

If h_1 = reading on LC (in mm or in number of fringes of wavelength λ_D)
h_0 = reading on SR at the time $t = 0$
h_t = reading on SR at the time $t = \Delta t$
$\Delta h = h_0 - h_t$

one has

$$h = h_1 + h_0 - \frac{\Delta h}{2} \tag{5}$$

$$\frac{dh}{dt} = \frac{\Delta h}{\Delta t} \tag{6}$$

The magnitude of $|\Delta h|$ is very small, between 1—4 % of h.

The next step was to fill the cell with the solution. All solutions were prepared by weighing from stock solutions. Specially designed flasks were used for this purpose. By means of a simple glass arrangement the required amount of stock solution was, under pressure from a rubber bulb, transferred into the weighing flask where dilution was carried out. In exactly the same manner the solution was transferred to the flask on the osmometer (at II). In this way changes in concentration due to evaporation (while handling the solutions) were avoided. This was very important as the concentrations were not measured afterwards (see below).

Because of the design of the cell the process of exchanging solvent was a somewhat delicate matter. The fairly large volume of solution (at least 100 ml) which is required must be passed very slowly through the cell chamber before reproducible measurements can be made. The rate of flow of solution through the cell was controlled by varying the amount of solution in the osmometer flask, the level in M being kept approximately constant during the exchange. The time required for this part of the operation was generally about one hour.

The sensitivity of the interferometric system was such that it was a relatively easy matter to find an approximate equilibrium position (within 0.2—0.3 mm) shortly after the cell was filled and the system was left for thermostating for about half an hour. Rate determinations were then made, as described above, on both sides of the equilibrium position. This procedure was repeated for all the solutions.

It was mentioned above that the concentrations of the solutions were determined before the osmotic measurements. This was done because for many reasons an accurate evaluation afterwards is difficult and would probably be

of doubtful significance (see *e.g.* Grassie[6]). Apart from possible evaporation during the transfer of solutions, error in this variable could arise from the passage of solvent through the membrane and from a possible adsorption of solute on the membrane. The amount of solvent passing through the membrane during thermostating is small and is estimated to be less than 0.5 % of the cell volume. If there is an appreciable adsorption on the membrane its influence on the concentration is considerably reduced or even eliminated by the following facts: a) the membrane was in contact with the solution during the time of exchange (about one hour) which makes it very probable that the membrane was saturated with solute when the measurements on the last part of the solution in the cell were started, b) measurements were made on solutions of increasing concentration.

The membrane which has been in continuous use for about half a year without any inconveniences was an Ultracellafilter from Membranfiltergesellschaft, Göttingen, labelled 30. The constant K (eq. 3) may be used to characterize its permeability or perhaps better the quantity τ defined as

$$\tau = \frac{\ln 2}{K} \qquad (7)$$

τ varies slightly with the solvent (*cf.* Breitenbach and Forster[7]). The whole membrane area gives a value of τ between 3 and 4 hours in our apparatus and for the solvents used. This means that if π is to be found statically and if the initial positions of the menisci differ from the equilibrium positions to the extent say, 0.1 mm which is in practice very difficult to achieve in a short time, it will take between 12 to 15 hours before π can be determined with an accuracy of 0.01 mm. By applying the dynamic technique, a rate determination at one position of the menisci could be carried out in 10 to 20 mins. and since measurements were generally made at four positions for each concentration and as the exchange of the solution and thermostating as mentioned above required about two hours a $\frac{\pi}{c}$ —c diagram containing data for four or five concentrations could be obtained within two days.

Fractions of three different kinds of polymers were examined. They covered a range of molecular weights from about 100 000 to 3 000 000. Methyl ethyl ketone (MEK) and ethyl acetate (Et Ac) were used as solvents. The samples are described as follows:

CNF—S, a cellulose nitrate fraction *, prepared by fractional precipitation in the system acetone-water. Molecular weight about 100 000. Nitrogen content: 12.40 %. Solvent: MEK.

PSF—12, a polystyrene fraction, prepared by fractional precipitation in the system carbon tetrachloride-methanol. The polymer synthesized by emulsion polymerisation. Molecular weight about 250 000. Solvents: MEK and Et Ac.

PSF—III, a polystyrene fraction, described in "Report on Molecular Weight Measurements of Standard Polystyrene Samples"[8]. Molecular weight about 500 000. Solvent: MEK.

* The author is indebted to fil.lic. Y. Sahlman for this sample.

Table 1. Experimental results for different samples in methyl ethyl ketone (MEK) and ethyl acetate (Et Ac)

Sample	Solvent	$c \cdot 10^2$ g/ml solution	π mm solution	$\frac{\pi}{c} \cdot 10^{-4}$ dyne g^{-1} cm
CNF–S	MEK	0.0462	1.20	20.6
		0.0983	2.70	21.7
		0.148	4.25	22.6
		0.231	7.39	24.9
		0.378	14.16	29.5
PSF–12	MEK	0.0367	0.47	9.9
		0.0667	0.85	10.1
		0.136	1.87	10.8
		0.172	2.33	10.8
		0.207	2.82	10.7
		0.294	4.07	10.9
		0.312	4.18	10.6
		0.350	4.95	11.2
		0.479	7.03	11.6
PSF–III	MEK	0.0939	0.59	5.00
		0.190	1.19	4.95
		0.286	1.81	5.01
		0.428	3.10	5.72
		0.566	4.57	6.40
PMF–EI	Et Ac	0.0419 *	0.69	14.8
		0.0825 *	1.39	14.9
		0.119	1.98	14.6
		0.211	3.64	15.2
		0.294	5.26	15.8
PSF–12	Et Ac	0.0743	0.86	10.2
		0.102	1.20	10.5
		0.108	1.28	10.5
		0.225	2.64	10.4
		0.345	4.25	10.9
PMF–EII	Et Ac	0.236	0.37	1.4
		0.293 *	0.58	1.8
		0.310 *	0.56	1.7
		0.407	1.01	2.2

* Static measurements.

PMF—EI and PMF—EII, two polymethylmethacrylate fractions * prepared by fractional precipitation in the system acetone-methanol. The polymer synthesized by block polymerisation. Molecular weights about 200 000 and 3 000 000. Solvent: Et Ac. All samples were dried to constant weight at elevated temperature (about 50° C) in vacuum. The solvents have been made free from water and purified by distillation. Refractive indices: MEK : n_D (25° C): 1.3764; Et Ac : n_D (25° C): 1.3697.

* The author is indebted to fil.lic. F. Eriksson for these samples.

Table 2. Values of the constants in eqs. (8) and (9) and sedimentation molecular weights (UC)

Sample	Solvent	$\left(\frac{\pi}{c}\right)_0 \cdot 10^{-4}$ extrapolated	$B\ 10^4$	M osmot.	M UC
CNF–S	MEK	19.0	10.8	130 000	132 000
PSF–12	MEK	10.1	1.2	245 000	–
PSF–III	MEK	4.6	1.0	540 000	–
PMF–EI	Et Ac	14.4	1.9	172 000	182 000
PSF–12	Et Ac	10.2	0.7	243 000	–
PMF–EII	Et Ac	0.8	1.2	3 100 000	2 100 000

All measurements were carried out at 25.00° C. All experimental data have been treated and constants computed in accordance with the equations

$$\frac{\pi}{c} = RT\ (A + Bc + \ldots) \tag{8}$$

$$A = \frac{1}{M} \tag{9}$$

All units and symbols used here are those recommended in "Report on Nomenclature in the Field of Macromolecules"[9].

Tables 1 and 2 give a survey of the experimental results and of the constants calculated from eqs. (8) and (9). Table 2 also includes some data from sedimentation and diffusion measurements. Figs. 1 and 2 show the corresponding $\frac{\pi}{c}$—c plots, where open circles indicate dynamic and filled circles static measurements the latter having been made in a few cases for comparison. Fig. 5 in the preceding paper[1] is a typical rate diagram with the corresponding $\frac{\pi}{c}$—c plot. The concentrations are given in g/ml solution but were originally determined in g/g solution and converted into the former unit using the density of the solvent*. For $\frac{\pi}{c}$, however, the same value is obtained whether π is expressed in cm solution and c in g/g solution or π in g/cm^2 and c in g/ml solution. It has not been considered necessary to apply any special corrections (see *e.g.* Lang[10]) to the data. It might also be mentioned here that the difference in refractive index between solvent and solution in the concentration range used is so small that error due to this is far less than other experimental errors.

The dispersion of the points in the rate diagram is small and the dynamic equilibrium value can be interpolated with an accuracy of about ten fringes, *i.e.* between 0.02 and 0.01 mm. Within the limits of experimental error the

* No density measurements were carried out. For the conversion of the unit, density data of the solvents from international tables were used.

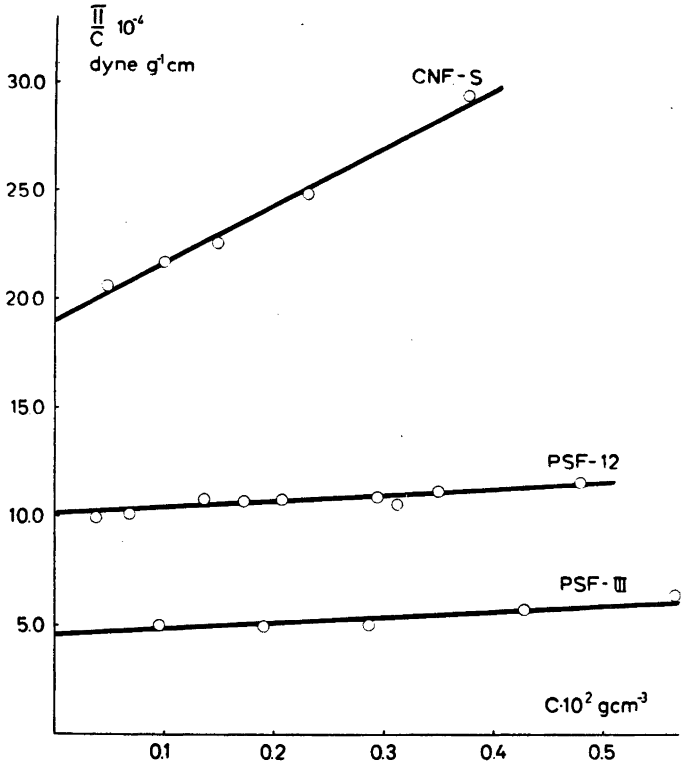

Fig. 1. $\frac{\pi}{c}$ *—c relationship for cellulose nitrate (CNF) and polystyrene (PSF) fractions in methyl ethyl ketone.*

lines are parallel; the slopes are the same for different polymers in the same solvent. There is, however, a tendency for the slope to decrease with increasing concentration, which might be explained by a diminishing "effective" membrane area with increasing amounts of polymer. The diagram also shows the sensitivity in the rate determination: the largest value of $\frac{dh}{dt}$ is 0.05 mm per 10 mins. It is not a coincidence that the extrapolated value for the reference position is zero. The screw SL was put in such a position as to give this value on the diagram. SL was of course not changed during the whole experiment. It corresponds in fact to a "cell constant" of about 0.05 mm. The reproducibility of the reference position was satisfactory. For this sample (PSF—III) it was checked immediately after the measurement on each solution and this is denoted in the diagram by the different sets of points.

In the $\frac{\pi}{c}$ —c plots (Figs. 1 and 2) all extrapolations to zero concentration have been made along straight lines and only slight tendencies for curvature upwards may be noticed in the relatively narrow concentration range in which

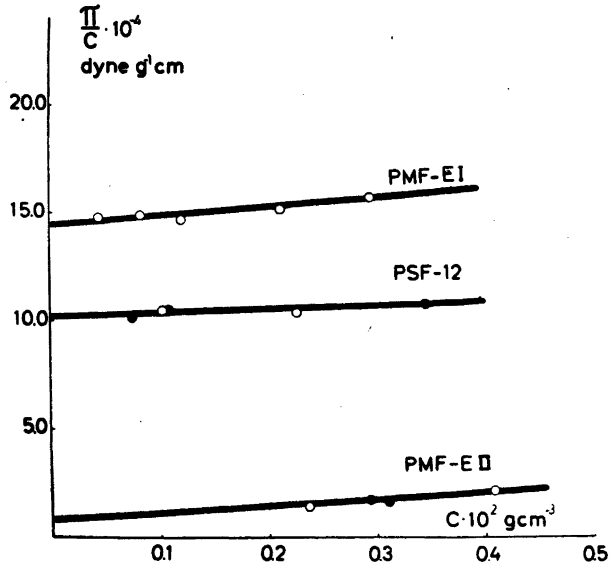

Fig. 2. $\frac{\pi}{c}$ *—c relationship for polystyrene (PSF) and polymethylmethacrylate (PMF) fractions in ethyl acetate. Filled circles denote static measurements.*

these measurements have been performed. The uncertainty in the determination of π can be particularly studied in Fig. 1 for the fraction PSF—12 where data from measurements on nine different concentrations are plotted. Furthermore, this fraction was examined in two different solvents (see also Fig. 2) giving results of good agreement. The deviation of the points from the straight line is in no case larger than about 5 % of the extrapolated $\frac{\pi}{c}$ value and this seems to be the upper limit of the dispersion of the data obtained in this investigation.

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Über katalytische Rhodanierung von aromatischen Kernen

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Aromatische Amine und Phenole werden, wie ich schon 1918 zeigte [1], leicht durch Dirhodan substituiert, aber die direkte Einführung der SCN-Gruppe in den Kern eines aromatischen Kohlenwasserstoffes ist bisher nur für gewisse polynucleare Kohlenwasserstoffe wie Anthracen und Benzpyren [2] gelungen. Es war jedoch anzunehmen, dass durch Zuhilfenahme von Katalysatoren die Rhodanierung auch von einfacheren Kohlenwasserstoffen wie Benzol und Naphthalin möglich sei. Nach einigem Suchen wurden in $AlCl_3$ und $Al(SCN)_3$ zwei effektive Katalysatoren gefunden, von welchen der letztere allerdings bisher nur als Ätherat $Al(SCN)_3 2(C_2H_5)_2O$ isoliert worden ist.

Die aromatischen Substanzen, die geprüft wurden, waren *Benzol*, *Mesitylen*, *Naphthalin* und *Thiophen* sowie zwei, die ohne Katalyse rhodanierbar sind, *Pyrrol* und *Phenol*. Als Lösungsmittel sind verschiedene verwendbar; es wurden *Benzol*, *Schwefelkohlenstoff*, *Chloroform* und *Äthyläther* gebraucht. Von diesen hat Äther nur eine beschränkte Verwendbarkeit, was zweifellos auf Solfatisierung zurückgeht. So lassen sich Benzol und Naphthalin nicht gut in Äther rhodanieren. Es bilden sich zwar nachweisbare Mengen von Phenyl- bezw. Naphthylrhodanid, aber die Hauptmenge des Rhodans wird als schwerlösliche, gefärbte Polymere abgeschieden. Für die Rhodanierung von Benzol ist dieses als Solvenz notwendig, diejenige von Naphthalin kann in Schwefelkohlenstoff oder Chloroform in glatter Reaktion ausgeführt werden, während Thiophen, Pyrrol und Phenol in allen oben genannten Solvenzien rhodanierbar sind.

Die Rhodanierung von Benzol kann nur mit $AlCl_3$ durchgeführt werden, mit $Al(SCN)_3$ wird die Hauptmenge des Rhodans als rotes Polymere abgeschieden. Diese Verschiedenheit ist sicher hauptsächlich dem Kristalläther des Rhodanids zuzuschreiben. Naphthalin, Thiophen und Phenol werden von $AlCl_3$ und $Al(SCN)_3$ unter gleichen Bedingungen mit nicht sehr verschiedener Geschwindigkeit substituiert.

Die Substitution kommt bei Benzol, Mesitylen, Naphthalin und Phenol mit der Bildung von Monorhodanderivat zum Stillstand, von Thiophen kann auch das Biderivat erhalten werden.

Die Reaktionsdauer variiert von etwa einer Stunde bei Benzol zu wenigen Minuten für Phenol. Wenn letzteres ohne Katalyse rhodaniert wird, beträgt die Reaktionszeit mehrere Tage, und die Ausbeute wird durch die Ausfällung einer

schwerlöslichen Verbindung von Rhodanwasserstoff und Rhodan stark beeinträchtigt [3].

Die Ausbeuten an aromatischen Rhodaniden sind im allgemeinen vorzüglich, von α-Naphthylrhodanid über 80 % und von p-Rhodanphenol über 90 %.

Die Reaktionen sind exotherm und die Wärmeentwicklung manchmal beträchtlich. Als Beispiel kann angeführt werden, dass wenn 0,1 Mol Naphthalin mit 0,1 Mol $(SCN)_2$ in 1-molarer Chloroformlösung mit 9 g $AlCl_3$ rhodaniert wird, die Temperatur bis auf den Siedepunkt des Chloroforms steigen kann. Man mässigt deshalb zweckmässig die Reaktion durch Zusatz von soviel Äther, wie es der Formel $AlCl_3 2(C_2H_5)_2O$ entspricht. $AlCl_3$ geht dann in Lösung; die Reaktion verläuft jedoch auch dann unter erheblicher Temperatursteigerung und ist binnen 30 Minuten abgeschlossen. Im allgemein wurde das Reaktionsgemisch anfänglich mit Eis oder kaltem Wasser gekühlt.

Das Reaktionsbild bei der Einführung von Rhodan in aromatische Kerne mit $AlCl_3$ und $Al(SCN)_3$ als Kontaktsubstanzen bietet gewisse Züge, die nicht bei den entsprechenden Reaktionen der elementaren Halogene zu beobachten sind. Sowohl Rhodan wie Rhodanwasserstoff haben eine ausgeprägte Tendenz, sich zu polymerizieren und $AlCl_3$ ausser Komplexbildungsvermögen auch einen polymerisierenden Effekt.

Wenn man z.B. Benzol rhodaniert, so bleibt nach beendeter Reaktion das in Benzol ziemlich schwerlösliche $AlCl_3$ nicht unverändert zurück, sondern es tritt während der Reaktion partielle oder vollständige Verflüssigung ein. Die orangefarbige, schwere Phase nimmt die Hauptmenge des bei der Substitution gebildeten Rhodanwasserstoffs auf und ausserdem Phenylrhodanid und Benzol. C_6H_5SCN ist zwischen beiden Phasen verteilt.

Eine ähnliche Schichtenbildung tritt auch bei Rhodanierung von Mesitylen, Naphthalin und Thiophen ein. Für die beiden ersten variiert die Farbe der Bodenschicht zwischen orange und braun, für Thiophen sind stark verschiedene Farben beobachtet worden, rot oder violettrot in Schwefelkohlenstoff und gelb oder grün (allmählich in braun übergehend) in Äther. Wenn Chloroform als Lösungsmittel dient, bildet die $AlCl_3$-Schicht die obere leichtere.

Durch Zusatz von Äther nach beendeter Reaktion kann die Schichtenbildung aufgehoben werden, was die Bearbeitung des Reaktionsgemisches vereinfacht.

Wenn die homogene Lösung oder die abgetrennte Bodenschicht mit Wasser behandelt wird, scheiden sich sofort gelbe oder gelbbraune feste Körper aus, die keine nennenswerte Al-Mengen enthalten und in Wasser und organischen Solvenzien unlöslich sind. Diese Körper sind höchst wahrscheinlich aus primär gebildetem HSCN entstanden und sind allem Anschein nach polymer.

Das Phenol nimmt unter den untersuchten aromatischen Substanzen in der Weise eine Sonderstellung ein, dass bei seiner Rhodanierung mit $AlCl_3$-Katalyse die Verflüssigung von $AlCl_3$ ausbleibt und der Rhodanwasserstoff quantitativ in der Lösung als HSCN auftritt, was durch einen Versuch mit Benzol als Lösungs mittel konstatiert wurde.

Das verflüssigte $AlCl_3$ kann indessen auch $(SCN)_2$ aus der Lösung aufnehmen. Wenn z.B. Naphthalin in Schwefelkohlenstoff oder Chloroform mit $(SCN)_2$ im Molverhältnis 2 : 1 rhodaniert wird, so ist nach einigen Stunden alles Rhodan aus der Lösung verschwunden, obwohl nur das Monoderivat gebildet

wird. Hydrolysiert man dann die $AlCl_3$-Schicht mit Wasser, so ist in diesem von den normalen Hydrolysenprodukten des $(SCN)_2$ nur HSCN nachweisbar, weder HCN noch H_2SO_4 können wiedergefunden werden. Es ist dazu zu bemerken, dass aus einer Lösung, die nur $(SCN)_2$ enthält, dieses nicht von $AlCl_3$ unter Verflüssigung aufgenommen wird, wohl aber bei Gegenwart von HSCN oder einem normalen organischen Rhodanid. Das durch Aufnahme von Rhodan nebst Rhodanwasserstoff verflüssigte $AlCl_3$ löst sich nicht vollständig in Äther, und in Übereinstimmung damit setzt eine ätherische Lösung von äquimolekulare Mengen $(SCN)_2$ und HSCN bei Gegenwart von $AlCl_3$ eine rotbraune flüssige Schicht ab, die längere Zeit haltbar ist und durch Wasser, wie oben beschrieben, hydrolysiert wird.

Das $Al(SCN)_3$ geht im Gegensatz zu $AlCl_3$, wenn es katalysierend wirkt, nicht in eine flüssige Phase über, sondern wird ganz oder teilweise aufgelöst. Es kann aber wahrscheinlich einen gewissen polymerisierenden Effekt haben, wenigstens wird bei Rhodanieren von Naphthalin in Chloroform das zunächst in Lösung auftretende HSCN ziemlich rasch als gelbe, schwerlösliche Substanz ausgefällt.

Die neuen Verbindungen, zu denen meine Rhodanierungsversuche geführt haben, sind *Rhodanmesitylen*, Schmp. 71°, *α,α-Dirhodanthiophen*, Schmp. 87—88°, und *α,α-Dirhodanpyrrol*, Schmp. 114°, sowie sein N-Methylderivat, Schmp. 118—119°.

Das Dirhodanpyrrol wird ohne Katalyse in recht glatter Reaktion gebildet. Versuche mit $(SCN)_2$ und Pyrrol im Molverhältnis 4 : 1 und $AlCl_3$ gaben kein Tetraderivat, was in Anbetracht der Leichtigkeit, womit Pyrrol tetrajodiert wird, ziemlich auffallend ist. Ebenso scheiterten Versuche, das Thiophen zu tetrarhodanieren. Es scheint überhaupt nicht möglich zu sein, mehr als zwei Rhodangruppen in einen aromatischen Kern durch direkte Substitution von Wasserstoff einzuführen, wenigstens nicht mit den bisher geprüften Katalysatoren.

Das Dirhodanpyrrol ist, wie viele andere am Stickstoff nicht substituierte Pyrrolderivate, in Alkalihydroxid löslich. Wenn zu der eisgekühlten Lösung Salzsäure sofort gesetzt wird, fällt das Dirhodanpyrrol unverändert aus. Bei Stehen färbt sich die alkalische Lösung schnell rot und setzt allmählich einen rotbraunen Körper ab. Nach einigen Stunden ist sie wieder farblos. Trotz dieser Unbeständigkeit in alkalischer Lösung ist es mir gelungen, die Methylierung des Dirhodanpyrrols mit Dimethylsulfat durchzuführen. Das Methylderivat hat eine grössere Löslichkeit in Benzol, aber eine geringere in Wasser und Äther als die Muttersubstanz.

Die Leichtigkeit, womit Pyrrol ohne Katalyse substituiert wird, erinnert an das Benehmen aromatischer Amine unter ähnlichen Bedingungen.

Kaufmann und Oehring [4] konnten durch Einwirkung von Brom und Natriumrhodanid auf Anilin in 95 %-iger Essigsäure das 2,4-Dirhodananilin, Schmp. 107—108°, darstellen, und ich habe selber das analoge 2,4-Dirhodandimethylanilin aus *p*-Rhodandimethylanilin und $(SCN)_2$ in Überschuss in Benzol gewonnen, Schmp. 92°. Das Rhodanid wird dabei nicht als Hydrorhodanid ausgefällt, sondern bleibt als freie Base in Lösung. Offenbar ist durch den Eintritt des zweiten SCN in den Kern die Basenstärke derart abgeschwächt worden, dass Salzbildung mit der starken Säure Rhodanwasserstoff unmöglich

wird. Das *p*-Rhodandimethylanilin hingegen gibt mit Rhodanwasserstoff in Benzol Ausfällung von Hydrorhodanid.

Die organischen Rhodanide können bekanntlich leicht durch Reduktion in Mercaptane und durch Oxydation in Sulfonsäuren übergeführt werden. Die katalytische Rhodanierung bietet deshalb eine neue Methode zur direkten Einführung von Schwefel in einen aromatischen Kern. Selbstverständlich wird die Verwendbarkeit der Methode unter den sehr schonenden Bedingungen, die das Arbeiten mit dem leicht veränderlichen Rhodan erfordert, durch den hemmenden Effekt vieler Substituenten eine rechte beschränkte.

EXPERIMENTELLER TEIL

Über Darstellung von Bleirhodanid

Das für die Rhodanierungsversuche benutzte Dirhodan wurde wie gewöhnlich aus Bleirhodanid und Brom gewonnen. Das Bleirhodanid war aus heissem Wasser kristallisiert und dann mehrere Tage bei etwa 60° getrocknet worden.

Das Rhodanid des Handels ist meistens durch das basische Salz Pb(OH)SCN stark verunreinigt, da es aus Bleiacetat durch Fällen mit Alkalirhodanid dargestellt wird. Dieses führt zwei Nachteile mit sich. Erstens ist das ausgefällte Salz, wie genannt, mit basischem stark bemengt und muss durch Kristallisation aus Wasser davon befreit werden. Wie von mir angestellte Versuche zeigen, enthält das mit äquivalenten Mengen der Komponenten dargestellte Salz aus 1-molarer Lösung etwa 22, aus 0,5 molarer 28 und aus 0,2 molarer etwa 43 mol-% basisches Bleirhodanid (Temperatur 22—23°). Solche Präparate werden von den chemischen Fabriken ohne Bedenken als chemisch rein charakterisiert.

Ferner wird die Ausbeute bei Fällen aus Bleiacetat schlecht, was auf den bekannten lösenden Effekt des Acetations zurückgeht. Die Ausbeute an reinem $Pb(SCN)_2$ fällt demzufolge immer unterhalb 50 % der theoretischen.

Wenn aber die Ausfällung aus dem *Nitrat* geschieht, werden beide Übelstände hinfällig, das ausgefällte Salz ist frei von basischem Salz, es löst sich vollständig in Wasser und wird in einer Ausbeute von über 90 % erhalten. Die starke Hydrolyse bei Ausfällung aus Bleiacetat erklärt sich durch den puffernden Effekt des Acetations und die Schwerlöslichkeit des basischen Salzes. Noch stärkere Hydrolyse konnte ich bei Ausfällen von Bromid beobachten. Wenn aus 1-molarer Lösung dargestellt, besteht das Produkt zu mehr als 80 % aus basischem Bromid.

Es geht aus diesen Versuchen hervor, dass Bleiacetat für die Darstellung von nicht extrem schwerlöslichen Salzen durch Umsetzung mit Neutralsalzen überhaupt nicht brauchbar ist. Angaben in der Literatur ohne analytische Belege über Eigenschaften von aus Bleiacetat dargestellten Bleisalzen sind entschieden nachzuprüfen.

Darstellung von Aluminiumrhodanid

Die Darstellung von Aluminiumrhodanid $Al(SCN)_3 2(C_2H_5)_2O$ ist schon in meinen ersten Mitteilungen über Dirhodan [5] beschrieben worden. Bei den jetzigen Versuchen wurden statt Aluminiumspäne dünne Al-Folien benutzt und halbmolare Ätherlösungen von Dirhodan. Die Reaktion setzt nicht sofort ein, es kann bis 2 Stunden dauern, bevor die ersten Zeichen der Einwirkung sich einstellen. Diese Zeit kann wesentlich (bis auf eine halbe Stunde) reduziert werden, wenn die Lösung von vornherein mit Rhodanid gesättigt wird, weshalb die Reaktion offenbar autokatalysiert wird. 100 ml Äther lösen bei 20° 1.4 g. Die Reaktion verläuft unter so starker Wärmeentwicklung, dass für Kühlung gesorgt werden muss. Die Ausbeuten sind keineswegs quantitativ und übersteigen nicht 55 % an reinem Rhodanid. Die stark eingeengte Mutterlauge besitzt den penetranten Geruch des Cyansulfids $(CN)_2S$, weshalb sich gleichzeitig mit der Rhodanidbildung eine Zersetzung des Rhodans abspielt. Der Kristalläther ist sehr fest gebunden, weshalb das stark hygroskopische Salz mehrere Tage in Vakuum über konz. H_2SO_4 oder P_2O_5 ohne merkbare Veränderung verträgt.

Phenylrhodanid

Aus 32 g Br_2 und 70 g $Pb(SCN)_2$ in 100 ml thiophenfreiem Benzol wurde eine Rhodanlösung bereitet, und das Bleihalogenid auf einem Glasfilter von passender Feinheit durch Ausrühren mit 100 ml Benzol in Portionen ausgewaschen. Zu der Lösung wurden 18 g pulverisiertes $AlCl_3$ gesetzt und umgeschüttelt. Binnen einer halben Stunde trat Zusammenbacken und dann Zerfliessen des Chlorids unter so grosser Wärmeentwicklung ein, dass mit Wasser gekühlt wurde. Nach einer halben Stunde enthielt die Lösung, die nun farblos geworden war, nur Spuren von Rhodan, eine orangefarbige schwere Schicht von ungefähr 90 ml hatte sich abgeschieden. Nach einer zweiten Stunde war die Lösung völlig rhodanfrei und enthielt nur geringe Mengen von HSCN. Es wurde nun 50 ml Äther zugesetzt, wodurch die Schichtenbildung aufgehoben wurde. Die homogene Lösung wurde in Portionen und unter lebhaftem Umrühren in eine eisgekühlte Lösung von 100 g KOH in 300 ml Wasser gegossen. Die Benzol-Ätherschicht wurde enthoben, zweimal mit 50 ml Wasser gewaschen und dann einige Stunden über $MgSO_4$ getrocknet. Das Lösungsmittel wurde dann im Vakuum abgetrieben, wobei die Badtemperatur auf 60° gesteigert wurde. Der Rückstand war 17 g einer völlig klaren, gelben Flüssigkeit, die bei Fraktionieren im Vakuum von 15 mm konstant bei 109° siedete und 14 g reines Phenylrhodanid lieferte = 50 %.

Rhodanmesitylen

Aus 16 g Br_2 in 200 + 100 * ml Schwefelkohlenstoff und 36 g $Pb(SCN)_2$ wurde eine Rhodanlösung dargestellt und zu dieser 12,0 g destilliertes Mesitylen sowie 9 g $AlCl_3$ gesetzt. Zusammenbacken von $AlCl_3$ nach etwa 5 Minuten und dann allmähliches Zerfliessen zu einem roten Sirup, dessen Farbe bald dunkler wurde. Nach einer halben Stunde war die Lösung rhodanfrei, enthielt aber etwas HSCN. Sie wurde vom Bodenkörper separiert, zweimal mit Wasser gewaschen und dann mit Na_2SO_4 getrocknet. Beim Abdestillieren des Schwefelkohlenstoffs blieb als Rückstand eine rötliche Flüssigkeit zurück, die im Vakuum über konz. H_2SO_4 kristallisierte. Ausbeute 3,9 g. Die Substanz wurde zweimal aus warmen Petroläther kristallisiert und schmolz dann bei 71—72°; Prismen, die bisweilen grosse, warzenförmige Aggregate bilden.

Der braune Bodenkörper wurde in 100 ml Äther gelöst und mit 100 ml Wasser digeriert, wobei Ausfällung einer gelbbraunen Substanz geschah, deren Gewicht nach Waschen und Trocknen 4,7 g betrug. Aus der Ätherlösung konnte nach Waschen mit Wasser und Bicarbonat und Verjagen des Äthers 8 g Substanz isoliert werden, die nach Kristallisieren aus siedendem Petroläther bei 71° schmolz. Totalausbeute an Rohprodukt 11.9 g = 67 %. (Gef. S 18,36; C 67,55; H 6,17. Ber. für $C_{10}H_{11}SN$ (177,15): S 18,10; C 67,74; H 6,26).

Versuche sind angestellt worden, durch Rhodan in Überschuss höhere Rhodanderivate von Mesitylen darzustellen, jedoch ohne Erfolg. Nur Monorhodan-mesitylen konnte isoliert werden.

α-Naphthylrhodanid

1. Katalysator $AlCl_3$. Aus 8,3 g Br_2 in 30 + 40 ml Chloroform (über P_2O_5 destilliert) wurde eine Rhodanlösung hergestellt und zu dieser 6,4 g Naphthalin sowie eine Lösung von 3,0 g $AlCl_3$ in 4,7 ml Äther und 10 ml $CHCl_3$ gesetzt. Mischflüssigkeit blassgelb und klar. Nach 5 Minuten war die Temperatur der anfänglich eisgekühlten Lösung über Zimmertemperatur gestiegen und nach 30 Minuten war die Lösung rhodanfrei, jedoch stark HSCN-haltig. 50 ml Wasser wurden in Portionen zugesetzt, was unter Wärmeentwicklung die Ausfällung einer gelbbraunen Substanz bewirkte, deren Gewicht nach Waschen und Trocknen 3,0 g betrug. Die Chloroformlösung wurde mit Wasser bis zum Verschwinden der HSCN-Reaktion gewaschen und dann mit $MgSO_4$ getrocknet. Abdestillieren des Lösungsmittels lieferte als Rückstand eine blassgelbe Flüssigkeit, die beim Stehen allmählich kristallisierte. Ausbeute 7,6 g; Theor. 9,2. Nach Naphthalin riechend; der Geruch verschwand bei Stehen in Luft. Nach Kristallisieren aus warmen Petroläther war das Präparat farblos und schmolz bei 54°. (Gef. S 17,75; C 71,18; H 3,87. Ber. für $C_{11}H_7SN$ (185,12): S 17,32; C 71,36; H 3,81).

2. Katalysator $Al(SCN)_32(C_2H_5)_2O$. Rhodanlösung aus 16,8 g Br_2 in 50 + 65 ml $CHCl_3$. Zusatz von 12,8 g Naphthalin und 8,4 g $Al(SCN)_32(C_2H_5)_2O$. Das Rhodanid

* Das zweite Volumen ist das Waschvolumen.

wurde nicht sofort aufgelöst, sondern in einen gelben Niederschlag verwandelt. Nach etwa 10 Minuten setzte die Reaktion unter Wärmeentwicklung ein, und der Niederschlag wurde rasch aufgelöst. Kühlung mit kaltem Wasser. Nach einer halben Stunde Probe auf $(SCN)_2$ mit negativem Resultat, starke Reaktion hingegen für HSCN. Die sehr hygroskopische Lösung wurde mit 100 ml Wasser in Portionen behandelt, was ohne nennenswerte Wärmeentwicklung die Ausfällung einer hellgelben Substanz bewirkte. Nach Abfiltrieren von dieser wurde die Chloroformlösung mehrmals mit Wasser gewaschen und über $MgSO_4$ getrocknet. Rückstand bei Abdestillieren des Lösungsmittels flüssig, erstarrte aber rasch bei Stehen. Gewicht nach Trocknen in Vakuum 16,5 statt 18,5 g. Durch Umkristallisieren aus Petroläther wurden 15,4 g farblose Kristalle ausgewonnen. Schmp. 54°.

Das α-Naphthylrhodanid wurde 1922 von Challenger und Wilkinson [6] aus α-Trinaphthylwismut und Dirhodan in Äther dargestellt, die entsprechende β-Verbindung 1875 von Billeter [7] aus β-Naphthylmercaptan und Chlorcyan. Schmp. 35°.

α-Rhodanthiophen

1. Katalysator $AlCl_3$. Aus 16 g Br_2 in 75 + 90 ml wasserfreiem Äther wurde eine Rhodanlösung bereitet und zu dieser 10,7 g Thiophen (25 % Überschuss) gegeben, sowie unter Kühlung 9 g AlCl, das sich vollständig auflöste. Die Lösung färbte sich sofort grün und eine schwere flüssige Schicht wurde abgeschieden, in der sich der grüne Farbstoff konzentrierte *. Nach beendetem Zusatz von $AlCl_3$ war die Lösung praktisch rhodanfrei. Nach einigem Stehen in der Kälte wurden 15 ml Wasser zugesetzt, wodurch die Bodenflüssigkeit verschwand und ein weisser kristallisierter Körper, aus hydratisiertem $AlCl_3$ bestehend, ausgeschieden wurde. Nach Abfiltrieren von diesem wurde der bei der Substitution gebildete Rhodanwasserstoff durch Ausschütteln mit Wasser erschöpfend entfernt und aus der wässrigen Lösung mit $CuSO_4$ und SO_2 als CuSCN ausgefällt, dessen Gewicht nach Waschen und Trocknen 11,75 statt berechnet 12,16 g betrug. Die gewaschene Ätherlösung wurde nach Trocknen mit $MgSO_4$ zunächst bei gewöhnlichem Druck und dann im Vakuum bei einer Badtemperatur von 70° eingeengt. Gewicht des Rückstands 13,3 g statt der theor. 14,1 g. 63 g in dieser Weise gewonnenes Präparat wurden mit Wasserdampf destilliert, wodurch 62 g = 98 % zurückgewonnen wurden. Nach kurzem Trocknen mit $MgSO_4$ wurde im Vakuum fraktioniert, wobei die Flüssigkeit ohne nennenswerten Rückstand unter 12 mm bei 108—110° siedete. (Gef. S 45,40. Ber. für $C_5H_3S_2N$ (141,17): S 45,13).

2. Katalysator $Al(SCN)_3$ 2 $(C_2H_5)_2O$. Der Versuch wurde in demselben Masstabe wie der vorige ausgeführt mit 7,6 g $Al(SCN)_3$, das zum grösstem Teil aufgelöst wurde. Keine Grünfärbung der Lösung und keine Schichtenbildung. Die Wärmeentwicklung war gering und nach 1/4 Stunde war die Reaktion abgeschlossen. Die Lösung wurde, wie oben beschrieben, behandelt, und die Ausbeute war ungefär dieselbe. 31 g lieferten bei Wasserdampfdestillation 29 g, die bei Fraktionieren bei 12 mm bei 108—110° siedeten.

Das α-Rhodanthiophen ist früher von Cherbuliez und Giddey [8] durch Einwirkung von Rhodan auf Chloromercurithiophen oder Quecksilberdithienyl gewonnen worden. Die katalytische Rhodanierung ist aber die weitaus bequemste Methode und gestattet eine schnelle Herstellung in grösseren Quantitäten.

α,α_1-Dirhodanthiophen

1. Katalysator $AlCl_3$. Rhodan aus 16 g Br_2 in 75 + 80 ml Äther. 4,2 g Thiophen und 9 g $AlCl_3$ wurden eingewogen. Anfänglich wurde mit Eis gekühlt. Eine blassgelbe schwere Bodenschicht wurde schnell ausgebildet und in dieser bei Stehen über Nacht Kristalle. Nach zweitägigem Aufbewahren im Eisschrank wurden die Kristalle abseperiert, mit kleinen Äthermengen gewaschen und luftgetrocknet. Gewicht 2,3 g. Die ätherische Mutterlauge, die noch etwas $(SCN)_2$ enthielt, wurde nach einigen Tagen mit Wasser von HSCN befreit, mit $MgSO_4$ getrocknet und der Äther abgetrieben. Dabei trat Kristallisation von 3,0 g Substanz ein; Totalausbeute 5,3 g = 53 %. Die Substanz wurde in warmem Benzin gelöst und kristallisierte bei Abkühlen in glänzenden Blättern.

* Wenn zu einer ätherischen Lösung von HSCN $AlCl_3$ gesetzt wird, tritt sofort Schichtenbildung ein, ebenso aus einer ätherischen HCl-Lösung und $Al(SCN)_3$.

Schmp. 87—88°. (Gef. S 48,64; C 36,22; N 14,35; H 1,18. Ber. für $C_6H_2S_3N_2$(198,26): S 48,52; C 36,32; N 14,13; H 1,02).

2. Katalysator Al(SCN)$_3$2 (C_2H_5)$_2$O. Rhodan aus 16 g Br_2 in 75 + 95 ml Äther und Zusatz von 4,2 g Thiophen und 8 g Al-Rhodanid. Dieses wurde bei Umschütteln teilweise aufgelöst. Die Lösung färbte sich allmählich rot. Nach 3-tägigem Verwahren in der Kälte enthielt die Lösung nur kleine Mengen von $(SCN)_2$. Nach weiteren 3 Tagen wurde vom Bodenkörper abfiltriert, dieser mit Äther gewaschen und der Waschäther zusammen mit der Hauptlösung erschöpfend mit Wasser gewaschen. Nach Trocknen mit $MgSO_4$ wurde der Äther abdestilliert, wobei Kristallisation eintrat. Ausbeute 4,1 g. Wurde zunächst aus siedendem Benzin und dann aus Tetrachlorkohlenstoff kristallisiert und daraus in grossen Blättern erhalten. Schmp. 87—88°.

Das Dirhodanthiophen ist mit einer gewissen Vorsicht handzuhaben, da es schwere Hautentzündungen mit Blasenbildung hervorrufen kann.

Die Dirhodanierung von Thiophen hat einen wesentlich glatteren Verlauf in Schwefelkohlenstoff als in Äther, und dieser Umstand veranlasste mich, eine Rhodanierung in diesem Solvenz mit Rhodan und Thiophen im Molverhältnis 4 : 1 zu prüfen. Binnen 4 Stunden waren sowohl $(SCN)_2$ wie HSCN aus der Lösung verschwunden und von dem lichtroten Bodenkörper von sirupöser Konsistenz aufgenommen. Weder aus diesem noch aus der Lösung konnte aber ein Tetrarhodanthiophen isoliert werden, sondern lediglich das Dirhodanid. Katalysator war bei diesem Versuch $AlCl_3$.

α,α_1-Dirhodanpyrrol

Eine Rhodanlösung wurde aus 16 g Br_2 in 75 + 80 ml thiophenfreiem Benzol bereitet und dazu die äquivalente Menge, 3,35 g, vakuumdestilliertes Pyrrol gegeben. Die anfänglich blassgelbe Farbe der Mischflüssigkeit wurde allmählich vertieft und nach etwa 1/4 Stunde trat Kristallisation ein. Nach etwa 3 Stunden wurde die Mutterlauge von den Kristallen abgesogen, diese mit etwas Benzol gewaschen und luftgetrocknet. Gewicht 3,1 g. Die stark HSCN-haltige Mutterlauge wurde mit Wasser dreimal ausgeschüttelt und mit $MgSO_4$ getrocknet. Durch Einengen der Lösung im Vakuum konnten weitere 3,1 g isoliert werden. Die beiden Fraktionen wurden getrennt umkristallisiert, zunächst aus warmen Wasser, dann aus Tetrachlorkohlenstoff, wodurch der Schmelzpunkt zuletzt auf 114° erhöht werden konnte. Kleine, farblose, verfilzte Prismen. Die Substanz ist bei Ausschluss von Licht gut haltbar. (Gef. S 35,31; C 39,86; N 23,20; H 0,91. Ber. für $C_6H_3S_2N_3$ (181,19): S 35,39; C 39,74; N 23,37; H 1,66).

Das Dirhodanpyrrol ist auch aus Äther mit guter Ausbeute erhalten worden. Da die Löslichkeit in Äther wesentlich grösser als in Benzol ist, tritt in diesem Fall kein direktes Auskristallisieren ein.

N-Methyl-dirhodanpyrrol

1,8 g Dirhodanpyrrol, Schmp. 113—114°, wurden in 20 ml H_2O suspendiert, eisgekühlt und dann 10 ml ebenfalls gekühlte 1-molar NaOH zugegeben. Das Rhodanid löste sich teilweise unter schwacher Rotfärbung. Es wurde nun sofort 1,26 g Dimethylsulfat zugegeben und energisch umgeschüttelt. Allmählich Ausscheidung von farblosen Kristallen, die flüssige Phase verschwand, ebenso die rote Farbe der Lösung. Der Kristallniederschlag wurde allmählich sehr voluminös. Nach einigen Stunden wurde abfiltriert, mit Wasser gewaschen und vakuumgetrocknet. Zwei solche Versuche gaben 3,56 statt 3,9 g = 91 %. Das Rohprodukt wurde wiederholt umkristallisiert, zunächst aus Wasser, in dem die Löslichkeit gering ist, und dann aus Äther. Die Auflösung von 1,37 g verlangte 100 ml Äther. Bei Verjagen des Äthers trat Kristallisation in farblosen Prismen ein; Ausbeute 0,92 g, Schmp. 118—119°. (Gef. S 32,75; C 43,08; H 2,63. Ber. für $C_7H_5S_2N_3$ (195,18): S 32,85; C 43,04; H 2,58).

Rhodanierung von Phenol

Katalysator Al(SCN)$_3$2 (C_2H_5)$_2$O. Rhodanlösung aus 16,8 g Br_2 in 75 + 90 ml Äther und 9,4 g Phenol, sowie 3,5 g Al-Rhodanid. Momentane Einsetzung der Reaktion unter starker Wärmeentwicklung, weshalb in Eisbad gekühlt wurde. Probe auf $(SCN)_2$ nach 5 Minuten mit negativem Resultat. Der Katalysator war dann zum grössten Teil aufgelöst und die Lösung fast farblos. Wurde von HSCN durch wiederholtes Ausschütteln mit

Wasser befreit. Das Waschwasser wurde bis zur Trockenheit vakuumdestilliert und das Destillat mit $AgNO_3$ vollständig gefällt. Gewicht des AgSCN nach Waschen und Trocknen bei 60° 16,59 g = 0,1 Mol. Das bei der Substitution gebildete HSCN war also quantitativ als solches zugegen. Die gewaschene Ätherlösung lieferte nach Trocknen und Verjagen des Äthers als Rückstand eine blassgelbe Flüssigkeit, die bei Aufbewahren im Vakuum bald erstarrte. Gewicht 14,1 statt theor. 15,1 g für Rhodanphenol. Zwecks Reinigung wurde zunächst aus warmem Benzin kristallisiert, aus dem die Substanz (Anhydrid) als blassgelbe kleine Prismen von Schmp. 61° fiel. Die weitere Reinigung geschah über das Halbhydrat $NCS \cdot C_6H_4OH$ 0,5 H_2O. 11 g Substanz wurden in 100 ml Benzol gelöst und das doppelte der für das Halbhydrat berechneten Wassermenge zugesetzt. Beim Umrühren trat bald Auskristallisieren ein. Nach zweitägigem Stehen wurden die farblosen Kristalle abgetrennt, mit etwas Benzol gewaschen und an der Luft getrocknet. Ausbeute etwa 80 %. Zur vollständigen Reinigung wurde einmal aus warmen Wasser umkristallisiert und an der Luft getrocknet. Schmp. 53–54°. (Gef. S 19,08; C 52,29; H 3,89. Ber. für $C_7H_6O_{1,5}SN$ (160,12): S 20,02; C 52,46; H 3,78).

Das Entwässern des *p*-Rhodanphenolhydrats kann entweder durch längeres Aufbewahren im Vakuum über konz. H_2SO_4 oder Trocknen der ätherischen Lösung durchgeführt werden. Schliesslich wird einmal aus siedendem Benzin umkristallisiert. Schmp. 61–62°. (Gef. S 21,34. Ber. für C_7H_5OSN (151,12): S 21,22).

Die Rhodanierung von Phenol mit Al-Rhodanid als Katalysator kann auch gut in Benzol geschehen, nur tritt hier die Komplikation ein, dass das Rhodanphenol sich teilweise mit dem Katalysator zu einer schwerlöslichen Substanz verbindet. Die Ausbeute ist vorzüglich.

Statt des Rhodanids kann $AlCl_3$ verwendet werden, und die Reaktion ist genau wie oben beschrieben durchzuführen. In Benzol bleibt jedoch die Ausfällung einer schwerlöslicher Verbindung von $AlCl_3$ und Rhodanphenol aus.

ZUSAMMENFASSUNG

Es wurde eine Methode zur katalytischen Rhodanierung von aromatischen Stoffen ausgearbeitet. Als Katalysatoren dienten $AlCl_3$ und $Al(SCN)_32(C_2H_5)_2O$, von denen das Chlorid am wirksamsten ist. Höchstens zwei Rhodangruppen konnten in einen aromatischen Kern eingeführt werden.

Die Komplexbildungstendenz der Al-Halogenide sowie die Neigung von Rhodan und Rhodanwasserstoff zur Polymerisation bewirken gewisse Erscheinungen, die die eigentliche Substitution begleiten.

An zwei auch ohne Katalyse rhodanierbaren Substanzen, *Pyrrol* und *Phenol*, wurde festgestellt, dass durch Katalyse der Rhodanierungsgrad nicht erhöht werden kann.

Da die Rhodangruppe leicht durch Reduktion in die Mercaptogruppe und durch Oxydation in Sulfonsäure übergeführt werden kann, bietet die neue Methode ein Verfahren zur Einführung von Schwefel in einen aromatischen Kern.

Für die Ausführung von Mikroanalysen danke ich Herrn Lic. Phil. *L. Schotte*.

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Effects of Some Enzyme Inhibitors on Fructosidase and Amylase

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The effect of some protease nhibitors on the activity of fructosidase and amylase was studied. Generally speaking acid compounds were inhibitory in acid reactions, while basic compounds inhibited in alkaline reactions. The reactions were reversible and suggested a salt formation between enzyme and inhibitor. Protease inhibitors of protein type were generally inactive.

The effects of a number of enzyme inhibitors on the fibrinolytic activity of some proteases have been recorded recently[1,2]. Great differences were observed between the effects of the different protease inhibitors on the fibrinolytic activity of various proteases and the results could be used in the characterization of the different enzymes. It was of interest to study the effects of the inhibitory substances used in these investigations on some non-proteolytic enzymes. Two enzymes acting on uncharged carbohydrates were chosen for this preliminary investigation. They were β-h-fructosidase, acting on a low molecular weight carbohydrate, and salivary amylase, acting on a high molecular weight carbohydrate. These two enzymes were available as purified preparations.

MATERIAL

The following enzyme inhibitors were used: *1. Acids:* D,L-α-tocopherylphosphate (Hoffman-La Roche & Co. Ltd., Basel); laurylsulphate (sodium salt with 30 % sodium sulphate from Lundbeck & Co., Copenhagen); heparin (Løvens kemiske Fabrik, Copenhagen); chitin disulphuric acid and cellulose trisulphuric acid (prepared as previously described[3]). *2. Bases:* Cetylpyridinium chloride (Bionova Ltd., Copenhagen); toluidine blue and janus green (Grübler & Co. Ltd., Leipzig); laurylamine (Ferro Metal & Chem. Corp., New York). *3. Ampholytes:* Pulmin, the protease inhibitor isolated from ox lung[4] (a partially purified preparation was used[5]); soya bean trypsin inhibitor of Kunitz (about 70 % pure, prepared by a simplified method[6]); serum from cattle blood (a freeze dried preparation of spontaneously coagulated fresh blood). The substances were used as 2 % solutions in buffer with the exception of toluidene blue and janus green, which were used as 0.1 % solutions. The solutions were pH-corrected by addition of dilute sulphuric acid or sodium hydroxide and checked electrometrically.

Table 1. The effect of enzyme inhibitors on fructosidase. Enzyme activity expressed as change in rotatory power per min. ($\Delta\alpha/\Delta t$).

Inhibitor	$\Delta\alpha/\Delta t$			
	pH 3.5 ± 0.1 acetate	pH 4.5 ± 0.1 acetate	pH 6.5 ± 0.1 phosphate	pH 6.8 ± 0.1 citrate
None	0.15	0.15	0.13	0.08
D,L-α-Tocopherylphosphate	0.00	0.14	0.14	
Laurylsulphate	0.00	0.05	0.14	
Heparin	0.08	0.15	0.14	
Chitin disulphuric acid	0.06	0.15	0.13	
Cellulose trisulphuric acid	0.12	0.14		0.07
Cetylpyridinium chloride	0.14	0.14	0.05	
Toluidine blue	0.16	0.13	0.08	
Janus green	0.16	0.14		0.05
Laurylamine	0.14	0.16	0.10	0.07
Pulmin	0.09	0.14	0.12	
Soy bean inhibitor	0.13	0.15	0.13	
Serum	0.16	0.15	0.13	

Fructosidase was prepared from brewer's yeast and purified more than 1 000 times[7]. The amylase was prepared from human saliva and purified 5 times by precipitation with acetone[8]. The preparations contained no α-glucosidase.

EXPERIMENTS AND RESULTS

Effect on fructosidase: The experiments were performed in a water bath at 30.0° C and all solutions were preheated to this temperature and had identical pH values. The strength of the enzyme solution was chosen so that the reaction could be followed polarimetrically for 6 minutes[9].

6 ml fructosidase solution was mixed with 1 ml 0.2 *M* of an appropriate buffer solution (see the tables). 2 ml of an inhibitor solution was added to the mixture, followed after 30 secs. by 1 ml 0.2 *M* buffer solution. After a further 30 secs. 5 ml of the resulting mixture was added to a substrate solution consisting of 2.375 g saccharose dissolved in 38.3 ml dist. water and 5 ml 0.2 *M* buffer solution (saccharose concentration: 4.75 %). In some experiments the saccharose concentration was 9.50 % and in others 14.25 %. The hydrolysis was followed polarimetrically every minute for 6 minutes. From the straight line graph obtained the change in rotatory power per min. was calculated ($\Delta\alpha/\Delta t$). The values read were corrected for the small blank values of the solutions used. The results appear from Table 1.

As the activity optimum for the enzyme is pH 4.5 and the isoelectric point is at pH 5[10] it is seen that on the acid side (pH 3.5) very acid compounds only appear to inhibit, the reaction with cellulose trisulphuric acid being an excep-

Table 2. Reversibility of enzyme inhibition.

Inhibitor	$\Delta a/\Delta t$	
	pH 3.5 ± 0.1 acetate	pH 6.5 ± 0.1 phosphate
None	0.15	0.13
Heparin,	0.08	
» followed by cetylpyridinium chloride	0.13	
Chitin disulphuric acid,	0.06	
» » » followed by cetylpyridinium chloride	0.11	
Cetylpyridinium chloride,		0.05
» » followed by laurylsulphate		0.14

tion. Pulmin has a slight inhibitory action but too little is known about the chemistry of this compound to warrant any speculations. At pH 4.5 inhibition is less marked, occurring only with laurylsulphate. At pH 6.5 and 6.8 only alkaline substances act as inhibitors. Laurylamine has only slight effect.

Some of the inhibitors used in these experiments are surface active agents. They are known to have a denaturing effect on proteins, and it was thus of interest to find out whether the inhibition observed was caused by a simple denaturation of the enzyme, or if it was caused by a reversible combination with the inhibitor. Use was here made of the neutralization of surface active amines with surface active acids. These experiments were performed in principle as above but an appropriate concentration of the neutralizing agent was dissolved in the 1 ml 0.2 *M* buffer solution which was added after 30 secs. incubation of the enzyme inhibitor solution. Thus in one experiment (pH 6.5) the inhibition

*Table 3 *. Reversibility of enzyme inhibition.*

Inhibitor	$\Delta a/\Delta t$		
	Saccharose conc. %	pH 3.5 ± 0.1 acetate	pH 6.5 ± 0.1 phosphate
None	4.75	0.15	0.10
Heparin	4.75	0.05	
Chitin disulphuric acid	4.75	0.04	
Cetylpyridinium chloride	4.75		0.05
None	9.50		0.10
Cetylpyridinium chloride	9.50		0.04
None	14.25	0.13	0.10
Heparin	14.25	0.06	
Chitin disulphuric acid	14.25	0.05	
Cetylpyridinium chloride	14.25		0.06

* These experiments were performed with a fructosidase preparation purified about 200 times.

Table 4. Effect of enzyme inhibitors on amylase. Enzyme activity expressed as change in titration value per min ($\triangle ml/\triangle t$).

Inhibitor	$\triangle ml/\triangle t$					
	pH 5.9 ± 0.1		pH 6.9 ± 0.1		pH 7.9 ± 0.1	
	phos-phate	acetate	phos-phate	citrate	phos-phate	NH_3—NH_4Cl
None	0.20	0.20	0.25	0.31	0.17	0.18
D,L-α-Tocopherylphosphate	0.07	0.07	0.25		0.18	
Laurylsulphate	0.12		0.16		0.10	
Heparin	0.16		0.28		0.16	
Chitin disulphuric acid		0.20		0.30		0.17
Cellulose trisulphuric acid		0.21		0.29		0.17
Cetylpyridinium chloride	0.13		0.13		0.08	
Toluidine blue		0.16	0.19	0.23	0.14	0.13
Janus green		0.21	0.19	0.28	0.15	0.14
Laurylamine	0.18		0.19		0.15	0.17
Pulmin	0.21		0.28		0.15	
Soy bean inhibitor	0.21		0.26		0.15	
Serum		0.20	0.24		0.15	

with cetylpyridinium chloride was made reversible with 1 ml buffer solution containing 0.12 g laurylsulphate. In other experiments (pH 3.5) the inhibitory effect of heparin and chitin disulphuric acid could be reversed by the addition of 0.12 g cetylpyridinium chloride in 1 ml buffer. These results are summarized in Table 2.

The addition of an excess of substrate did not suffice to prevent the inhibition. This was the case when the substrate concentration was changed from the ordinary 4.75 to 9.50 and 14.25 %. Results of such experiments are presented in Table 3.

Effect on amylase: The experimental arrangement was as described above for fructosidase. 4 ml amylase solution was mixed with 2 ml 0.2 *M* buffer solution and incubated for 30 secs. with 2 ml inhibitor solution and 2 ml dist. water. 5 ml of the mixture was added to a substrate solution containing 25 ml fresh prepared 1 % solution of soluble starch (Baker's), 5 ml 0.2 *M* buffer solution and 1 ml 0.8 *M* sodium chloride. The enzymatic reaction was followed every minute for 6 mins. by the iodine method as described by Blom *et al.*[11] The change in ml 0.1 *N* thiosulphate solution per min. ($\triangle$ ml/$\triangle$ *t*) was constant and was calculated from the straight line graph obtained. Blank values were small and were used for correction. Table 4 shows the results.

On the acid side (pH 5.9) of the activity optimum (pH 6.9)[12] and near the isoelectric point (pH 5.1—5.9)[13] of the enzyme, inhibition was produced by tocopherylphosphate, laurylsulphate, (heparin) and cetylpyridinium chloride. With a neutral reaction (pH 6.9) inhibition was found with laurylsulphate and cetylpyridinium chloride. A slight inhibition was possibly also produced by the other bases tried. Very similar results were seen at pH 7.9.

DISCUSSION

These results deviate considerably from those obtained in the previous investigations of the inhibitory effects on proteases[1, 2]. Such a difference was expected as the nature of the enzymes and their substrates differed so much in the two series. The difference was apparent especially in the case of the ampholytes, which are very potent inhibitors of proteases, but which were without effect here, with the exception of pulmin, which had an inhibitory action on fructosidase in acid reaction (Table 1). It is also interesting that the polysaccharide sulphuric acid esters (heparin, chitin disulphuric acid, cellulose trisulphuric acid), which are known as potent inhibitors towards a number of proteolytic enzymes, were almost without effect in the systems here investigated. Only in very acid reaction (pH 3.5) with the fructosidase system was a pronounced effect observed. The acids were the most potent inhibitors in acid reactions. Similarly the bases were most active in the alkaline region. These results are in agreement with the inhibitory experiments of Myrbäck[14] and Quastel *et al.*[15] This may be an indication that the inhibition is caused by compound formation between the enzyme and the inhibitory substance by means of an ionic reaction. This is further strengthened by the velocity of the reaction, which is complete in a very short time. The reversibility of the inhibition on addition of an oppositely charged compound as seen in Table 2 is also evidence in these cases of salt formation between the enzyme and its inhibitor. A simple protein denaturation does not seem to occur under the conditions used here. — The interaction times were kept short in order to avoid secondary denaturing reactions. — When the reaction times were prolonged it was found that some denaturation of the enzyme could occur, because the decrease in activity could not be completely reversed by the addition of the appropriate neutralizing agent. Acid reaction and the surface active acids especially promoted this non-specific decrease in activity.

It was found that the inhibitory effect of the carbohydrate sulphuric acids, which may react with amino groups could be reversed by the addition of cetylpyridinium chloride (fructosidase pH 3.5). This was not so pronounced with an excess of substrate, though it was shown by Myrbäck[14] that an amino group in the enzyme is of importance in the enzyme substrate binding of fructosidase.

Though this investigation was of an orientating nature one may say that carbohydrate splitting enzymes, supposed to act on uncharged carbohydrate molecules, behave differently towards a number of inhibitory substances than is the case for proteases and protease inhibitors.

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Die UV-Absorption von Titandioxyd

Ein Beitrag zur Frage der Photosensibilisierung durch Titandioxyd

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Nach einer Übersicht über das lichtschädigende Verhalten von Titandioxyd-Pigment wird das Absorptions- und Reflexionsverhalten von Titandioxyd im sichtbaren und ultravioletten Spektralbereich näher untersucht. Es wird eine Absorptionsstelle bei etwa 3 500 Å beobachtet; das zugrundeliegende chromophore System ist jedoch nicht eindeutig identifizierbar. Ein unmittelbarer und eindeutiger Zusammenhang zwischen Reflexionsverhalten und Aktivität — hinsichtlich Lichtschädigungswirkung — scheint nicht zu bestehen. Es wird noch zu prüfen sein, ob Licht der Wellenlänge 3 500—3 750 Å, welches im Sonnenlicht vorkommt und auch Fensterglas noch durchdringt, eine erhöhte Lichtschädigung auf spinnmattierte Fasern ausübt.

Titandioxyd hat in den letzten Dezenien steigende Anwendung in der keramischen Industrie sowie als Weiss- bezw. Mattpigment in der Lack-, Kunstharz- und Reyonfabrikation sowie Papierindustrie [1] gefunden.

Für die Reyonmattierung stellt z.B. Titandioxyd heute immer noch ein unübertroffenes Mattpigment dar, doch darf jetzt wohl als erwiesen gelten, dass dasselbe eine mehr oder minder starke Beschleunigung der Lichtschädigung der Fasern hervorruft. Ausser Zweifel tritt dieser Effekt in Erscheinung, wenn Mattfasern eines feinen Titers vorliegen und die Gewebe stärkerer Lichteinwirkung ausgesetzt sind, wie dies z.B. bei Gardinenstoffen der Fall ist [2].

Bei Lack ist das Pigment für das »Kreiden» des Anstriches [3] verantwortlich und auch hier weiss man, dass verschiedene Titandioxyd-Pigmente sich unterschiedlich verhalten können. (Nicht nur nichtkreidene [freikreidende] Titandioxyd-Farben sind heute gesucht, sondern auch »kontrolliert kreidende» [3].) Zweifellos bestehen zwischen beiden Effekten — der Lichtschädigung spinnmattierter Fasern und dem Kreiden — enge Zusammenhänge, in die man allerdings noch wenig Einblick hat. Auch in der elektrokeramischen Industrie werden starke Güteunterschiede bei Titandioxyd-Rohstoffen beobachtet, ohne dass man die letzten Ursachen einwandfrei kennt. Bei keramischen Glasuren treten öfter Phototropieeffekte [4] auf. Kunstharzartikel, die Titandioxyd enthalten, können u. U. irreversible Verfärbungen durch Sonnenbestrahlung erleiden.

Im Vordergrund unseres Interesses stand die Aufklärung der Ursachen der *erhöhten* Lichtschädigung der Cellulose durch Titandioxyd-Mattpigmente [5] und die Möglichkeit einer raschen Klassifizierung der Pigmentaktivität [6] sowie eine eventuelle Herabsetzung derselben. Eng mit dieser Frage verbunden sind allgemeine Beobachtungen einer starken Oxydationswirkung von Titandioxyd auf viele organische und anorganische Substanzen, wenn sie mit UV-Licht bestrahlt werden. (z. B. Photolyse der Aminosäuren nach Giri u. Mitarb.)

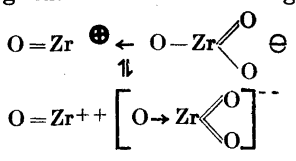
Im Zusammenhang mit diesen Fragestellungen sei zusammenfassend vorausgestellt dass in der Sprache von Jacobsen, Goodeve und Kitchener [5,7] sowohl Anatas alsauch der strukturdichtere, stabile (Goldschmidt) und somit etwas weniger aktive Rutil [8] »Photosensitizer» sind. Möglicherweise besteht die Auffassung von Renz [9] zu Recht, dass eine partielle Photoreduktion zu einem niederen Oxyd vorausgeht (die Energieunterschiede zwischen den Wertigkeitsstufen sind gering) unter Abgabe aktiven (atomaren) Sauerstoffs, wobei das Pigment im Sinne eines phototropen Prozesses wieder in den Ausgangszustand zurückkehren kann. (Ähnliche Effekte treten auch bei anderen Oxyden auf. Gut studiert ist das photosensibilisierende Verhalten von ZnO.) Für die graduellen Unterschiede dürfte das Ausmass der Gitterstörungen (inkl. O-Defizit) und des Fremdstoffeinbaues in den Kristall bei vorgegebener innerer Oberfläche (die bei den normalen Mattpigmenten nach unseren Messungen etwa 140 m^2/cm^3 beträgt [10] und bestimmter Modifikation massgebend sein.

Da die Effekte nur bei Belichtung auftreten, ist es immerhin denkbar, dass gewisse Beziehungen zwischen der Absorption bezw. Reflexion des Pigments und der Lichtschädigung bestehen. So wird von Goodeve, Kitchener und Rao angenommen, dass die Sensibilisierung am wirksamsten im Absorptionsgebiet ist und Dantuma [11] diskutiert eine solche Relation zwischen Reflexionsverhalten und dem Kalken (Kreiden) des Pigments.

Über die Absorption von Titandioxyd ist jedoch wenig bekannt. Cronemeyer [12] hat Rutil im Sichtbaren und UR vermessen (Abb. 1) und findet zu Beginn des UV-Gebietes einen starken Absorptionsanstieg, auf welchen qualitativ auch einige andere Autoren hingewiesen haben. So ist nach Jura Titandioxyd zwischen 2 100 and 3 200 Å undurchsichtig. Nach anderen Angaben liegt die langwellige Absorptionsgrenze bei etwa 3 970 Å. Einen Abfall des Reflexionsvermögens im UV beschreibt Lukisch [13].

Bei sehr oberflächlicher Betrachtung würde man Farblosigkeit bis ins fernere Quarz-UV für Titandioxyd annehmen und annähernd konstante Reflexion erwarten. Nun darf aber nicht übersehen werden, dass die Elemente Ti und Zr »Übergangselemente» sind, als solche der »Eisenreihe» bezw. »Palladiumreihe» (nach Thomson-Bohr) angehören und Farblosigkeit daher nicht unbedingt im UV-Gebiet angenommen werden darf. Insbesonders wird bei TiO_2 ein merkbares Sauerstoffdefizit gefunden (bis $TiO_{1,9}$) und mit spurenweisen Auftreten niederer Oxydationsstufen ist stets zu rechnen. Ti^{3+} ist aber bereits in konzentrierten Lösungen (unsymmetrisches, umgebendes elektrisches Feld) bekanntlich nicht mehr farblos [14]. $TiO_{1,9}$ zeigt starke Gitterdeformation und die Farbe stahlblau bis dunkelblau (vgl. auch [15]).

Im Gegensatz dazu ist Zirkonoxyd sehr stabil und unter irdischen Verhältnissen ist *nur* ZrO_2 bekannt. Bei den Lang'schen Testmethoden [6] wird praktisch keine Aktivität beobachtet. Für Zirkonoxyd wird folgende Strukturformel vorgeschlagen [16]:



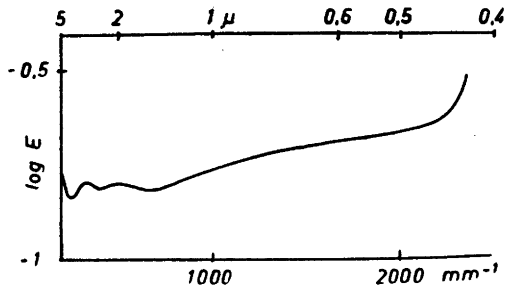


Abb. 1. UR-Aufnahme eines synth. Rutilplättchens nach Cronemeyer [12]. (E = Extinktion).

Ähnliche Bindungsverhältnisse dürfen vielleicht auch bei Titandioxyd in Betracht gezogen werden.

Die Frage nach der eigentlichen Absorption von Titandioxyd wird aber auch noch dadurch experimentell sehr erschwert, dass die Anwesenheit von Fremdmetallspuren kaum auszuschliessen ist. Nach Weyl und Förland [14] werden solche Ionen unter starker Deformation eingebaut, wodurch weitere

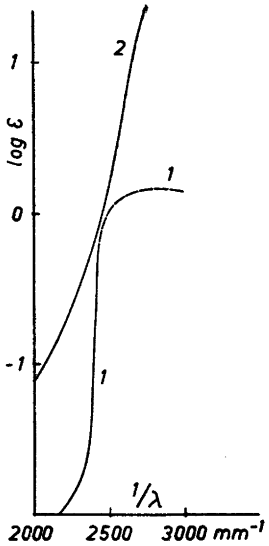


Abb. 2. UV-Absorption von: Kurve 1. synth. Rutil (Code MP-927). Kurve 2. natürl. Anataskristall, Dünnschliff, mit Fremdmetallen verunreinigt. (ε = molarer, dekadischer Extinktionskoeffizient).

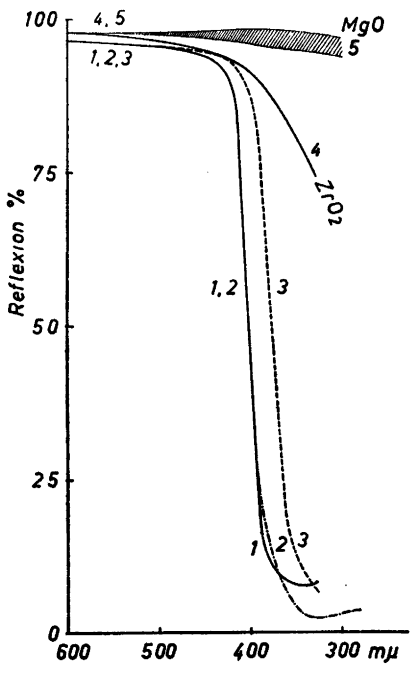


Abb. 3. Reflexionsmessungen an Titandioxyd-Pigmenten: Kurve 1. Titandioxyd-Pigment RN. Kurve 2. Titandioxyd-Pigment R. Kurve 3: Titandioxyd-Pigment A. Kurve 4. Zirkonoxyd. Kurve 5. Bezugssubstanz MgO nach [17].

sehr starke Farbeffekte entstehen. Kationen mit einer Wertigkeit < 4 sollen z.B. für die Gelbfärbung verantwortlich sein.

Wir mussten uns daher begnügen, Messungen an optisch einwandfreien Kristallplättchen synthetischer reinster Rutile durchzuführen. Die Reflexionsmessungen wurden an handelsüblichen Mattpigmenten gemacht. Wieweit die gefundene Absorption dem reinen Titandioxyd zukommt, ist aus obigen Gründen schwer entscheidbar; doch glauben wir, dass diese Entscheidung für manche Fragen im Zusammenhang mit der Lichtschädigung nicht unbedingt nötig ist.

EXPERIMENTELLES

Zur Messung kamen 2 synthetische, reinste, optisch völlig klare Rutilplättchen mit 1 mm Dicke. Die Farbe in dicker Schicht war schwach gelblich. Die Dichte betrug 4,237. Die Bestimmung der Absorption erfolgte nach verschiedenen Messmethoden.

Für die Reflexionsmessungen wurden 3 Pigmente der deutschen Titangesellschaft mit den Bezeichnungen A, R und RN herangezogen, welche im Reflexionszusatz zum Beckman-Spektrophotometer vermessen wurden. Als Bezugsnormal diente reinstes Magnesiumoxyd, welches im Messbereich stets mehr als 93 % Reflexion aufweist [17].

ERGEBNISSE

Die Absorption setzt, wie Abb. 2 zeigt, am kurzwelligen Ende des sichtbaren Spektralgebietes sehr steil ein und wird mit normalen Messmethoden bei obiger Schichtdicke zu Beginn des Quarz-UV bereits unmessbar. Mit einer besonderen Messanordnung wurde noch der Bereich von 2 400 bis 2 900 mm^{-1} vermessen, doch sind die erhaltenen Werte (gestrichelt gezeichnet) mit grosser Unsicherheit behaftet. Trotzdem glauben wir daraus entnehmen zu dürfen, dass ein sehr flaches Absorptionsmaximum bei 2 530—2 800 mm^{-1} vorhanden ist. Es wird nun zu prüfen sein, ob Licht dieser Wellenlänge eine besondere Schädigung titanmattierter Spinnfasern hervorruft.

Analog diesem Befund wurde am kurzwelligen Ende des sichtbaren Spektralbereiches eine sehr starke Abnahme der Reflexion gefunden mit einem mutmasslichen, schwach ausgeprägtem Minimum bei etwa 350 $m\mu = 2\,860$ mm^{-1}. Die drei Pigmente, die sich hinsichtlich der Aktivität unterscheiden (A: $\sim$ 99,5 % Titandioxyd, kreidend; RN: $\sim$ 97,5 % Titandioxyd mit Zusätzen [Aluminiumhydroxyd?), inaktiv; R: sehr reines Titandioxyd mit $\sim$ 99,8 % Titandioxyd, inaktiv), zeigen merkbare Differenzierungen (Abb. 3), jedoch ist ein unmittelbarer Zusammenhang zwischen Ausmass der Aktivität und dem Reflexionsvermögen nicht erkennbar.

Annähernd derselbe Kurvenverlauf, den Abb. 3 (Kurve 3) zeigt, wurde auch bei einem Titandioxydpräparat von Baker, USA (99,98 % TiO_2) erhalten und dieser änderte sich bei weiterer Reinigung nur unwesentlich ($\sim$ 3 %). (Das gereinigte Pigment erwies sich als ziemlich inaktiv).

Bemerkenswert ist der Umstand, dass das inaktive Zirkonoxyd im vermessenen Spektralbereich einen wesentlich schwächeren Reflexionsverlust aufweist, so dass die Vermutung besteht, dass die Reflexionsminderung bei Titandioxyd vornehmlich dem 0-Defizit und Fremdmetallspuren zuzuschreiben ist.

Wir danken zum Schluss der National Lead Comp., Titanium Div., South Amboy, USA, der deutschen Titangesellschaft Leverkusen, der österreichischen Gesellschaft für Holzforschung und den optischen Werken C. Reichert, Wien, für die gewährte Unterstützung.

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The Oxidation of Glycosides

IV*. Oxidation of Methyl β-D-Glucoside with Dichromate

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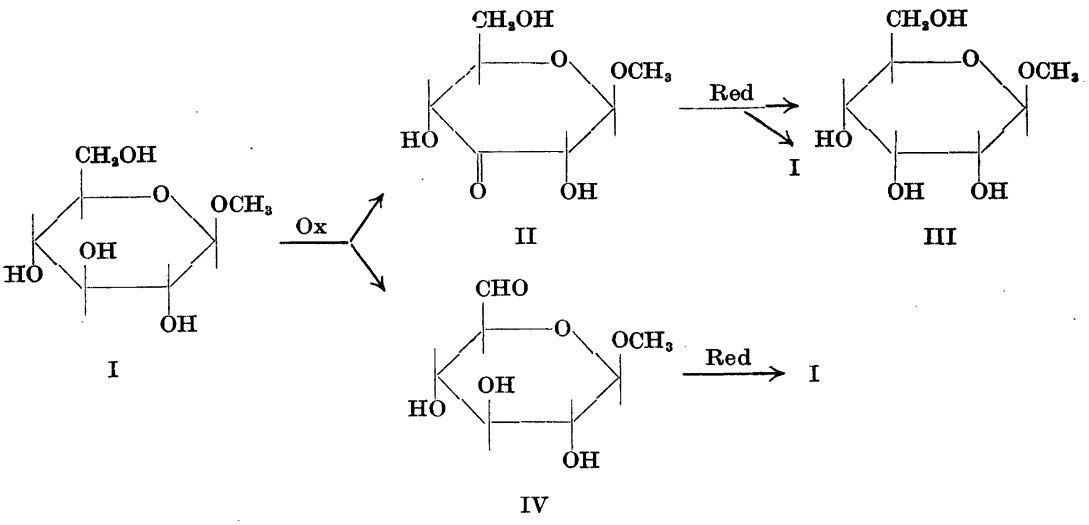
Methyl β-D-glucopyranoside has been oxidized with dichromate in the presence of oxalic acid, and from the resulting mixture two carbonyl derivatives have been isolated. One of these, a crystalline product, has the structure of methyl β-D-3-ketoglucopyranoside. On reduction it yields a mixture of methyl glucoside and methyl alloside, which proves the position of the keto group. The other substance, methyl β-D-6-aldehydoglucopyranoside, is amorphous and on reduction yields methyl β-glucoside only. The substances are of interest as models for oxycelluloses.

As part of our program on the study of oxycelluloses, the oxidation of methyl β-D-glucopyranoside (I) with dichromate has been investigated. It is known that when cellulose is oxidized with chromate in the presence of oxalic acid[1], an extreme type of oxycellulose with high copper number and practically no carboxyl groups is obtained, and as we were interested in the carbonyl derivatives, this oxidizing agent was used. From the neutral part of the reaction mixture, after separations on carbon and thick filter paper (See Experimental) two oxidized products were isolated.

The first product (II) was crystalline, m.p. 127—128° and specific rotation −68° in water. It was strongly reducing and gave positive reactions with 2,4-dinitrophenylhydrazine as well as with typical sugar reagents. The analyses were consistent with the formula $C_7H_{12}O_6$, that is a methyl glucoside with one of the hydroxyl groups oxidized to a carbonyl group. On reduction with Raney nickel[2] it yielded a mixture of methyl β-glucoside and another substance (III), m.p. 150—151° and specific rotation −53° in water. With the carbonyl group in C_2, C_3 or C_4, a mannoside, alloside or galactoside, respectively, should be formed, but the data for the substance do not agree with those for methyl β-mannoside or methyl β-galactoside. On hydrolysis the substance (III) yielded a reducing sugar with colour reactions and R_F-values in different

* Part III. The Chlorine Oxidation of Glycosides. *Acta Chem. Scand.* **7** (1953) 280.



solvents indistinguishable from those of allose. Methyl β-D-allopyranoside (III) has not been described previously in the literature, but the data in Table 1 show that the rotation observed for the substance is in good agreement with that expected.

Table 1. Specific rotations of some β-hexoses and their methyl β-glucosides.

Hexose	$[\alpha]_D$ of free sugar in β-form	$[\alpha]_D$ of methyl β-glycoside	Δ
D-Glucose	19	−34	53
D-Galactose	54	0	54
D-Allose [a]	1	−53 [b]	54

[a] Ref.[3] [b] Present investigation.

It is consequently proved that II has the structure of methyl β-D-3-keto-glucopyranoside, and by these reactions an interconversion from the D-glucose to the D-allose series has been accomplished.

The other product (IV), an amorphous powder, was also strongly reducing and gave positive reactions with 2,4-dinitrophenylhydrazine and sugar reagents. It gave two spots and considerable trailing between them on the paper chromatogram, but when either of these was cut out, extracted and rechromatographed, the same picture was obtained. A probable explanation of this behaviour is that the substance occurs in different forms, the equilibrium between which is set up rather slowly. This rendered the purification of it difficult and it is questionable whether the substance is quite pure. In contra-

distinction to the first substance (II) it gave positive reaction with Schiff's reagent and on reduction yielded methyl β-glucoside only. These facts, as well as the result of the periodate oxidation of the substance, are in agreement with the structure of methyl β-D-6-aldehydoglucopyranoside (IV). The percentage of IV in the amorphous powder, which probably contained small amounts of salts and water, was estimated to be 79 % by hypoiodite oxidation. Similar substances, methyl glycosides of D-mannohexodialdose, have recently been prepared by Ballou and Fischer[4].

As considerable losses could not be avoided during the isolation of the substances described above, it is difficult to estimate how much of the total oxidized, non-acidic material they represent. It seems, however, as if they are the two most important constituents and that the yield of the aldehyde derivative is the higher. The presence of both aldehyde and keto groups in oxidized cellulose and starch have been demonstrated analytically by Purves and coworkers[5,6]. As they point out, there is no definite proof that the aldehyde groups occupy the sixth position, but they regard it as very probable, and this is strongly supported by the result of the present investigation. They have also proved that a considerable part of the carbonyl groups, at least 17 % in an oxidized starch, occurred as 2-keto glucose residues. In the present investigation no 2-keto glucoside was isolated but the corresponding 3-keto derivative isolated amounted to about 15 % of the total carbonyl derivatives. Both substances described above are thus of interest as model substances for oxycelluloses and will be further investigated in this respect.

EXPERIMENTAL

Melting points uncorrected. Evaporations performed under reduced pressure and a bath temperature of 40°. Whatman 1 and Whatman 3 MM filter papers used for paper chromatography. Solvents used:

A. Butanol-ethanol-water, 5:1:4.
B. Ethyl acetate-acetic acid-water, 3:1:1.
C. Butanol-pyridine-water, 3:1:1.5.

Oxidation of methyl β-glucoside and fractionation of product. Methyl β-glucoside (30 g) was dissolved in a mixture of 0.1 *N* potassium dichromate (1 300 ml) and 0.5 *N* oxalic acid (1 300 ml). After 20 hours at room temperature excess of calcium carbonate was added and the mixture stirred overnight, filtered, the solids washed with water of 50° and the combined filtrates concentrated to 100 ml. The solution was then diluted with 90 % ethanol (300 ml) and kept at 5° for 4 hours. The precipitated chromium salts were removed by filtration and washed with 75 % ethanol. By this treatment practically all the chromium salts were removed and no neutral carbonyl compounds could be found in the precipitate. The filtrate and washings were concentrated to a thick sirup, from which unchanged methyl β-glucoside (22 g) could be recovered by crystallisation from ethanol. From the mother liquors a thick sirup (8 g) was obtained by concentration. The recovered methyl β-glucoside plus fresh material (8 g) was oxidized and worked up as described above. This procedure was repeated a third time.

The combined sirups (25 g) were dissolved in 1 % aqueous ethanol (100 ml) and added to the top of a column (50 × 6 cm), built up of equal parts of carbon (Darco 60) and Celite. The column was eluted with aqueous ethanol, 6 000 ml, the concentration of which was continuously increased from 1 % to 8 %, then 8 000 ml, 8—20 %, then 8 000 ml, 20—30 %, and finally 4 000 ml 75 % ethanol. The eluate was divided into fractions and investigated by paper chromatography. The main amount of the sirup (19.1 g) eluted with 1—6 % ethanol, consisted of unchanged methyl-β-glucoside, which was almost

completely separated from the carbonyl compounds. There were also small amounts of glucose and chromium salts in this fraction. The carbonyl compounds (1.7 g) were eluted with 6—10 % ethanol. The material which appeared after 10 % ethanol (1.0 g) was a complex mixture, probably containing only small amounts of mono-carbonyl compounds. Attempts to separate the carbonyl compounds on cellulose columns were not successful. However, a separation was accomplished using thick filterpaper. About 2 mg per cm paper (Whatman 3 MM) was used and the chromatograms were run for 7 hours in solvent B, dried and then run for 7 hours in solvent A. The substances were located and isolated in the ordinary manner. From the separation on thick paper the ratio of substance II to IV was calculated to be 1:4.

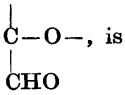
Methyl β-D-3-ketoglucopyranoside (II). The eluate from the thick papers containing methyl β-D-3-ketoglucopyranoside was deionised by passing it through the ion exchange resins Amberlite IR 120 and IR 4B and concentrated to dryness. The residue, which crystallised and amounted to about 15 % of the total carbonyl compounds, was recrystallised from ethanol. M. p. 127—128°. $[\alpha]_D^{20}$ −68° (water, c = 2). (Found: C 43.9; H 6.25; OCH_3 15.1. Calc. for $C_7H_{12}O_6$ (192.2): C 43.7; H 6.29; OCH_3 16.1).

On periodate oxidation with 0.1 *M* solution, buffered with acetate to pH 4.0, at room temperature, the substance overnight consumed 0.7 moles of reagent. By oxidation with sodium metaperiodate under similar conditions, 1.0 moles of acid were formed. (These oxidations, as well as those described below, were made with a very small amount of material and thus a better agreement with the calculated values, 1 mole periodate and 1 mole acid, could hardly be expected.)

The $R_{glucose}$-values for the substance were 1.98 (Solvent A), 1.82 (B) and 2.70 (C). The corresponding values for methyl β-glucoside were 1.77, 1.67 and 2.02. Colour reactions: Anisidine phosphate, first yellow then greyish brown; Aniline hydrogen phthalate, greyish brown; Resorcinol-hydrochloric acid, dark brown.

Hydrogenation of II. II (45 mg) was dissolved in 70 % ethanol (5 ml) and Raney nickel (500 mg) was added. The mixture was refluxed on the steam bath for 3 hours, filtered and concentrated. A small amount of the reaction product was hydrolysed with 0.1 *N* hydrochloric acid at 100° overnight. The hydrolysate gave two spots on the chromatogram, indistinguishable from those of glucose and allose. Crystalline methyl β-D-alloside separated when the product of hydrogenation was dissolved in ethanol and was purified by recrystallisation from the same solvent. Yield, 16 mg. M. p. 150—151°. $[\alpha]_D^{20}$ −53° (water, c = 2). From the mother liquors a small amount of pure methyl β-glucoside (6 mg) was isolated. M. p. 109—110°, undepressed on admixture with authentic material.

The methyl alloside on hydrolysis yielded only one reducing sugar, chromatographically indistinguishable from allose. On periodate oxidation the alloside consumed 1.7 moles of reagent, under the formation of 0.9 moles of acid. The required values are 2 and 1 respectively.

Methyl β-D-6-aldehydoglucopyranoside (IV). The purification of IV was rather difficult as it gave several spots with trailing between them on the chromatogram. The eluates from the thick filter paper were concentrated without previous ion exchange and thus the amorphous product contained some salts from the paper in addition to water. The amount of this substance was about 60 % of the total carbonyl compounds. The product consumed hypoiodite[7] corresponding to a purity of 79 %. On periodate oxidation it consumed 4.6 moles of reagent (corrected to pure substance). As a group containing "active" hydrogen,  , is formed during the oxidation[8], a consumption of 5 moles is required by IV.

The $R_{glucose}$-values for the substance were 0.73, 2.35 (A), long band with centre at 1.05 (B) and 0.98, 2.90 (C).

Colour reactions: Anisidine phosphate, first yellow, then reddish brown; Aniline hydrogen phthalate, reddish brown; Resorcinol-hydrochloric acid, pink.

IV (20 mg) on reduction with Raney nickel as described above, yielded pure methyl β-glucoside (6 mg), identical with authentic material. Glucose was the only reducing sugar detected in the hydrolysate of the crude reaction product.

Acknowledgement. The authors are indebted to *Statens Tekniska Forskningsråd* for financial support and to Dr. Emma J. McDonald, National Bureau of Standards, Washington, for a gift of D-allose.

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ACTA CHEMICA SCANDINAVICA 8 (1954) 1875—1876

Low-molecular Carbohydrates in Algae

VI *. Laminitol, a New C-Methyl Inositol from *Laminaria cloustoni*

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A C-methylinositol, laminitol, has been isolated from the brown alga *Laminaria cloustoni*. It is isomeric but not identical with mytilitol, another C-methylinositol, obtained from the mussel *Mytilis edulis*.

An investigation of the low-molecular carbohydrates of *Laminaria cloustoni* was reported in Part V of this series. One of the substances isolated was not identified. It was fast on the carbon column but had a low R_F-value, only slightly higher than that of *d*-inositol with the ethylacetate-acetic acid-water (3 : 1 : 1) mixture. Finally it reduced the silver nitrate-sodium ethoxide reagent[1] strongly, though not spontaneously like the reducing sugars. We suggested that the substance might be a cyclitol, and it has now been subject to further investigations.

The analysis of the substance, after drying, was consistent with the formula $C_7H_{14}O_6$. When the substance was dried, water, corresponding to two moles of water of crystallization, was lost. It consumed 6.5 moles of periodic acid with formation of 5.2 moles of acid. Its acetate was shown by analysis to be a hexaacetate. Finally, the presence of a C-methyl group was demonstrated by analysis according to Kuhn-Roth[2]. The only formula in agreement with these analyses is that of a C-methylinositol.

Two C-methylinositols have been described previously. One, mytilitol (I) has been isolated from the mussel *Mytilis edulis*[3] and the configuration has been determined by Posternak[4]. The other, *iso*mytilitol (II) is a synthetic product, prepared by Posternak[4]. From the melting points of our substance, mytilitol, *iso*mytilitol and their hexaacetates, 266—269°, 266—268°, 225—226°, and 151—152°, 181°, 188—189°, respectively, it is evident that our substance is not identical with either of these, but is a new natural product, for which we suggest the name laminitol. Further, laminitol is much faster than mytilitol on the paper chromatogram and the m.p. of its hexaacetate was not raised on recrystallisation and seeding with mytilitol hexaacetate.

* Part V, *Acta Chem. Scand.* 8 (1954) 1547.

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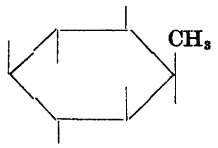
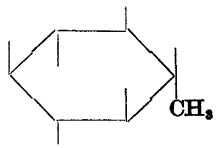
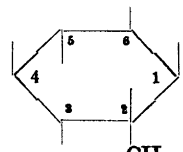
Four of the nine possible *cyclo*hexanehexols are naturally occurring, *d*- and *l*-inositol, *meso*-inositol and scyllitol. Of these *meso*-inositol is the most widespread. It seems reasonable to assume



I



II



III

that laminitol should have the same configuration as one of these. It is optically active, $[\alpha]_D - 3°$, but the low value for the rotation agrees better with an unsymmetrically substituted *meso*-inositol (2-methyl *meso*-inositol, III, or the corresponding 3-methyl derivative, IV) than with a *d*- or *l*-inositol derivative. This possibility is especially attractive, because it provides us with a potential biochemical relationship between laminitol and mytilitol. The hydroxyl group at C_1 is axial and known to be oxidized under biochemical conditions (*Cf. e.g.* Ref.[5]) and the carbonyl derivative formed could give a mixture of mytilitol (I) and III or IV, resp., on subsequent reduction.

The configuration of laminitol and its potential occurrence in other algae is under investigation. Provided that laminitol is a constituent of microscopic algae serving as food for *Mytilus* there may be a direct casual relation between laminitol and mytilitol.

EXPERIMENTAL

(Melting points uncorrected.)

Pure laminitol was prepared by deacetylation of the acetate. Paper chromatograms and m. p. determinations showed that the substance was not affected by the acetylation-deacetylation. It was recrystallised from aqueous ethanol. M. p. 266–269° (Slight decomp.) $[\alpha]_D^{20} - 3°$ (water, $c = 2$). On drying at 110° 15.9 % water was lost. Calc. for $C_7H_{14}O_6 \cdot 2H_2O$ (230.2) 15.6 %. (Found: C 42.9; H 7.27; C–CH_3 4.92. Calc. for $C_7H_{14}O_6$ (194.2): C 43.3; H 7.27; C–CH_3 7.73).

By analysis of lauric acid, under identical conditions[6] 4.52 % C-methyl was obtained, compared to the calculated value, 7.52 %. On periodate oxidation with 0.1 *M* solution, buffered with acetate to pH 4.0, at room temperature and over-night, the substance consumed 6.5 ± 0.1 moles periodate. By oxidation with sodium metaperiodate under similar conditions, 5.2 ± 0.1 moles acids were formed.

Laminitol hexaacetate. By acetylation of laminitol with acetic anhydride, using either pyridine (equal part) or conc. sulphuric acid (1 %) as catalyst, the same acetate was obtained in good yield. It was purified by recrystallisation from ethanol. M. p. 151–152° $[\alpha]_D^{20} - 19°$ (chloroform, $c = 2$). (Found: C 51.1; H 5.70; $COCH_3$ 59.0. Calc. for $C_7H_8O_6(OCCH_3)_6$ (446.4): C 51.1; H 5.87; $COCH_3$ 57.8).

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ACTA CHEMICA SCANDINAVICA 8 (1954) 1877—1879

Ion Exchange Chromatography of Nucleoside Polyphosphates

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Ion-exchange chromatography of the mono-, di- and triphosphates of adenosine, guanosine, inosine and uridine on Dowex-1 formate columns by elution with formic acid containing successively increased amounts of formate, is described.

Separation of the different adenosine-5′-phosphates, *viz.* adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP) by ion-exchange chromatography, has been achieved by Cohn and Carter[1] by using a column of the strong base resin Dowex-1 in the chloride form and elution by dilute hydrochloric acid, containing successively increased amounts of alkali chloride. Application of the method to the resolution of analogous series of polyphosphates, derived from other nucleosides, gives equally satisfactory results, as shown in the case of the inosine polyphosphates[2]. Resolution of mixtures containing both series of polyphosphates could even be accomplished by slight modification of the original method[2].

The isolation[3] of guanosine triphosphate (GTP) and uridine triphosphate (UTP) from rabbit skeletal muscle made further changes in the method necessary, in order to render it adaptable to the chromatography of mixtures containing polyphosphates of the different nucleosides, as they occur in muscle extracts. As previously reported, GTP and UTP — although clearly separated from ATP — appear in the effluent in the form of a mixture of both triphosphates on chromatography in the chloride system; in the presence of ITP*, all three triphosphates are eluted at the same anion concentration in the eluent. As no effective resolution could be achieved in the chloride system, recourse was made to the use of Dowex-1 formate columns and elution with formic acid solutions, containing progressively increased amounts of formate, an anion system introduced by Cohn and Carter[4] for the chromatography of nucleic

* ITP = inosine triphosphate; IDP = inosine diphosphate; IMP = inosine monophosphate; GDP = guanosine diphosphate; GMP = guanosine monophosphate; UDP = uridine diphosphate; UMP = uridine monophosphate.

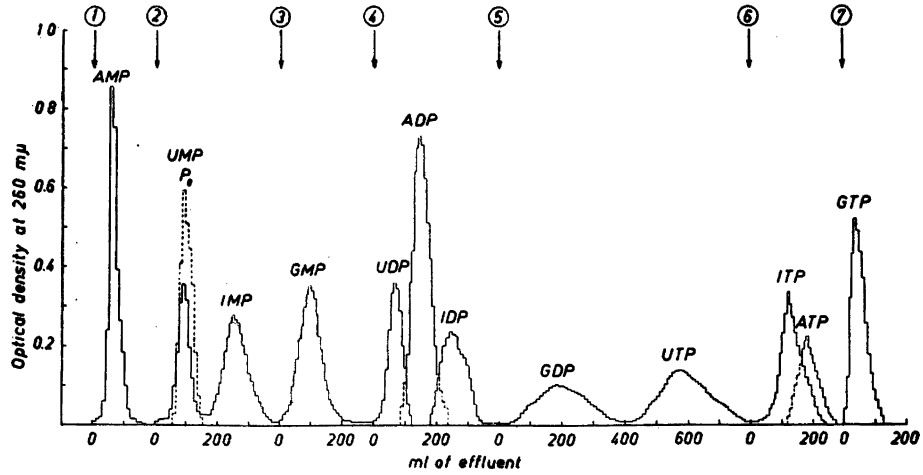

Fig. 1. Ion-exchange chromatography of nucleoside polyphosphates (for details see Experimental part).

acid fragments. Clean separation of GTP and UTP was accomplished in this system as briefly reported at the 2nd meeting of the Swedish Biochemical Society (Uppsala, November 28, 1953). Subsequently, a procedure for the separation of the mono-, di- and triphosphates of adenosine, guanosine, inosine and uridine was worked out, establishing the elution positions of the individual compounds, using Dowex-1 formate columns and formic acid—formate eluents. UMP* and orthophosphate are not separated by this method, but this can easily be achieved by other means. The ineffective separation of ATP and ITP on the other hand, necessitates a second chromatographic procedure in the chloride system for the preparative resolution of mixtures of these two triphosphates.

An ion-exchange procedure by means of a mechanically operated two-column system of anion-exchange with Dowex-1 in the formate form has been developed by Hurlbert, Schmitz, Drumm and Potter[5] for the separation of acid soluble nucleotides from tissue extracts including polyphosphates of cytidine, guanosine and uridine.

EXPERIMENTAL

Materials. The adenosine and inosine polyphosphates were prepared and purified as described previously[2]. GTP and UTP were isolated from rabbit muscle, purified by the present method and precipitated as the barium salts. GDP* and UDP* were obtained by the action[6] of actomyosin gel on the triphosphates and isolated as the barium salts. GMP* was a preparation of the barium salt, obtained by courtesy of Prof. A. R. Todd, Cambridge, and UMP was prepared by acid hydrolysis[7] of UTP. The barium salts were converted to the ammonium salts prior to chromatography, as previously described[2]. All chemicals used were of analytical grade.

Chromatography (Fig. 1). A column of Dowex-1 (250—500 mesh) in the formate form, 0.6 cm^2 × 6 cm, was used, with a flow rate 0.5 ml/min. A mixture of orthophosphate (1.160 mg), AMP (0.780 mg), ADP (1.380 mg), ATP (0.620 mg), GMP (0.970 mg),

GDP (0.780 mg), GTP (1.375 mg), IMP* (1.200 mg), ITP (1.160 mg), IDP* (1.480 mg), UMP (0.600 mg), UDP (0.770 mg) and UTP (1.320 mg) as ammonium salts at pH 11.0 was put on the column and, after washing with water, the following eluting agents were used in succession: (1) 0.1 *M* formic acid, pH 2.5; (2) 0.1 *M* formic acid + 0.05 *M* sodium formate, pH 3.4; (3) 0.1 *M* formic acid + 0.1 *M* sodium formate, pH 3.6; (4) 0.1 *M* formic acid + 0.3 *M* sodium formate, pH 4.1; (5) 0.1 *M* formic acid + 0.4 *M* sodium formate, pH 4.2; (6) 0.1 *M* formic acid + 0.5 *M* sodium formate, pH 4.3; (7) 0.1 *M* formic acid + 1.0 *M* sodium formate, pH 4.5. Recoveries, based on optical density measurements and in addition in some cases on phosphorus analysis, were at least 95 %.

Analytical procedures. Phosphorus determinations were performed as previously described[3] and optical densities were measured in a Beckman Universal Spectrophotometer, Model DU, the molecular extinction coefficients of 14 200 at 257 mμ for adenosine phosphates, 10 850 at 256 mμ for guanosine phosphates, 13 200 at 250 mμ for inosine phosphates and 10 050 at 262 mμ for uridine phosphates being used in the identification of the different fractions in the effluent (purine:total phosphorus:labile phosphorus).

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The Isolation of Uridine-5′-Triphosphate from Muscle

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The isolation of uridine-5′-triphosphate from rabbit skeletal muscle is described. The identification is based on the results of paper chromatography and ultraviolet absorption measurements on the products of hydrolysis, together with enzymic evidence and chemical analysis, electrometric titration and ultraviolet absorption spectra at different pH values, of the compound before hydrolysis.

The occurrence of ribonucleosides in the form of polyphosphate derivatives — as distinct from that in nucleic acids — was first established for adenosine, by the isolation of adenosine triphosphate (ATP) from muscle[1, 2]. Similar derivatives of the other ribonucleosides, present in nucleic acids, were not known until the isolation of uridine-diphosphate-glucose (UDPG) by Caputto, Leloir, Cardini and Paladini[3], followed by the demonstration of other derivatives[4, 5, 6] of uridine diphosphate (UDP) in biological material. The natural occurrence of uridine triphosphate (UTP), previously obtained from UDP[7] and UDPG[8] in enzymic reactions, has been established by its isolation from muscle[9]; moreover, UTP has been isolated from yeast[10] and demonstrated in various tissues[11].

UTP from muscle was first obtained by ion-exchange chromatography of ATP preparations originating from rabbit skeletal muscle. Ion-exchange chromatography of ATP on Dowex-1 or Dowex-2 chloride columns, using the chloride elution system according to Cohn and Carter[12], gave regularly a contaminating phosphate fraction amounting to 2—4 % of the ATP and appearing in the effluent by elution with *N* hydrochloric acid, after the ATP had been removed from the column by the eluting solvent of next lower anion concentration. Phosphorus analysis showed the presence of acid-labile phosphate amounting to two-thirds of the total phosphorus indicating a triphosphate of ATP type. Ultraviolet absorption measurements showing a maximum around 260 mμ, suggested the presence of a nucleoside derivative.

By application of the ion-exchange procedure directly to trichloroacetic acid extracts of muscle, similar amounts of the unknown triphosphate fraction were obtained.

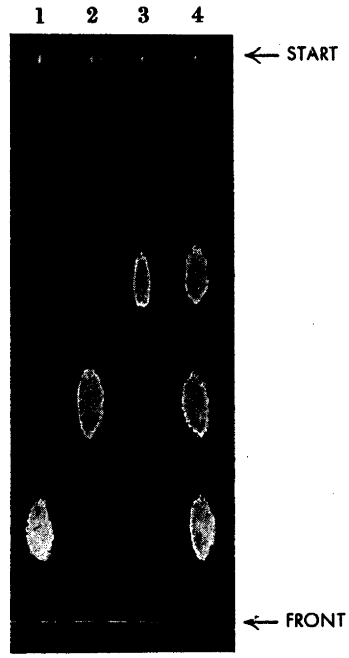

Fig. 1. Separation of ATP, GTP and UTP by paper chromatography in saturated ammonium sulphate-water-isopropanol (79:19:2), 10 hours. (1) UTP; (2) GTP; (3) ATP; (4) mixture of ATP, GTP, and UTP; approx. 50 μg of each compound.

Paper chromatography in the saturated ammonium sulphate solution-water-*iso*-propanol (79 : 19 : 2) solvent system resolves the unknown triphosphate material into two components, both triphosphates, according to phosphorus analysis, and later identified as UTP and guanosine triphosphate (GTP). The same solvent system is equally suitable for the separation of mixtures containing ATP besides GTP and UTP (Fig. 1). A separation of GTP and UTP can also be achieved by fractional reprecipitations of the mixture of barium salts, as obtained from pooled fractions of the effluent from ion-exchange columns. Ion-exchange chromatography on Dowex-1 formate in the formate system[13], however, is the method which has been used by preference for the preparation of the pure triphosphates in quantity.

The first indication as to the nature of the triphosphate ultimately identified as UTP, was obtained by measurement of the ultraviolet absorption spectra in acid and in alkali, resulting in absorption curves typical for uridine derivatives. Subsequent hydrolysis in 70 % perchloric acid for 1 hour at 100° C, followed by paper chromatography, gave one ultraviolet absorbing spot with the same R_F value as uracil, chromatographed side by side with the experimental solution in four different solvent systems (Table 1). The ultraviolet absorption curves in acid and in alkali were likewise identical with those of uracil (Fig. 2).

Hydrolysis of UTP in *N* hydrochloric acid for 1 hour at 100° C resulted in the splitting off of two-thirds of the bound phosphorus in the form of ortho-phosphate under formation of a nucleoside monophosphate, in agreement with the expected behaviour of a pyrimidine nucleoside-5′-polyphosphate. Paper

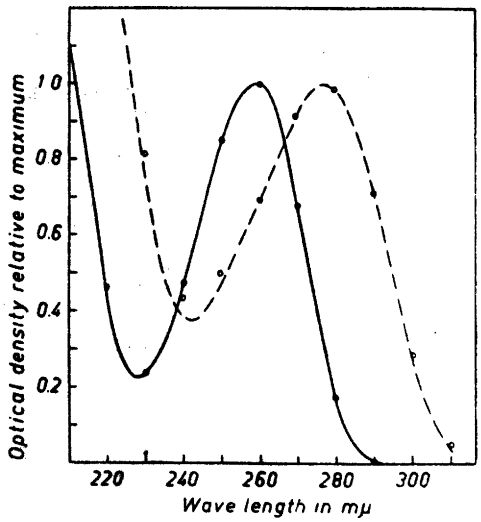

Fig. 2. Ultraviolet absorption curves for uracil obtained from UTP by hydrolysis in 70 % perchloric acid (1 hour at 100 °C) in comparison with authentic uracil in 0.1 N acid and 0.1 N alkali. ——— *uracil in acid and* — — — *in alkali.* ● *uracil from UTP in acid and* ○ *in alkali.*

chromatography of the hydrolysis products gave an ultraviolet absorbing spot with the R_F value of 5′-uridylic acid (UMP) in four different solvent systems (Table 1). Identity with UMP was likewise demonstrated by comparison of the ultraviolet absorption curves in acid, in alkali and after treatment with bromine,

Table 1. R_F values of the ultraviolet-absorbing base obtained from UTP by hydrolysis in 70 % perchloric acid (1 hour at 100 °C) in comparison with uracil, and of the nucleotide obtained by hydrolysis in N hydrochloric acid (1 hour at 100 °C) in comparison with 5′-UMP on paper chromatography. Solvent systems: (A) hydrochloric acid (sp.gr. 1.180)-isopropanol-water (17:65:18); (B) n-butanol-formic acid (sp.gr. 1.220)-water (77:10:13); (C) n-butanol-ammonia (sp. gr. 0.880)-water (84:5:14); (D) 5 % potassium dihydrogen phosphate in isoamyl alcohol. Descending chromatography was used in systems A, B and C, and ascending chromatography in D.

	R_F			
	A	B	C	D
Ultraviolet absorbing spot from UTP	0.68	0.40	0.19	0.72
Uracil	0.68	0.39	0.19	0.72
Nucleotide from UTP	0.79	0.03	0	0.84
5′-UMP	0.79	0.03	0	0.84

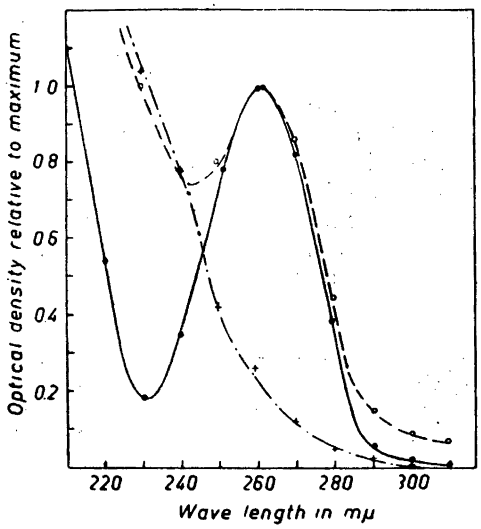

Fig. 3. Ultraviolet absorption curves for UMP obtained from UTP by hydrolysis in N hydrochloric acid (1 hour at 100 °C) compared with those for 5'-UMP in 0.1 N acid, in 0.1 N alkali and after treatment with bromine. ———— *5'-UMP in acid,* — — — *in alkali and* —·—·— *after treatment with bromine.* ● *UMP from UTP in acid,* ○ *in alkali and* + *after treatment with bromine.*

followed by aeration (Fig. 3). Location of the phosphate group in the 5'-position of UMP derived from muscle UTP was confirmed by experiments with 5'-nucleotidase[14] from snake venom (Table 2).

Pentose estimations with the orcinol reagent[15] on UTP before and after treatment with bromine[16] show colour development only in the latter case, suggesting the presence of pyrimidine-bound pentose. Hydrolysis of UTP for 2 hours in 2 *N* hydrochloric acid after treatment with bromine and paper

Table 2. Action of 5'-nucleotidase[14,6] on UMP obtained from UTP. 1 μmole nucleotide in 0.1 ml M *glycine buffer (pH 8.5) + 0.1 ml 0.1* M *magnesium chloride + 0.1 ml of a solution of Crotalus adamanteus venom (2 mg/ml) in a total volume of 1 ml were incubated for 45 minutes at 37 °C. Phosphorus was estimated after addition of 1 ml ethanol and centrifugation.*

Compound (1 μmole)	Orthophosphate in μmoles		μmoles orthophosphate formed
	time of incubation		
	0 min.	45 min.	
Adenosine-5'-phosphate	0.01	0.92	0.91
Adenosine-3'-phosphate	0.005	0.005	0
Uridine-3'-phosphate	0.007	0.013	0.006
Uridine phosphate from UTP	0	0.98	0.98

chromatography of the resulting solution in the *n*-butanol-acetic acid-water (4 : 1 : 5) system, gave a spot, indistinguishable from that obtained from D-ribose ($R_F = 0.32$) on development with aniline hydrogen phthalate.

The identification of uracil, D-ribose and the 5′-UMP moiety establishes the UTP isolated from muscle as a derivative of 5′-UMP. Chemical analysis (base : pentose : total phosphorus : acid labile phosphorus) shows it to be a triphosphate, containing two acid-labile phosphate groups attached to 5′-UMP.

Experiments on copper complex formation show that UTP forms a copper complex in analogy to other 5′-substituted nucleoside compounds[17] without substituents in the 2′- and 3′-positions of the molecule (Table 3).

Table 3. Copper complex formation[17,3]. *To 1 μmole of the sample, about 3 mg of copper phosphate and 0.5 ml of 20 % trisodium phosphate were added. The suspension was centrifuged after 90 minutes and the supernatant mixed with 1 ml of 0.5 % sodium diethyl dithiocarbamate. The copper complex was extracted with 6 ml amyl alcohol and measured in a Klett-Summerson colorimeter with filter No. 42.*

Compound (1 μmole)	Colorimeter reading
Adenosine-3′-phosphate	23
Adenosine-5′-phosphate	330
Adenosine-5′-triphosphate	320
Uridine-3′-phosphate	26
Uridine	333
Uridine triphosphate	360

Similar results were obtained in experiments on the oxidation of UTP with periodate, giving evidence of unsubstituted 2′- and 3′-hydroxyl groups in the ribose moiety of the molecule, under conditions where no splitting off of the labile phosphate groups takes place (Table 4). The results of periodate oxidation provide at the same time a quantitative measure of the pentose content of UTP.

The results of copper complex formation and periodate oxidation show that the two labile phosphate groups present in UTP cannot be attached to 5′-UMP in the 2′- and 3′-positions. Although location of the labile phosphate groups in the pyrimidine part of the molecule is not excluded by these experiments, the rates of acid hydrolysis (Fig. 4) as compared with ATP, make the formulation of UTP as a 5′-triphosphate most likely. Furthermore, electrometric titration of UTP shows the presence of three primary and one secondary phosphate groups, in harmony with the expectations for a 5′-triphosphate (Fig. 5).

Finally, comparison of the reaction[19] with actomyosin gel of UTP prepared from muscle with that of synthetic[20] 5′-UTP shows complete identity of the two compounds both in regard to dephosphorylation of UTP to the diphosphate and volume constriction of the actomyosin gel.

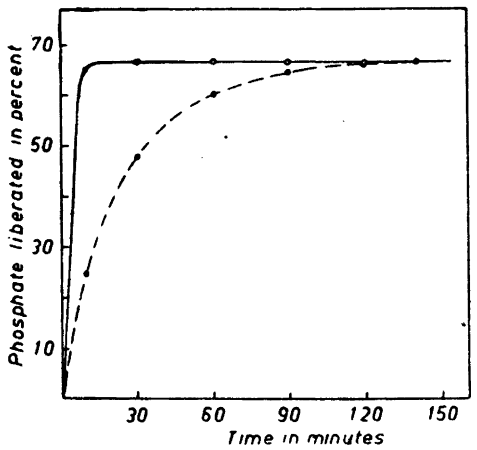

Fig. 4. Acid hydrolysis of UTP at 100 °C. ———— in N hydrochloric acid and — — — in 0.1 N hydrochloric acid.

Table 4. Periodate oxidation.[18] 0.50 μmole potassium metaperiodate was added to approx. 0.3 μmole of the sodium salt of the nucleotide in a total volume of 3 ml. The amount of periodate consumed was measured at 227 mμ against a blank containing periodate of the same concentration.

Compound	μmoles periodate		μmoles periodate consumed per μmole nucleotide
	added	consumed	
Adenosine-3′-phosphate, 0.31 μmole	0.50	0.04	0.13
Adenosine-5′-phosphate, 0.27 μmole	0.50	0.26	0.96
Adenosine-5′-triphosphate, 0.30 μmole	0.50	0.29	0.97
Uridine-3′-phosphate, 0.33 μmole	0.50	0.03	0.09
Uridine triphosphate, 0.36 μmole	0.50	0.36	1.00

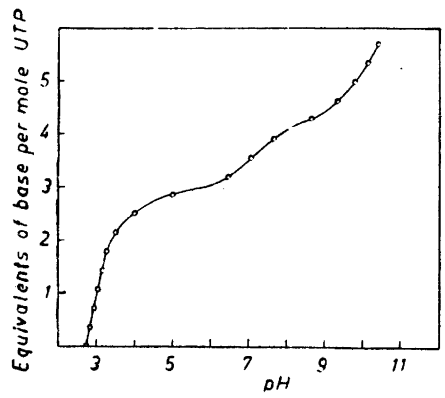

Fig. 5. Electrometric titration of UTP. 5 μmoles UTP titrated with 0.10 N sodium hydroxide.

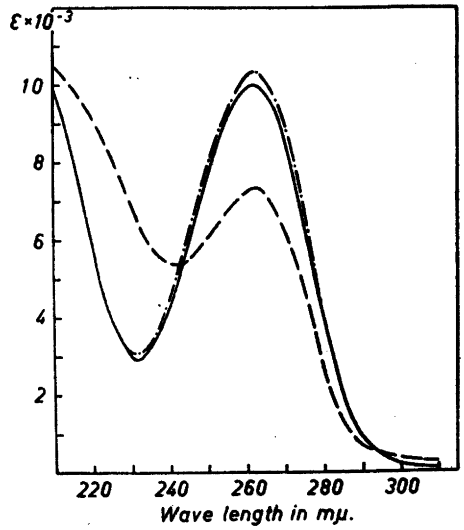

Fig. 6. Ultraviolet absorption curves for UTP from muscle. ——— *at pH 1.0,* — — — *at pH 6.5 and* —·—·— *at pH 11.0.*

On the basis of the evidence obtained, we feel justified in formulating UTP isolated from muscle as uridine-5′-triphosphate:

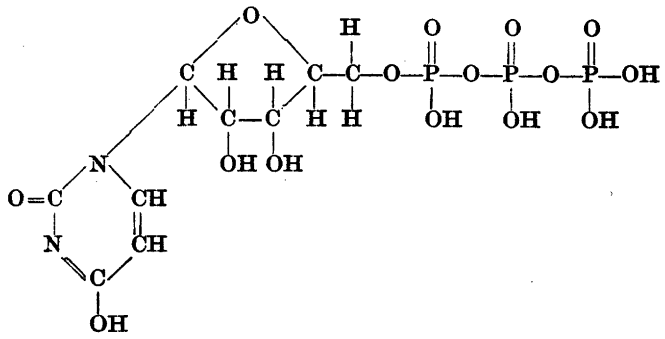

Fig. 6 gives the ultraviolet absorption curves for 5′-UTP obtained from muscle and purified by ion-exchange chromatography. Molar extinction coefficients of 10 050 at pH 1.0, 7 330 at pH 6.5 and 10 350 at pH 11.0 were obtained at 262 mμ (± 2 %).

EXPERIMENTAL

Isolation of UTP. ATP, used as the starting material for the preparation of UTP, was obtained as the barium salt from the muscles of the hind limbs and the back of rabbits by the method described by D. M. Needham[21]. In several experiments, the crude barium salt obtained from the neutralized trichloroacetic acid extract of muscle in the first step of the preparation, was used directly.

For ion-exchange chromatography, the barium salt was converted to the free acid with Dowex-50 resin in the hydrogen form[22] and absorbed from dilute ammoniacal solution at about pH 11 on columns of the anion-exchange resins Dowex-1 or Dowex-2 (250—500 mesh) in the chloride form. Columns of bed size 8 cm^2 × 30 cm were used for amounts of approx. 500 mg of total phosphorus, with a flow rate of 4 ml/min. The eluting solvents of Cohn and Carter[12] were employed, 1) 0.003 *M* hydrochloric acid, 2) 0.02 *M* sodium chloride in 0.01 *M* hydrochloric acid, 3) 0.2 *M* sodium chloride in 0.01 *M* hydrochloric acid and 4) *M* hydrochloric acid. Fractions of 100 ml were taken with an automatic fraction collector adjustable to different time intervals and the fractions were analysed for total phosphorus or/and by optical density measurements. For the isolation of the nucleoside triphosphate fraction appearing in the *M* hydrochloric acid effluent, appropriate fractions of this effluent were pooled and the barium salt precipitated by addition of a small excess of 2 *N* barium acetate solution to the neutralized pooled fractions at 0 °C, followed by 1—2 volumes of ethanol in some experiments, when rapid precipitation of the barium salt was desired.

2—8 (mostly 4—8) mg (calc. as the free acid) of nucleoside triphosphate beside 100—200 (mostly 150—200) mg ATP were obtained from 100 g muscle in different experiments.

For the preparation of the nucleoside triphosphate fraction directly from deproteinized muscle extracts, the trichloroacetic acid extract from muscle was extracted with ether in order to remove the bulk of the acid, neutralized with ammonia and freed from ether by aeration, whereupon the solution was subjected to ion-exchange chromatography. The yield of nucleoside triphosphate obtained was practically identical with and in no case higher than that prepared *via* the isolated barium salts.

In the first experiments the resolution of the ATP-free nucleoside triphosphate mixture was achieved by fractional reprecipitations of the barium salts. The barium salts were treated with 0.1 *N* hydrochloric acid at 0 °C by thorough mixing with successive portions of acid for a few minutes and centrifuging after each treatment. The acid solutions were neutralized with ammonia first to pH 4.5 and then to pH 7.5, followed by centrifugations. By pooling similar fractions and repeating the procedure, UTP and GTP of over 95 % purity were ultimately obtained, as judged by paper chromatography; UTP being accumulated in the most soluble and GTP in the least soluble fractions.

For the separation of UTP and GTP by paper chromatography, the barium nucleoside triphosphate mixture was freed from barium with Dowex-50 and neutralized with ammonia. Chromatography was performed in the saturated ammonium sulphate-water-*iso*-propanol (79:19:2) solvent system on Whatman No. 1 paper after equilibration overnight, as described previously[23]. For the quantitative evaluation of the chromatograms, the spots were cut out from the paper, cut into pieces of 2 × 2 mm and extracted with 0.1 *N* hydrochloric acid for 8 hours. Optical densities were read against an extract from a paper blank of the same area cut out from beside the spot; the same blank was used for phosphorus estimations.

Separation of UTP and GTP by ion-exchange chromatography was performed on Dowex-1 formate columns, using the formate elution system, as described elsewhere[13]. UTP was isolated as the barium salt by addition of 2 *N* barium acetate solution to the pooled effluent fractions brought to pH 7 with ammonia. The barium salt was washed with water, ethanol and ether and dried *in vacuo* over phosphoric oxide. Yields of 1—4 mg pure UTP (calc. as the free acid) were obtained from 100 g muscle. (Found: P 11.0; uracil: pentose: total P: acid-labile P, 1:1:3:2. Calc. for $(C_9H_{11}O_{15}N_2P_3)Ba_2$, $4H_2O$: P: 11.3 %; uracil: pentose: total P: acid labile P 1:1:3:2).

Analytical procedures. Phosphorus was determined by the method of Allen[24] using a Klett Summerson photoelectric colorimeter with a red filter. Acid-labile phosphorus was determined after 10 minutes hydrolysis in *N* hydrochloric acid at 100 °C. Pentose estimations were carried out with the orcinol reagent according to Albaum and Umbreit[15] or before and after treatment with bromine according to Massart[16]. Optical densities were measured in a Beckman Universal Spectrophotometer Model DU with Photomultiplier.

Copper complex formation was measured by applying the procedure described by Caputto, Leloir, Cardini and Paladini[3].

Oxidation with periodate was first carried out by colorimetric estimation of the excess of periodate after reaction for 24 hours at 20° C, whereby the oxidation of *p*-phenylene diamine to a black pigment (Bandrowski's base) by periodate was used for the colori-

metric procedure. Later, the method of Dixon and Lipkin[18] was used exclusively and all previous analysis were repeated with this method.

Electrometric titration. The pure barium salt of UTP was freed from barium by Dowex-50 in the hydrogen form, and the solution of the free acid thus obtained was used for titration after analysis. 5 μmoles were titrated with 0.10 *N* sodium hydroxide in an atmosphere of nitrogen. Stirring was effected by bubbling nitrogen through the solution. A Beckman pH meter, Model G was used for the measurements.

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The Isolation of Guanosine-5′-Triphosphate from Muscle

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The isolation of guanosine-5′-triphosphate from rabbit skeletal muscle is described. The identification is based on the results of paper chromatography and ultraviolet absorption measurements on the products of hydrolysis, together with enzymic evidence and chemical analysis, electrometric titration and ultraviolet absorption spectra of the compound before hydrolysis. Xanthosine-5′-triphosphate was prepared by deamination of guanosine-5′-triphosphate.

The isolation of guanosine triphosphate (GTP) from rabbit skeletal muscle has been recently reported[1] from this laboratory. This, together with the isolation of uridine triphosphate (UTP) from muscle[1] and yeast[2], followed by the demonstration of cytidine triphosphate (CTP), besides GTP and UTP in various tissues[3], establishes the natural occurrence of ribonucleoside triphosphates other than adenosine triphosphate (ATP), the only previously known representative of this type of compound in nature.

It was to be expected that if, besides ATP, additional nucleoside triphosphates are present in muscle, they would be separated from other types of acid-soluble phosphorus compounds by the methods used for the isolation of ATP from deproteinized tissue extracts, appearing as contaminants in the final ATP preparation. According to expectations, the presence of a contaminating triphosphate fraction was revealed upon subjecting ATP, which had been prepared from muscle, to chromatographic purification.

Ion-exchange chromatography on the strong base anion-exchange resins Dowex-1 or Dowex-2 in the chloride form, according to Cohn and Carter[4], gave a fraction appearing in the effluent by elution with *N* hydrochloric acid, after the ATP has been removed from the column by the eluting solvent of next lowest anion content. This fraction, regularly contaminating our ATP preparations, has been subsequently recognized as a mixture of GTP and UTP. In later experiments, a further contaminating phosphate fraction was observed on ion-exchange chromatography of muscle ATP preparations. This fraction appeared in the effluent by elution with 0.01 *M* hydrochloric acid containing 0.02 *M* sodium chloride, following the adenosine diphosphate fraction and prior to the removal of ATP from the column. Phosphorus analysis indicated a tri-

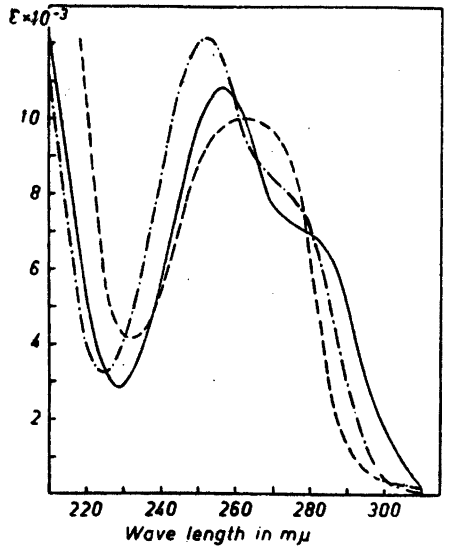


Fig. 1. Ultraviolet absorption curves for GTP from muscle. ——— *at pH 1.0,* — — — — *at pH 6.5 and* —·—·—·— *at pH 11.0.*

phosphate containing two acid-labile phosphate groups and paper chromatography after hydrolysis for one hour in *N* hydrochloric acid at 100° C gave R_F values identical with those obtained with synthetic cytidine-5′-phosphate (CMP). Ultraviolet absorption measurements on the eluted spots showed likewise identity of the product of hydrolysis with synthetic 5′-CMP. Our results indicate identity of this contaminating phosphate fraction with CTP, found in amounts corresponding to 0.2—0.3 % of the ATP, as compared to the nearly ten times larger quantities of GTP and UTP obtained from the same muscle extract. Our findings confirm the previously reported[3] occurrence of CTP in muscle and show at the same time the suitability of the barium salt precipitation for the separation of all the four ribonucleoside triphosphates from muscle extracts.

Application of the ion-exchange procedure directly to trichloroacetic acid extracts of muscle yields similar amounts of the four nucleoside triphosphates.

For the isolation of pure GTP from the GTP—UTP mixture obtained on ion-exchange chromatography, fractional reprecipitation of the barium salts and — in later experiments exclusively — ion-exchange on Dowex-1 formate columns[5] was employed, GTP being isolated as the barium salt from pooled fractions of the effluent.

Ultraviolet absorption measurements on the pure triphosphate in acid and in alkali indicate a guanosine derivative (Fig. 1) and pentose estimations with the orcinol reagent[6] show the presence of directly estimable pentose as expected for a purine nucleoside derivative. Phosphorus analysis gives values corresponding to three phosphate groups for each pentose unit in the molecule, two-thirds of the phosphorus being present in the form of acid-labile phosphate groups.

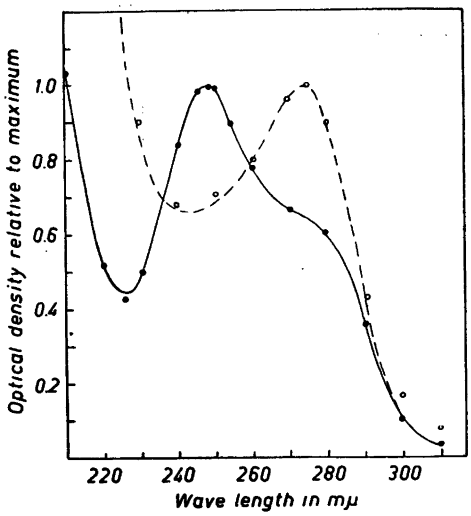


Fig. 2. Ultraviolet absorption curves for guanine obtained from GTP by hydrolysis in 70% perchloric acid or N hydrochloric acid (1 hour at 100 °C) in comparison with authentic guanine in 0.1 N acid and 0.1 N alkali. ———— guanine in acid and — — — — in alkali. ● guanine from GTP in acid and ○ in alkali.

Identification of the purine base obtained on hydrolysis of the triphosphate with 70 % perchloric acid or with *N* hydrochloric acid for one hour at 100° C, was performed by paper chromatography. R_F values identical with those of authentic guanine were obtained in four different solvent systems (Table 1). The ultraviolet absorption curves of the product of hydrolysis in acid and in alkali were likewise found to be identical with those of guanine (Fig. 2). On hydrolysis of the triphosphate with *N* sulphuric acid, guanine could be isolated as the sulphate in the form of colourless needless, indistinguishable under the microscope from the sulphate prepared from authentic guanine under identical conditions. The ultraviolet absorption curve, identical for both preparations, gave the calculated guanine content.

Table 1. R_F values of the ultraviolet absorbing base obtained from GTP (1) by hydrolysis in 70 % perchloric acid (1 hour at 100 °C) and (2) by hydrolysis in N hydrochloric acid (1 hour at 100 °C) in comparison with guanine. Solvent systems: (A) hydrochloric acid (sp. gr. 1.180)-isopropanol-water (17:65:18); (B) n-butanol-formic acid (sp. gr. 1.220 -water (77:10:13); (C) n-butanol-ammonia (sp. gr. 0.880)-water (84:5:14); (D) 5% potassium dihydrogen phosphate in isoamyl alcohol. Descending chromatography was used in systems A, B and C, and ascending chromatography in D.

	R_F			
	A	B	C	D
Base (1)	0.25	0.14	0.12	0
Base (2)	0.25	0.14	0.12	0
Guanine	0.25	0.14	0.12	0

Table 2. R_F values of pentose derivatives obtained from GTP on hydrolysis with Dowex-50 (hydrogen form) for 2 hours at 100 °C, followed by descending chromatography in n-*butanol-acetic acid-water (4:1:5).*

Compound	R_F	
	Developed with aniline hydrogen phthalate	Developed with molybdate reagent
Pentose from GTP	0.32	—
D-Ribose	0.32	—
Pentose-phosphate from GTP	0.06	0.06
D-Ribose-5′-phosphate	0.06	0.06

For the identification of the pentose part of the molecule, the triphosphate was hydrolysed in *N* hydrochloric acid for one hour or with Dowex-50 resin acid for two hours at 100° C; in the last case the base formed during hydrolysis is absorbed on the resin, the solution containing the products formed from the pentose — phosphate part of the molecule. Paper chromatography revealed the presence of ribose and ribose-5′-monophosphate in the hydrolysed solution (Table 2), no other spots being obtained on development of the chromatogram with *m*-phenylene diamine or aniline hydrogen phthalate.

The identification of guanine and ribose establishes the triphosphate as a derivative of guanosine; the presence of ribose-5′-phosphate among the products of hydrolysis indicates a derivative of guanosine-5′-monophosphate (5′-GMP). In confirmation of this, 5′-GMP was obtained on heating a solution of the free triphosphoric acid for two hours to 100° C with splitting off of two-thirds of the bound phosphorus in the form of orthophosphate. Fig. 3 shows the ultraviolet absorption curves for GMP from GTP in comparison with synthetic 5′-GMP. Location of the phosphate group in the 5′-position is shown by the action of 5′-nucleotidase from snake venom[7] (Table 3).

Table 3. Action of 5′-nucleotidase on GMP obtained from GTP. 1 µmole nucleotide in 0.1 ml M *glycine buffer (pH 8.5) + 0.1 ml* M *magnesium chloride + 0.1 ml of a solution of Crotalus adamanteus venom (2 mg/ml) in a total volume of 1 ml were incubated for 45 minutes at 37 °C. Phosphorus was estimated after addition of 1 ml ethanol and centrifugation.*

Compound (1 µmole)	Orthophosphate in µmoles time of incubation		µmoles ortho-phosphate formed
	0 min.	45 min.	
Adenosine-3′-phosphate	0.005	0.005	0
Adenosine-5′-phosphate	0.01	0.92	0.91
Guanosine-3′-phosphate	0.05	0.05	0
Guanosine-5′-phosphate	0.02	0.93	0.91
Guanosine phosphate from GTP	0	0.90	0.90

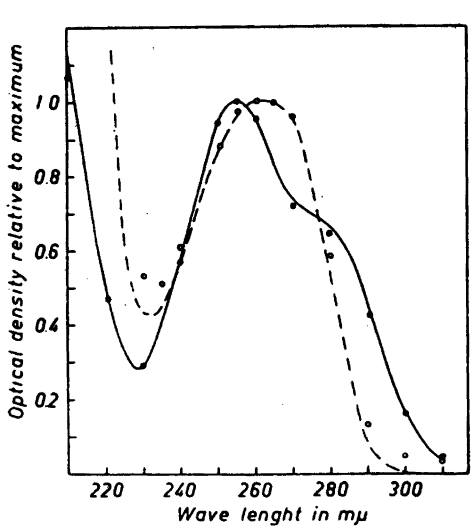


Fig. 3. Ultraviolet absorption curves for GMP obtained from GTP in comparison with synthetic 5′-GMP in 0.1 N acid and in 0.1 N alkali. ——— 5′-GMP in acid and — — — — in alkali. ● GMP from GTP in acid and ○ in alkali.

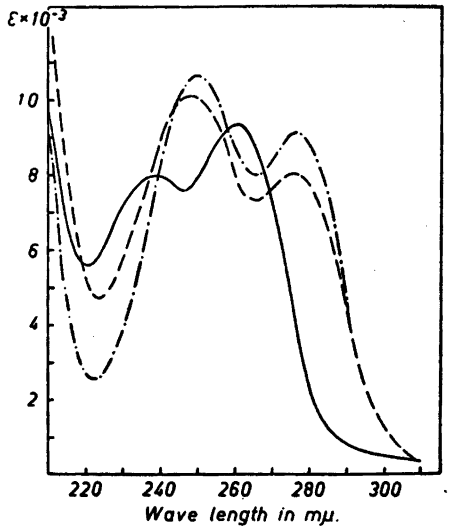


Fig. 4. Ultraviolet absorption curves for XTP, obtained by deamination of GTP. ——— at pH 1.0, — — — at pH 6.5 and —·—·—·— at pH 11.0.

As regards the attachment of the two acid-labile phosphate groups to the 5′-GMP moiety in GTP, the 2′- and 3′-positions are excluded on the basis of the results obtained in experiments on the formation of copper complex from GTP and on its oxidation with periodate. GTP forms a copper complex in analogy to 5′-substituted nucleoside derivatives[8] and in contrast to those substituted in the 2′- or 3′-positions (Table 4). The absence of substituting groups in the 2′- and 3′-positions of GTP is further demonstrated on oxidation

Table 4. Copper complex formation[9]. To 1 μmole of the sample, about 3 mg of copper phosphate and 0.5 ml of 20 % trisodium phosphate were added. The suspension was centrifuged after 90 minutes and the supernatant mixed with 1 ml of 0.5 % sodium diethyl dithiocarbamate. The copper complex was extracted with 6 ml amyl alcohol and measured in a Klett-Summerson colorimeter with filter No. 42.

Compound (1 μmole)	Colorimeter reading
Adenosine-3′-phosphate	23
Adenosine-5′-phosphate	330
Adenosine-5′-triphosphate	320
Guanosine-3′-phosphate	62
Guanosine-5′-phosphate	315
Guanosine	335
Guanosine triphosphate	318

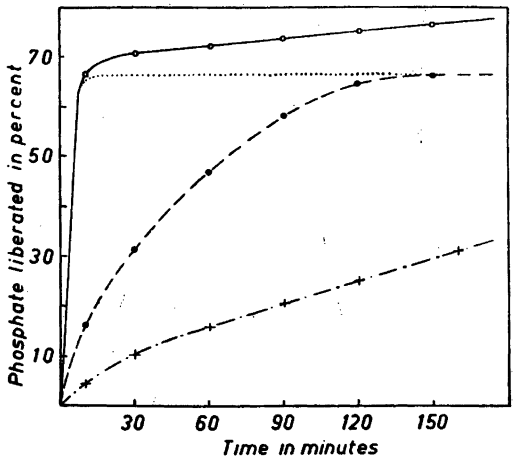


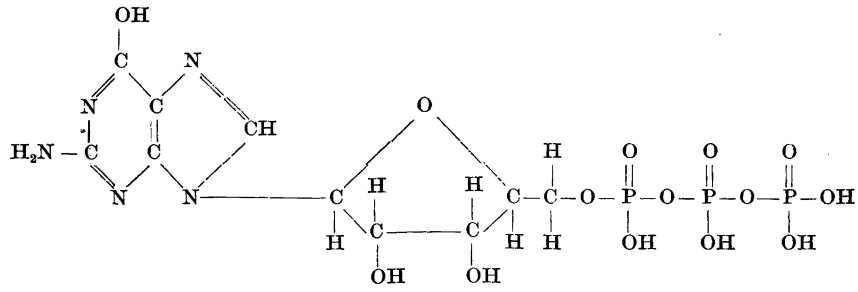
Fig. 5.
Acid hydrolysis of GTP at 100°C. ——— *in N hydrochloric acid,* – – – – *in 0.1 N hydrochloric acid and* — · — · — *synthetic 5′-GMP in N hydrochloric acid.* ····· *represents the hydrolysis of the labile phosphate groups in GTP and is obtained by subtraction of one-third of the % phosphorus liberated from 5′-GMP from the curve obtained in N acid for GTP.*

with periodate under conditions where no splitting off of the labile phosphate groups takes place (Table 5).

Deamination of GTP with nitrous acid results in the formation of xanthosine triphosphate (XTP), proving the absence of substituting phosphate groups in the amino-group of guanine in GTP. XTP was isolated as the barium salt and identified by phosphorus analysis and qualitative and quantitative evaluation of the xanthine spot obtained on paper chromatography from XTP after hydrolysis for one hour in *N* hydrochloric acid at 100° C, in comparison with authentic xanthine. The ultraviolet absorption curves for XTP at different pH values are shown in Fig. 4.

Our results, showing the presence of the 5′-GMP moiety and that of two acid-labile phosphate groups attached thereto in positions other than 2′-, 3′- and the amino-group of guanine, suggest the formulation of the GTP isolated from muscle as guanosine-5′-triphosphate.

The rates of acid hydrolysis for GTP (Fig. 5) as compared with ATP and the results of the electrometric titration (Fig. 6), showing the presence of three primary and one secondary phosphate groups in GTP, substantiate further this formulation:



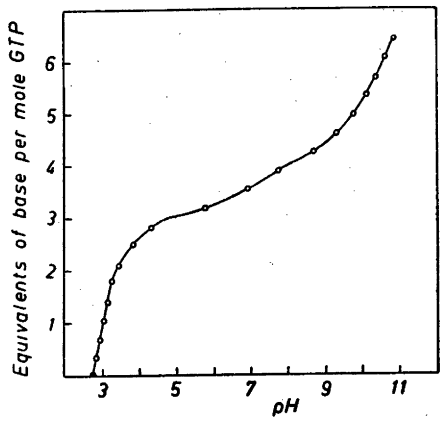


Fig. 6. Electrometric titration of GTP. 5 μmoles titrated with 0.10 N sodium hydroxide.

EXPERIMENTAL

Isolation of GTP. The preparation of barium GTP from rabbit skeletal muscle was carried out as described for UTP[11], by using as starting materials barium ATP preparations from muscle[12] or the crude mixture of barium salts obtained from the neutralized trichloroacetic acid extract of muscle in the first step of the preparation of ATP. Ion-exchange chromatography on Dowex-1 or Dowex-2 chloride columns, followed by resolution of the resulting GTP – UTP mixture by fractional precipitation of the barium salts, or, mostly, by ion-exchange on a Dowex-1 formate column in the formate system, gave pure GTP, isolated ultimately as the barium salt from pooled fractions of the effluent. In some experiments, ion-exchange chromatography in the formate system was applied directly to the starting material. Separation of GTP from UTP and ATP by paper chromatography has been described previously[11]. Yields of 1 – 4 mg of pure GTP (calc. as the free acid) were obtained from 100 g muscle; the same or a somewhat lower yield was obtained when the deproteinized muscle extract was subjected directly to ion-exchange chromatography.

The barium salt was washed with water, ethanol and ether and dried *in vacuo* over phosphoric oxide. (Found: P 10.2 %; guanine: pentose: total P: acid-labile P, 1:1:3:2. Calc. for $(C_{10}H_{12}O_{14}N_5P_3)Ba_2, 4H_2O$: P 10.7 %; guanine: pentose: acid-labile P, 1:1:3:2). Molar extinction coefficients of 10 850 at pH 1.0 (256 mμ), 12 150 at pH 6.5 (253 mμ) and 10 080 at pH 11.0 (260 mμ) were obtained (± 2 %).

Table 5. Periodate oxidation[10]. 0.50 μmole potassium metaperiodate was added to approx. 0.3 μmole of the sodium salt of the nucleotide in a total volume of 3 ml. The amount of periodate consumed was measured at 227 mμ against a blank containing periodate of the same concentration.

Compound	μmoles periodate		μmoles periodate consumed per μmole nucleotide
	added	consumed	
Adenosine-3′-phosphate, 0.31 μmole	0.50	0.04	0.13
Adenosine-5′-phosphate, 0.27 μmole	0.50	0.26	0.96
Adenosine-5′-triphosphate, 0.30 μmole	0.50	0.29	0.97
Guanosine-3′-phosphate, 0.38 μmole	0.50	0.05	0.09
Guanosine-5′-phosphate, 0.37 μmole	0.50	0.36	0.98
Guanosine triphosphate, 0.36 μmole	0.50	0.35	0.97

Analytical procedures. Phosphorus was determined by the method of Allen[13], and acid-labile phosphorus was determined after 10 minutes hydrolysis in *N* hydrochloric acid at 100 °C. Pentose estimations were carried out with the orcinol reagent according to Albaum and Umbreit[6]. Optical densities were measured in a Beckman Universal Spectrophotometer Model DU with Photomultiplier.

Copper complex formation was measured by applying the procedure described by Caputto, Leloir, Cardini and Paladini[9].

Oxidation with periodate was performed according to Dixon and Lipkin[10].

Electrometric titration. The pure barium salt of GTP was freed from barium by Dowex-50 (hydrogen form), and the analysed solution of the free acid thus obtained was used for titration. 5 μmoles were titrated with 0.10 *N* sodium hydroxide in a nitrogen atmosphere; stirring was effected by a continuous stream of nitrogen through the solution. A Beckman pH meter, Model G was used for the measurements.

Isolation of guanine sulphate. 25 mg barium GTP in 5 ml *N* sulphuric acid were heated to 100 °C for one hour. After removal of barium sulphate by centrifugation, the solution was left overnight at 0 °C. After filtration, washing with water and drying *in vacuo* over phosphoric oxide 2.3 mg of colourless needles were obtained, indistinguishable from the product obtained from guanine (Roche) under identical conditions. (Found: guanine 69.6 %. Calc. for $(C_5H_5ON_5)_2$, H_2SO_4, 2 H_2O: guanine 69.3 % by using the molecular extinction coefficient of 10 850 at 256 mμ).

Preparation of XTP. The preparation of XTP by deamination of GTP with nitrous acid was carried out in acetate buffer of pH approximately 4 by application of the procedure used by Kleinzeller[14] for the preparation of inosine triphosphate (ITP)from ATP. To 10 μmoles sodium GTP in 0.6 ml water, 0.25 ml of a solution, prepared from 2.85 g anhydrous sodium acetate, 10 ml glacial acetic acid and 10 ml water, was added, followed by the addition of 0.1 ml 60 % sodium nitrite solution. After standing for 6 hours at 20° C the solution was cooled with ice and 2 *N* ammonia was added until the pH was approx. 7, followed by 50 μl 2 *N* barium acetate solution. The precipitate was spun off, washed with water and dissolved in 1.5 ml 0.2 *N* hydrochloric acid. After addition of 10 mg urea the solution was left for one hour at 0 °C. The barium salt was then precipitated by addition of 2 *N* ammonia until the pH was approx. 7 and then spun off. After one more reprecipitation the barium salt was washed with water and dried *in vacuo* over phosphoric oxide. Paper chromatography in the saturated ammonium sulphate solution-water-*iso*propanol (79:19:2) solvent system[15,16] gave only one ultraviolet absorbing and phosphorus containing spot, with an R_F value of 0.56 as compared with the R_F values obtained for ATP, 0.40; GTP, 0.64; ITP, 0.73 and UTP, 0.84, when chromatographed side by side on the same paper.

On hydrolysis of XTP for one hour in *N* hydrochloric acid at 100 °C, followed by ascending paper chromatography in the 5 % potassium dihydrogen phosphate-*iso*-amyl alcohol solvent system only one ultraviolet absorbing spot was observed, with the same R_F value (0.56) as given by xanthine, chromatographed side by side with the experimental solution.

Phosphorus analysis before hydrolysis in conjunction with ultraviolet absorption measurements on the eluted xanthine spot from XTP (using a molecular extinction coefficient of 9 350 at 262 mμ) gives the calculated ratio of 1:3:2 for xanthine: total phosphorus: acid-labile phosphorus.

We wish to express our gratitude to Professor A. R. Todd, Cambridge, for samples of synthetic cytosine-5′-phosphate and guanosine-5′-phosphate. This work has been supported by the *Swedish Natural Science Research Council*, the *Lilly Foundation* and by *Kungliga Fysiografiska Sällskapet, Lund.*

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On the Dephosphorylation and Deamination of Adenosine Triphosphate by Actomyosin Gel

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Analysis by chromatographic methods shows rapid dephosphorylation of adenosine triphosphate to adenosine diphosphate by actomyosin gel, concurrent with the volume constriction of the gel. Dephosphorylation is followed by a slower process of deamination, yielding inosine diphosphate as the isolated end product of reaction. The deamination of adenosine diphosphate to inosine diphosphate represents a new type of deamination of adenine nucleotides by muscle preparations, in addition to the previously known route *via* adenosine monophosphate.

In previous investigations [1,2], the chemical aspects of the interaction of actomyosin and adenosine triphosphate (ATP) had been studied from the point of view of eventual chemical changes in the protein, accompanying the volume constriction of actomyosin threads. In the present work — short accounts[3,4] of which have already been published — the reaction between actomyosin gel and ATP has been investigated from the point of view of chemical changes in the nucleotide component of the reaction.

MATERIALS AND METHODS

Actomyosin gel. Rabbits were killed by stunning and packed in ice for 15 minutes. The muscles of the hind limbs and the back were excised and minced at 0° C, followed by extraction for 24 hours at 0° C with three times their volume of Weber's solution (0.6 mole potassium chloride, 0.01 mole sodium carbonate and 0.04 mole sodium hydrogen carbonate per litre). After dilution of the extract with the same volume of Weber's solution and filtering through madapolam, the actomyosin was precipitated by adding four volumes of distilled water to one volume of the extract. After standing for 12 hours at 0—3° C, the solution was decanted and the precipitate centrifuged for a few minutes at 8 000—10 000 g. The precipitate was brought into solution again by adding enough solid potassium chloride to make the resulting solution 0.5 molar. The viscous solution was centrifuged, thereby removing any insoluble protein and the clear solution reprecipitated by the addition of four volumes of distilled water to one volume of the solution. Three such reprecipitations were carried out. After the last reprecipitation, the precipitate was thoroughly washed with distilled water and centrifuged. Three such washings were carried out. Only glass-distilled water was used throughout the preparation.

The dry weight was determined for each preparation; it varied between 1 and 3 %. For the determination of the dry weight, duplicate samples were heated at 110° C to constant weight.

ATP was prepared as the barium salt from rabbit muscle by the method described by Needham[5] and purified by ion-exchange chromatography[6].

Adenosine diphosphate (ADP) was obtained from appropriate fractions of the effluent in the ion-exchange chromatography of ATP or prepared according to Bailey[7] as the barium salt and further purified by ion-exchange[6].

Adenosine-5'-monophosphate (AMP) and *inosine diphosphate (IDP)* were prepared and purified as described previously[6]. All the chemicals used were of analytical grade of purity.

Reaction between ATP and actomyosin gel. For the experiments, 10 g of actomyosin gel was placed in a centrifuge tube and after addition of 8 ml water or the buffer solution to be used and 2 ml of sodium ATP solution (20 mg/ml, calc. as the free acid) and thorough mixing with a glass rod, the tube was kept at 20° C for the desired reaction time. The reaction was interrupted by addition of 2 ml 50 % (w/v) trichloroacetic acid solution to the reaction mixture at 0° C. After centrifugation at 10 000 g, the ice-cold trichloroacetic solution was extracted with ether in order to remove the bulk of the acid, neutralized by addition of a drop of ammonia and freed from ether by aeration. The protein-free extract thus obtained was used for phosphorus analysis, paper chromatography and ion-exchange. Approximately 95 % of the total phosphorus in the reaction mixture were recovered in the protein-free extract.

For the purpose of direct spectroscopic examination of the protein-free extracts, perchloric acid was substituted for trichloroacetic acid in parallel samples in some experiments.

In each experiment, volume constriction of the actomyosin gel was measured in a duplicate tube containing the same concentrations of protein and ATP and compared with the gel volume in a third tube, in which ATP had been substituted by water or 0.04 *M* potassium chloride solution. The protein-free extract, prepared from the actomyosin gel without added ATP, gave on analysis nucleotide material not exceeding 2—3 % of that found in ATP-actomyosin mixtures.

Experiments on the reaction between ADP and actomyosin gel were performed analogously to those with ATP, ADP being used in the same molar concentration as ATP.

Paper chromatography[8]. One-dimensional chromatography in saturated ammonium sulphate solution-water-*iso*propanol (79 : 19 : 2) was used for the separation of the adenosine series of phosphates from the inosine series before and after hydrolysis to the corresponding purines for 1 hour in *N* hydrochloric acid at 100° C. Separation of the individual nucleotides was performed by two-dimensional chromatography, using *n*-propanol-ammonia (sp.gr. 0.880)-water (60 : 30 : 10) in the first direction and the ammonium sulphate-water-*iso*propanol solvent in the second.

50—100 μl of the protein-free extract containing 100—200 μg nucleotide material were applied to the paper in 5 μl portions, the spot being dried after each application in a current of cold air. The size of the starting spot did not exceed 1 cm in diameter. Descending chromatography on acid-washed Whatman No. 1 paper (46 × 53 cm) was used with developing times of 40—80 hours in the propanol-ammonia-water solvent and 6—8 hours — after previous equilibration overnight — in the ammonium sulphate-water-*iso*propanol system. The spots on the finished chromatogram were located by ultraviolet photography.

For the quantitative evaluation of the chromatograms the spots were cut out from the paper, cut into pieces of approx. 2 × 2 mm and extracted with 0.1 *N* hydrochloric acid for 4—8 hours. Optical densities were read against an extract from a paper blank of the same area, cut out from beside the spot; the same blank was used for phosphorus estimations. Prior to the application of the method to the analysis of protein-free extracts, adenosine and inosine phosphate preparations of known composition were subjected to analysis (Table 1).

Ion-exchange chromatography. The method of Cohn and Carter[9] in the modification previously described[6], was used.

Analytical methods. Phosphorus was determined by the method of Allen[10] using a Klett Summerson photoelectric colorimeter with a red filter and was differentiated in separate analyses into total phosphorus (P_T), inorganic phosphorus (P_0) and acid-labile

phosphorus (P_{10}) determined after 10 minutes hydrolysis in *N* hydrochloric acid at 100° C. In order to investigate whether the directly estimable phosphorus (P_0) in the protein-free extracts corresponded to inorganic orthophosphate or was partly liberated from labile organic phosphates during analysis, orthophosphate determinations were in addition carried out by the method of Lowry and Lopez[11].

Pentose was determined by the method of Mejbaum[12] as modified by Albaum and Umbreit[13]. A Klett Summerson photoelectric colorimeter was used with a red filter.

Optical densities were measured in 0.01 or 0.1 *N* hydrochloric acid solution in a Beckman Universal Spectrophotometer Model DU, the molecular extinction coefficients of 14 200 at 260 mμ for adenosine phosphates and 13 500 for adenine, 13 200 at 250 mμ for inosine phosphates and 11 500 for hypoxanthine being used.

RESULTS

Analysis of the deproteinised extract from the reaction between ATP and actomyosin gel shows that about one-third of the total phosphorus is present as orthophosphate, indicating that only the terminal phosphate group of ATP has been split off in the reaction. Orthophosphate values corresponding to 32—34 % of the total phosphorus are generally obtained after reaction for

Table 1. Analysis of adenosine and inosine phosphates by paper chromatography[8]. (For details see text).

Substance and chromatogr. method	μg Adenine		μg Hypoxanthine		μg Acid-labile P		μg Total P	
	found	calc.	found	calc.	found	calc.	found	calc.
ATP, 72 μg, one-dimensional	18.5 18.8	19.0			8.6 8.9	8.8	13.3 13.6	13.2
ATP, 56 μg one-dimensional after hydrolysis	14.4	14.8						
ATP, 119 μg two-dimensional	32.1	31.4			15.0	14.5	22.4	21.8
ATP, 160 μg two-dimensional					20.2	19.5	29.5	29.3
ADP, 70 μg + IDP, 104 μg one-dimensional	21.8 21.4	22.0	 32.5 31.3	 32.8	4.7 5.1 8.0 8.4	5.1 7.4	9.8 9.8 15.7 15.7	10.2 15.0
ADP, 54 μg + IDP, 104 μg one-dimensional after hydrolysis	17.0	17.0	32.5	32.8				
ADP, 157 μg + IDP, 104 μg, two-dimensional	47.6	49.3	 31.4	 32.8	10.5 8.1	11.4 7.5	22.1 15.6	22.8 15.0

Table 2. Analysis of the eluted spots from two-dimensional paper chromatography of the protein-free extract from the reaction (30 min.) between ATP (4 μmoles/ml) and actomyosin gel (10 mg dry weight/ml). The extract contained 450 μg total purine, 104 μg inorganic phosphorus, 104 μg acid-labile phosphorus and 313 μg total phosphorus.

	μg Purine	μg Acid-labile P	μg Total P	Purine:acid-labile P: total P (calc. 1:1:2)
ADP spot	336	74	148	1:0.95:1.90
IDP spot	102	25	50	1:1.07:2.14

5 minutes as well as after 4 hours, suggesting that dephosphorylation is complete within the first minutes of reaction, no significant amounts of orthophosphate being formed thereafter.

Measurements on the volume constriction of the gel after 5 and 30 minutes reaction with ATP show that up to 90 % of the final value is already obtained after 5 minutes.

Spectroscopic examination of extracts prepared with perchloric instead of trichloroacetic acid reveals a progressive shift of the absorption maximum towards shorter wavelengths in the course of reaction, suggesting deamination of the original adenine nucleotide.

Paper chromatography of the protein-free extract confirms the results of direct spectroscopic examination and shows the presence of inosine nucleotide in amounts increasing with increasing times of reaction. A reaction time as short as 5 minutes gives only slight deamination, but after 4 hours most of the purine is transformed into hypoxanthine (Fig. 1). Similar results are obtained by paper chromatography of the extract before or after hydrolysis in *N* hydrochloric acid for 1 hour at 100° C.

Separation of the reaction products by two-dimensional paper chromatography results essentially in two ultraviolet-absorbing spots in positions corresponding to those of ADP and IDP, the latter spot increasing at the expense of the former, as the reaction proceeds (Fig. 2). Analyses on the eluted spots by measurement of ultraviolet absorption and phosphorus analysis give the

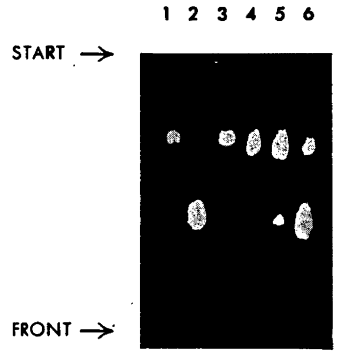

Fig. 1. Time course of deamination in the reaction between actomyosin gel (10 mg dry weight/ml) and ATP (4 μmoles/ml) as followed by paper chromatography of the protein-free extract, after hydrolysis for 1 hour at 100° C. Solvent: saturated ammonium sulphate solution-water-isopropanol (79 : 19 : 2). Time: 7 hours. (1) Adenine, 15 μg; (2) hypoxanthine, 30 μg; (3) hydrolysate of ATP employed, containing 20 μg adenine; (4), (5) and (6) hydrolysates of protein-free extract (containing 45 μg total purine) after 5 min., 30 min. and 4 hours reaction, showing spots for hypoxanthine corresponding to 0, 10 and 80 % of the total purine.

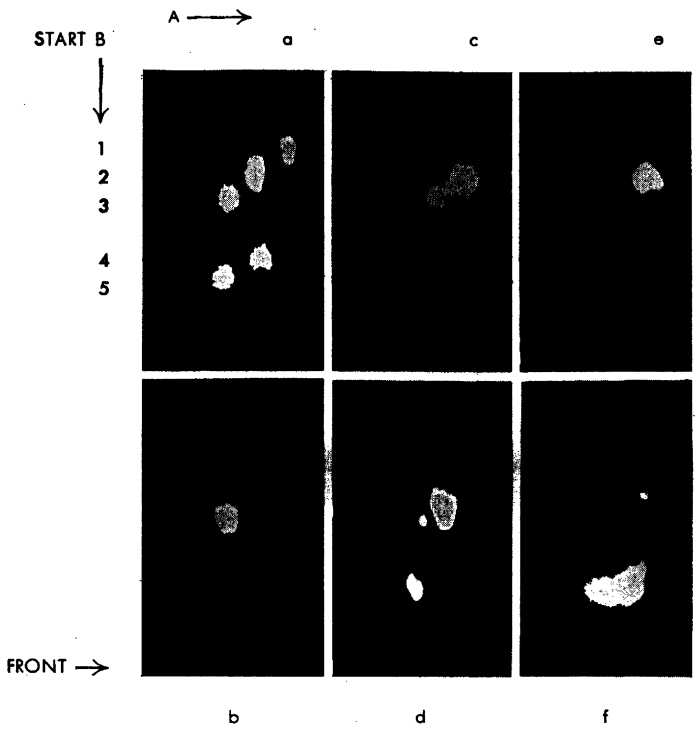

Fig. 2. The products of reaction between actomyosin gel (10 mg dry weight/ml) and ATP (4 μmoles/ml) as shown by two-dimensional paper chromatography of the protein-free extract. (a) first solvent: n-propanol-ammonia-water (60 : 30 : 10) 50 hours. (B) second solvent: saturated ammonium sulphate solution-water-isopropanol (79 : 19 : 2) 8 hours. (a) Mixture of (1) AMP, (2) ADP, (3) ATP, (4) IMP and (5) IDP, approx. 50 μg each; (b) ATP employed; (c) protein-free extract after 5 min. reaction, showing spots for ATP (38 μg), ADP (117 μg) and IDP (8 μg), corresponding to 80 % dephosphorylation and 5 % deamination; (d) protein-free extract after 30 min. reaction showing spots for ATP (8 μg), ADP (141 μg) and IDP (18 μg), corresponding to 95 % dephosphorylation and 11 % deamination; (e) and (f) extracts from another experiment, (e) after 8 min. reaction, showing a spot for ADP and a very faint spot for IDP, and (f) after 4 hours, with spots for ADP and IDP, corresponding to 94 % deamination.

values expected for ADP and IDP (Table 2). In some 5 and 30 min. experiments, a third spot in the position of ATP was observed (Fig. 2).

Ion-exchange chromatography gives three main fractions, corresponding in elution position and chemical analysis to orthophosphate, ADP and IDP (Table 3). In several experiments, however, ion-exchange chromatography of the protein-free extract from ATP-actomyosin reaction mixtures gave the IDP fraction in an elution position different from that of authentic IDP, the IDP fraction appearing first in the effluent by elution with the solvent of next highest anion concentration to the one which removes IDP from the column.

Isolation of the IDP fraction as the barium salt[14] from pooled fractions of the effluent from ion-exchange columns gave barium IDP, indistinguishable

Table 3. Analysis of the fractions obtained on ion-exchange chromatography of the protein-free extract from the the reaction (30 min.) between ATP (4 μmoles/ml) and actomyosin gel (10 mg dry weight/ml).

	μg Purine	μg Acid-labile P	μg Total P	Purine:acid-labile P:total P
Orthophosphate			1.26	
ADP	4.06	0.95	1.96	1:1.01:2.09
IDP	1.60	0.38	0.77	1:1.03:2.10
Calc. for conversion of ATP to diphosphate	5.52	1.27	3.80	1:1:2

from authentic IDP prepared by chemical deamination of ADP. The barium salt, isolated from IDP fractions with retarded elution position, was likewise identical with IDP, as judged by chemical analysis, paper chromatography and elution position on ion-exchange analysis performed on the isolated product, after removal of barium (Table 4).

Beside ADP and IDP, an adenine nucleotide fraction in the elution position of ATP was observed in the ion-exchange analysis in some 5 and 30 min. experiments, in agreement with the results of two-dimensional paper chromatography.

Dephosphorylation and deamination as measured by different methods of analysis, is seen from Table 5.

Essentially the same results were obtained by analysis of the reaction products from forty different actomyosin preparations after 30 minutes and 4 hours reaction with ATP. 20—30 % deamination was obtained after 30 minutes reaction, with the exception of three preparations which gave 50—65 %;

Table 4. Analysis of barium IDP ($C_{10}H_{11}O_{11}N_4P_2Ba_{1.5}$) isolated from the reaction between ATP and actomyosin gel, by precipitation with barium acetate and ethanol from the effluent in ion-exchange chromatography of the protein-free extract, followed by drying in vacuo over phosphoric oxide.

	Found %	Calc. %
Total phosphorus	9.76	9.96
Acid-labile phosphorus	4.88	4.95
Inorganic phosphorus	0	0
Pentose	25.2	23.8
Hypoxanthine	20.40	21.55
Total P:Acid-labile P:	2:1:	2:1:1:1
Pentose:Hypoxanthine	1.05:0.95	
Water (loss of weight at 110° C)	0	0

Table 5. Dephosphorylation and deamination of ATP (4 μmoles/ml) by actomyosin gel (10 mg dry weight/ml), as measured by different methods of analysis, on aliquots of the same protein-free extract after 30 min. reaction.

Method	% Conversion of ATP to the diphosphate	% Deamination
Direct analysis	96	
One-dimensional paper chromatography		22
One-dimensional paper chromatography after hydrolysis		18
Two-dimensional paper chromatography	94	18
Ion-exchange	94	22

4 hours reaction resulted in yields of 65—95 %. Only in two experiments was IMP detected among the reaction products, amounting to less than 5 % of the IDP fraction.

Reaction between ATP and actomyosin gel in buffer solutions between pH 6.0 and 8.0 results in dephosphorylation and deamination of ATP in all cases; considerably higher rates of deamination are found, however, at pH 6.0 than at higher pH values (Table 6), in analogy to the pH course of the deamination of AMP.

Substitution of ATP by ADP in the reaction with actomyosin gel results in deamination without dephosphorylation, and without any volume change

Table 6. Deamination in the reaction between ATP (4 μmoles/ml) and actomyosin gel (10 mg dry weight/ml) in buffer solutions, as measured by one-dimensional paper chromatography of the protein-free extract. Phosphorus analysis showed conversion of ATP to the diphosphate. The following buffer solutions were used: 0.1 M succinate at pH 6.0 and 6.6, 0.05 M acetate at pH 6.8, 0.1 M glycylglycine at pH 7.2 and 0.05 M Tris (hydroxymethyl)-aminomethane at pH 7.2 and 8.0.

Duration of the reaction	% Deamination at pH				
	6.0	6.6	6.8	7.2	8.0
60 min.	53		32	23	
4 hours	100	76		60	53

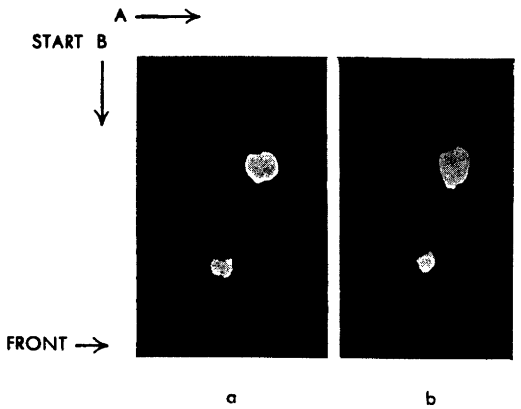

Fig. 3. Deamination in the reaction of actomyosin gel (10 mg dry weight/ml) with ADP (4 µmoles/ml) and ATP (4 µmoles/ml) as shown by two-dimensional paper chromatography of the protein-free extracts after 30 min. reaction. The solvents are the same as in Fig. 2. (a) Extract from the reaction with ADP (total nucleotide content 200 µg) and (b) extract from the reaction with ATP (total nucleotide content 150 µg), showing spots for ADP and IDP corresponding to (a) 37 % and (b) 27 % deamination.

in the protein gel. Similar results on deamination were obtained with ATP and ADP respectively, with somewhat higher deamination in the case of ADP in several 30 minutes experiments (Fig. 3).

DISCUSSION

The results of the present investigation establish two phases in the reaction between ATP and actomyosin gel, from the point of view of chemical changes in the nucleotide component of reaction.

In a first rapid phase, the terminal phosphate group of ATP is split off under formation of ADP. The dephosphorylation of ATP — concurrent with the volume constriction of the gel — is completed within the first minutes and even reaction times as short as a few seconds are reported[15] to result in complete dephosphorylation. In some of our experiments, however, only about 80 % dephosphorylation was obtained after 5 minutes reaction, as judged by chromatography.

The quick initial phase of dephosphorylation in the reaction between ATP and actomyosin gel is followed by a second slow phase, in which deamination of the diphosphate takes place. Deamination of adenine nucleotides by myosin and actomyosin has been repeatedly reported, whereby the reaction was assumed to take place *via* AMP giving IMP as the product of reaction. Deamination of ATP and ADP was interpreted as the result of the combined action of three enzymes, *viz.* 1) dephosphorylation of ATP to ADP by ATPase, 2) dismutation of ADP to AMP and ATP by adenylate kinase (myokinase) and 3) deamination of AMP to IMP by adenylate deaminase.

In our experiments, the absence of deamination *via* the AMP route to any noticeable degree is already indicated by the results of phosphorus analysis in the deproteinised reaction mixture, showing that only the terminal phosphate

group of ATP has been split off. Furthermore, the time course of deamination as contrasted with the rapid reaction found when AMP is added to our actomyosin preparations, points likewise towards a different type of deamination process. Finally, the fact, that IMP could not be detected in any quantity among the products of reaction, shows definitely that under the conditions of reaction the main route of deamination does not proceed through the AMP→IMP reaction, probably because our actomyosin preparations were practically free from adenylate kinase; in the presence of this enzyme, deamination *via* the AMP route would be the principal one at pH near 7.

Direct analysis of the protein-free reaction solution together with the results obtained by ion-exchange and paper chromatography show that deamination occurs at the diphosphate level; ADP formed by primary dephosphorylation of ATP is transformed by direct deamination into IDP. The direct proof of deamination at the diphosphate stage is furnished by isolation of IDP as the main product of reaction. In view of the diverging results as to the elution position of the IDP fraction at ion-exchange analysis in some of our experiments, the possibility of an IDP derivative not identical with, but easily transformed into IDP being the primary product of deamination, cannot be yet definitely excluded.

The demonstration of a new route of deamination, *viz.* direct deamination of ADP by actomyosin, as well as by washed myofibrils[16], in addition to the previously established route *via* AMP, necessitates further investigation of the specificity range of adenylate deaminase, and of the question of identity of the enzymes effecting the different types of deamination.

Investigations on these lines are now in progress in this laboratory; short reports [17,18] on some of the results have already been presented.

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Studies on the Hydrolysis of Metal Ions

X. The Hydrolysis of the Uranyl Ion, UO_2^{2+}

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Some five years ago, one of us (Ahrland [1]) published an investigation on the hydrolysis of the ion UO_2^{2+}. Uranyl solutions of various initial concentrations were titrated with OH^-, and the hydrogen ion concentration was measured with quinhydrone or glass electrodes at various points during the titration. The equilibria were studied at 20° C in 1 C $NaClO_4$ medium.

The data were more accurate and covered a wider concentration range than any published before. They showed quite obviously that the uranyl ion UO_2^{2+} does not attract protons even at the highest acidities studied, and that the splitting off of protons at low acidities is connected with the formation of mainly polynuclear complexes.

In the meantime, mathematical methods for dealing with polynuclear complexes have been published (ABI[2], ABII[3]), and applied to the hydrolysis of a number of ions (parts VIII[4] and IX[5] of this series). We thought it would be interesting to apply these methods to the data for UO_2^{2+} in order to see whether they could throw light on the mechanism of its hydrolysis.

Formula of the complexes

We shall use the same notation as in previous parts of this series; h = hydrogen ion concentration $[H^+]$; M = total (analytical) concentration of uranyl ion, free or bound in complexes; Z = average number of H^+ set free (or OH^- bound) per uranyl group. (The symbols in the original paper [1] were $[H^+]$ for h, C_M for M, and $\bar{n}$ for Z.)

Anrland's data can be expressed in the form $Z(h, M)$ — each titration gives Z values for a number of sets (h, M). Since M varied in each titration, one cannot simply measure the distances between curves $Z(\log h)_M$, as described in (VIII, 14 and following). However, by picking out a few values it was found by very rough calculations that the value for t in (VIII, 14 and 15) is probably 2 for UO_2^{2+}.

$$t = 2 \qquad (1)$$

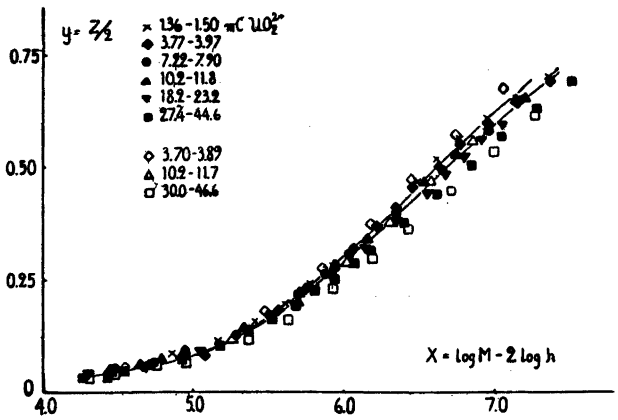

Fig. 1. Points, Ahrland's data on the hydrolysis of UO_2^{++}, reduced to the form $y(x)$; symbols see the figure. "Best curves": I (lower curve) equal weight to all points; II (upper curve) giving low weight to points for high concentrations. Points with open symbols (last three in list) were obtained with glass electrode, the others with quinhydrone electrode.

To test this value, we have plotted Z or rather

$$y = \frac{Z}{2} \tag{2}$$

as a function of

$$x = \log M - 2 \log h \tag{3}$$

As seen from Fig. 1, with this choice of coordinates the data for a wide range of concentrations seem to fall upon a single curve $y(x)$. The largest deviations are for the very highest M values, around 30—40 mC. They may be due either to deviations in the activity factors or to a small error in the determination of free acid ("C_H"), which was not so accurate for the highest M, with the method chosen. If one considers that the range in log M is almost 1.6, it is easily understood that other reasonable values for t, such as 1, 1.5, 2.5, or 3, would give a systematic spread of the data $y(x)$.

We may then conclude that, with good approximation, all complexes present in considerable amounts can be written in the form (*cf.* ABI [2], 16—19, 25—26, VIII [4], 15):

$$UO_2((OH)_2UO_2)_n^{2+} \tag{4a}$$

or, which is equivalent,

$$UO_2(OUO_2)_n^{2+} \tag{4b}$$

For structural reasons to be given below we find (4a) more probable. For the sake of brevity we shall, however, use the shorter (4b) in most of our equations.

Direct analysis of $y(x)$ curve

When the general formula (4) of the complexes has been established, the next questions are which complexes (which values for n in 4) occur and what are the equilibrium constants for the formation of these complexes.

As was demonstrated in ABII[3] and exemplified in part IX[5], the data $y(x)$ can be used directly for obtaining a power series $g(u)$, the coefficients of which are equilibrium constants k_n for the formation of the various complexes (10 below). From $g(u)$ one may then calculate $\bar{n}$, the average number of "links" per complex at any point of the $y(x)$ curve. We have (ABII[3], 14, 15, 73)

$$\log(1+g) = \int_{-\infty}^{x} y\, dx + \log(1-y) + y \log e \tag{5}$$

$$\log u = x - y \log e - \int_{-\infty}^{x} y\, dx \tag{6}$$

$$\frac{\mathrm{d} \log g}{\mathrm{d} \log u} = \bar{n} = \frac{\Sigma n c_n}{\Sigma c_n} \tag{7}$$

The variables u and g are (VIII, 31, 32; ABII, 2, 3, 3a):

$$u = mh^{-t} = [UO_2^{2+}]\ h^{-2} \tag{8}$$

$$g = f(u) = \Sigma\ k_n u^n \tag{9}$$

and k_n is the equilibrium constant for the reaction:

$$(n+1)UO_2^{2+} + n\ H_2O \rightleftharpoons UO_2(OUO_2)_n^{2+} + 2\ n\ H^+;\ \text{equil. const.} = k_n \tag{10}$$

For the calculations, a "best" curve $y(x)$ was drawn in two different ways, either: I) giving equal weight to the data for all M values or II) giving less weight to the very highest concentrations (Fig. 1); the latter seems preferable.

Table 1 gives the values for $\log(1+g)$ and $\log u$ obtained from (5) and (6) assuming the residue integral

$$\delta = \int_{-\infty}^{4.4} y\, \mathrm{d}x \tag{11}$$

to be zero.

Fig. 2 shows $\bar{n}$ as a function of x, using curve I with three values for δ and curve II with one δ value. The first part of the curve, for x less than about 6, is seen to be very sensitive to the choice of δ. It may be noted that the initial decrease in $\bar{n}(x)$ cannot be real because of the law of mass action. Moreover, the lowest possible value for $\bar{n}$ should be 1, since $\bar{n}$ is the average number of OUO_2 "links" per complex, and the uncomplexed ions UO_2^{2+} are not counted in the average (7). Since the first part of $\bar{n}(x)$ is also very sensitive to the way the "best" curve $y(x)$ is drawn through the experimental points, no great weight should be placed on its irregular behavior.

Table 1. Points from "best curves" I and II, and primary values for "direct analysis".

	Curve I			Curve II		
x	y	log $u + \delta$	log(1+g)-δ	y	log $u + \delta$	log(1+g)-δ
4.4	0.039	4.3831	−0.0004	0.040	4.3826	−0.0003
4.6	0.050	4.5694	0.0083	0.050	4.5693	0.0084
4.8	0.062	4.7530	0.0192	0.062	4.7529	0.0193
5.0	0.080	4.9310	0.0328	0.080	4.9309	0.0329
5.2	0.106	5.1011	0.0502	0.106	5.1010	0.0503
5.4	0.141	5.2612	0.0728	0.145	5.2589	0.0730
5.6	0.180	5.4121	0.1017	0.186	5.4080	0.1026
5.8	0.230	5.5494	0.1317	0.240	5.5420	0.1388
6.0	0.285	5.6740	0.1803	0.300	5.6619	0.1832
6.2	0.346	5.7844	0.2312	0.364	5.7677	0.2357
6.4	0.410	5.8810	0.2898	0.430	5.8597	0.2962
6.6	0.471	5.9665	0.3569	0.493	5.9400	0.3650
6.8	0.530	6.0407	0.4314	0.553	6.0093	0.4410
7.0	0.588	6.1037	0.5112	0.614	6.0661	0.5205
7.2	0.646	6.1551	0.5939	0.668	6.1145	0.6066
7.4	0.696	6.1992	0.6837			

The $\bar{n}(x)$ curve for x values higher than 6 is almost independent of the choice of δ. The "best" $y(x)$ curves I and II lead to $\bar{n}(x)$ curves which are only slightly shifted, and have the same general shape. The curve is seen to rise rather uniformly, without any "shelf", and has attained values of about $\bar{n} = 2.5$ at the end of the experimental range. There is no sign that the curve will bend off before $\bar{n} = 3$.

We may conclude that the complex with $n = 1$, $(UO_2)_2O^{2+}$, predominates at the lowest degree of hydrolysis, and that larger complexes are certainly formed, at least one with n not less than 3.

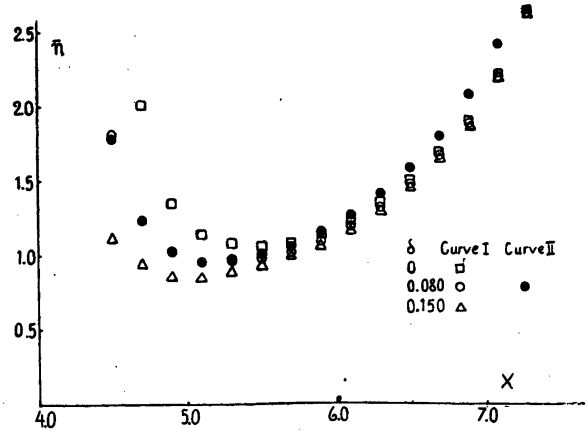

Fig. 2. Average number of links per complex, $\bar{n}$, calculated by direct analysis of $y(x)$ curve. Open symbols: from curve I with $\delta = 0$ (□). 0.080 (○), and 0.150 (△). Black dots ●; from curve II, $\delta = 0.080$.

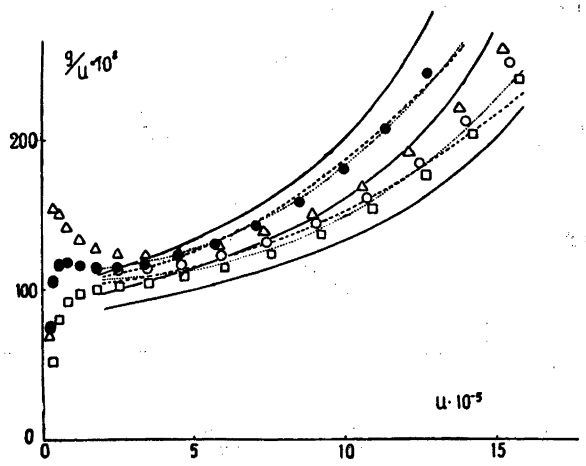

Fig. 3. Points: function gu^{-1}, calculated by direct analysis of $y(x)$ curve, symbols as in Fig. 2. Full-drawn curves: hypothesis IIIa, $k_0 = 2$, log $k = -6.40$ (lower), -6.35 (middle), -6.30 (upper). Broken curves: log $k_1 = -5.99$, log $k_3 = -18.30$ (lower); log $k_1 = -5.98$, log $k_3 = -18.10$ (upper). Dotted curves: log $k_1 = -5.975$, log $k_3 = -18.64$, log $k_4 = -24.70$ (lower), log $k_1 = -5.95$, log $k_3 = -18.30$, log $k_4 = -24.70$ (upper).

Limited series of complexes

In order to find possible values for the constant k_n we have plotted gu^{-1} as a function of u, as obtained by direct analysis of the $y(x)$ curves (Fig. 3, same symbols as in Fig. 2). According to (9) we should have

$$gu^{-1} = \Sigma k_n u^{n-1} = k_1 + k_2 u + k_3 u^2 + k_4 u^3 + \ldots \qquad (12)$$

We have tried to fit the empirical points in Fig. 3 with polynomials containing only a few members. For instance, the points obtained from curve I with $\delta = 0.080$ fit rather well with (12) assuming either of the sets:

$k_1 = 10^{-5.99}$; $k_2 = 0$; $k_3 = 10^{-18.30}$; $k_4 = 0$ (broken)
or $k_1 = 10^{-5.975}$; $k_2 = 0$; $k_3 = 10^{-18.64}$; $k_4 = 10^{-24.70}$ (dotted)

With these sets one obtains the lower broken and dotted curves in Fig. 3.

On the other hand, a good fit with the points from curve II with $\delta = 0.080$ is obtained for

$k_1 = 10^{-5.98}$; $k_2 = 0$; $k_3 = 10^{-18.10}$; $k_4 = 0$ (broken)
or $k_1 = 10^{-5.95}$; $k_2 = 0$; $k_3 = 10^{-18.30}$; $k_4 = 10^{-24.70}$ (dotted)

As seen from Fig. 3 the upward trend of the experimental data at the highest x indicates that there are terms of higher degree in (12) which have been neglected.

Fig. 4 shows the $y(x)$ curves calculated with these sets of constants — the differences are seen to be within the limits of experimental error. However,

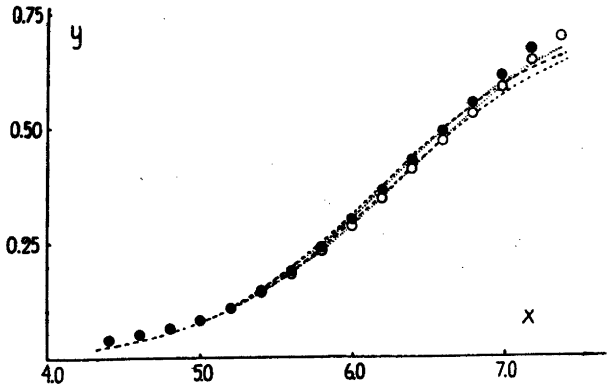

Fig. 4. Diagram y(x). Points from best curves I (circles) and II (dots). Curves: calculated with limited polynomials, symbols as in Fig. 3.

the experimental points for high y possess an upward trend relative to the curves calculated using polynomials with no higher constants than k_3 or k_4.

One can thus get a fair agreement assuming $k_2 = 0$. However we consider this fact as fortuitous.

Simple unlimited mechanisms

We have also compared the empirical $y(x)$ with curves calculated assuming the various mechanisms discussed in ABII. As could be expected already from the $\bar{n}$ curves, no mechanism involving a single complex (hypothesis II) gave an acceptable fit.

With the unlimited mechanisms, on the other hand, a reasonable fit was obtained with IIIa, $k_0 \approx 2$, $\log k = -6.35 \pm 0.10$. With either IIIb ($k_0 \approx 3 \rightarrow 4$) or IIIc ($k_0 \approx 0.5 \rightarrow 1.0$) the points for $y < 0.5$ could be made to agree, whereas the higher y deviated.

Mechanism IIIa would involve $k_n = k_0 k^n$. Thus we would have the equilibrium constant for the initial reaction:

$$UO_2^{2+} + H_2O + UO_2^{2+} \rightleftharpoons UO_2OUO_2^{2+} + 2\ H^+;\ kk_0 = 10^{-6.05 \pm 0.1} \qquad (13)$$

and the constants for all following reactions would be equal:

$$UO_2(OUO_2)_n^{2+} + H_2O + UO_2^{2+} \rightleftharpoons UO_2(OUO_2)_{n+1}^{2+} + 2\ H^+;\ k = 10^{-6.35 \pm 0.1} \qquad (13a)$$

Fig. 3 gives, as full-drawn curves, gu^{-1} calculated with IIIa, $k_0 = 2$ assuming $\log k = -6.30$, -6.35 or -6.40. The fit with any set of empirical points could be further improved by small adjustments of k_0 and k. However, we think this would mean no gain in real accuracy.

The $y(x)$ curves calculated with IIIa and the same k and k_0 values are given in Figs. 5 and 6. The agreement is satisfactory. The small deviation at the lowest values of y is not serious. In this range the solution is

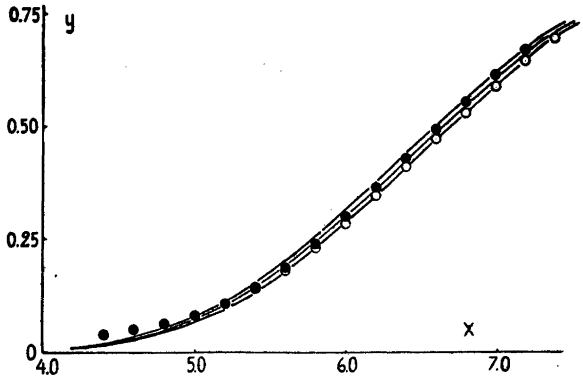

Fig. 5. Diagram y(x). Points from "best curves" I (circles), II (dots). Curves: calculated for mechanism IIIa with $k_0 = 2$ and log $k = -6.30$ (upper), -6.35, and -6.40 (lower).

fairly acidic, and y is less accurate partly because it is the difference between two large quantities, partly because of the liquid junction potential.

One can thus obtain a reasonable agreement with the experimental data assuming either a limited number of complexes $UO_2(OUO_2)_n^{2+}$, or an unlimited series of such complexes.

However, it seems hard to understand why Nature should form only the complexes with $n = 1$ and 3, or 1, 3 and 4 in the series (4) and then abruptly break the series at $n = 3$ or 4. To us it seems more plausible that the series goes on to high n, and that an extension of the experimental range would give

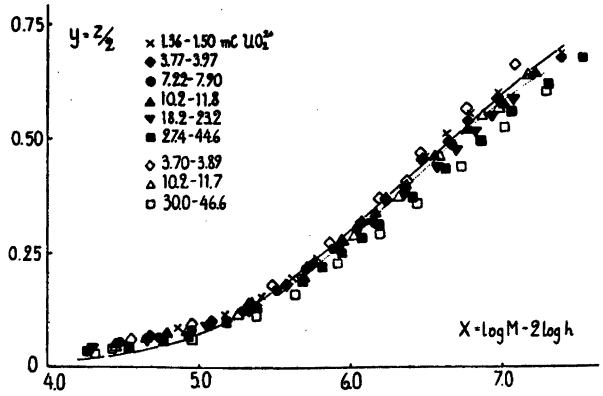

Fig. 6. Diagram y(x). All experimental points, symbols as in Fig. 1. Full-drawn curve: calculated for mechanism IIIa, $k_0 = 2$ and log $k = -6.35$. Dotted curve: calculated with log $k_1 = -5.975$, log $k_3 = -18.64$, log $k_4 = -24.70$.

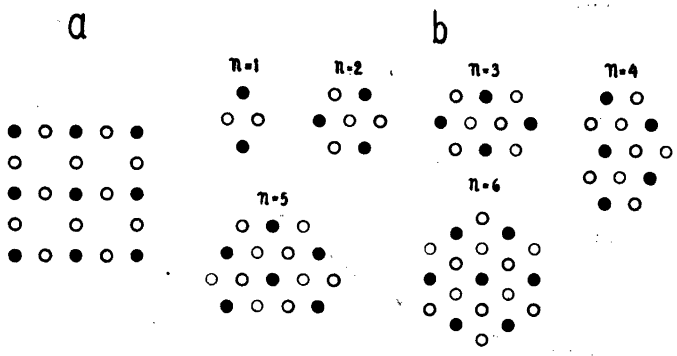

Fig. 7. Slightly idealized structure of uranyl-hydroxide (or -oxide) sheets: a) coordination UO_2 — 4 OH, OH — 2 UO_2, b) coordination UO_2 — 6 OH, OH — 3 UO_2. Possible structures of complexes $UO_2((OH)_2UO_2)_n^{2+}$ with n = 1 to 6 are shown as increasing fragments of a sheet. Black circles = uranyl groups with U in the plane of the paper and the two uranyl O (not shown here) above and below, perpendicular to the paper. Circles = oxygen atoms a) in the plane of the paper b) above (thick) and below (thin) the plane of the paper.

still higher $\bar{n}$ values. Also a consideration of possible structures of the complexes (4) gives little indication as to why a few n values should be preferred to all others (See below).

Comparison with other data

The formation of the complex $(UO_2)_2O^{2+}$ has been indicated by Faucherre[6] and Sutton[7]. Our equilibrium constant, $k_1 = 10^{-6.05}$ for ionic strength $I = 1.0$, may be compared with Faucherre's: $10^{-5.97}$ for $I = 0.60$ and $10^{-5.72}$ for $I = 0.06$. Sutton also found evidence for the complex $U_3O_8^{2+}$ and gave $k_1 = 10^{-5.94}$ for $I = 0.1$ and $k_2 = 10^{-12.9}$ (compare $k_2 = 10^{-12.4}$ with mechanism IIIa). He also proposed complexes like $U_3O_8(OH)_i^{2-i}$ (with $i = 1$, 2, 3, 4, and 5) but, unlike us, found no evidence for complexes with more than three U. — It would be interesting to have data obtained with a constant ionic medium and a wide range of M and Z also from the alkaline range, where uranium forms anions, according to Sutton.

Structure of the complexes

The equilibrium data have indicated that the general formula of the hydrolysis products of UO_2^{2+} can be described by the formula (4), $UO_2((OH)_2UO_2)_n^{2+}$, or $UO_2(OUO_2)_n^{2+}$. From X-ray investigations, several crystal structures are known where UO_2^{2+} groups are joined by O^{2-} or OH^- groups to form sheets.

In the crystal structure of $BaUO_2O_2$ (Samson and Sillén[8]), the coordination is UO_2—4 O, O—2 UO_2; in $CaUO_2O_2$ (Zachariasen[9]), we have UO_2—6 O, O—3 UO_2. These and a number of other metal uranyl oxides studied by

Zachariasen and coworkers were prepared from melts. Sheets with a coordination UO_2—4 OH, OH—2 UO_2 or UO_2—6 OH, OH—3 UO_2 have also been found in uranyl hydroxides prepared from aqueous solutions and studied in this laboratory.

One can imagine complexes of formula $UO_2(OUO_2)_n^{2+}$ as fragments of sheets with coordination UO_2—4 O, O—2 UO_2, or complexes $UO_2((OH)_2UO_2)_n^{2+}$ as fragments of sheets with coordination UO_2—6 OH, OH—3 UO_2 (Fig. 7). The latter structure seems to us to be more plausible because first it gives a more compact structure and second it would give a direct connection between the structure of the complexes and the structure of uranyl hydroxides which can be precipitated by increasing the pH of uranyl solutions.

Of course, we would not claim that no other complexes are found than those of the series (4). However, since it gives a good approximation to the equilibrium data in the range studied, and is easy to combine with the structure data, we hope that the mechanism proposed will contain some essential features of the process.

SUMMARY

Ahrland's data [1] on the hydrolysis of UO_2^{2+} in 1 C $NaClO_4$ medium at 20° C have been recalculated using methods for treating polynuclear complexes which have since been published [2-4]. It was found that the data can be explained assuming that all complexes formed in appreciable amounts can be written in the form (4), $UO_2((OH)_2UO_2)_n^{2+}$, or, which is equivalent, $UO_2(OUO_2)_n^{2+}$. This follows from the fact that Z (the number of H^+ split off per UO_2^{2+}) is, within the experimental error, a function of the single variable $x = \log M - 2 \log h$. No certain indication of mononuclear complexes could be found

A direct analysis of the $y(x)$ curve — $y = \frac{1}{2} Z$ — showed that several complexes with different n are formed. A reasonable agreement with the experimental data could be obtained either by limited mechanisms, assuming complexes with $n = 1 \rightarrow 3$ or $1 \rightarrow 4$, or with an unlimited mechanism, assuming that complexes with all integral values for n are formed. The authors are inclined to prefer the latter type of explanation.

A quite good approximation to the experimental data is obtained by the very simple assumption that the equilibrium constant for the first reaction: $UO_2^{2+} + H_2O + UO_2^{2+} \rightleftharpoons (UO_2)_2O^{2+} + 2\ H^+$ is $10^{-6.05 \pm 0.1}$, and that the equilibrium constants for all the following reactions: $UO_2(OUO_2)_n^{2+} + H_2O +$ $+ UO_2 \rightleftharpoons UO_2(OUO_2)_{n+1}^{2+} + 2\ H^+$ is $10^{-6.35 \pm 0.1}$.

In view of X-ray evidence on the structure of compounds that may be precipitated from uranyl solutions, it seems reasonable that the complexes are sheet-like, probably with double OH bridges (Fig. 7b). The most correct formula would thus be $UO_2((OH)_2UO_2)_n^{2+}$.

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Studies on the Hydrolysis of Metal Ions

XI. The Aluminium Ion, Al^{3+}

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In 1952, one of us (Brosset [1]) published the results of extensive studies on the behavior of the aluminium ion in perchlorate solutions of varying acidity at 40° C. The ionic medium was kept constant by means of 2 C $NaClO_4$, and the concentration of free H^+ (or OH^-) was measured by means of a hydrogen electrode.

Considerable difficulties were met with because equilibrium was attained rather slowly, especially in the region where precipitation occurred. It is however thought that the values finally given were not far from those at real equilibrium.

From the data it was obvious — contrary to what had been stated by many authors — that the main products of Al^{3+} hydrolysis are *polynuclear* complexes, and not mononuclear ones, even at rather low degrees of hydrolysis. To explain the data, the formation of an infinite series of complexes was assumed: $Al((OH)_3Al)_n^{3+}$ in the acid range, and $(Al(OH)_3)_nOH^-$ in the alkaline range. A satisfactory fit with the data was then obtained in a considerable part of the experimental range.

In the meantime, general methods for dealing with polynuclear complex equilibria have been given [2,3,4]. We have now applied these methods to the same data [1] in order to find out whether an equally good or perhaps better agreement could be obtained with some other polynuclear mechanism than those mechanisms originally proposed.

We shall use the same notation as in other papers of this series [4,5,6]: M = total Al concentration, h = hydrogen ion concentration, Z = average number of H^+ split off (OH^- bound) per Al. In the original paper [1], M and h were denoted by $[Al_t]$ and $[H^+]$, and Z by $\bar{n}$. In the original tables and figures, pOH was given as a measure of the acidity; it is easily recalculated to h using the pK_w value measured by Brosset [1] for the temperature and ion medium in question:

$$\log h = \text{pOH} - 13.52 \qquad (1)$$

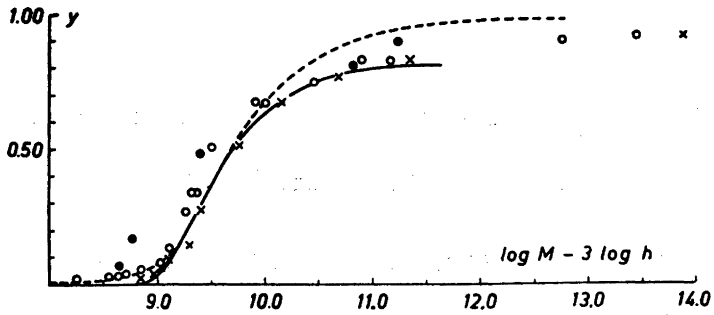

Fig 1. Acid range y(x) for reactants $-H^+$ and Al^{3+} with t = 3.0. Experimental data: × 2.209 mC, ○ 11.07 mC, ● 55.41 mC. Full-drawn curve: hypothesis II, N = 5. Dotted curve: hypothesis IIIa, log k = − 9.4, k_0 = 0.1.

The data were plotted (Fig. 7 [1]) in the form $Z(\mathrm{pOH})_M$ which is equivalent to $Z(\log h)_M$ because of (1). Three values for M were used: 55.41, 11.07, and 2.209 mC. In the alkaline range only the two lower concentrations were studied. As seen from Fig. 7 in the original paper[1], Z changes rapidly with $\log h$ in two ranges: in an *acid range*, $-\log h \approx 3$—5, with positive Al complexes (Z from 0 to about 2.5), and in an *alkaline range*, $-\log h \approx 9$—11, with negative Al complexes (Z from 3 to 4). The intermediate range, $Z = 2.5$—3, is characterized by very slow attainment of equilibrium.

We shall now discuss the acid and alkaline ranges separately.

ACID RANGE

General formula of the complexes

In the acid range, the $Z(\log h)_M$ curves for the three M values are approximately parallel, which would indicate that the complexes can be written in a "core + links" form

$$\mathrm{Al((OH)_tAl)_n} \qquad (2)$$

where t is a constant whereas n may assume several values. From the distance between the curves we obtain values for the derivative

$$\left(\frac{\partial \log M}{\partial \log h}\right)_Z \qquad (3)$$

between 2.5 and 3, which should be equal to t, the number of OH groups in the "link" of the complexes (part VIII[4], 14, 15).

To check the value for t, we plotted the function $y(x)$ where

$$y = \frac{Z}{t} \qquad (4)$$

$$x = \log M - t \log h \qquad (5)$$

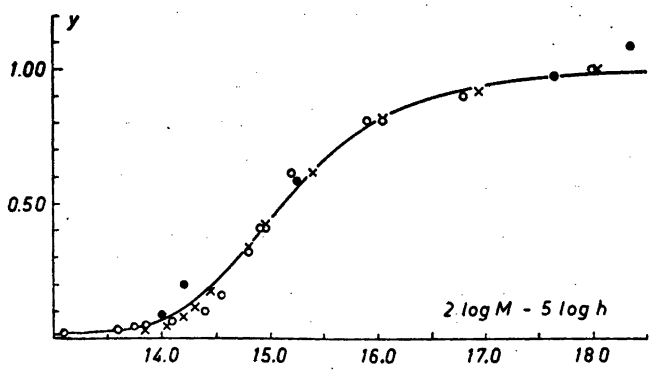

Fig 2. Acid range y(x) for t = 2.5. Experimental data: × 2.2 mC, ○ 11.07 mC, ● 55.41 mC. Curve calculated for IIIa, $k_0 = 0.5$, log k = − 14.8.

assuming t to be 3.0 (Fig. 1), 2.75, or 2.5 (Fig. 2). For the correct t value the points should be on the same curve. This is approximately so in both Fig. 1 and Fig. 2. Considering the general spread of the data, we thought it safe not to exclude any part of the range $t = 2.5 \rightarrow 3$. With $t > 3$, the spread becomes too great — mechanisms with $t < 2.5$ could not explain that the Z curves bend off close to $Z = 2.5$.

We shall thus assume that all complexes formed in appreciable amounts can be written in the form (2). However, we shall for the present leave the question open whether t is 2.5 or 3.0, or has some intermediate value.

Comparison with simple mechanisms

In the previous papers [5,6] on Th^{4+} and UO_2^{2+} a "direct analysis" of the $y(x)$ curve was made. Integrating $y(x)$ and applying equations (ABII[3], 14, 15, 73) a function $g(u)$ could be calculated which should be a polynomial with coefficients equal to the equilibrium constants for the formation of the various complexes. By differentiation of g one could moreover obtain the average number of "links" in the complexes at any value for x. Unfortunately, the spread of the data for Al^{3+} is too great to allow this procedure to be carried out with any accuracy.

We must then restrict ourselves to studying certain simple mechanisms, I—III, described in ABII [3] and involving only one or two parameters to be determined. Following ABII we compared the experimental $y(x)$ data for the tentative t values with families of curves $y(x)$, calculated for the simple mechanisms I—III.

Mechanisms with a single complex

With hypothesis I (no soluble complex, precipitate formed), no satisfactory agreement was found.

With hypothesis II (only one complex formed, with $n = N$) an acceptable agreement was found

for $t = 3.0$ with $N = 5$ $Al_6(OH)_{15}^{3+}$, $Z_{max} = 2.50$
for $t = 2.75$ with $N = 8$ $Al_9(OH)_{22}^{5+}$, $Z_{max} = 2.44$
for $t = 2.75$ with $N = 12$ $Al_{13}(OH)_{33}^{6+}$, $Z_{max} = 2.54$
for $t = 2.5$ with no value for N.

The agreement was better for $t = 3$ than for 2.75. With $t = 3$, $N = 4$ or 6 might also be possible.

With intermediate values for t one may expect that an acceptable agreement with the data would be obtained assuming complexes like the following

$Al_6(OH)_{14}^{4+}$ ($t = 2.8$, $N = 5$, $Z_{max} = 2.33$)
$Al_8(OH)_{20}^{4+}$ ($t = 2.86$, $N = 7$, $Z_{max} = 2.50$)
$Al_9(OH)_{23}^{4+}$ ($t = 2.88$, $N = 8$, $Z_{max} = 2.55$)

From the equilibrium data alone it is not possible to decide between these complexes and a few other similar ones. On the other hand, the data allow one to conclude that, if a single complex is formed in the acid range, it cannot be $Al_2(OH)_2^{4+}$, nor $Al_3(OH)_6^{3+}$, which have previously been proposed [7,8,9].

Assuming that the main product is the complex $Al_6(OH)_{15}^{3+}$, we may read off the x value where $y = \frac{1}{2}$, $x_{\frac{1}{2}} = 9.7 \pm 0.1$, and apply (ABII, 24), which gives $\log k = -9.4 \pm 0.2$ and the equilibrium constant (k^5)

$$6\ Al^{3+} + 15\ H_2O \rightleftharpoons Al_6(OH)_{15}^{3+} + 15\ H^+;\ [Al_6(OH)_{15}^{3+}]\ [H^+]^{15}\ [Al^{3+}]^{-6} = 10^{-(47\pm1)}\ C^{10} \qquad (6)$$

Repeated mechanisms

The hypotheses IIIa-c, involving the formation of complexes with all integral values for n, would have a physical meaning only for $t = 3$. For $t = 2.5$, complexes are possible only for even values of n ($n = 2\ n^*$): $Al((OH)_{2.5}Al)_{2n^*}$ or $Al((OH)_5Al_2)_{n^*}$. Since the link would be $Al_2(OH)_5$, it is easily seen that one can use the same arguments as in ABII after a few simple transformations. The expression for the concentration of the n^*:th complex, c_{n^*}, in (VIII, 30) will then always contain a power of $u^* = m^2h^{-5} = u^2$, and one may use the equations given in ABII (8, 8a, 9, 9a etc.), with the only difference that x is replaced by $x^* = 2 \log M - 5 \log h = 2\ x$.

Likewise, to compare the data recalculated for $t = 2.75$ with infinite mechanisms, one should plot y as a function of $x^* = 4 \log M - 11 \log h = 4\ x$.

For $t = 3$, the lower part of the experimental curve could be made to agree with the calculated curve for IIIa with $k_0 = 0.1$ if placed at $x_{\frac{1}{2}} = 9.7$, which gives $\log k = -9.4$ (Fig. 1). (The mechanism proposed in the earlier paper [1] was IIIa with $k_0 = 0.08$, and $\log k = -9.22$.) However, for $y > 0.5$ the experimental values were systematically lower than those calculated. A similar partial agreement could be obtained with IIIb ($k_0 \approx 0.1$) and IIIc ($k_0 \approx 0.01$).

For $t = 2.75$, IIIa ($k_0 = 5 \rightarrow 10$) was acceptable for the lowest y, but not for the high y values. Both IIIb ($k_0 \approx 10$) and IIIc ($k_0 \approx 2$) were acceptable in a somewhat wider range. We have $x^*_{\frac{1}{2}} = 34.5$.

For $t = 2.5$, an acceptable agreement was found with both IIIa ($k_0 \approx 0.5$) and IIIb ($k_0 \approx 0.1 \rightarrow 0.5$) in both cases with $x^*_{\frac{1}{2}} = 15.1 \pm 0.1$, whereas IIIc gave no good fit.

We conclude that an unlimited mechanism with $t = 3$ (complexes $Al((OH)_3Al)_n^{3+}$) is not likely but that, with the present data, one cannot exclude unlimited mechanisms with the formation of $Al((OH)_5Al_2)_n^{3+n}$ ($t = 2.5$) or $Al((OH)_{11}Al_4)_n^{3+n}$ ($t = 2.75$). With intermediate values for t one might get agreement with, *e. g.*, $Al((OH)_8Al_3)_n^{3+n}$ ($t = 2.67$).

If we assume the main product to be a series of complexes $Al((OH)_5Al_2)_n^{3+n}$, we would have the equilibrium constant for the initial reaction

$$3\ Al^{3+} + 5\ H_2O \rightleftharpoons Al_3(OH)_5^{4+} + 5\ H^+;\ kk_0 = 10^{-(15.1 \pm 0.1)}\ C^3 \qquad (7a)$$

and for all following n

$$Al_{2n+1}(OH)_{5n}^{3+n} + 2\ Al^{3+} + 5\ H_2O \rightleftharpoons Al_{2n+3}(OH)_{5n+5}^{4+n} + 5\ H^+;\ k = 10^{-(14.8 \pm 0.1)}\ C^3 \qquad (7b)$$

The $y(x)$ curve so calculated is given in Fig. 2.

Discussion

The data [1] for Al^{3+} hydrolysis thus allow us to exclude a number of mechanisms that have been proposed or might otherwise be conceived. Even if we keep to the simple one- or two-parameter mechanisms there remain, however, a certain number of mechanisms which would all give an acceptable agreement within the accuracy of the data.

To decide among them, or to discuss more complicated mechanisms (say, the formation of several complexes) one must have equilibrium data of higher accuracy and perhaps in a wider range of concentrations. To get such data, however, would involve difficult experimental problems. There are, fortunately, other data which may have some value as evidence.

A number of authors have studied Al-OH solutions which have been obtained by mixing Al^{3+} and OH^- in various proportions and allowing the mixture to age for different times. Clear chloride or nitrate solutions can be obtained with a ratio of up to 2.5 OH/1 Al ($Z = 2.5$, "$\frac{5}{6}$ basic solution") [7,10,11,12,13]. The formation of the Al^{3+} —OH^- complexes takes considerable time — of the order of 10—30 hours at about 20° C — and an intermediate precipitate is often observed which dissolves gradually. On the other hand, if H^+ is added to an aged $Al(OH)_{2.5}$ solution, the H^+ ions react very slowly. On titration with OH^-, an aged Al—OH solution gives a distinct pH change at about $Z = 2.5$.

This behavior is consistent with the slow formation of either a series of complexes $Al((OH)_5Al_2)_n^{n+3}$ (limiting ratio $Z = 2.5$) or a single complex such as $Al_6(OH)_{15}^{3+}$ or $Al_8(OH)_{20}^{4+}$.

Now a series of salts are known which can be written as containing the group $Al_2O_3H^+aq$ or $Al_2(OH)_5^+aq$ (thus $Z = 2.5$) and some anion. The chloride and nitrate are very soluble and hard to crystallize but several other salts, such as the sulfate, chromate, phosphate, silicate and oxalate, are only sparingly soluble (order of magnitude 0.05 mC[15]). The sulfate is described as forming nice tetrahedral crystals [12].

It is remarkable that on addition of sulfate ions to solutions with Z between 1.7 and 2.5, the only salt precipitated is this well-crystallized sulfate of gross composition $(Al_2(OH)_5)_2SO_4aq$ [12]. Likewise, oxalate ions, on addition to solutions with $Z = 0.8$ to 2.5 always give the oxalate $(Al_2(OH)_5)_2C_2O_4$ [7].

Now if there were a series of complexes in the solution, say $Al((OH)_5Al_2)_n$, and the transformations between them were very slow reactions, it is hard to conceive that a well-crystallized compound of composition $(Al_2(OH)_5)_2SO_4$ could precipitate quickly out of such a solution.

If, on the other hand, only one single complex with 2.5 OH/Al is formed, then the formation of a crystalline precipitate is very natural, once the complex has had time to form in the solution.

So we are led, on the basis of the present evidence, to prefer mechanisms with a single complex of ratio OH/Al close to 2.50 — the analytical data on the solid salts may allow a small deviation, say to 2.44 or 2.54.

The equilibrium data allow no certain decision between the several conceivable formulas $Al_6(OH)_{15}^{3+}$, $Al_8(OH)_{20}^{4+}$ etc. However, it may be said that the agreement gradually gets worse when the number of Al per complex is increased beyond 6. We will not put undue stress on the fact that Kohlschütter and Hantelmann [7], by cryoscopic measurements on solutions with 2.5 OH/1 Al, have found a molecular size corresponding to 6 Al, thus $Al_6(OH)_{15}^{3+}$. However, the complex with 6 Al seems to be more likely because of the crystal structure of γ-$Al(OH)_3$, the stable form. It consists of sheets, in each of which the Al atoms form 6-membered rings (Fig. 3) [16], each Al being joined to the two neighbouring Al by double OH bridges. An ion $Al_6(OH)_{15}^{3+}$ may be pictured as such a ring where additional OH^- groups are attached to alternate Al in the ring, and the remaining coordination places are filled up with H_2O.

It would be interesting to study the crystal structure of one of the $Al(OH)_{2.5}$ salts, in order to find the size and structure of the complex ion. The chromate is perhaps the most promising salt to start with.

On the basis of the present evidence we thus consider ring-like complexes $Al_6(OH)_{15}^{3+}$ as the most probable main reaction product of the hydrolysis of Al^{3+} in the acid range.

ALKALINE RANGE

In the alkaline range, Brosset [1] observed a slow reaction which made the pOH of a mixture shift by as much as one unit during the measurements. Three series of data were given: 1) the "first values", for experimental reasons measured about half an hour after the solution was prepared, 2) the "final values" measured where the emf did not seem to change any more (often after 60—90 hours), 3) the "zero values" extrapolated to the time zero.

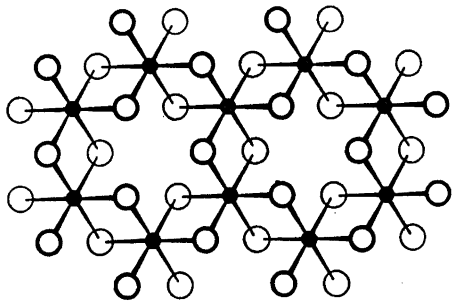

Fig 3. Part of the crystal structure of γ-Al(OH)$_3$ showing 6-membered Al rings. From A. F. Wells, Structural Inorganic Chemistry, second edition, Oxford University Press 1950, p. 417) by permission.

In Brosset's original Fig. 7, and in his calculations, only the zero values were considered. Clearly the "first values" represent a very arbitrary point in the slow process, and are not worth any special attention. Even the "final values" seem to spread strongly in a plot $Z(\log h)_M$, indicating that real equilibrium had perhaps not been reached.

The "zero values", on the other hand, form two reasonably parallel curves. The distance between them gives us a value —1 for the derivative (3), so that the sign is reversed in comparison with the acid range.

Since the concentration of free Al^{3+} is negligible, the argument in (ABI[2], p. 308 and VIII[4], 19—21) can be applied. From ABI 20, or VIII 19, we conclude that the general formula of the complexes in this range may be written $OH(Al(OH)_t)_n$. Since the limiting values for Z are 3 and 4, we conclude that $t = 3$, and that the complexes may be written in the form $(Al(OH)_3)_nOH^-$ It is then convenient to proceed as outlined in (ABI, p. 311 and VIII 20—21), namely to consider the smallest complex, $Al(OH)_4^-$, as the core. The link, $Al(OH_3)$, is a complex of $Al(OH)_4^-$ and H^+. The reactants A and B in the equations of ABI and ABII thus correspond to H^+ and $Al(OH)_4^-$, and the general complex $B(AB)_n$ is formed by a reaction

$$\begin{aligned} &Al_n(OH)_{3n+1}^- + Al(OH)_4^- + H^+ \rightleftharpoons Al_{n+1}(OH)_{3n+4}^- + H_2O \\ &B(AB)_{n-1} + B + A \rightleftharpoons B(AB)_n \end{aligned} \qquad (8)$$

The total concentration of B is M, and the average amount of A bound per B is Z', which is related to the Z defined with Al^{3+} and $-H^+$ as reactants by

$$Z' = 4 - Z \qquad (9)$$

This follows from

$$Z = \frac{\Sigma(3n+4)c_n}{\Sigma(n+1)c_n}\,;\; Z' = \frac{\Sigma n c_n}{\Sigma(n+1)c_n} \qquad (9a)$$

The data can be reduced to the form $y(x)$ by the transformations

$$\begin{aligned} y &= Z'/t' = 4 - Z \\ x &= \log B + \log a = \log M + \log h \end{aligned} \qquad (10)$$

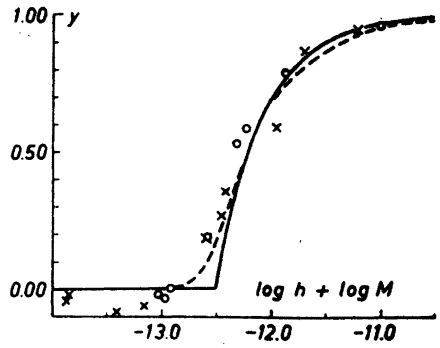

Fig 4. Alkaline range, reactants H^+ and $Al(OH)_4^-$, $y(x)$ for $t = 1$. Experimental data, "zero values": × 2.209 mC, ○ 11.07 mC. Full-drawn curve calculated for mechanism I, broken curve for IIIa with $k_0 = 0.01$, log $k = 12.5$.

The "zero values" are given in the form $y(x)$ in Fig. 4, and are seen to lie roughly on the same curve. A certain spread in the experimental values makes it impractical to use the "direct analysis".

A comparison with the calculated $y(x)$ curves from ABII shows that the experimental points, within the limits of error, fall on the curve for the limiting hypothesis I: no soluble complex, precipitate formed (full-drawn curve in Fig. 4). One may also find a fair agreement with hypothesis IIIa using a very small value for k_0 (IIIa with $k_0 = 0.01$, log $k = 12.63$ was the hypothesis proposed in the original paper [1]; broken curve in Fig. 4). This is quite natural since I is the limiting case of IIIa for $k_0 \to 0$.

Since, with the present accuracy, we cannot definitely state that k_0 is different from 0, we shall assume the simplest hypothesis I ($k_0 = 0$) which implies that we have only the ion $B = Al(OH)_4^-$ and solid $AB = Al(OH)_3$ (or AlOOH; they cannot be distinguished by equilibrium measurements). The equilibrium constant comes out as

$$Al(OH)_3(s) + H_2O \rightleftharpoons Al(OH)_4^- + H^+;\ K_s = k^{-1} = h\,[Al(OH)_4^-] = 10^{-12.5\pm0.2} \tag{11}$$

By using Brosset's value 13.52 for pK_w in this medium we find for the reaction

$$Al(OH)_3(s) + OH^- \rightleftharpoons Al(OH)_4^-;\ K = [Al(OH)_4^-][OH^-]^{-1} = 10^{1.0\pm0.2} = 10 \pm 6 \tag{11a}$$

Of course, it is by no means proved that polynuclear anions are not formed in the solution but obviously the present data can, within the limits of accuracy, be explained by means of the simple reaction (11).

The "final values", if treated in the same way, give no smooth $y(x)$ curve (Fig. 5). Assuming that $Al(OH)_4^-$ and $Al(OH_3)$ (s), the latter in some other modification, are the only species present, one may draw a curve for hypothesis I

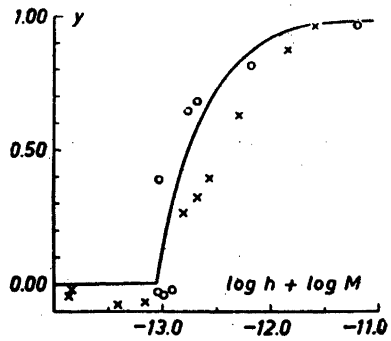

Fig 5. Alkaline range, "final values", × 2.209 mC, O 11.07 mC. Curve calculated for I with log k = 13.0.

through the points $y(x)$ and find a rough average value for log $k = 13.0 \pm 0.2$, and thus the constant in (11a) $K = 10^{0.5 \pm 0.2}$. The solid phase would then have been partly transformed into a less soluble modification. However, it is also conceivable that soluble complexes have been formed.

SUMMARY

Brosset's [1] data on the hydrolysis of Al^{3+} have been recalculated, using the general methods for treating polynuclear equilibria that have recently been published [2,3].

In the acid range ($Z = 0 \rightarrow 2.5$) Brosset's data can be explained assuming either a single and rather large complex, such as $Al_6(OH)_{15}^{3+}$, or an infinite series of complexes $Al((OH)_5Al_2)_n^{3+n}$. Mononuclear complexes ($AlOH^{2+}$, $Al(OH)_2^+$) and such polynuclear complexes as $Al_2(OH)_2^{4+}$ and $Al_3(OH)_6^{3+}$, if they exist, are certainly by no means the main product. The equilibrium data alone do not permit one to decide whether a single complex or a series of complexes is formed. However, judging from the behavior of aged solutions and crystallographic evidence, the complex $Al_6(OH)_{15}^{3+}$ is thought to be the most likely main product. The main reaction, and its equilibrium constant, are given in equation (6).

In the alkaline range ($Z = 3 \rightarrow 4$) Brosset's "zero values" can be explained assuming the existence of a single complex $Al(OH)_4^-$ and solid $Al(OH)_3$. For $Al(OH)_3(s) + OH^- \rightleftharpoons Al(OH)_4^-$, log $K = 1.0 \pm 0.2$. The aging process may be the transformation of the originally precipitated $Al(OH)_3$ to a more stable form for which log $K = 0.5 \pm 0.2$.

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Phase and Structural Relations in the System Iron Tellurium

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Only a few investigations have dealt with the reaction between iron and tellurium and the formation of iron tellurides. Tammann and Schaarwächter [1] subjected a mixture of equivalent amounts of iron and tellurium to thermal analysis and examined the reaction product. It was found to consist of an iron tellurium compound surrounded by tellurium, indicating the existence of an iron subtelluride. Moser and Ertl [2], as well as Oftedal [3], also only obtained heterogeneous preparations. Oftedal was, nevertheless, able to demonstrate by X-ray methods that his quenched preparations contained a phase Fe_xTe_y of the $B8$ (NiAs) structure type, having a composition which most likely deviated from the simple stoichiometric formula FeTe. Brukl [4] on the other hand, reported the existence of an iron monotelluride with stoichiometric composition formed by the action of hydrogen telluride on ferrous salt solutions.

Tengnér [5] prepared iron ditelluride, $FeTe_2$, and determined its crystal structure to be of the $C18$ (marcasite) type. $FeTe_2$ was later found in nature by Thompson [6], who gave it the name Frohbergite.

Our study of the system iron tellurium was started several years ago, and only a short note [7] in Norwegian giving some preliminary results has thus far been published.

PREPARATION OF THE ALLOYS

The *iron* used was prepared from Fe_2O_3, Merck "Ferrum oxydatum sec. L. Brandt", by reduction with dry, purified hydrogen gas, first for 4 hours at 400°—600° C and then for 2 hours at 1 000° C. Heating at the latter temperature was prolonged until constant weight was obtained.

One part of the *tellurium* used was "Tellurium, reinst in Stangen" from Kahlbaum. Another part was prepared by dissolving tellurium metal in nitric acid (d = 1.25) at 70° C and recrystallizing the basic nitrate. The nitrate was dissolved in nitric acid and hydrated tellurium oxide precipitated by hydrolysis. The oxide was washed and heated to 600° C and then dissolved in hydrochloric acid. From this solution tellurium was precipitated, partly with hydrazine hydrate and partly with sodium hypophosphite. Finally, the tellurium was subjected to repeated vacuum distillations in silica vessels. A spectro-

graphic analysis, kindly carried out by sivilingeniør S. Rutlin, Statens Råstofflaboratorium, Oslo, revealed no impurities *, and this material was used for the determination of the lattice constants and the composition of the phases.

The alloys were prepared by heating calculated amounts of iron and tellurium in evacuated and sealed silica tubes. The heating of the preparations was done in various ways, but the preparations used for phase analysis were first heated at 900° C for 24 hours. At this temperature all preparations with more than 44.5 atomic % tellurium were molten. After they had been cooled, the grey, metallic reguli were finely ground and parts of each regulus were annealed, respectively, at 300° C, 600° C and 750° C for 24 hours in evacuated and sealed silica tubes. The samples were quenched from these temperatures in ice water.

Tellurium-rich preparations, which were made in order to determine the composition of the iron ditelluride, were annealed for 14 days at 425° C and slowly cooled to room temperature. Single crystals of one of the iron telluride phases (the β-phase) were obtained by heating $FeTe_{0.5}$ and $FeTe_{0.67}$ for 34 days at 800° C. In all, iron tellurium alloys with 29 different compositions were made in the range $FeTe_{0.5}$ to $FeTe_3$.

Some FeTe alloys were prepared at 1 000° C. In that case the walls of the silica tubes got covered with a thin black film, and a small condensate was found in the top of the tube. This black coating was avoided by holding the temperature at 900° C, and the distillation process was avoided by keeping the top part of the silica tube slightly hotter than the bottom.

PHASE INVESTIGATION BY X-RAY DIFFRACTION

All the alloys prepared were examined by the X-ray powder method. Cameras with 5.75 cm diameter were used for phase identification and a Philips 11.48_3 cm diameter camera, with Straumanis asymmetric film mounting, was used for lattice constant determinations. Fe$K\alpha$ radiation was employed and all the lattice constants are expressed in Å units (λ Fe$K\alpha_1$ = 1.93597 Å). The error in the lattice dimensions is less than 0.001 Å for values given to the fourth decimal, and less than one part in two thousand when three decimals are given, unless otherwise stated. Lattice constants from earlier investigations have been transformed from kX to Å units by multiplication by the factor 1.00202.

The iron used was found to have the lattice constant $a = 2.8663 \pm 0.0002$ Å at room temperature (20 ± 2° C). This value is in perfect agreement with the precision value $a = 2.86635$ Å recently reported by Thomas [8]. The lattice constant found for iron in equilibrium with the first intermediate iron telluride phase was $a = 2.8669 \pm 0.0002$ Å in the preparation $FeTe_{0.60}$ quenched from 900° C. The slight difference between the two values indicates the range of homogeneity of the α-phase in the system iron tellurium to be very narrow.

a. Phase relations below 425° C

Phase analysis of preparations quenched from 300° C led to the conclusion that the intermediate iron telluride phase richest in iron exists in the composition range $FeTe_{0.8}$ to $FeTe_{0.9}$. X-ray powder photographs of this phase — *the β-phase* — could be indexed assuming a tetragonal unit cell. For a preparation

* Some tellurium of another origin (Tellurium lump, B.D.H.) was distilled without chemical purification. In this case the spectrographic analysis showed the presence of some Fe and traces of Mg and Pb.

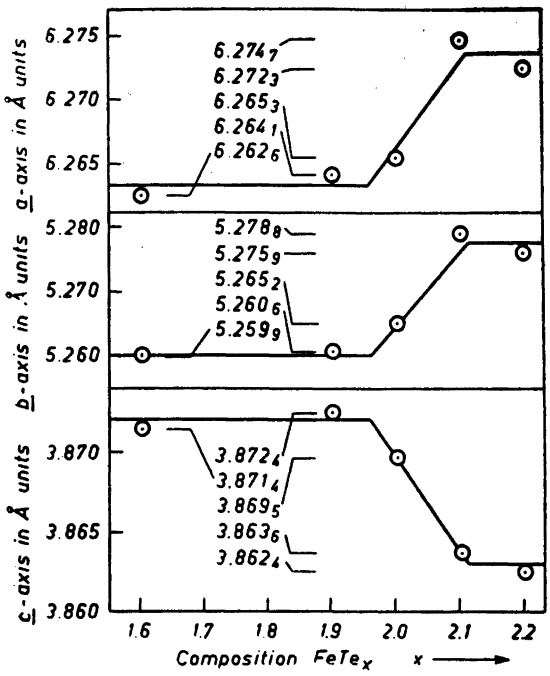

Fig. 1. Lattice constants of the orthorhombic ε-phase in the range $FeTe_{1.60}$ to $FeTe_{2.20}$.

$FeTe_{0.8}$ where the β-phase exists in equilibrium with the α-phase, iron, these lattice constants were computed:

$$a = 3.823_0 \text{ Å}, \quad c = 6.276_7 \text{ Å}.$$

In equilibrium with the phase richer in tellurium the following lattice constants were found for the β-phase:

$$a = 3.819_8 \text{ Å}, \quad c = 6.280_5 \text{ Å}.$$

The slight variation in the lattice constants leads us to assume that the β-phase has only a narrow range of homogeneity, and as the ferromagnetism* of $FeTe_{0.88}$, which is probably due to iron, has disappeared almost completely at $FeTe_{0.90}$, the range of homogeneity of the α-phase should be close to the composition $FeTe_{0.90}$.

At the stoichiometric composition FeTe many additional interferences from a phase richer in tellurium are observed. There exists thus, at lower temperatures, no iron telluride phase with the composition FeTe. At the composition $FeTe_{1.50}$ all interferences belonging to the tetragonal β-phase have disappeared and interferences from a new intermediate phase — *the ε-phase* —

* The magnetic properties of the iron tellurides will be discussed in another communication.

Table 1. Lattice constant determinations of orthorhombic $FeTe_2$ in Å units. Axes in standard setting (Pnnm) of the International Tables [10]. *Transformation to the old (Pmnn) marcasite orientation in terms of the present: $00\bar{1}/010/100$ and to the orientation after Buerger* [11] *(Pnnm) → present: $010/100/00\bar{1}$.*

a	b	c	Authors
6.273	5.352	3.857	Tengnér [5]
6.27	5.29	3.86	Thompson [6]
6.265_3	5.265_2	3.869_5	Present

which is identical with the $FeTe_2$-phase with marcasite structure found by Tengnér [5], exist alone on all photographs of preparations up to the composition $FeTe_{2.5}$. In this preparation the strongest tellurium interferences are observed.

In order to establish the composition limits of the ε-phase at "room temperature" a series of preparations was made, annealed for 14 days at 425° C and slowly cooled to room temperature. X-ray photographs show that $FeTe_{1.60}$ and $FeTe_{1.90}$ have practically the same cell dimensions. The two phase region between the β- and ε-phase thus extends at least to the composition $FeTe_{1.90}$. With further increase in the tellurium content the lattice dimensions start to change at about $FeTe_{1.95}$. As shown in Fig. 1 the a- and especially the b-axis increase with increasing tellurium content until the composition $FeTe_{2.10}$ is reached, while the c-axis shows a slight decrease in the same concentration range. The ε-phase is accordingly assumed to be homogeneous in the range $FeTe_{1.95}$ to $FeTe_{2.10}$ (66.1 to 67.7 atomic % Te). This is the first marcasite-like phase which has been shown to have a metalloid content exceeding the formula AB_2.

In Table 1 are listed the results of our lattice constant determination of $FeTe_2$, together with the results of Tengnér [5] and Thompson [6]. The agreement between Tengnér's results and ours is very good for the a- and c-axis, but there is a remarkable disagreement regarding the length of the b-axis. The lattice constants of the mineral Frohbergite as determined by Thompson [6] agree well with our values for synthetic $FeTe_2$.

No indications of the existence of iron tellurides higher than $FeTe_2$ have been found. In Table 2 are listed the lattice constants determined for tellurium

Table 2. Lattice constants of tellurium in Ångström units.

Sample	a-axis	c-axis	T °C
$FeTe_2$ + Te	4.454_9	5.922_9	20 ± 2
Tellurium	4.456_0	5.925_1	20 ± 2
Te, Straumanis[9]	4.45591 ±0.00012	5.92687 ±0.00002	20

in a two-phase preparation consisting of the ε-phase and tellurium. In Table 2 is also found our lattice constant determination of pure tellurium, together with the precision determination made by Straumanis [9]. The results indicate that the solubility of iron in tellurium is small.

b. Phase relations in preparations quenched from 600° C

When we consider the phase relations in preparations quenched from 600° C, we find that the β- and ε-phases still appear on the photographs. In addition to these, another intermediate phase — *the δ-phase* — is found in the composition range $FeTe_{1.40}$ to $FeTe_{1.50}$. A closer study revealed that the quenched δ-phase has a pseudo-hexagonal structure at the composition $FeTe_{1.40}$ (58.33 atomic % Te) which is close to the iron rich limit of the phase. The structure was tentatively indexed as monoclinic, and the corresponding lattice constants are listed in Table 3. The monoclinic deformation disappears with increasing tellurium content and the phase becomes hexagonal at the composition $FeTe_{1.50}$. No two phase region has been observed and, consequently, the monoclinic and the hexagonal structures probably belong to the same phase.

Table 3. Lattice constants of the δ-phase in Ångström units.

Sample	a	b, $(a\sqrt{3})$	c	β
$FeTe_{1.40}$	3.846	6.661	5.641	90.2°
$FeTe_{1.50}$	3.816_2	(6.610_1)	5.654_8	(90°)
Fe_xTe_y, Oftedal [3]	3.808 $\pm$0.003	(6.596)	5.662 $\pm$0.005	(90°)

The lattice constants of $FeTe_{1.50}$ are also listed in Table 3, together with the values found by Oftedal [3]. The agreement between the cell dimensions and intensities of our δ-phase and the Fe_xTe_y-phase with $B8$-like structure found by Oftedal shows that they are no doubt identical.

It is known from the investigation of chromium sulfides, selenides and tellurides by Haraldsen *et al.*[12] that a $B8$ (NiAs)-like phase exists in the composition range $CrX_{1.40-1.50}$ (X = S, Se or Te). The same deviating composition has now been found for the hexagonal iron telluride. The analogy between the iron tellurium δ-phase and the chromium chalcogen phases goes even further, as a continuous change from monoclinic to hexagonal structure is observed for these phases in the same composition range. The δ-phase is, however, only obtained in quenched preparations, while the corresponding chromium chalcogenides are also obtained in slowly cooled preparations.

A series of FeTe samples was annealed and quenched from successively lower temperatures starting with 600° C. When the annealing temperature was 500° C, interferences from the δ-phase could still be observed, while at 480° C the δ-phase had disproportionated into the β- and ε-phases. This is in

good agreement with a thermal analysis made on $FeTe_{1.50}$ by which the decomposition temperature was determined to be 488° ± 2° C.

Some experiments were carried out at 600° C to see how quickly the hexagonal δ-phase was formed from the β- and ε-phases. Even after an annealing time of 15 minutes the δ-phase appeared with as sharp and well defined interferences as it did after 24 hours annealing time. On the other hand, experiments with five minutes annealing time proved insufficient to obtain sharp interferences.

c. Phase relations in preparations quenched from 750° C

The β-, as well as the δ- and ε-phases, are found at their respective places in preparations quenched from 750° C. In addition to this, the existence of still another phase was noted. The phase was observed on powder photographs of the preparations FeTe, $FeTe_{1.05}$, $FeTe_{1.10}$ and $FeTe_{1.15}$, when quenched from 750 ± 30° C. The composition of this phase — *the γ-phase* — is judged to be around $FeTe_{1.10}$. The composition is, however, somewhat uncertain since the photographs have blurred interferences and high background blackening.

During annealing experiments made in order to obtain single crystals of the γ-phase condensates were sometimes observed in the top of the silica tube *. X-ray photographs of the condensates showed a mixture of the γ- and δ-phases.

It is difficult to decide if the γ-phase is appreciably volatile under the present conditions. Another possibility is that the β-phase volatilizes and condenses in the cooler part of the tube, where it reacts with tellurium vapor from dissociated iron telluride. In this way also a mixture of the γ- and the δ-phases could be obtained.

To summarize the results of the phase analysis of the system iron tellurium we may describe the four intermediate phases which were found as follows:

1) The tetragonal β-phase with composition close to $FeTe_{0.90}$ exists in the quenched and slowly cooled preparations.
2) The γ-phase with unknown structure exists close to the composition $FeTe_{1.10}$ and is only stable around 750° C.
3) The monoclinic-hexagonal δ-phase with a composition range $FeTe_{1.40}$ to $FeTe_{1.50}$ is only stable above 488° C.
4) The orthorhombic ε-phase with composition $FeTe_{1.95}$ to $FeTe_{2.10}$ is found both in quenched and slowly cooled preparations.

CRYSTAL STRUCTURES OF THE IRON TELLURIDE PHASES

a. The β-phase, $FeTe_{0.90}$

The crystals obtained of the β-phase consisted of layers, partly forming rectangular pyramides, often double, with a slight angular distortion of the

* An attempt to obtain larger quantities of this distillate by one months annealing of $FeTe_{1.2}$ at 750° C was unsuccessful as the silica tube was strongly attacked, and the condensate in the upper part of the tube had been subject to various reactions.

layers, and partly forming tiny needles growing in the layer plane. Some of the needles had small excrescences normal to the needle axis.

Rotation photographs have been taken of many of these crystals and also of a tabular crystal with metallic luster. One photograph was taken with the rotation axis normal to the plate and two others with the rotation axis at right angles to the first. The last two photographs were identical and different from the first one. Accordingly, the crystal should have tetragonal structure with the c-axis normal to the plate. Photographs of the needle-shaped crystals showed that the a-axis was the needle axis of these crystals. The following identity periods were found: $a = b = 3.8$ Å, $c = 6.3$ Å.

The photographs were examined closely with regard to superstructure interferences, but no indications of a larger unit cell were found on photographs of crystals grown at 800° C.

In order to establish the cell content of the β-phase, a density determination was carried out on $FeTe_{0.90}$ in vacuum with kerosene as pycnometer liquid. The density was found to be 6.77_7 g/cm^3 at 20° C. Using $1.660 \cdot 10^{-24}$ as the atomic weight unity in gram, a cell content of 2.19 Fe atoms and 1.98 Te atoms is found. This result shows that the structure of $FeTe_{0.90}$ can be considered as an AB-interstitial structure with the formula $Fe_{1.11}Te$ or Fe_9Te_8.

Table 4. Observed and calculated intensities of interferences from tetragonal $FeTe_{0.90}$. $z_1 = 0.285$, $z_2 = 0.70$.

$\sin^2\Theta \cdot 10^4$	*hkl*	I_{obs}	I_{calc}	$\sin^2\Theta \cdot 10^4$	*hkl*	I_{obs}	I_{calc}
238	001	1	0.2	—	005	—	0.2
—	100	—	0	6012	301	3	3.2
880	101	8	7.4	6084	222	1	0.9
952	002	1	0.3	6368	204	6	4.7
1282	110	1	1.1	6397	310	1	1.4
1521	111	2	2.9	6580	105	1	0.9
1593	102	1	1.0	6659	311	2	3.7
2136*	003	2	1.6	—	302	—	0.7
2235	112	*10*	*10*	7010	214	4	4.9
2563	200	4	4.8	7223	115	8	9.6
—	201	—	0.2	7262	223	4	6.0
2780	103	3	2.4	7363	312	18	20
—	210	—	0	7908	303	5	3.7
—	113	—	0.2	—	320	—	0
3441	211	7	6.5	8505	205	2	2.1
3511	202	1	0.9	—	313	—	0.8
3802	004	2	1.0	8572	321	15	15
4151	212	1	1.1	—	006	—	0.1
4445	104	2	1.8	8929	224	15	14
4701	203	3	4.0	9146	215	6	5.6
—	114	—	0.2	9192	106	18	13
5129	220	2	3.6	9287	322	3	4.2
5344	213	5	4.0	9575	304	11	10
—	221	—	0.1	9836	116	18	15

* At this and higher angles the α doublet is resolved and the $\sin^2\Theta$ value refers to the α_1 component.

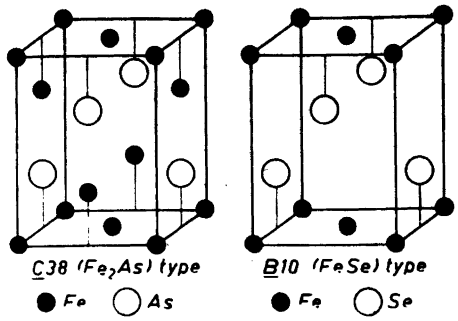

Fig. 2. Atomic arrangement for the B10 and C38 structure types.

The only systematic missing reflections on our Weissenberg photographs are those of the type $hk0$ with $h + k = 2n + 1$. They characterize the space groups $P4/n$ (C_{4h}^3) and $P4/nmm$ (D_{4h}^7). Apparently, the structure of the β-phase is closely related to the structure of tetragonal FeSe, found by Hägg and Kindström [13]. This structure is of the tetragonal PbO type ($B10$) with iron atoms in the oxygen positions: (a) 0,0,0; 1/2,1/2,0, and selenium in the lead positions: (c) 0,1/2, z; 1/2,0, $\bar{z}$. The intensities on the single crystal photographs showed accordingly that two iron atoms occupy positions (a) while the two tellurium atoms are in (c) with $z = 0.285$.

Intensities, determined photometrically for all interferences occurring on a powder photograph of $FeTe_{0.90}$ taken with Fe$K\alpha$ radiation, are listed in Table 4 together with the calculated intensities and the $\sin^2\Theta$ values. To facilitate comparison, the observed and calculated values of 112 have been set equal. The agreement between observed and calculated intensities is seen to be very satisfactory.

The tetragonal β-phase has a typical layer structure. This caused a pronounced, preferred orientation in the powder samples used originally, and too high intensities for the reflections from the basal planes and all other planes forming small angles with them, relative to the reflections from the prism planes.

The formation of crystal flakes was best prevented by letting the iron and tellurium react at 500° C and allowing only a short time for homogenization. Prepared in this way, the sample did not consist of flakes and the irregular intensity relationship was avoided.

The tetragonal $FeTe_{0.90}$ contains 0.2 interstitial iron atoms per unit cell. There are two reasonable positions for these atoms in the space group D_{7h}^4: (b) 0,0,1/2; 1/2,1/2,1/2, or (c) 0,1/2, z; 1/2,0, $\bar{z}$, with $z \approx 0.70$. If we assume that the excessive iron atoms are distributed on the (c) positions — the intensities in Table 4 have been calculated for that assumption — the structure can be regarded as a transition link between the $B10$ structure type and the $C38$ type, found by Elander, Hägg and Westgren [14] for Fe_2As and Cu_2Sb. The difference between these two structure types is seen from Fig. 2, in that two additional metal atoms located are in (c) 0,1/2, z; 1/2,0, $\bar{z}$, with $z \approx 0.70$ in the $C38$ structure.

A transition between the *B*10 and *C*38 structure type similar to that for tetragonal $FeTe_{0.90}$ was found by Forman and Peacock [15] for Rickardite, $Cu_{4-x}Te_2$ (x = 1.2). The structure is described as being of the *C*38 type, but grossly defective, with an almost invariant proportion of vacant Cu-positions in (*c*). Therefore, Rickardite $Cu_{1.40}Te$ may also be counted as a link between the *B*10 and *C*38 types. It can furthermore be mentioned that Haraldsen and Grønvold [16] found that tetragonal FeSe has a composition which deviates slightly from the stoichiometric ratio, and that Forman and Peacock [15] also observed a defective composition for Cu_2Sb. A study of the nickel tellurium system, which has been carried out in this institute by Jan Barstad has likewise revealed a phase with similar tetragonal structure close to the composition Ni_3Te_2.

In the *B*10 structure the Te atoms are arranged in a distorted cubic close packing (ideal for $c/a = 1.414$ and $z = 0.25$) with eight of the twelve Te atoms at a distance of 3.82 Å and the rest at 4.49 Å. This gives a Te radius of 1.91 Å, which is definitely shorter than the ionic radius of Te^{-2} (2.11 Å according to Goldschmidt [17] or 2.21 Å according to Pauling [18]). Each Te atom is further surrounded by 4 Fe atoms at a distance of 2.60 Å or far shorter than the sum of the ionic radii for Fe^{+2} and Te^{-2} (which is 0.83 Å + 2.11 Å = 2.94 Å according to Goldschmidt [17]), but close to the sum of the elemental radii (1.24 Å + 1.43 Å = 2.67 Å).

The deviation from the composition FeTe of the iron telluride led us to investigate whether or not the tetragonal PbO also had a non-stoichiometric composition. LeBlanc and Eberius [19] ascribed a range of homogeneity to PbO between PbO and $PbO_{1.10}$. However, Byström [20] found no signs of a range of homogeneity for the tetragonal PbO-phase in his thorough X-ray work on lead oxides. In addition to X-ray studies, which confirmed Byströms results at this point, we have also carried out chemical analyses of PbO samples carefully prepared in different ways. By reduction with dry, purified hydrogen gas, the lead was determined as metal and the oxygen as water, absorbed in phosphorus pentoxide. The results of these analyses are listed in Table 5. They indicate that the composition of tetragonal PbO does not deviate appreciably from the stoichiometric ratio.

Table 5. Analyses of four lead monoxides.

Oxide sample	Oxide weight in gram	Found (gram)		PbO_x
		Pb	O	x
PbO, red, tetragonal from NaOH solution	1.3915$_6$	1.2907$_0$	—	1.012
PbO, tetragonal	1.2534$_2$	1.1632$_2$	—	0.998
PbO, tetragonal, from $PbCO_3$	1.1999$_2$	1.1133$_6$	0.0857$_3$	0.997
PbO, orthorhombic, from PbO tetr. heated 24 hours at 700° C	1.2947$_3$	1.2003$_4$	0.0917$_2$	0.990

b. The γ-phase, $FeTe_{1.1}$

The structure of the γ-phase has not yet been established, and the phase can therefore only be characterized by means of the position and relative intensities of the interferences. In Table 6 are listed the results from the best X-ray photograph obtained in a 5.75 cm diameter camera.

Table 6. Interferences observed on a photograph of the γ-phase, $FeTe_{1.10}$, heated for 7 days at 750° C and quenched. Fe$K\alpha,\beta$ radiation.

I_{obs}	Θ	$\sin^2\Theta \cdot 10^4$	I_{obs}	Θ	$\sin^2\Theta \cdot 10^4$
m	17.3	884	w +	40.6	4235
st +	19.2	1082	vw	41.5	4391
v w	19.8	1147	vw	42.3_5	4538
w	20.6	1238	w—	43.1	4669
v w	22.4	1452	w	44.5	4913
v w	23.5	1590	w—	46.7	5297
w	24.0	1654	vw	48.2_5	5566
v st	26.7	2019	w—	48.7	5661
v w	27.5	2132	w—	49.4_5	5773
st	28.1_5	2226	vw	50.0_5	5876
st +	28.8	2321	w—	52.6	6311
w	30.3	2545	w—	53.4	6445
w—	31.6_5	2753	w	54.5	6628
v w	32.6	2903	w	55.6	6808
w +	35.1_5	3314	w—	56.5	6954
m	35.8_5	3430	w—	56.8	7002
v st	36.5	3538	w +	57.9	7176
w	37.9	3773	m	58.2_5	7231

c. The δ-phase, $FeTe_{1.5}$

The density determination of a preparation $FeTe_{1.50}$ with kerosene as pycnometer liquid gave the value 7.63_8 g/cm³ at 20° C. This density corresponds to 1.99 Te atoms and 1.33 Fe atoms per unit cell, and confirms the idea that the $B8$-like structure can be considered as an AB subtractive structure. The X-ray density of $FeTe_{0.667}$ is 7.674 g/cm³.

Compared to the ordinary $B8$ (NiAs)-structure, 0.67 Fe atoms are missing per unit cell Fe_2Te_2. These atoms can either be missing at random from the positions (a) 0,0,0, and (b) 0,0,1/2, or position (a) can be fully occupied while position (b) is only filled to one third with iron atoms. The latter distribution constitutes a transition stage between the $B8$ and the $C6$ structure types.

In Table 7 are listed the observed intensities of a $FeTe_{1.50}$ preparation together with those calculated using the assumption of random missing (I_1) and preferential missing of iron atoms (I_2). In both cases the atomic parameters are assumed to remain unchanged compared to those of the $B8$ type. The first alternative seems to give the best agreement between the observed and calculated intensities, *i.e.* the iron atoms are probably distributed at random over the twofold (a) positions of the space group $C\ 6/mmc$. This is in contrast

Table 7. Observed and calculated intensities of $FeTe_{1.50}$. Assumption I_1: 0.67 Fe atoms in 000; 0.67 Fe atoms in 00½. Assumption I_2: 1 Fe atom in 000; 0.33 Fe atoms in 00½.

$\sin^2\Theta \cdot 10^4$	*hkil*	I_{obs}	I_1	I_2	$\sin^2\Theta \cdot 10^4$	*hkil*	I_{obs}	I_1	I_2
—	0001	—	0	0.2	—	$11\bar{2}3$	—	0	0.2
856	$10\bar{1}0$	2	0.5	0.5	5549	$10\bar{1}4$	2	0.4	0.4
1154	$10\bar{1}1$	12	14	9.4	6005	$21\bar{3}0$	2	0.4	0.4
1173	0002	2	1.5	1.5	6071	$20\bar{2}3$	8	6.9	4.8
2034	$10\bar{1}2$	10	13	13	6294	$21\bar{3}1$	20	14	10
2574	$11\bar{2}0$	*10*	*10*	*10*	7171	$21\bar{3}2$	13	12	12
—	0003	—	0	0.0	7262	$11\bar{2}4$	20	20	20
—	$11\bar{2}1$	—	0	0.3	—	$000\bar{5}$	—	0	0.0
3438	$20\bar{2}0$	1	0.3	0.3	7713	$30\bar{3}0$	13	11	11
3497 *	$10\bar{1}3$	10	7.3	9.7	—	$30\bar{3}1$	—	0	0.3
3723	$20\bar{2}1$	6	7.0	9.6	—	$20\bar{2}4$	—	0.7	0.7
3740	$11\bar{2}2$	6	4.6	4.6	8179	$10\bar{1}5$	13	13	8.5
4601	$20\bar{2}2$	6	5.5	5.5	8631	$21\bar{3}3$	35	31	42
4695	0004	3	2.4	2.4	8881	$30\bar{3}2$	17	12	12
					—	$11\bar{2}5$	—	0	2.0

* At this and higher angles the α-doublet is resolved and the listed $\sin^2\Theta$ value refers to the α_1-component.

to the view of Tengnér [5] on the CoTe-$CoTe_2$ transition and of Hoschek and Klemm [21] on the VSe—VSe_2 transition according to which the subtraction of metal atoms from the MeX ($B8$) structure, needed to give the MeX_2 ($C6$) structure, is supposed to cause vacant positions in every other metal layer in the hexagonal plane, except for compositions close to MeX. The Fe_2Te_3 phase is, however, only stable at high temperatures where random distribution is more probable.

In the δ-phase, each Te atom is surrounded on the average by 4 Fe atoms at the corners of a right trigonal prism at a distance of 2.62 Å. Each Fe atom is surrounded by 6 Te atoms at the same distance. The iron atoms have furthermore 2 Fe atoms as neighbors at $c/2 = 2.83$ Å. The tellurium atoms form an approximately hexagonal close-packing, the shortest distance between atoms in adjacent layers (3.60 Å) being smaller than between atoms within the layer (3.82 Å). Although the Te—Te distance is smaller than it is in the tetragonal structure, and the ionic radius of Fe^{+3} is smaller than it is for Fe^{+2}, the Fe—Te distance remains practically unchanged, indicating the presence of metallic (covalent) bonds.

d. The ε-phase, $FeTe_2$

In accordance with Tengnér's [5] results, the X-ray powder photographs of the ε-phase could be indexed assuming an orthorhombic unit cell. The extinctions are of the type $h0l$ absent when $h + l = 2n + 1$ and $0kl$ absent when $k + l = 2n + 1$ and they characterize the space groups $Pnnm$ (D_{2h}^{12})

Table 8. Observed and calculated intensities of the ε-phase ($FeTe_{1.00}$), assuming marcasite structure with parameters $x = 0.365$ and $y = 0.225$.

$\sin^2\Theta \cdot 10^4$	*hkl*	I_{obs}	I_{calc}	$\sin^2\Theta \cdot 10^4$	*hkl*	I_{obs}	I_{calc}
—	110	—	0.7	6277	141	2	1.8
866	101	3	4.1	—	510	—	0.7
964	{200 / 011}	3	{ 0.1 / 3.0	6326	402	5	4.9
				—	240	—	0.1
1204	111	13	19	6510	232	18	14
1295	210	13	17	6598	501	3	4.2
1359	020	5	4.3	6667	412	2	1.1
1591	120	2	0.8	—	430	—	0.5
1918	211	2	1.1	—	213	—	0.8
2218 *	121	13	18	6940	511	18	15
2311	220	3	1.4	7005	241	8	6.7
2499	{310 / 002}	7	{ 1.4 / 8.4	7222	123	18	15
				—	520	—	0.8
2774	301	16	12	7496	431	9	9.0
2935	221	3	2.5	—	340	—	1.3
—	112	—	0.2	7675	422	35	32
3113	311	6	5.9	—	332	—	0.6
3285	130	3	2.7	7777	303	15	14
—	202	—	0.1	7917	{042 / 223}	15	{ 15 / 2.5
—	320	—	0.3				
3675	031	2	0.5	—	521	—	0.1
3782	212	15	16	8115	313	6	9.0
—	400	—	2.4	8152	142	6	5.6
3853	022	6	4.0	8193	341	35	23
3911	131	8	7.5	8597	600	5	3.0
4002	230	7	6.5	8671	033	2	1.0
4095	122	2	0.8	8700	150	2	1.1
4129	321	7	5.0	8806	512	3	2.5
—	410	—	0.5	—	242	—	0.6
4622	231	2	1.2	8906	133	24	21
—	411	—	0.1	8939	610	20	20
4808	222	2	1.8	—	530	—	0.0
4988	312	2	1.9	9088	051	15	24
5183	420	15	10	9126	323	30	18
—	330	—	0.2	9239	440	13	9
5423	040	6	4.1	9332	151	13	16
5666	140	2	1.6	9370	432	7	2.9
5782	132	4	4.4	9423	250	16	17
—	421	—	0.1	9561	611	3	4.8
5817	331	3	4.1	9622	233	8	6.2
5860	103	2	1.9	9642	531	46	53
5954	013	2	1.3	—	413	—	0.5
—	322	—	0.5	9822	522	7	9
6207	113	*10*	*10*				

* At this and higher angles the α-doublet is resolved and the $\sin^2\Theta$ value refers to the α_1-component.

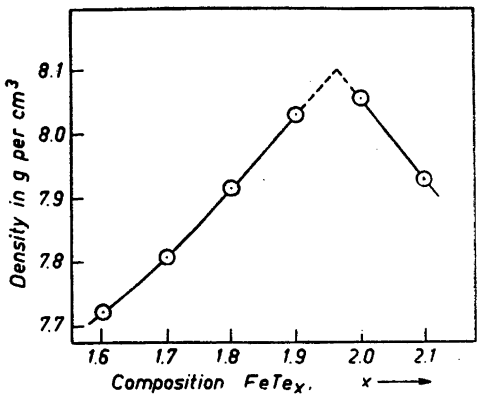

Fig. 3. Observed densities in the composition range $FeTe_{1.60}$ to $FeTe_{2.10}$.

and *Pnn* (C_{2v}^{10}). In the space group *Pnnm* the marcasite grouping is realized with the atoms in the following positions:

2 Fe atoms in (*a*) 0,0,0; 1/2, 1/2, 1/2,
4 Te atoms in (*g*) $x, y, 0$; $\bar{x}, \bar{y}, 0$; 1/2—x, 1/2 + y, 1/2; 1/2 + x, 1/2—y, 1/2.

By a systematic variation of the x and y parameters the best agreement with observed intensities was obtained with $x = 0.365$ and $y = 0.225$. These values are very close to the values $x = 0.36$ and $y = 0.22$ found by Tengnér. In Table 8 are listed the results of the calculations, together with the observed relative intensities of the interferences and the $\sin^2\Theta$ values.

In $FeTe_2$ each Fe atom is surrounded by 6 Te atoms at the corners of a deformed octahedron at the distances Fe—2Te = 2.58 Å and Fe—4Te = 2.56 Å, while each Te atom is surrounded by one and two iron atoms at the same distances. The tellurium radius is found from the shortest Te—Te distance to be 1.45 Å. This is appreciably larger than the tetrahedral Te radius [18], 1.32 Å.

The homogeneity range of the ε-phase $FeTe_2$ is not only noted from changes in the position of the X-ray interferences but also from changes in their intensity. As the changes are especially noticeable on high angle interferences with low l values they are probable due to alterations in the parameter values, and the observed intensities of the $FeTe_{2.10}$ interferences are in fact reproduced better with the parameter values $x = 0.36$ and $y = 0.22$ than with the values used for $FeTe_2$.

Marcasite-like phases with metalloid deficit have been known since Buerger[22] in 1932 found by chemical analysis that loellingite had the composition $FeAs_{1.78}$*. Two years later Buerger [24] concluded from available analytical data that marcasite itself had a sulfur deficient composition which corresponded to the

* Chemical analysis of a loellingite from Kuortane, Finland, and a comparison with seven other analyses reported in the literature led Pehrman [23], in contrast to Buerger, to the conclusion that the composition of loellingite is usually close to MeX_2.

Table 9. Calculated densities for $FeTe_{1.95}$ and $FeTe_{2.10}$.

Sample	Type of solid solution	Formula	Calc. density in g/cm³
$FeTe_{1.95}$	Interstitial	$Fe_{1.026}Te_2$	8.131
	Substitutional	$Fe_{1.017}Te_{1.983}$	8.063
	Subtractive	$FeTe_{1.950}$	7.928
$FeTe_{2.10}$	Interstitial	$FeTe_{2.100}$	8.402
	Substitutional	$Fe_{0.968}Te_{2.032}$	8.130
	Subtractive	$Fe_{0.952}Te_2$	8.002

formula $FeS_{1.985}$. By means of magnetic measurements and density determinations Haraldsen, Rosenqvist and Grønvold [25] found that chromium diantimonide is homogeneous at the composition $CrSb_{1.85}$. In all these phases the metal excess was combined with a substitution of metal for metalloid atoms, giving the formulae: $Fe_{1.08}As_{1.92}$, $Fe_{1.01}S_{1.99}$, $Cr_{1.05}Sb_{1.95}$.

A metal excess was also observed in the iron ditelluride and density determinations were carried out to find the composition limits and cell content of the ε-phase. The densities were determined as usual by means of kerosene displacement in vacuum at 20° C. The results are shown in Fig. 3. Extrapolation of the two parts of the curve gives a maximum density close to the composition $FeTe_{1.95}$. This agrees very well with the X-ray results and supports the conclusion that the ε-phase is homogeneous at this composition.

In many cases it is possible to decide between the different types of solid solution in non-stoichiometric compounds by means of density determinations. The three types of solid solution to be taken into account are interstitial, substitutional and subtractive. The densities calculated for these three possibilities and the corresponding formulae for $FeTe_{1.95}$ are listed in Table 9.

The density of $FeTe_{1.95}$ has not been determined experimentally but from the densities of the adjacent samples a density of 8.09 g/cm³ is interpolated. It is therefore evident that the solid solution does not take place by subtraction of tellurium atoms. As a rule, densities found by the liquid displacement method used here, are about 0.5 % too low. Accordingly, the interstitial solid solution type is considered as the most probable.

At the stoichiometric composition $FeTe_2$ the calculated density 8.092 g/cm³ is in good agreement with the observed value 8.05_1 g/cm³. The observed density for $FeTe_{2.10}$ is 7.92_7 g/cm³ and a comparison with the calculated values in Table 9 shows that the assumption of a subtractive solid solution is needed to account for the low density observed. Thus, within the whole range of homogeneity of the ε-phase from $FeTe_{1.95}$ to $FeTe_{2.10}$, a removal of iron atoms from the lattice takes place with increasing tellurium concentration.

SUMMARY

Iron tellurium alloys with compositions ranging from Fe_2Te to $FeTe_3$ have been studied by means of X-ray diffraction. The existence of four intermediate phases was revealed.

1. The β-phase, $FeTe_{0.9}$ or Fe_9Te_8, with a narrow homogeneity range, is found to have tetragonal structure with the lattice constants:

$a = 3.823_0$ Å, $c = 6.276_7$ Å at the iron rich limit,
and
$a = 3.819_8$ Å, $c = 6.280_5$ Å at the tellurium rich limit.
Space group: $P4/nmm$, with 2 Fe in (a) and 2 Te in (c) with $z_1 = 0.285$. Excessive 0.2 Fe atoms are probably located in (c) with $z_2 \approx 0.70$. The observed density of $FeTe_{0.90}$ is 6.77_7 g/cm^3.

2. The γ-phase, $FeTe_{1.1}$, is stable in the temperature range around 750° C. Its structure is not yet known.

3. The δ-phase, $FeTe_{1.4}$—$FeTe_{1.5}$, has a structure of $B8$ (NiAs)-like type and is stable above 488° C. The lattice constants of the hexagonal $FeTe_{1.50}$ are:
$a = 3.816_2$ Å, $c = 5.654_8$ Å.
The observed density 7.63_3 g/cm^3 shows that the phase is of the AB subtractive type with missing iron atoms.

A monoclinic deformation is observed at the iron rich side of the homogeneity range.

4. The ε-phase, $FeTe_2$, with orthorhombic structure of the $C18$ (marcasite) type, has a range of homogeneity between $FeTe_{1.95}$ and $FeTe_{2.10}$. The lattice constants are:

$a = 6.264_1$ Å, $b = 5.260_6$ Å, $c = 3.872_4$ Å for $FeTe_{1.90}$,
$a = 6.274_7$ Å, $b = 5.278_8$ Å, $c = 3.863_6$ Å for $FeTe_{2.10}$.

For $FeTe_2$, the best agreement between the observed and calculated X-ray intensities was obtained using the parameters $x = 0.365$ and $y = 0.225$.

The density determinations indicate that some iron atoms are in interstitial positions in $FeTe_{1.95}$ and are missing in $FeTe_{2.10}$.

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Short Communications

The Ozonization of *p*-Benzoquinone

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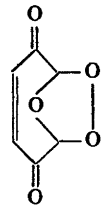
In the course of our investigations on the reactions between quinones and β-diketones it became desirable to subject some of the substances prepared to ozonolysis. Searching of the literature disclosed the surprising fact that very little was known about the action of ozone on quinones and it was therefore decided to look more fully into this question.

For the sake of simplicity *p*-benzoquinone was chosen as a model substance. The ozonolysis of this quinone was reported by Fichter, Metzer and Leepin [1]. These authors worked in chloroform solution but gave no information about working temperatures or concentrations of ozone. They did, however, claim to have isolated an ozonide (or "perozonide") with the composition $C_6H_4O_9$ contrary to the expected normal diozonide of *p*-benzoquinone $C_6H_4O_8$. When this ozonide was decomposed by water it yielded formic acid and another acid the identity of which they did not establish.

In the present investigation *p*-benzoquinone was ozonized in chloroform at 0° with 3 to 3.5 % ozone. An ozonide was formed and being almost insoluble in chloroform it separated as a gel-like mass varying in colour from almost white to yellow. Purification of the ozonide for analysis was very difficult because of its sensitivity to moisture, the risk of thermal decomposition and because it retained chloroform very tenaciously. No analytical data will therefore be presented on this occasion. The ozonide was (as Fichter *et al.* also observed) not explosive but burned vigorously when heated on a spatula in a Bunsen flame.

Water decomposed the ozonide very rapidly without noticeable evolution of heat. Hydrogen peroxide could not be detected among the decomposition products. The aqueous solution containing these was treated in a column with an ion exchange resin in order to remove some acidic compounds. The neutral eluate reduced Fehling's solution at room temperature and reacted with the common carbonyl reagents. There must consequently have been an aldehydic compound present. If the solution was boiled for some minutes with dilute hydrogen peroxide and then evaporated to dryness maleic acid could be isolated in good yield. These results point towards the following structure for the aldehydic compound:

CHOCOCH=CHCOCHO (I).



II.

As will be seen this is a compound to be expected if benzoquinone reacted with ozone to give the monozonide (II) and this again was decomposed by water to a dialdehyde. A further indication that (I) is the correct structure of the carbonyl compound is found in the fact that it reacts with 2,4-dinitrophenylhydrazine to give a derivative of the approximate composition $C_{30}H_{20}N_{16}O_{16}$ corresponding to a tetrakis-(dinitrophenylhydrazone) of (I). (Found: C 40.76, H 2.51, N 26.04. Calc. for $C_{30}H_{20}N_{16}O_{16}$: C 41.86, H 2.34, N 26.05.) The highly interesting compound (I) will be the subject of further investigations. Experiments on the ozonization of other quinones and quinonoid substances are in hand.

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Synthesis of Dodeca-1:11-diene-3:5:7:9-tetrayne

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One of the substances identified by Sörensen and Sörensen [1] in the roots of several *Coreopsis* species shows a characteristic absorption spectrum, which was found to be very similar to that of synthetic tetradeca-2:12-diene-4:6:8:10-tetrayne[2]. The volatility of the natural product, and the relative positions of the absorption maxima, suggested that the natural product might be even simpler than this hydrocarbon. Accordingly we have now synthesized dodeca-1:11-diene-3:5:7:9-tetrayne by oxidative coupling of hex-1-ene-3:5-diyne [3]. This extremely unstable hydrocarbon has m.p. 38—40°, whereas the *Coreopsis* product subsequently [1] obtained pure, darkens on heating above 43° without melting. A comparison of infra-red absorption spectra left little doubt that the two hydrocarbons are not identical. The ultra-violet absorption maxima of the synthetic compound lie at wavelengths 16—28 Å shorter than those of the *Coreopsis* compound, whereas the estimated experimental error of our values is about 10 Å, and of Prof. Sörensen's only about 5 Å. That these small differences are real was confirmed by measurements made at Manchester on a sample of the natural hydrocarbon kindly supplied by Professor Sörensen. The ultra-violet absorption spectrum, intermediate between those of the two synthetic hydrocarbons, suggests a monoalkyl-diene-tetrayne chromophore. The presence of three linear C_{12} polyenynes[1,4,5] in related species supports the view that the *Coreopsis* hydrocarbon is trideca-1:11-diene-3:5:7:9-tetrayne.

This conclusion contrasts with the earlier one of Sörensen and Sörensen [1], based upon extremely difficult experiments with minute quantities of a very unstable substance, that the natural compound was the C_{12} hydrocarbon now synthesised. It is hardly necessary to mention that the identification of saturated hydrocarbons such as dodecane and tridecane on a milligramme scale is by no means an easy task.

Experimental. A solution of hex-1-ene-3:5-diyne in liquid ammonia was prepared from 5.85 g of 1:6-dichlorohex-4-ene-2-yne [3], and extracted with *iso*butane, and the *iso*butane was replaced by acetone (10 ml). Cuprous chloride (10 g) and ammonium chloride (16 g) in water (50 ml) were added, giving a clear red solution. Hydrogen peroxide (15 ml, 30 %) was introduced [6] below the surface of the liquid, with external cooling to below 10°, during 20 minutes, followed by 50 ml of hydrochloric acid (1:3). From this point all operations were carried out at —5° in weak artificial light. The mixture was extracted with ether, and the extract at once evaporated under reduced pressure to less than 5 ml. The residue was dissolved in pure hexane (30 ml), and the mixture chromatographed on 50 g of alumina, which was eluted with 150 ml of hexane. Evaporation of the eluate to 10 ml and cooling to —75° gave crystalline dodeca-1:11-diene-3:5:7:9-tetrayne, which after four recrystallisations formed pale yellow needles (*ca.* 0.3 g), m.p. 38—40° in an evacuated capilliary tube. It blackened at once in light even at —5°, and absorbed oxygen so rapidly that analysis or even normal weighing for spectrographic examination was impossible; for the latter purpose a little was sublimed at 30° (bath temp.)/10^{-5} mm on to a cold finger and dissolved at once in hexane, absolute intensities being found approximately by evaporating an aliquot and weighing the residue in nitrogen. Hydrogenation gave *n*-dodecane, m.p. —10- —9°, in agreement with literature values [1]. The observed uptake (8.8 moles) was low, probably because of partial polymerisation.

U.V.-spectrum in hexane:

ε_{max}	8 600	14 000	12 000	7 350
λ_{max}	3 880	3 600	3 350	3 140 Å
ε_{max}	115 000	160 000	99 000	85 500
λ_{max}	2 850	2 690	2 560	2 440 — 2 460 Å

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On the Intestinal Absorption of Phospholipids in the Rat

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Recently Artom and Swanson [1], after feeding ^{32}P-labelled phospholipids to rats and then studying the distribution of radioactivity and phosphorus in the plasma and liver, obtained results, which suggested that phospholipids may be absorbed at various stages of hydrolysis. They suggested that even a small part might be absorbed as intact molecules. Bloom *et al* [2]. have recently made similar experiments with phospholipids labelled with ^{14}C-palmitic acid.

The purpose of the present investigation was to further elucidate the mechanisms concerned with the absorption of phospholipids by studying the composition of lymph lipids after feeding phospholipids labelled in the fatty acid, glycerol or phosphate portions of the molecule.

Experimental. Phospholipids containing labelled fatty acids were biosynthesized by feeding rats palmitic acid-1-^{14}C. After 5 hours the rats were killed and the total fat from the liver was extracted with alcohol-ether and subjected to chromatography on silicic acid [3]. The labelled phospholipids were dissolved in olive oil (Mixture A, Table 1).

Phospholipids containing ^{14}C-labelled glycerol were biosynthesized by incubating rat liver slices with glycerol-1-^{14}C in a medium of Krebs-Ringer phosphate buffer, pH 7.4, for 2 hours. Free radioactive glycerol was removed from the total fat by dilution with inactive glycerol and washing several times with water. The labelled phospholipids were isolated by precipitating with ice-cold acetone and then dissolved in appropriate amounts of olive oil (Mixture B, Table 1). After hydrolysis almost all of the activity was found in the glycerol portion of the phospholipids and only negligible amounts in the fatty acids. In order to obtain triglycerides labelled in the glycerol portion the neutral fat from these experiments was further purified from phospholipids by chromatography on silicic acid [3] and then dissolved in olive oil (Mixture C, Table 1).

In the same way phospholipids containing ^{32}P were isolated from samples of blood from a patient suffering from polycythemia vera and treated with ^{32}P (Mixture D, Table 1).

These mixtures were then fed in an amount of 0.5 ml to rats with a cannulated thoracic duct and the lymph collected for the following 24 hours. The lymph fat was then subjected to chromatography on silicic acid [3] and the distribution of the activity between the neutral fat and phospholipids determined.

Results. In the experiments with phospholipids containing ^{14}C-labelled fatty acids 52—98 % of the administered activity was absorbed. An average of more than 65 % of the absorbed activity was recovered in the lymph lipids. The major part of this activity was obtained in the neutral fat and about 16 % in the phospholipids. If palmitic-1-^{14}C acid is fed in the form of glyceride or as the free acid only about 4 % of the lymph activity is obtained in the phospholipids [2,4,5].

In the experiments with phospholipids labelled in the glycerol portion more than 60 % of the fed activity was recovered in the lymph lipids. From 57 to 72 % of the lymph activity was found in the neutral fat and 28—43 % in the phospholipids. In similar experiments with triglycerides containing ^{14}C-labelled glycerol about 52 % of the administered activity was recovered in the lymph lipids. From 76 to 84 % of the lymph activity was found in the neutral fat and the remainder of the activity in the phospholipids.

Table 1. Recovery of activity in lymph lipids after feeding phospholipids containing A. ^{14}C fatty acids, B. ^{14}C-glycerol, C. triglycerides containing ^{14}C-glycerol and D. phospholipids containing ^{32}P, to rats with lymph fistula. Each rat received 0.5 ml of olive oil containing about 100 mg of labelled phospholipids.

Diet administered	Number of rats	% absorbed activity recovered in lymph lipids	% fed activity recovered in lymph lipids	% of lymph activity recovered as Neutral fat	% of lymph activity recovered as Phospholipids
A	5	50—81		75—88	12—25 (m = 16)
B	4	—	55—73	57—72	28—43
C	2	—	45—59	76—84	16—24
D	2	—	5—10	—	100

After feeding phospholipids containing ^{32}P only about 7 % of the fed activity was recovered in the lymph.

Discussion. The results of the first experiments show that fatty acids fed in the form of phospholipids are well absorbed mainly *via* the lymph. After absorption most of the labelled acid in the fed phospholipids was recovered in the lymph glycerides, but a significantly greater amount of activity was obtained in the phospholipids than after feeding palmitic-1-^{14}C acid as glyceride or as the free acid [2,4,5]. That these findings mean that a small part of the phospholipids is absorbed completely unchanged seems unlikely but final conclusions must await further experiments.

The results of the experiments with phospholipids labelled in the glycerol portion also suggest that the phospholipids are hydrolyzed to a considerable extent although somewhat less than the triglycerides. This suggestion is supported by experiments with free-labelled glycerol [6,7] and with glycerol-phosphate [1] which have shown that these substances are only utilized to a very small extent for synthesis of triglycerides or phospholipids in the intestinal contents or cells when free fatty acids are absorbed and transported from the intestine as triglycerides to the lymph.

Since certain amount of activity was found in the lymph after feeding phospholipids containing ^{32}P, these results also seem to indicate the possibility that some phospholipids were absorbed without splitting of the glycerol phosphate bond. However, as the phospholipids in the last-mentioned experiments were of human origin, these experiments are not quite comparable. The preliminary experiments with ^{32}P containing phospholipids support the suggestion that most of the ester linkages in the phospholipid molecules between the glycerol and the phosphorus are split during the absorption process. The remaining part of the phospholipid molecule, *i. e.* a mono- or diglyceride, can then be reutilized for the formation of phospholipids or triglycerides or else degraded further to free fatty acids and glycerol.

The experiments in this investigation with triglycerides labelled in the glycerol portion suggest that a significant part of the glyceride glycerol must have been utilized for synthesis of lymph phospholipids. It is probable that this has been done from a partial glyceride. From data published by Reiser *et al.* it appears that they also recovered some of the labelled glycerol from fed triglyceride in the lymph phospholipids (*cf.* Table 2).

Table 2. Comparison of recovery and distribution in lymph lipids after feeding free labelled glycerol, triglycerides containing labelled glycerol or phospholipids containing labelled glycerol or palmitic acid.

Labelled substances dissolved in olive oil and fed to rats with lymph fistula	% fed isotope recovered in lymph lipids	% of isotope recovered as		References
		Neutral fat	Phospholipids	
Free ^{14}C-glycerol	<2			Bernhard *et al.*[7]
Glyceride ^{14}C-glycerol	26—35	94—95	5—6	Reiser *et al.*[6]
Glyceride ^{14}C-glycerol	45—59	76—84	16—24	Blomstrand 1954
Phospholipide ^{14}C-glycerol	55—73	57—72	28—43	Blomstrand 1954
PL-^{14}C palmitic acid		80	20	Bloom *et al.*[2]
		75—88	12—25 (m = 16)	Blomstrand 1954

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Continuous Fractionation of Serum by Zone Electrophoresis

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By electrophoresis in a supporting medium complete separation of components of a mixture can be obtained. By arranging a simultaneous transport of the liquid medium in a direction perpendicular to the electric field the fractionation can be performed as a continuous process, which allows the application of greater quantities of material than those conveniently used in a batch procedure. According to these principles an apparatus has been developed [1–3] and applied to the study of the proteins of blood serum [4]. Using a veronal buffer of the ionic strength 0.03 and pH 8.4 as the conducting medium, the dialyzed serum solution is injected as an 1 cm wide zone. In the velocity field this zone, which contains about 2 % of protein, is split into its component zones and finally covers a distance of about 20 cm horizontally. The fractions are collected at intervals of 0.5 cm. The protein contents of the fractions give a distribution curve, where the γ-, β-, α-globulins and serum albumin form the four main peaks (Fig. 1). By the fractionation of 20–30 ml of whole serum enough material is obtained for extensive analysis of the fractions. The rate of fractionation usually is about 0.8 ml of whole serum per hour.

The homogeneity of the fractions has been studied electrophoretically and in the ultracentrifuge. By paper electrophoresis it can be shown that material from the principal maxima of the distribution curve migrate as singular spots at rates characteristic of the γ-, β-, α-globulins and albumin, respectively. When material from successive fractions of the γ group are simultaneously submitted to electrophoresis on the paper, the line connecting the centres of the spots gradually becomes inclined, demonstrating that the differentiation achieved in the separation column is not an artefact. The two maxima in the γ peak can be shown to contain material of distinctly different mean mobility. In the ultracentrifuge the fractions containing the γ group and the maxima of the β-globulin and albumin zones are homogeneous, whereas the α peak always contains three main components and the dilute fractions between the γ and β groups two components each. On both sides of the α maximum the fractions are also heterogeneous in the ultracentrifuge, but those fractions which by paper electrophoresis can be split into albumin and α_1-globulin only contain one boundary, although the heterogeneity is apparent from the skewness in the optical pattern. By ultracentrifugations of solutions containing approximately 0.5 % of protein in a phosphate buffer of the ionic strength 0.05 and pH 6.83 the following sedimentation constants have been computed (*cf.* Fig. 1):

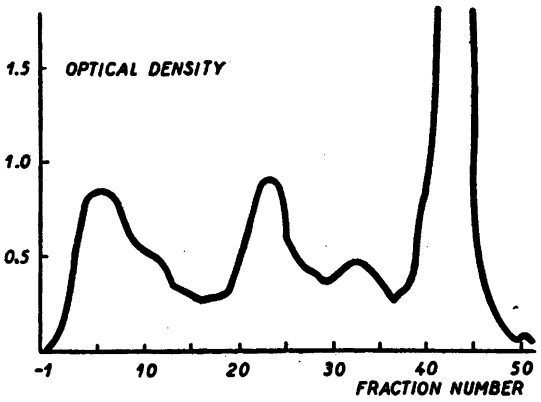

Fig. 1. Fractionation of normal human serum. The optical densities of the fractions at 284 mμ have been plotted against the fraction numbers.

Fraction No.	5	11	13	15, 16, 17	20	23	28	33	39	43
s_{20}	7.0	7.03	6.90 19.3	6.68 19.2	(5.23) 6.29	5.30	6.36 11.8	4.01 7.83 (11.6) 18.3	4.56	4.54

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4. Brattsten, I. *Arkiv Kemi (In press)*.

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*epi*Friedelinol and Derivatives, a Re-investigation

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The occurrence of *epi*friedelinol in *Ceratopetalum apetalum* D. Don (Cunoniaceae)[1] and the lichen *Cetraria nivalis* (L.) Ach.[2] has recently been reported simultaneously and independently by us. The rotations recorded in the two communications were, however, so widely differing (compare Table 1) that it could not be accounted for by experimental errors alone. The difference could conceivably be due to a small content of a contaminant in one of the samples. To settle this question a re-investigation was undertaken, the details of which are found in the experimental part. We believe that the rotations now found (see Table 1) should be regarded as the correct ones. The differences in the m.p.'s are not significant in view of their range and different measurement conditions.

As is seen from the experimental part, there is a strong indication that *epi*friedelinol from *Cetraria nivalis* did contain a small amount of some other material, but further investigations were prevented by lack of material.

Experimental. M.p.s are not corrected. Rotations were measured at room temperature (approximately 20°) in chloroform in a 1 dm tube, unless specified to the contrary.

The *epi*friedelinol used in these investigations, except in one case, was the original sample obtained from *Ceratopetalum apetalum* D. Don[1], $[\alpha]_D$ +19° (*c*. 0.91), +21° (*c*, 1.02), m. p. 272—275°, admixture with a sample of the lichen *epi*friedelinol (m. p. 273—276°) m. p. 272—276°, all three taken at the same time. Similarly, in evacuated tubes, the m.p.s were, respectively, 280—281°, (281—282°) and 281—282°.

epi*Friedelanyl acetate.* The acetate was prepared with acetic anhydride in pyridine with standing at room temperature for 4 days in the same way as previously used by Bruun[2]. Sufficient pyridine was used to keep the alcohol in solution. The product was filtered through alumina in benzene and crystallised from chloroform-methanol, m. p. 284—285°, admixture with the original acetate (prepared in the same way) of *epi*friedelinol from lichens (m. p. 273—274°)[2] m. p. 270—285°, all three taken at the same time, $[\alpha]_D$ +37° (*c*, 1.02). Recrystallised the acetate melted at 282—283°, $[\alpha]_D$ +38° (*c*, 0.96). After drying at 115° for 6 hours in a water-pump vacuum it melted at 282—285°, $[\alpha]_D$ +34° (*c*, 1.76).

A sample of the acetate was also prepared by refluxing for 1 hour 20 mg of *epi*friedelinol from *lichens* with acetic anhydride as used by Jefferies[1]. The crystals which deposited on cooling were filtered, m. p. 276—279°, admixture with the previously prepared acetate from lichens (m. p. 271—274°) m. p. 271—274°, all three taken at the same time, $[\alpha]_D$ +33° (*c*, 1.24). Recrystallised it melted at 281—283°, admixture with the sample above from *Ceratopetalum apetalum* (m. p. 282—285°) m. p. 282—285°, all three taken at the same time, $[\alpha]_D$ +33° (*c*, 1.11).

Table 1.

	Ceratopetalum apetalum D. Don		*Cetraria nivalis* (L.) Ach.		Present investigation	
	m.p.	$[\alpha]_D$	m.p.	$[\alpha]_D$	m.p.	$[\alpha]_D$
*epi*Friedelinol	279—283°	+24°	281—282° (vac.)	+15°	272—275° 280—281° (vac.)	+ 20°
Acetate	290—294°	+ 45°	275—276° (vac.)	+ 12°	282—285°	+ 35°
Benzoate	254—257°	+ 40°	232—233° (vac.)	+ 25°	246—248°	+ 34°

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The acetic anhydride filtrate (immediately above) was decomposed with water and organic material extracted with ether. Acetic acid was removed by sodium hydrogen carbonate solution. Evaporation of the ether left a yellow resin that partly crystallised. The amount was estimated at 2 mg or 10 % of the starting material. From acetone colourless crystals were obtained, which melted at 230—245°. A mixture with *epi*friedelanyl acetate melted at 230—255°.

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We thank Professor N. A. Sörensen for the interest he has taken in this work. One of us (T. B.) thanks *Norges Almenvitenskapelige Forskningsråd* for a Research Fellowship.

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The Structure of Liquid Carbon Disulphide

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Several investigators have noted a departure from normal behaviour of liquid carbon disulphide. Hildebrand [1] as early as in 1919 found that the internal pressure calculated from the coefficients of expansion and compressibility gave a comparatively low relative value. Benson [2] observed that the ratio of the density at the boiling point to the density at the critical point was abnormally large. This he concluded to be due to a dense packing in the liquid structure, arising from the chain-forming tendency of sulphur atoms with available 3d orbitals. Stavely and Tupman [3] in a discussion of entropies of vaporization and internal order in liquids, found the entropy of vaporization of liquid carbon disulphide surprisingly low. According to their general theory, a nearly unrestricted rotation or a low degree of order should be expected in such cases. The Kerr effect, however, indicates a considerable degree of hindrance to free rotation in this liquid, as pointed out by Debye [4].

Information about the structure of liquid carbon disulphide would consequently be of interest. Hence the electronic radial distribution curve has been determined (at 15° C) for this system. A procedure according to Finbak [5] was employed. The X-ray intensity measurements have been obtained using Mo$K\alpha$-radiation only. For low scattering angles a Guinier camera was used, permitting observations to be made down to $s = 4\pi(\sin\nu/\lambda) = 1.3$ Å^{-1}. The resulting distribution curve is reproduced in Fig. 1. Maxima are found at 1.55 Å, 4.0 Å, 6.5 Å and 7.6 Å. At about 3.1 Å a superimposed peak can be distinguished.

The peaks at 1.55 Å and 3.1 Å obviously correspond to the C—S and S—S distances within the molecule. They agree very well with the results obtained on gaseous carbon disulphide by X-ray diffraction [6] and electron diffraction [7,8].

The remaining peaks reflect the packing of the molecules. In order to interpret these peaks, a model of the liquid structure will be considered. The molecule itself can be regarded approximately as a cylinder, with a radius r and a length $d + 2r$. Here d is the S—S distance within the molecule. A closepacking of these cylinders requires the volume:

$$V = 2\sqrt{3}\, r^2(2r + d)$$

Inserting in the formula above the observed liquid volume extrapolated to 0° K [9], r is found to be 1.85 Å. This value equals the van der Waals radius of sulphur, as tabulated by Pauling [10]. At room temperature the formula gives r equal to 2.0 Å and a corresponding packing distance of 4.0 Å for the molecules. The largest peak in the experimental distribution curve occurs at just this distance.

The peaks at 6.5 Å and 7.6 Å are more uncertain because of the experimental errors. The "end to end" packing of the molecules should give a peak at about 7.1 Å according to the previous discussion. The second coordination shell is found at 6.9 Å (and 14.2 Å for the "end to end"

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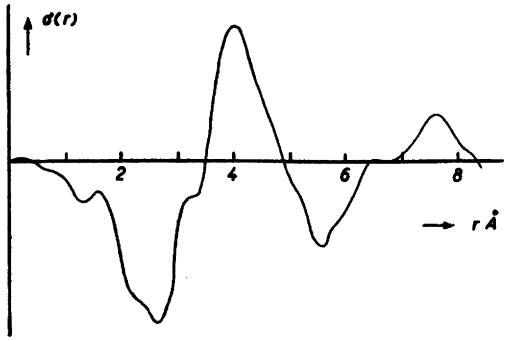

Fig. 1. Electronic radial distribution curve, σ(r), for liquid carbon disulphide.

packing). These packing distances are probably not so definite and may very well account for the observed peaks.

Eventually it can be concluded from these considerations that rotation about the two short axes of the molecule is very restricted. Free rotation would have displaced the major peak from 4.0 Å to at least 5.0 Å. The molecules thus tend to orient themselves with their long axis parallel, corresponding to the proposed closepacking.

Any tendency for the molecules to be mutually displaced along the parallel major axes can not be settled by the present experimental distribution function. A crystal structure investigation of solid carbon disulphide would be of great interest in this connection. (The determination by de Smedt [11] seems questionable with a C—S distance of 1.77 Å and packing distances varying from about 2.2 Å to 8.0 Å. The result is also inconsistent with symmetry observations by Washer [12].) Kast and Stuart [13] have discussed various possibilities for the liquid structure. They suggest that the quadrupole moment of the molecule may give rise to parallel displacements as discussed above, (and even to crossed positions of the major molecular axis).

The defect in the entropy of vaporization of liquid carbon disulphide is explained neither by a low degree of order in the structure, nor by unrestricted rotation. The explanation must be sought in an intermolecular potential energy function differing from normal, as suggested by Frank [14].

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Structure of some 1,2,3,4-Tetrahalogeno*cyclo*hexanes

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Tetrahalogeno*cyclo*hexanes obtained from 1,3-*cyclo*hexadiene and halogens are usually considered to be 1,2,3,4-derivatives. This is probably correct, but the first step is not necessarily a Thiele addition and when different halogens are used in the two steps the structure formula of the final product must be determined in each separate case. We are interested also in the steric forms of tetrahalogeno compounds and have carried out some preliminary investigations in the field. The only member of the series the structure of which has been definitively settled is the 1,2,3,4-tetrabromo*cyclo*hexane of m. p. 142° investigated by E. Wang Lund[1] and which has the configuration eeee. Besides this compound we had four more representatives of this group at our disposal (*cf.* Table 1) and the results of the determination of the dipole moments of all five substances are listed in Table 1.

Table 1.

Substances	Br_4	Br_4	Br_4	Br_3Cl_3	Cl_4
Melting point	142°	90°	156°	128°	112°
Dipole moment	2.89	2.82	3.66	3.81	2.95

If "theoretical" dipole moments are calculated assuming the partial electric moments of both C—Cl and C—Br bonds to be 2.1 D and all valency angles "tetrahedral" the following moments are found: eeee (3.4), eeea (4.9), eaee (3.4), eeaa (3.4), eaae (0) aeea (3.4), aeae (6.8). Considering the results earlier obtained for halogenated *cyclo*hexanes it seems very probable that the substances with a measured dipole moment of about 2.9 D all belong to the group with theoretical values 3.4 D. This is substantiated by the fact that the tetrabromo*cyclo*hexane m. p. 142° has been shown to be the eeee compound. The two compounds with dipole moments of 3.7—3.8 D would then be expected to represent the configuration *eeea*. The alternative aeae would probably demand a still higher experimental dipole moment and would also be less stable and more unlikely to result from the reaction mentioned above. Furthermore, it has already been shown by X-ray analysis that the tetrabromo compound m. p. 156° and the dichlorodibromo compound m. p. 128° form isomorphous crystals [2].

Before starting X-ray crystallographic work we have tried to elucidate the structures in question using electron diffraction technique. The $\sigma(r)/r$-curves thus obtained for the tetrabromo*cyclo*hexane (156°) and the dichlorodibromo*cyclo*hexane (128°) had important features in common. In Fig. 1 the $\sigma(r)/r$-curve of the tetrabromo compound has been reproduced. The pronounced peak at $r = 5.8$ Å can only be due to a 1e, 3e-Br-Br-distance which would exclude the conformations aaee, eaae and aeea. If we consider the fact that no indication of a 1e, 4e-distance ($r = 6.7$ Å) is observed, the two conformations eeee and eaee should also be excluded. The two remaining possibilities, aeae and aeee, cannot easily be distinguished on the basis of electron diffraction experiments as both may be brought in good agreement with the observed $\sigma(r)/r$-curves.

In order to decide whether the first reaction step is a Thiele addition or not it appears necessary to complete the crystal structure determination of the 128° substance. In the first case only *one* com-

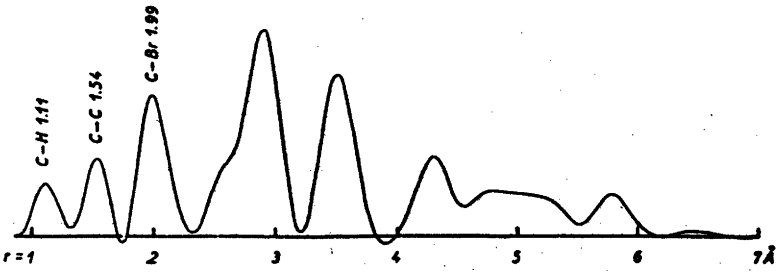

Fig. 1.

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Derivatives of β-10-Phenothiazinepropionic Acid

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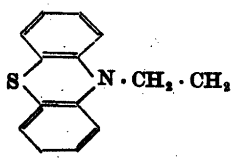
Previous investigations in this Laboratory have shown that certain derivatives of phenothiazine-10-carboxylic acid containing basic substituents possess strong spasmolytic and nicotinolytic properties [1]. As an extension of this work some new derivatives of the easily accessible β-10-phenothiazinepropionic acid were prepared (I—VI).



I. R = Cl
II. R = N⟨ ⟩
III. R = O—CH_2—CH_2—$N(CH_3)_2$
IV. R = O—CH_2—CH_2—$N(C_2H_5)_2$
V. R = S—CH_2—CH_2—$N(C_2H_5)_2$
VI. R = NH—CH_2—CH_2—$N(C_2H_5)_2$

The esters and amides were obtained *via* the acid chloride (I). The compounds III—VI were tested for cholinolytic and antihistaminic effect but their activity was rather weak.

Experimental. β-10-Phenothiazinepropionyl chloride (I). A mixture of β-10-phenothiazinepropionic acid [2] (5.42 g, 0.02 mole), pyridine (1.58 g, 0.02 mole), and ether (60 ml) was cooled to —5° and thionyl chloride (2.38 g, 0.02 mole) was added drop by drop with stirring. The mixture was kept at room temperature overnight. The separated pyridine hydrochloride was then filtered off and the ether was evaporated *in vacuo*. The residue (5.4 g, 93 %) was recrystallised twice from ether; m. p. 117—119°. (Found: C 62.6; H 3.97; Cl 12.0. $C_{15}H_{12}ClNOS$ requires C 62.2; H 4.18; Cl 12.2 %).

N-(β-10-Phenothiazinepropionyl)-piperidine (II). The acid chloride obtained above (1.45 g) was dissolved in ether (15 ml) and treated with piperidine (1.1 g) at room temperature. The mixture was filtered and the filtrate washed with water and evaporated to dryness. The residue (0.9 g, 53 %) was recrystallised from ethanol; m. p. 127—128°. (Found: C 70.4; H 6.23; N 8.09. $C_{20}H_{22}N_2OS$ requires C 70.9; H 6.55; N 8.28 %).

β'-Dimethylaminoethyl β-10-phenothiazinepropionate (III). A solution of I (2.9 g, 0.01 mole) and β-dimethylaminoethanol (2.2 g, 0.025 mole) in toluene (25 ml) was refluxed for two hours. After cooling the mixture was filtered and the filtrate washed with water and extracted with 2 *N* hydrochloric acid. The extract was made alkaline with sodium carbonate solution and the oily base extracted with ether. The ether was then evaporated giving a solid residue (2.0 g, 60 %) which melted at 81—83° after recrystallisation from ether. (Found: C 66.5; H 6.46; N 8.14. $C_{19}H_{22}N_2O_2S$ requires C 66.6; H 6.48; N 8.18%).

β'-Diethylaminoethyl β-10-phenothiazinepropionate oxalate (IV). Prepared by the same method as III. The oily base was isolated as the oxalate. Yield 55 %; m. p. 118—120° (from acetone). (Found: C 59.8; H 6.21. $C_{23}H_{28}N_2O_6S$ requires C 60.0; H 6.13 %).

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N-(β-10-Phenothiazinepropionyl)-N^1,N^1-diethylethylenediamine oxalate (VI). Prepared from I and N,N-diethylethylenediamine [4] by the same method as for the esters. Yield 87 %; m. p. 130—131° (from acetone). (Found: C 59.8; H 6.14; N 8.78. $C_{23}H_{29}N_3O_5S$ requires C 60.1; H 6.36; N 9.14 %).

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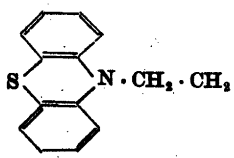

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Structure of Compounds between 1,4-Dioxan and Inorganic Substances

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The crystal structure of the compound formed by 1,4-dioxan and bromine has recently been determined [1]. The O—Br bond distance is very short — 2.71 Å — and the bond *direction* nearly corresponds to an "*e*" bond in *cyclo*hexane. At the same time the Br—Br bond distance nearly coincides with the distance in a free bromine molecule.

A structure investigation of the corresponding compound containing equal numbers of dioxan and mercuric chloride molecules has later been carried out. The structure is *triclinic*, not monoclinic, with a unit cell (space group $P\bar{1}$):

$a = 7.37$ $b = 7.12$ $c = 4.05$ $\alpha = 91.7°$
$\beta = 98.7°$ $\gamma = 67.9°$

containing *one* molecule of each species but geometrically related to the monoclinic C-centered cell of the bromine compound. The mercury atoms are in unique positions and determine the signs of the structure factors. A direct computation of electron density maps is therefore possible. In Fig. 1 a projection along the short (*c*) axis is reproduced. Besides the tilting of the dioxan rings (*cf.* the corresponding map for the bromine compound) the two structures differ also with respect to the position of the centers of the inorganic molecules along the *c*-axis: In the bromine compound the centers of bromine molecules are situated in planes midway between planes containing the centers of dioxan rings, whereas in the mercuric chloride compound mercury atoms and dioxan centers are all in the same levels. The planar arrangement of the two oxygen atoms and the two chlorine atoms surrounding a mercury atom thus corresponds to the arrangement of oxygen and chlorine atoms around the copper atom in addition compounds containing cupric chloride. The Hg—O distance is 2.66 Å, the Hg—Cl distance 2.34 Å.

It appears tempting to investigate structures of compounds containing more easily ionized halides. Some progress has already been made in the structure determination of compounds of dioxan with oxy-acids in which hydrogen bonding will be expected to play a decisive part. Furthermore, we have prepared a compound containing the substance of m. p. 135° C, believed to be a "naphtodioxan" [2], and mercuric chloride. A crystal structure investigation of this compound has been started in order to check the correctness of the assumed structure formula.

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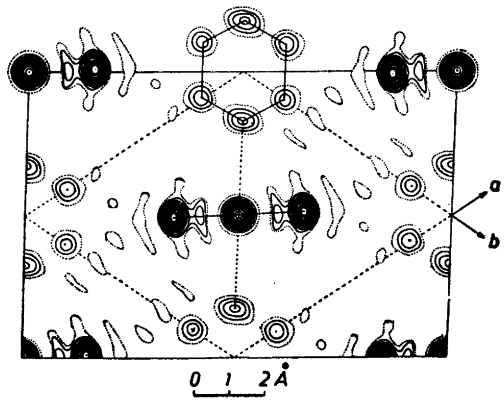

Fig. 1. Electron density map projected along the c*-axis. The* a*- and* b*-axes of the primitive unit cell are indicated by broken lines.*